Developmental Cell
Perspective

A Hitchhiker's Guide to Mechanobiology

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DOI 10.1016/j.devcel.2011.06.015

More than a century ago, it was proposed that mechanical forces could drive tissue formation. However, only recently with the advent of enabling biophysical and molecular technologies are we beginning to understand how individual cells transduce mechanical force into biochemical signals. In turn, this knowledge of mechanotransduction at the cellular level is beginning to clarify the role of mechanics in patterning processes during embryonic development. In this perspective, we will discuss current mechanotransduction paradigms, along with the technologies that have shaped the field of mechanobiology.

Introduction

Why should we study mechanical forces in cell biology? This is a reasonable question given the daunting task of adopting a plethora of theoretical approaches and experimental tools that would be necessary to address the problem. The answer, however, is rather fundamental: a vast body of research has accumulated to illustrate that forces are ubiquitous in vivo and that these forces directly impact cell function. Although the biological effects of forces are perhaps most evident in the context of physical activity—breathing, heart pumping, blood flow, and physical exercise—such forces also regulate morphogenesis, cell migration, and even cell adhesion to extracellular matrix. Importantly, it is now apparent that such forces can regulate a wide variety of biological processes, from cell proliferation and differentiation to tissue mass homeostasis and complex inflammatory cascades.

The idea that forces can regulate tissue remodeling and development was articulated more than a century ago. In 1892, the surgeon and anatomist Julius Wolff postulated that bone tissue adapts its structure to the mechanical environment based on the observation that trabeculae matched the principal stress lines in bones caused by daily physical loading (Wolff, 1892). Although the alignment of trabeculae could have arisen strictly during prenatal development, he reported this remodeling occurred even after healing of misaligned fractures. In the same era, mechanical forces were proposed to shape tissues and organs during embryonic development (Roux, 1895; Thompson, 1917), but the tools were not available to directly test such ideas experimentally. Nearly a century passed before these concepts began to captivate the scientific community once again.

Interestingly, the development of mechanobiology as a field appears to be closely connected to the advent of enabling technologies. For example, the earliest observations suggesting that mechanical forces drives embryogenesis and bone structure were a natural result of newfound microscopy methods. Mechanobiology received relatively little attention for much of the 20th century as scientists focused on developing molecular biology tools to catalog the genetic basis for life. The recent renaissance in studying mechanics primarily in cell culture has largely been enabled by a suite of tools to measure and manipulate such forces in vitro. For example, in vitro application of strains that would be experienced by bone during physical activity increases cell proliferation (Raab-Cullen et al., 1994), osteogenic differentiation (el Haj et al., 1990; David et al., 2007), and bone matrix deposition (Bancroft et al., 2002), which are all characteristic for mechanically induced anabolic bone growth in vivo. Mechanical stretch that mimics the effect of pulsating blood flow has been shown to trigger many alterations in endothelial and smooth muscle cell signaling (Chien et al., 1998; Tzima et al., 2005), vascular cell proliferation (Davies et al., 1984; Haudenschild et al., 1985; Sumpio et al., 1987), and expression of inflammatory markers (Yamawaki et al., 2005). Methods to apply shear flow on cell cultures have shown that shifts between steady and turbulent shear flow can prevent or promote inflammatory activation, respectively, and may explain the localization of atherosclerotic plaques to specific regions along the vascular tree (Davies et al., 1986; Shyy et al., 1994). Flow rates during development also can drive arterial versus venous phenotype (le Noble et al., 2004). Bioreactor-type devices have been developed also to model the compression experienced by chondrocytes in the articular joint, tension in muscles, ligaments, and tendons, as well as impact forces associated with trauma (Freed et al., 1993; Molnar et al., 1997; Garvin et al., 2003; Frieboes and Gupta, 2009).

In addition to such externally applied forces, nonmuscle cells generate contractile forces on their own. This was first illustrated by Harris and Stopak, showing that cells cultured on soft polymer substrates would wrinkle the substrate surface (Harris et al., 1980, 1981). The implication that forces could be ever present, even in settings without an explicit mechanical stimulus, seemed heretical at the time. However, over the past three decades, it has become clear that (1) most eukaryotic cells can generate intracellular forces that act on the surrounding extracellular matrix (ECM) or neighboring cells, and that (2) this contractile activity is critical for a number of biological processes such as cell migration, mitosis, as well as stem cell differentiation and self-renewal. For physical functions such as mitosis and migration, force is clearly "essential" in the same way that oxygen is essential for life. That is, without force, mitosis and migration cannot proceed (Civelekoglu-Scholey and Scholey, 2010; Renkawitz and Sixt, 2010). The role of force in genetic responses such as proliferation and differentiation, however, appears to be "regulatory" in the same manner as cytokines might be. Although we have some clues to how forces exert these regulatory functions, clarifying these mechanisms remains a central question for mechanobiology.

