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More Than a Feeling: Discovering, Understanding, and Influencing Mechanosensing Pathways

Andrew W. Holle and **Adam J. Engler***
Department of Bioengineering, University of California, San Diego; La Jolla, CA 92093

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Mechanosensing; Cell Mechanics; Extracellular Matrix; Focal Adhesions

Introduction

Mechanotransduction is the complex process where a cell converts a mechanical stimulus into a biochemical signal. Although all adherent cell types participate in this, the specific mechanical input and the nature of the corresponding output varies widely. As a result, there are two different regimes of mechanotransduction: passive or ‘outside-in’ sensing, in which the cell responds to a force imparted upon itself, e.g. shear stress [1], extension [2], compression [3], and pressure [4], and active or ‘inside-out’ sensing, in which the generation of internal forces allows for ‘measurement’ of the extracellular environment, e.g. cell traction forces feeling changes in stiffness [5], surface topography [6], and ligand density [7] (Figure 1). Despite two decades of serious scientific inquiry, a consensus on the signaling pathways that are necessary and sufficient to undergo mechanosensation and the resulting behaviors that it elicits has yet to be realized. Here, we discuss the context in which mechanotransduction occurs, the categories of known mechanosensitive pathways within the cell, the systems used to perturb these pathways, and provide an opinion on where consensus can be found within the mechanotransduction community.

Controlling the Physical Microenvironment of the Cell

To appreciate mechanotransductive mechanisms, it is first necessary to review the systems used that impart “outside-in” forces or modulate “inside-out” cell traction forces by exposing cell populations to a controlled physical microenvironment. Fluid flow assays, which mimic blood or ECM fluid flow, are the historical standard for controlling shear forces and examining “outside-in” mechanosensing [8]. Extracellular pressure variations [4] or pulsatile flow [9] have also been utilized to more specifically recreate a physiological environment. Controlled extension of silicone [10] or hydrogel [11] substrates can mimic ECM stretching in vivo. More targeted methods of physically perturbing cells include the use of optical tweezers [12], atomic force microscopy [13], and magnetic twisting cytometry

*Corresponding author: Phone: (858) 246-0678, Fax: (858) 534-5722, aengler@ucsd.edu, Mailing address: University of California, San Diego, 9500 Gilman Drive, MC 0412, La Jolla, CA 92093.

[14]. Some of these tools have also been used to assess “inside-out” mechanosensing by measuring the force with which a cell pulls on its extracellular environment [15].

To induce “inside-out” changes via adjusting the microenvironment, hydrogels like polyacrylamide [16], pNIPAAM [17], PDMS [18] and PEG [19] have been used to vary substrate stiffness. This change in the physical microenvironment has been shown to influence cell migration [16], cell stiffness [20], and adult stem cell differentiation [21]. These substrates can also be patterned with ligands favorable or unfavorable for cell attachment, effectively controlling cell shape [22], or molded into pillars with different heights, which not only provides a variable topography for cells, but also varies stiffness and provides a method for measuring cell traction forces generated [23]. As previously mentioned, gradients in substrate stiffness, ligand patterning, or topography also serve as mechanical inputs to cells, with changes in directed cell migration as a common output, i.e. “durotaxis” [24]. While many durotactic studies mimic pathological gradients [25], even those using physiological gradients found at the interfaces of tissue types have detected durotaxis [26,27]

Regardless of the system used or whether focusing on “inside-out” or “outside-in,” understanding the conversion of physical to chemical signals requires one to focus on where intracellular sensors could exist. Sensors have typically been proposed where clusters of structural and adaptor proteins exist, namely focal adhesions (FA) [28], the complexes that bind the ECM to the cell’s cytoskeleton, and the perinuclear cytoskeleton [29], which binds the cell’s cytoskeleton to the nucleus. Given that cells dramatically change their membrane tension in response to changes in microenvironmental stiffness [21], we will focus our discussion on focal adhesion-based sensing, though a complete picture of overall sensing should likely include both means of signaling.

The Usual Suspects: Known Mechanosensing Pathways

Cell contractile forces result in varying levels of ECM deformation depending upon its physical characteristics. The force to which FA complex structural proteins are exposed changes as well as they are connected in series between force-generating units within cytoskeleton and the ECM [30]. Here we review the evidence of how 3 separate means of responding to such forces have been proposed (Figure 2).