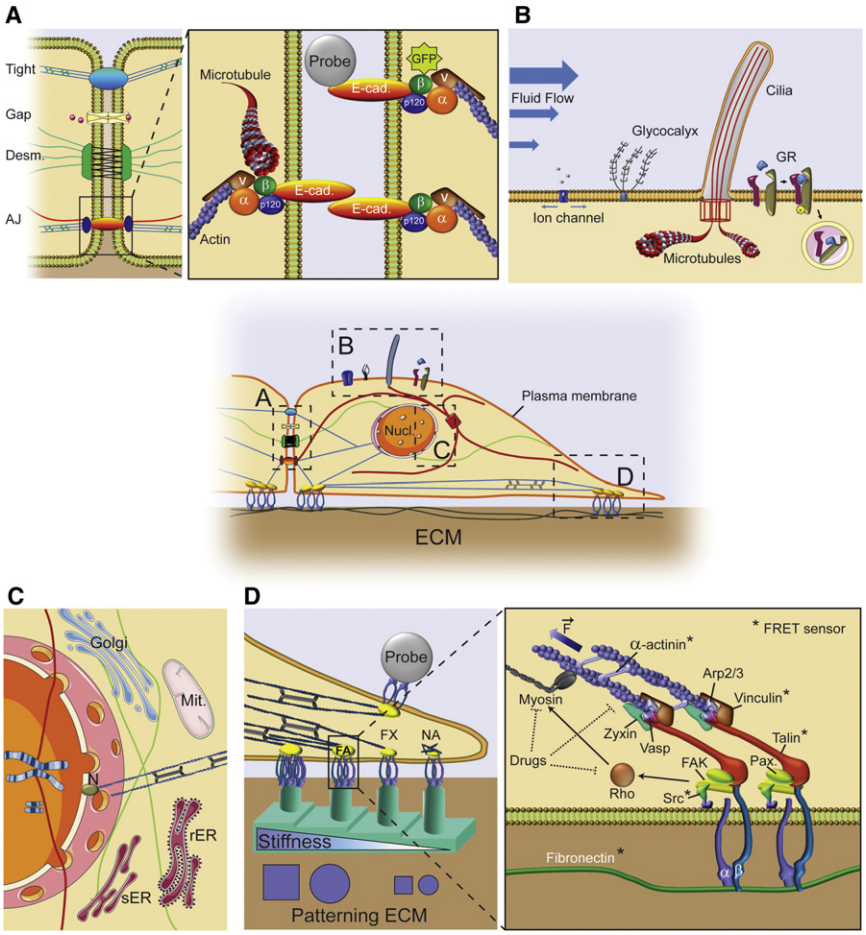

Figure 1. Mechanotransduction in a Cell-ECM Unit

Center: An overview of a cell connected to ECM and a neighboring cell. The boxes (A–D) zoom in on areas of interest where mechanotransduction in the cell-ECM unit occurs. The blue lines represent actomyosin filaments, green lines embody intermediate filaments and the red lines correspond to microtubules. This color code is maintained in all panels. The blue structures linking the cell with the ECM represent integrins (detailed in D). nucl.: nucleus.

(A) Mechanotransduction at adherens junctions. Left: Different cell-cell junctions connect neighboring cells. Tight junctions (Tight) constitute of sealing strands of protein complexes (blue ellipse) in the intercellular space that are anchored to the actomyosin skeleton in the cytoplasm. Gap junctions (GAP) are clusters of connexon channels that allow ion exchange (pink circles) and electrochemical communication between two cells. Desmosomes (Desm.) link intermediate filaments through adhesion plaques (green). Adherens junctions (AJ) tether actomyosin skeleton and microtubules via cadherin complexes (orange). Right: Molecular structure of AJ: E-cad: E-Cadherin, α: Alpha Catenin, β: Beta Catenin, p120: p120 Catenin, v: Vinculin, Probe: Atomic Force Microscope, Optical tweezers, and Magnetic tweezers can be used to manipulate and measure force at the AJ. In addition, labeling molecules such as beta catenin with GFP allows measuring of the size of the AJs, which can be used to estimate the force acting on the AJs.

(B) Mechanoreceptors at the cell membrane. Fluid flow or stretch deforms the plasma membrane which can lead to activation of ion channels that results in an influx of ions. In addition, fluid flow directly impacts glycocalyx and cilia movement which triggers diverse downstream signaling cascades. Moreover, mechanical forces mediate growth factor receptor (GR) clustering and endocytosis, and thus affect GR signal transduction as well.

(C) Mechanotransduction at the nucleus. The nucleus and surrounding organelles (Golgi apparatus, Mit: Mitochondria, rER/sER: rough and smooth endoplasmic reticulum) are interconnected by intermediate filaments and microtubules. Nesprins (N) tether the nucleus with the actomyosin cytoskeleton. Changing cell shape/contractility can alter spatial localization of organelles and induce conformational changes of nuclear pores.

(D) Mechanotransduction at the focal adhesion (FA). Left: Integrin clustering develops nascent adhesions (NA) that mature to focal complexes (FXs) and focal adhesions (FA), a process controlled by actomyosin contractility which can be modified by stiffness (step rigidity posts or crosslinked polymer substrates, in green), cell shape (patterning, in blue) or external application of force with atomic force microscopy, optical tweezers, or magnetic tweezers (Probe). Right: simplified molecular structure of FA. α/β: alpha and beta unit of integrins, Pax: paxillin, F: Force delivered by actomyosin contraction. Clustering of integrins can induce RhoA signaling, thereby increasing myosin contractility which leads to unfolding of proteins as observed by FRET. The molecules for which a FRET sensor has been developed are marked with an asterisk (*). Pharmacological drugs can be used to alter myosin contractility and actin polymerization.

Given the dependence of this field on enabling technologies, this perspective will present (1) our current understanding of the role of mechanical forces in cell biology, (2) the techniques that are being developed to enable such studies, and (3) recent efforts to consider mechanical forces in development. Due to space constraints, this perspective is not an exhaustive review of mechanobiology, but rather examines the current thinking and directions in the field. We begin by describing the cellular context in terms of the physical structures through which forces are transmitted.

The Mechanotransduction Machinery in a Cell-ECM Unit

Mechanical forces are calculated in engineering devices by measuring deformation of materials, be they membranes under pressure or changes in electrical current in a narrowing metallic strip under stretch (strain gauge). By analogy, knowing which cellular structures bear force is a starting point for understanding where forces are transmitted and potentially transduced into biochemical signals. In discussing how mechanical forces regulate cells, it is essential that we do not consider the cell in isolation, but rather in direct physical contact with the ECM. This is because adhesion of cells to matrix results in the structural organization of the cell itself. Integrin binding and clustering against ECM ligands leads to changes in cell shape and cytoskeletal architecture, anchoring the actin cytoskeleton to sites of adhesion. The cytoskeleton is linked to the nuclear envelope, thus forces experienced or generated by the cell-ECM module (Figure 1) are therefore transmitted and sensed throughout this module as a coordinated system. In this section, we will examine where and how those forces might be transduced to regulate cell function. Along the way, we will describe the tools used to study these mechanotransduction processes.

Structural Basis of Force Transmission in Cells

In the cytoplasm, the cytoskeleton is a fundamental structure for mediating force transmission (Wang et al., 1993). The cytoskeleton is a highly dynamic cellular scaffolding structure composed of filamentous actin (6 nm in diameter), intermediate filaments (10 nm), and microtubules (23 nm). These three cytoskeletal elements are not single proteins, but consist of many monomers able to span large distances within the cell. Tubulins polymerize to form hollow cylinders known as microtubules and provide a structure for motor proteins such as kinesins and dyneins to travel between different cell compartments (Sheetz, 1996). Vimentin, keratin, and lamin monomers form intermediate filaments that connect the nucleus with the endoplasmic reticulum, mitochondria, and Golgi apparatus, providing structural integrity to the cell. Actin monomers assemble into filamentous actin (F-actin) and together with myosin filaments, form the cytoskeletal contractile apparatus. The actomyosin cytoskeleton connects multiple parts of the cell membrane as well as the cell membrane to the nucleus (Sims et al., 1992). At the cell membrane, these filaments anchor into clusters of proteins that include focal adhesions (FAs) which link the cytoskeleton through transmembrane integrin receptors with the ECM. In the extracellular space, the ECM materializes as a mesh of crosslinked proteins and carbohydrates, and depending on the tissue, can include different constituents including collagen, laminin, elastin, and fibronectin fibers interlocked with hyaluronic acid and proteoglycans.

From a mechanical standpoint, applying force to this cell-ECM unit leads to structural deformations and rearrangements of the ECM, force transmission through the FA, and (given the highly interconnected nature of the cytoskeleton) deformation of nearly every aspect of intracellular structure, including the position of mitochondria, endoplasmic reticulum, and the nucleus (Figure 1C). However, ''outside-in'' force transmission is only half the tale, as cells also generate force. Polymerization and depolymerization of microtubules drive pushing and pulling forces, respectively, to control the position of mitotic spindles, chromosomes, and nuclei (Dogterom et al., 2005). The head domain of myosin II pulls on actin filaments to generate traction forces, which are transmitted to focal adhesions and deforms the ECM via an ''inside-out'' transmission path (Vicente-Manzanares et al., 2009). Hence, mechanical forces are experienced throughout the cell via the integrated cytoskeleton-focal adhesion-ECM architecture.

Structural Basis for Mechanotransduction

The integrated nature of this cell-ECM structure raises the possibility that many anatomical sites could be involved in transducing such forces into biochemical signals. Beginning with the ECM itself, it has been shown that forces can induce opening of quaternary domain-domain structures, as well as direct unfolding of unstable domains, such as in fibronectin (Krammer et al., 1999). These events, in turn, can reveal cryptic sites for engagement and signaling of cellular receptors. Similarly, it has been shown that ECM-bound growth factors such as TGF-β can be activated and released via mechanical forces to trigger cellular signaling (Wipff et al., 2007). Given the complexity of characterizing changes in molecular structure and multimolecular organization, especially in disordered materials like the ECM, it remains a major challenge to systematically examine the role of forces in modulating the ''bioactivity'' of ECM.

Intracellular mechanisms of mechanotransduction have been even more difficult to demonstrate. For example, it has been postulated that force-induced nuclear deformations can directly alter genomic structure and accessibility of transcription factors to specific genetic targets, but no direct demonstration of such a mechanism has yet been described. Nonetheless, circumstantial evidence for a nuclear mechanosensor is provided by recent studies showing that lamins and nesprins—scaffolding proteins critical to the integrity of nuclear structure—can impact force transduction (Chancellor et al., 2010; Lammerding et al., 2004). New molecular-resolution imaging approaches (e.g., super-resolution microscopy) that can track chromatin changes following force application may soon be ready to begin to address this impasse. One recent approach to provide an unbiased examination of mechanotransduction involves ''shotgun'' labeling of cryptic cysteines that are revealed upon mechanical loading. Inspired perhaps by the findings of cryptic site unmasking in fibronectin, Discher and colleagues used this approach to identify intracellular proteins that undergo conformational change when in cells that are mechanically stretched (Johnson et al., 2007). The Sheetz group also screened for stretch-dependent binding of cytoplasmic proteins on Triton X-100-insoluble cytoskeletons (Sawada and Sheetz, 2002) before using more targeted approaches to demonstrate force-induced unfolding of talin and the Src family kinase substrate p130cas (del Rio et al., 2009; Sawada et al., 2006). It remains to be seen to what extent such unfolding events contribute to the many force-induced changes in cellular signaling.