Rho/ROCK Signaling Pathway

Due to its prevalence in cellular processes associated with the cytoskeleton and cellular contractility, the Rho/ROCK signaling pathway has been extensively studied [31]. Upon binding of integrins to the ECM, guanidine exchange factors (GEFs), which associate near FAs, catalyze a number of Rho GTPases, including RhoA and Rac [32,33]. The amount of contractile force the cell exerts is translated into ROCK phosphorylation via RhoA [34]. Activated ROCK effects several cellular processes, most notably actin organization via LIM kinase and Cofilin activation and cell contractility via phosphorylation of Myosin Light Chain (MLC) [35] and inactivation of MLC Phosphatase [36]. Another RhoA effector protein is mDia, which is responsible for the creation of new actin stress fibers by catalyzing actin nucleation and elongation [37]. Altogether, the Rho/ROCK signaling pathway is the ‘muscle’ responsible for upregulating and stabilizing the amount of stress fibers the cell displays in response to an increase in ‘inside-out’ force.

Stretch Activated Channels (SACs)

Mechanosensitive channels that become more permeable to soluble ions in response to contractile force are hypothesized to function through a protein ‘gate’ which is physically separated under force [38]. Although the specific family of SACs integral to the

mechanosensing process has not yet been clearly identified, TRP channels have received the most attention due to their calcium ion permeability [39] and ability to permit transient calcium influx in stretched cells [40]. It has been shown that cells on substrates of differing stiffness exhibit changes in the amplitude of calcium ion oscillations, proving that SAC permeability is related to active sensing of the physical microenvironment of the cell [41]. Furthermore, cells plated on substrates with a stiffness gradient showed even stronger calcium ion oscillations, indicative of a sensing role for calcium signaling kobayashi [41]. While changes in calcium ion concentration can affect or effect a number of pathways, one particularly important downstream role is an increased activation of Calmodulin and Myosin Light Chain Kinase (MLCK), which increases cellular contractility by phosphorylating MLC [42]. Other mechanosensitive channels, such as the TREK-1 potassium channel, are currently being investigated both as a model for understanding the protein ‘gate’ mechanism and as a robust touch sensor [43].

Force-induced Protein Unfolding

The existence of force-inducible protein unfolding, which we refer to as ‘molecular strain gauges’ (Figure 3), provides a third potential, FA complex-based mechanosensing mechanism. These proteins are capable of unfolding under physiological force, exposing binding domains for other proteins and signaling molecules further down the pathway. Talin, a FA protein that binds to integrins and actin, has been shown to unfold under stretching forces as low as 12 pN and expose up to 11 binding domains for vinculin, another FA protein [44]. Vinculin itself has been shown to have a force-dependent activation event required for Talin binding [45], which exposes a predicted binding domain for MAPK1 (unpublished), a prominent signaling kinase. Furthermore, the FA protein p130Cas, which binds to FAK and associates with Talin, has been shown to unfold under force to activate the Crk/C3G-Rap1 signaling cascade that results in phosphorylation of a different form of MAPK [46]. Another protein, receptor-like tyrosine phosphatase alpha (RPTPα) has been shown to associate with α integrins, catalyzing binding to fibronectin and vitronectin in a force-dependent manner via the Src family kinase cascade [47]. Thus, a connection can be made between force-dependent unfolding of FA proteins and signaling cascades that may ultimately affect gene expression.

While most research focuses on one particular potential regime of mechanosensing, it is becoming more apparent that they interconnected in several respects. If the Rho/ROCK pathway is utilized mainly to strengthen and reinforce adhesions in response to the physical microenvironment, then that increase in contractile force caused by greater MLC activity will likely result in a greater number of SAC’s being activated and molecular strain gauges unfolding. The SAC activity can serve to reinforce the activity of the Rho/ROCK cascade, while the molecular strain gauges work more directly in the signaling cascade that ultimately alters gene expression patterns. Furthermore, the MAPK1 signalling cascade associated with vinculin activation has been found to be upregulated in concurrence with an increase in Rho/ROCK activity [19], lending further evidence to a synergistic mechanosensing system utilizing all three paradigms.