Although cytoskeletal and ECM-based mechanotransduction has been difficult to demonstrate, receptor-mediated transduction of forces has been convincingly shown for stretch-activated ion channels (Lansman et al., 1987; Sadoshima et al., 1992) and for integrins (Wang et al., 1993). For stretch-activated receptors, mechanical forces appear to alter the conformation of the Transient Receptor Potential (TRP) family of channels, allowing for rapid signaling responses such as calcium influx (<4 ms) following mechanical perturbations (Figure 1B) (Matthews et al., 2010). In contrast to this relatively straightforward mechanism, integrins appear to be involved in a complex response to force, perhaps resulting from their central position in dynamically regulating the interactions between the cytoskeleton and the ECM.

ECM-bound integrins cluster to initiate the formation of highly organized protein assemblies composed of multiple protein-specific strata. At the cell membrane, the integrin layer is oriented with the head domains connecting to the ECM and the cytoplasmic tails binding to focal adhesion kinase (FAK) and paxillin. Recently revealed with super-resolution methods, this cytoplasmic layer assembles with a stratum containing talin and vinculin, and an uppermost actin-regulatory sheet consisting of zyxin, VASP (vasodilator stimulating phosphoprotein) and α-actinin which tethers the FA to the actomyosin cytoskeleton (Kanchanawong et al., 2010) (Figure 1D). Nascent adhesions consisting of integrin clusters either turn over rapidly ($\sim$60 s) or mature into larger Focal Complexes (FX) of which some assemble into larger FAs (Zimerman et al., 2004; Vicente-Manzanares et al., 2008; Choi et al., 2008).

Importantly, the morphogenesis of these adhesions requires force (Parsons et al., 2010). Force appears to unfold several

protein domains of talin, paxillin and p130cas exposing cryptic binding and/or phosphorylation sites (del Rio et al., 2009; Sawada et al., 2006; Zaidel-Bar et al., 2007) which leads to stabilization of nascent adhesions into FXs. Further application of force drives the maturation of FXs into FAs, now tethered to thick actin stress fibers. Recent evidence suggests that the integrin receptor itself switches to a high-affinity state in response to force, at least for the ubiquitous $\alpha_5\beta_1$ isoform (Friedland et al., 2009). Conversely, inhibition of myosin II and reducing cytoskeletal tension also leads to disassembly of FAs (Kirfel et al., 2004; Rid et al., 2005). Although it remains largely unclear whether nascent adhesions, FXs, and FAs are actually distinct states or lie on a single continuum, it is apparent that small and large adhesions have distinct molecular signatures. Recent proteomic studies of blebbistatin-treated (myosin inhibited) small adhesions compared to native adhesions display a large number of compositional differences (Byron et al., 2011; Kuo et al., 2011). These early studies will likely provide important handles to begin to explore the nature of these complex force-responsive structures.

Perhaps the most interesting feature of these adhesions is that they not only perform a physical-anchoring function, but also are biochemical signaling centers. FAs contain many signaling proteins such as FAK, ERK, JNK, Src, MEK, Ras, and Raf (Miyamoto et al., 1995), all of them involved in a myriad of pathways regulating diverse cell functions including migration, proliferation, and differentiation. As such, changes to composition of such adhesions mediated by contractility can impact localization of members within a signaling cascade, thereby orchestrating cellular responses to force.

Because adhesion dynamics and related signaling are regulated by mechanical stress at the cell-ECM interface, it appears that one can modulate adhesion signaling through multiple distinct means. That is, manipulating the density of ECM ligand deposited on a substrate impacts adhesion signaling by controlling the amount of integrin ligation and clustering from the outside-in. Adhesion signaling can also be modulated by mechanical stresses at adhesions, for example, by manipulating cellular contractility or the mechanical stiffness of the ECM. Interestingly, functional studies thus far would suggest that these different approaches to impact adhesions lead to indistinguishable consequences. Increasing ECM density, RhoA-mediated myosin activity, or ECM stiffness all suppress cellular apoptosis and enhance proliferation (Chen et al., 1997; Wozniak et al., 2003; Pirone et al., 2006). Although such links would suggest the possibility that focal adhesions use a common mechanism to integrate adhesive and mechanical cues, a clearer molecular mechanism describing the transduction of ligand density, traction force, and substrate mechanics is required to convincingly explain the basis for similarity or uniqueness of each of these cues.

Even with these mechanisms described, it is equally important to not simply describe what cells could transduce, but what cues they do transduce in different physiologic or pathologic settings. Indeed, recent studies have begun to link these fundamental mechanotransduction processes to clinically important settings. For example, the stiffening of arteries associated with atherosclerosis appears to be involved in promoting neointimal smooth muscle hyperproliferation and vascular lumen narrowing (Klein et al., 2009). Similarly, the enhanced tissue stiffening due to the fibrotic response of tumors plays a role in tumor proliferation, progression, and metastasis (Paszek and Weaver, 2004; Levental et al., 2009). In contrast, tissue angiogenesis appears to be enhanced with decreased tissue stiffness and cellular contractility (Ingber et al., 1995). Together, these studies are beginning to illustrate the ubiquitous and critical role of mechanical forces in biological systems.

Mechanotransduction in a Multicellular Context

In vivo, cells generally are not solitary but are tightly connected to each other via cell-cell junctions (Figure 1A). Adherens junctions (AJ) link the cytoskeletons of adjacent cells via clusters of cadherins, of which the cytoplasmic tails are tethered to cortical actin filaments by protein complexes harboring vinculin, p120, alpha and beta catenin (Yamada et al., 2005). From a mechanical point of view, AJs are well placed to act as mechanosensing complexes for multicellular architectures. Indeed, similar to FAs, AJs grow when an external force is applied (outside-in) or when actomyosin contraction is enhanced (inside-out), leading to strengthening of the AJ (le Duc et al., 2010; Liu et al., 2010; Smutny et al., 2010). Such a positive feedback mechanism may be in place in order to prevent cell-cell ruptures. Part of this feedback mechanism may involve force-induced unfolding of alpha catenin, exposing cryptic binding sites to vinculin, which in turn supports recruitment of more actomyosin fibers (Yonemura et al., 2010). In addition, Rac1 activated by cadherin-catenin clustering (Nakagawa et al., 2001) can recruit p190RhoGAP to p120 catenin, resulting in inhibition of Rho activity (Wildenberg et al., 2006). Hence, the ultimate size of AJs is partially determined by the balance between Rho and Rac signaling at the AJ (Liu et al., 2010).

The presence of a cell-cell pathway for transmitting force has numerous implications. In the context of multicellular sheets, uniform contractile activity of cells can lead to nonuniform distributions of cell-ECM stress that are determined by the geometry of the cell sheet (Nelson et al., 2005). Furthermore, these underlying stress distributions triggered spatially localized patterns of cell proliferation. These patterns of proliferation were disrupted when cells expressed a dominant negative cadherin, suggesting that changes in AJs could guide multicellular patterning. Similarly, geometrically imposed patterns of intercellular stress direct lineage commitment of mesenchymal stem cells which preferentially differentiate to adipocytes in low tensile zones while regions of higher tension are characterized by alkaline phosphatase positive preosteoblasts (Ruiz and Chen, 2008). Recent work suggests that these localized patterns of force are also present in models of 3D morphogenesis (Nelson et al., 2006; Gjorevski and Nelson, 2010).

Thus, these early studies suggest that our insights into multicellular mechanics and mechanotransduction will require a deeper understanding of the mechanical and signaling interdependencies between cell-cell and cell-ECM adhesions. Recent studies have begun to illustrate examples of direct mechanical connections between these two types of adhesions, as well as indirect connections including cadherin engagement-induced activation of RhoGTPases to impact focal adhesions (Maruthamuthu et al., 2011; de Rooij et al., 2005; Nelson et al., 2004; Weber et al., 2011). Taken together, it appears that long-range force transmission through AJs can potentially play a distinct

role in guiding patterning processes during embryonic development (Martin et al., 2010), a topic we will examine momentarily after discussing some of the techniques that have been developed to measure and to manipulate force in cells.

Approaches to Measure and to Manipulate Cellular Forces

Cells are constantly probing, pushing, and pulling on their microenvironment. A growing body of work is investigating what regulates the cellular generation of contractile forces. To identify when such forces are invoked, several approaches have been developed that allow quantitative measurement of cellular traction forces. In addition, numerous methods are being used to manipulate cell-ECM forces, all of which have been critical in enabling the growing field of mechanobiology. In this section, we will examine the tools used to measure and manipulate cellular forces, and how they have contributed to our understanding of the role of mechanical events in both physiological and pathological settings.

Approaches to Measure Cell-Generated Traction Forces

Owing to the evolution of biomaterials and polymer chemistry, the conception of soft substrates linked with ECM matrices has enabled the measurement of forces generated by even single cells. The basic concept of using an elastic substrate for force measurement was originally conceived by Harris et al.: when adherent to a thin silicone membrane, nonmuscle fibroblasts can cause wrinkles in the substrate (Harris et al., 1981). However, due to the inherent nonlinearity of wrinkling and the complexity of the displacement field generated by a single cell, this technique was not applicable to accurately quantify cell forces. The development of traction force microscopy by Dembo and Wang has been a significant improvement to measure cellular forces. This method uses fluorescent microbeads embedded in a polyacrylamide hydrogel as markers for tracking the deformation of the gel caused by the adherent cell. After obtaining the displacement vector for every bead, the inverse problem is solved to calculate the cell generated force field (Dembo and Wang, 1999; Butler et al., 2002; Yang et al., 2006). With this technique, several groups have demonstrated that in addition to tangential forces, cells also exert forces normal to the 2D planar adherent surface (Hur et al., 2009; Delanoë-Ayari et al., 2010; Maskarinec et al., 2009). However, the calculation of forces is computationally intensive, because deformations propagate on these continuous substrates. Furthermore, changing the crosslinking chemistry to manipulate the rigidity of hydrogels may inadvertently affect surface hydration, chemistry, and adhesiveness. To address these limitations in the design of soft substrates, microfabricated arrays of elastomeric cantilever posts have been developed to quantify cell forces by measuring the deflection of the posts under cell tension (Tan et al., 2003; du Roure et al., 2005).

These two force measurement techniques have demonstrated that (1) cells pull harder on stiffer surfaces, affecting cell shape, motility, growth rate, and intracellular signaling and that (2) adherent cells continuously apply tensile forces to substrates that are directed toward the centroid of the cell (Tan et al., 2003; Dembo and Wang, 1999; Balaban et al., 2001). Interestingly, similar observations have recently been reported in cells embedded in a 3D hydrogel, suggesting that mechanotransduction mechanisms might be conserved between 2D and 3D (Legant et al., 2010).

Ultimately, these methods were critical in identifying a role for cellular forces in several important settings. For example, it had been observed that cells restricted from spreading against extracellular matrix become growth arrested, regardless of whether that restriction is due to cell crowding upon reaching confluence (Nelson and Chen, 2002), decreased ECM ligand coating density on a surface (Ingber and Folkman, 1989), or micropatterning to define the area of spreading of a cell (Chen et al., 1997). These changes in cell spreading were later found to impact cell contractility. Restricting cell spreading on micropost arrays revealed that decreased spreading prevented cells from generating traction forces (Tan et al., 2003). Conversely, upregulating contractility by activating RhoA rescued proliferation even in unspread cells (Pirone et al., 2006), thus demonstrating that the mechanism by which cell shape regulates contractility is a mechanotransduction event. One could consider these contractile forces as being part of an "autocrine" loop.