A Hard Path To Follow: Confounding Factors in Mechanosensitive Pathway Research

One cannot underscore enough the complexity of chemical signaling within FAs, as is illustrated in Figure 4. Many mechanotransduction studies identify a specific mechanism(s) by analyzing the cell’s response to perturbations via chemical inhibition to block activation events (e.g. phosphorylation) or by altering the expression of signaling proteins. Perhaps the most widely used inhibitor towards a mechanosensing pathway element is the molecule

Y-27632, which inhibits ROCK by competitively binding to its catalytic site [48]. Reducing or even eliminating mechanosensing proteins by siRNA or knockout systems, and their corresponding 'add back' experiments, can also provide some level of confirmation. However, there is a critical need for developing techniques which monitor real-time changes in the mechanical state of proteins as current methods mentioned here have significant problems associated with their use. For example, Y-27632 is a valid inhibitor for studying ROCK disruption, but it also has potency as an inhibitor of Protein Kinase C-Related Kinase (PRK2) [49]. The cellular effect of PRK2 inhibition is unknown. However, the use of Y-27632 alone potentially opens the door for misleading results caused by unintentional inhibition of other pathways. The same is true for any type of protein knockdown or knockout. In FAK-null cells, cellular adhesion strength has been shown to be over 40% higher than in wild type cells, an effect believed to be due to reduced vinculin recruitment to FAs [50]. Thus, cells that are FAK-null, due to their higher adhesion force, can be expected to transduce force differently than their wild type counterparts, not because FAK is a sole mediator of mechanosensitivity, but because of a disruption in the general structure of the FA. It is important, then, to understand the difference between a heavy-handed FA disruption and the precise, elegant excision of a pathway element that serves one purpose. Whether or not such an elegant element even exists is still an unsettled issue, but the development of more refined inhibitors and the adoption of more specific approaches are both key to moving this question forward.

Pathfinders: Future Directions for Mechanotransduction

The complexity of FA-based mechanosensing necessitates the drive for more complex tools. At this point, it may not be enough to perturb one pathway, to knock down one protein, or to test one inhibitor. With each new variable, such as knocked down protein, inhibitor and inhibitor concentration, substrate stiffness, and time, comes an exponential increase in the number of cell populations and observations. To fully understand the mechanosensing ability of the cell, and thus more capably influence it, the use of high throughput systems capable of ascertaining the effect of combinatorial treatments of cell populations will be required.

As bioinformatics and proteomics advance, it is easy to foresee the development of a new field of mechanomics, in which the predicted structure of a protein is related to the predicted chemical forces inherent to its structure. Thus far, to learn about the properties of a protein unfolding under physiological force, researchers have had to use a number of costly, time consuming tools, including AFM [51], constitutively active FRET sensors [52], and laser tweezer [53]. With the development of mechanomics, the behavior of proteins and signaling molecules in response to physiological force can be modeled *in silico*, and thus it is reasonable to believe that one day, entire mechanosensing pathways could be modeled instantly and accurately, then verified experimentally.

The ultimate goal of work on discovering and understanding mechanosensing pathways is to be able to effectively influence them for the desired outcomes of a clinical treatment. There are dozens of clinical conditions arising from deficits in mechanosensing, including muscular dystrophy, cardiomyopathies, developmental disorders, and cancer, as reviewed by Jaalouk [54]. While inhibitory molecules have been suggested to address these deficits, such as Y27632 in the treatment of bronchial asthma [55], successful clinical implementation relies on a fully characterized system. In addition, diseased tissue often exhibits different mechanical properties than healthy tissue, which means that any treatment utilizing cells in such areas necessitates a change in physical microenvironment for the therapeutic cell population. Cellular myoplasty with MSCs on infarcted rat heart has been shown to improve scarred heart muscle compliance but also cause small cardiac calcifications [56], presumably

due to the increased stiffness of the physical microenvironment of the infarct. This response illustrates the dangers of cellular misinterpretation of tissue mechanics. By influencing the mechanosensing pathways of these cells to temporally ignore the stiffness of the extracellular environment, and only take cues from the chemical factors present, errant differentiation could be avoided, increasing the clinical relevance of such an approach.

Abbreviations

ECM	extracellular matrix
TLN	talin
Cas	p130Cas
VCL	vinculin
CaM	calmodulin
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
CFL	cofilin
LIMK	LIM Kinase
αA	alpha actinin
PXN	paxilin
GEF	guanidine exchange factor

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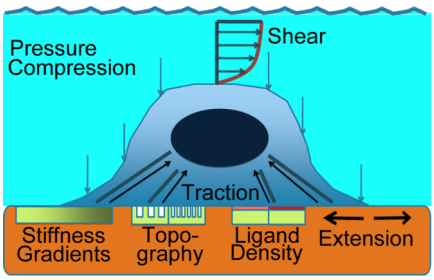

Figure 1. The physiological physical microenvironment of the cell

Outside-in forces are depicted acting on the cell. On the other hand, inside-out transduction of cellular contractility can be affected by changes in stiffness gradients, ECM topography, and ligand density.