Approaches to Manipulate Cell-ECM Forces

Intracellular forces can be modulated by using traditional molecular methods to directly target the force-generating apparatus. Several pharmacologic agents are available for inhibiting modulators of contractility, including the molecular motor myosin II (blebbistatin), the upstream regulators of myosin phosphorylation Myosin Light Chain Kinase (ML-6, ML-9), and the Rho/ROCK signaling pathway (fasudil, Y27639, C3 botulinum exotoxin), as well as the polymerization processes of actin (latrunculin, cytochalasin D). Similarly, molecular-genetic methods have also been used effectively to target these pathways, including the nonmuscle myosin II isoforms themselves, and have been a mainstay in identifying force in a regulatory pathway (Figure 1D).

In addition to molecular methods, recent attention has turned toward the use of biophysical approaches to manipulate cell-ECM mechanics. One such method is to vary the mechanical stiffness of the substrate to which cells attach. Most commonly, stiffness is manipulated by changing the degree of crosslinking of polymeric hydrogels, the most widely available of which is polyacrylamide. Depending on the chemistry, crosslinking is controlled by the ratio of polymer to crosslinking agents, the duration of exposure to a light source (photopolymerization) or heat, among other factors. Interestingly, whereas the stiffness of most substrates is determined upfront, substrate stiffness of collagen can be altered during the course of the experiment by supplementing ribose to the medium (Girton et al., 1999).

Recent studies have used these methods to reveal a role for ECM stiffness in regulating cell function. ECM stiffness has been shown to affect migration, proliferation, and differentiation (Peyton and Putnam, 2005; Lo et al., 2000; Yeung et al., 2005). Whereas increasing stiffness enhances spreading and proliferation of many cell types (Pelham and Wang, 1997; Yeung et al., 2005; Thompson et al., 2005) and facilitates tumor growth (Wozniak et al., 2003; Georges and Janmey, 2005; Levental et al., 2009), compliant substrates appear to promote branching of neurons (Flanagan et al., 2002; Willits and Skornia, 2004) or adhesion of and albumin secretion by hepatocytes (Chen et al., 2009; Semler et al., 2005). The mechanism by which cells

transduce alterations in substrate stiffness is not fully understood, but it has been shown that cells attached to more compliant substrates exhibit suppressed integrin activation, decreased traction force generation, and immature focal adhesions. As such, it is thought that at least in part, the effects of ECM stiffness on cells are similar to effects of decreased integrin-mediated adhesion and cellular force generation. Several technical challenges, however, remain. Besides stiffness, cross-linking chemistry can also alter the internal structure, surface topology, and growth factor adhesion of the hydrogels (Houseman and Mrksich, 2001; Keselowsky et al., 2005; Crouzier et al., 2011). An alternative to overcome these limitations is to seed cells on posts of different height. By varying the height of the microposts, the stiffness is altered even though the material properties are held constant (Fu et al., 2010) (Figure 1D).

A large array of mechanotransduction studies have relied on the local application of force at single sites of adhesion. Magnetic tweezers (MTs), optical tweezers (OTs), and atomic force microscopy (AFM) are the most common tools for investigating the mechanical properties of biological molecules, cells, and tissues. MTs can exert calibrated forces from as little as 0.05 pN up to 150 pN, and as such has mostly been used to manipulate single molecules and to apply forces to transmembrane integrins (Alenghat et al., 2000; Matthews et al., 2004; Wang et al., 1993; del Rio et al., 2009). OTs, exerting forces from 0.1 to 100 pN, have been utilized to characterize molecular motors as kinesin, myosin, and dynein (Asbury et al., 2003; Altman et al., 2004; Gross et al., 2000), and to restrain movement of membrane bound beads on fibroblasts, demonstrating that cells strengthen their cytoskeleton linkages corresponding to the force needed to restrain the beads (Choquet et al., 1997). Because of its wide force range (5–10,000 pN), AFM has been applied to study force induced unfolding of proteins (Rief et al., 1999; Dietz and Rief, 2004; Puchner et al., 2008), as well as to characterize the mechanical and adhesive properties of single cells on substrates or cell-cell adhesion (Puech et al., 2006; Helenius et al., 2008). Recently, Webster et al. have developed an AFM feedback algorithm to rapidly change the stiffness presented to a cell while accurately measuring force and deformation (Webster et al., 2011). This "stiffness clamp" demonstrated that fibroblasts can exhibit a subsecond change in traction rate and contraction velocity in response to step changes in stiffness. This response was independent of the absolute contractile force and cell height, demonstrating that cells can react directly to changes in stiffness alone.

Mechanotransduction in Stem Cells and Development

Multicellular organisms develop distinct shapes that emerge from continuous pulling, buckling, twisting, and bending interactions between cells and matrix. Following the genomic revolution, biologists have begun to consider the importance of forces during development as many of the developmental mechanisms involved in sculpting the metazoan body plan remain unexplained by genetics alone. A number of recent studies suggest that mechanical context may be a critical cue in guiding stem cell behavior, perhaps underscoring the link between stem cell function and the mechanical changes occurring during the development of the body plan. Here, we will discuss these stem cell studies as well as traditional embryological studies.

Mechanotransduction in Stem Cells

Although often generalized in a single term, stem cells represent a wide range of cell types that can be ranked on a continuum of stemness with the zygote and morula-derived cells as totipotent stem cells on one end, and terminally differentiated cells, such as red blood cells and osteocytes, on the other. In between, a full range of pluripotent, multipotent, and bipotent progenitor cells have been described with different proliferation and differentiation capacities. Importantly, much attention has recently focused on transcriptional and microenvironmental contexts that can drive stem cells into different states, including the reprogramming of a differentiated cell into a more naive cell (Gurdon et al., 1958; Takahashi and Yamanaka, 2006).

Given the surprisingly plastic nature of stemness and that stem cells live through a constantly changing dynamic mechanical environment (whether in embryogenesis or in the transit of adult stem cells through different tissues), a number of studies have begun to explore whether mechanical forces may be involved in the regulation of stem cell processes. Indeed, recent evidence has shown that compliant but not stiff substrates create permissive environments promoting self-renewal and proliferation of hematopoietic stem cells (Holst et al., 2010), muscle-derived stem cells (Gilbert et al., 2010), and embryonic stem cells (ESCs) (Chowdhury et al., 2010). Note, earlier studies had shown increased proliferation on stiffer surfaces for lineage-committed cells as mentioned above. In fact, Gilbert et al. observed within a mixed population enrichment of stem versus nonstem cells by increasing cell survival depending on substrate mechanics. Furthermore, addition of ROCK inhibitors such as Y27632 to freshly isolated ESCs in culture also increases survival and self-renewal (Watanabe et al., 2007; Harb et al., 2008), as well as increases the efficiency of reprogramming of dermal fibroblasts into induced pluripotent stem cells (iPSC) (Lai et al., 2010). These studies highlight the role of low contractility in the preservation of stemness, though the mechanistic basis for this effect remains to be defined.

Stiffness and contractility also modulate commitment of mesenchymal stem cells (MSCs) into different lineages. Depending on the soluble factors in medium, MSCs will adopt the adipogenic (McBeath et al., 2004; Sordella et al., 2003) or chondrogenic (Gao et al., 2010) phenotype when cell size is restricted or when contractility is reduced. Conversely, spreading and high contractility promotes osteogenic (McBeath et al., 2004; Kilian et al., 2010) and myogenic differentiation (Gao et al., 2010), albeit through different mechanotransduction pathways. Whereas the adipo/osteo switch is driven through RhoA/ROCK, the decision making at chondro/myo switch depends on the activation of Rac1. In analogy to previous studies, stiffness also dictates differentiation of MSCs toward a neurogenic or adipogenic phenotype on compliant substrates (<1 kPa), myogenesis at medium stiffness (8–17 kPa), and osteogenesis at high stiffness (>25 kPa) (Engler et al., 2006; Fu et al., 2010). Early studies now also show that the lineage fates of neuronal stem cells are also regulated by substrate stiffness and cell spreading (Saha et al., 2008).

MSCs are a heterogeneous cell population; not all MSCs undergo lineage commitment with equal efficiency. Intriguingly, MSCs that ultimately differentiate in response to soluble osteogenic supplements display significantly enhanced contractility

one day after initial stimulation, predicting osteogenic differentiation that only can be confirmed 7 days later (Fu et al., 2010). In addition, microscopy-based quantitative metrics describing changes in cell shape and actin cytoskeletal structure have been able to discriminate osteogenically induced MSCs from untreated or adipogenically induced controls 24 hr after stimulation, demonstrating early changes in cytoskeletal integrity preceding osteogenic differentiation (Treiser et al., 2010). The notion that contractility and cytoskeletal responses can act as harbingers of a differentiation response suggests that biophysical measures of stem cells could one day have utility in selection of clones with differential response characteristics.

Mechanotransduction in Embryogenesis

Two popular animal models to study embryogenesis are *Drosophila melanogaster* and *Caenorhabditis elegans*. Time-lapse microscopy of developing *Drosophila* eggs reveals a remarkable sequence of mechanical events during early fly development. During gastrulation, the transcription factors Snail and Twist induce a pulsed actomyosin contraction in ventral midline cells which invaginate according a ratchet-like mechanism, forming the ventral furrow and presumptive mesoderm (Martin et al., 2010; Pouille et al., 2009). At the same time, cells flanking the ventral furrow undergo passive cell shape changes (Butler et al., 2009). Under control of polarized actomyosin dynamics, cellular convergence-extension movements and cell intercalation are regulated along the anterior-posterior axis (Irvine and Wieschaus, 1994; Bertet et al., 2004; Blankenship et al., 2006; Fernandez-Gonzalez et al., 2009). This process, also called germ band extension, elongates the axial body to 2.5 times of its original length. When fully extended, the caudal end of the germ band retracts and pulls the amnioserosa over the dorsal epidermal gap that becomes exposed. At this stage, dorsal closure is initiated by the formation of a supracellular contractile ring in the epidermal cells at the leading edges of the opening and the pulsed contractions of amnioserosa cells pulling the leading edges together (Kiehart et al., 2000; Solon et al., 2009; Gorfinkiel et al., 2009). At the time when the lateral flanks of the epidermis come together, the remaining amnioserosa cells undergo apoptosis and involute, apparently distorting the neighboring cells and exerting a pull on surrounding cells. This apoptotic force contributes to the total force generation and speed of dorsal closure (Toyama et al., 2008).