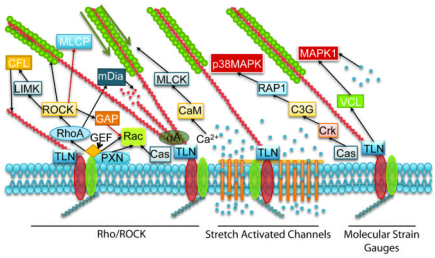

Figure 2. Signaling events of three major candidate mechanosensing pathways

To the left, the **Rho/ROCK** system is summarized, showing the mechanisms for actin nucleation, assembly, and stabilization, as well as myosin contractility. A positive feedback loop results in increasing cellular contractility, visually verified by the robust amount of stress fibers present at focal adhesions of cells on stiff microenvironments. In the center, a network of **mechanosensing ion channels** or "stretch activated channels" (SACs) is responding to membrane stretching, which forces open the channels. An increase in intracellular calcium concentration also provides positive feedback through favorable activation of MLCK, causing further increases in cellular contractility. Finally, **molecular strain gauges**, e.g. p130Cas and talin, are shown responding to increases in cellular contractility by exposing binding sites for Crk and vinculin, respectively. These pathways progress to the activation of two types of MAPK signalling kinases, which may influence changes in cell behavior further downstream. It is of note that the degree of interconnectivity between these paradigms of mechanotransduction, even as briefly overviewed in this figure, is high. Abbreviations: TLN- talin, Cas- p130Cas, VCL-vinculin, CaM-calmodulin, MLCK-myosin light chain kinase, MLCP-myosin light chain phosphatase, CFL-cofilin, LIMK-LIM Kinase, AA-alpha actinin, PXN- paxilin, GEF- guanidine exchange factor.

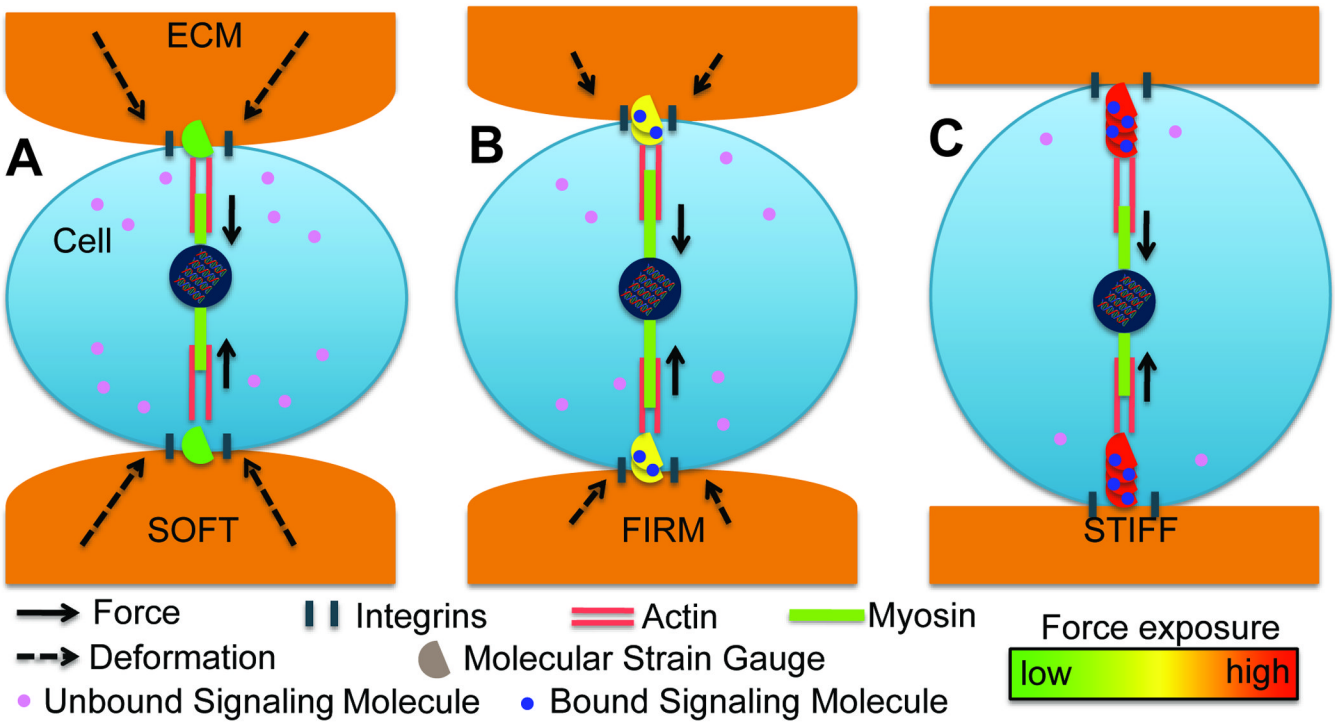

Figure 3. Changes in substrate stiffness result in a spectrum of linker protein force exposure

Because some focal adhesion proteins have been shown to undergo force-responsive unfolding events, it is feasible that changes in ECM stiffness may be a cause of discretized unfolding. Differences in unfolding at focal adhesions may result in modulation of downstream signalling molecules responsible for effecting changes in cell behavior. **A**) On soft substrates (~0–4 kPa), the force generated by cellular contractility is transferred to the ECM through linker proteins, but because of compliance of the ECM, linker proteins are not exposed to a high degree of force. **B**) On firm substrates (~8–15 kPa), compliance of the ECM decreases but does not disapear, resulting in an incremental increase in force exposure at focal adhesions. **C**) On firm substrates (~25 kPa-50 GPa) matrix compliance approaches zero, causing nearly the full force of cellular contractility to be focused on linker proteins in focal adhesions.

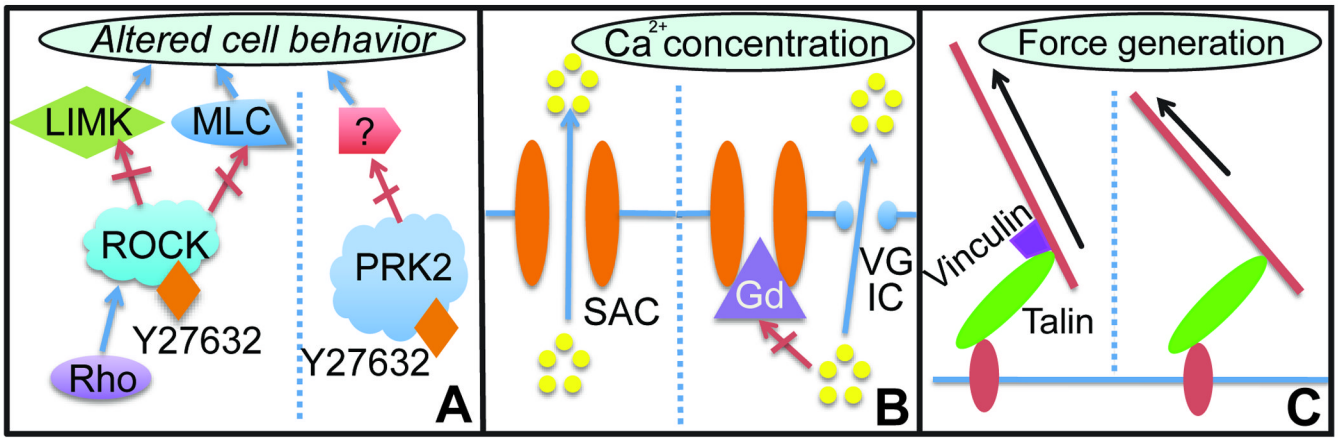

Figure 4. Confounding factors in manipulating mechanosensitive pathways

To further understand the mechanosensitive pathways of the cell, interfering with a hypothesized pathway and observing a change in mechanosensing can provide evidence for function. However, for a number of these interferences, confounding factors which may affect cell behavior may exist. **A**) The inhibitor Y27632 is a classic inhibitor of ROCK, and is used to study the role of the Rho/ROCK pathway on mechanosensing. However, Y27632 has also been shown to inhibit PRK2, whose role in mechanosensing is currently unclear. **B**) Intracellular calcium concentration has been shown to be important to substrate stiffness-based mechanosensing, with reduction in calcium ion concentration resulting in a decrease in stiffness-based differentiation. However, blocking stretch activated channels with the potent inhibitor gadolinium did not result in any changes in differentiation (unpublished), suggesting that calcium ions can enter the cell via other routes, including voltage gated ion channels. **C**) siRNA mediated knockdown of vinculin has been shown to reduce stiffness-based differentiation (unpublished), but if perturbing focal adhesions by knocking down constituents has an impact on cellular force generation, any change in cellular behavior may be attributed to a loss of contractility.