Most of aforementioned findings are based on careful observation of the dynamics of fluorescently tagged proteins with time-lapse laser scanning confocal or two-photon microscopy. Hypotheses are tested with genetic and biochemical manipulations, such as silencing myosin-regulating gene expression with siRNA, or introducing drugs to affect contractility. In addition, laser ablation has been utilized as a "mechanical manipulation" to abolish long-range force transduction between cells (Kiehart et al., 2000; Fernandez-Gonzalez et al., 2009). Such techniques fall short of quantitative characterization of the endogenous magnitudes of force experienced in these settings, but they do provide a clear demonstration that such processes are at least partially driven by actomyosin contractility (Mammoto and Ingber, 2010; Kasza and Zallen, 2011). In addition, it is known that application of forces can directly impact myosin itself (Fernandez-Gonzalez et al., 2009). Despite these limitations, these approaches have contributed to our understanding of how genetics and biochemical signaling initiate mechanical events, such as the regulation of invagination of the ventral furrow by Snail and Twist. Far fewer studies, however, have been able to address the question of whether force is critical for inducing biochemical signaling that is required for subsequent stages of development.

Elegant experiments from the Farge group have given insight in to how compression forces between two cell types during *Drosophila* germ band elongation can induce the expression of Twist, a transcription factor that regulates the differentiation of the anterior midgut. Using a custom-built microscope (two-photon microscope with a third harmonics generation module and a femto-second titanium/sapphire oscillator), dorsal cells of the germ band were laser ablated, preventing the germ band from pushing the stomodeal primordium at the anterior side of the embryo (Supatto et al., 2005). To restore deformation, anterior pole stomodeal cells were indented with a micropipette, or a ferrofluid was injected and concentrated with an electromagnet in approximately 50 cells surrounding the stomodeal primordium. Then magnetic microtweezers were used to push the magnetized patch against the stomodeal cells with a force of 60 ± 20 nN. These mechanical manipulations rescued Armadillo/beta catenin translocation from the cell junctions to the nucleus which restored transcription of Twist (Desprat et al., 2008).

A slightly different approach was ventured by Zhang et al. to reveal a role for force in the maturation of hemidesmosomes and its impact on embryonic elongation in *C. elegans* (Zhang et al., 2011). Embryonic elongation is regulated by both the epidermis and muscles as muscle-defective mutants arrest midway through elongation. *C. elegans* hemidesmosomes (CeHD) are anchoring structures found at the apical and basal plasma membrane of the epidermis, connecting the exoskeleton (apical side) with intermediate filaments to the ECM at the muscle-epidermis interface (basal side). Zhang and colleagues identified p21 activated kinase (PAK1), G protein-coupled receptor kinase interactor (GIT1), and PAK interacting exchange factor (PIX1) to be three key signaling proteins involved in CeHD biogenesis and intermediate filament phosphorylation. Interestingly, during growth arrest in defective muscle mutants, PAK1 and PIX1 remained localized around the CeHDs, whereas GIT1 gradually disappeared from the CeHDs as embryos stopped elongation. By compressing muscle-defective mutant embryos between a blunted microneedle tip and a programmable microscope stage, the authors were able to rescue GIT1 signaling and CeHD maturation, thus providing a link between muscle-generated tension and longitudinal growth in *C. elegans*. Hence, CeHDs might be mechanosensors, in the same way as FAs and AJs are.

Similar to *Drosophila* and *C. elegans*, zebrafish (*Danio rerio*) and *Xenopus laevis* embryos can be easily manipulated and monitored under a microscope making these lower vertebrate animals attractive for mechanotransduction research. Indeed, scientists have focused on the role of myosin II-driven cortical forces in sorting out progenitor cells into three germ layers in zebrafish (Krieg et al., 2008; Schötz et al., 2008), and regulation of convergence-extension cellular movement (Skoglund et al., 2008) or fibronectin fibril assembly in the blastocoel roof in *Xenopus* embryos (Dzamba et al., 2009). Despite the fact that zebrafish and *Xenopus* are vertebrates, surprisingly little data

are available addressing mechanoregulation of skeletal development in these models. In chicken, however, abolishment of muscle contractions by injecting a neuromuscular blocker reagent during limb development impairs cartilage synthesis and tenascin-c expression, suggesting a role for force in developmental skeletogenesis (Mikic et al., 2000, 2004).

The function of myosin II and its isoforms has been explored to some extent in mouse development. Because direct manipulation is experimentally difficult, mechanotransduction studies in mice often restricted to examination of phenotypic abnormalities observed in knockout or transgenic mice. Deletion of myosin IIB in mice results in death from cardiac malformation and brain defects at 14 days post coitus (dpc) (Tullio et al., 2001), a surprising finding considering its role during gastrulation in other species (Skoglund et al., 2008). Moreover, myosin IIA knockout mice are also embryonic lethal but at 6.5/7.5 dpc because they fail to develop a normal visceral endoderm associated with mislocation of E-cadherin and beta catenin and are therefore unable to assemble AJs (Conti et al., 2004). Interestingly, knocking in myosin IIB driven by a myosin IIA promoter can rescue this phenotype. However, death occurs in the knockin mice at 12.5–14.5 dpc due to abnormal angiogenesis and migration defects in the placenta which may indicate a unique role for myosin IIA in placenta development (Wang et al., 2010). Despite the lack of technology to apply forces directly on cells in utero, knockout/knockin strategy, which controls for the total amount of myosin II, might suggest that localization of beta catenin and E-cadherin in presumptive visceral endodermal cells may be driven by contractile forces, independent of the myosin isoform producing the force. As conditional knockouts are introduced for these myosin isoforms, we will begin to better define their roles more completely in tissue-specific physiologic or pathologic responses to force.

The severe phenotypes observed in knockout mice lacking the genes encoding for members of the Kinesin Family 3 and Polycystic Kidney Disease have directed the interest to explore mechanotransduction in cilia, motile cilia, and primary cilia in mouse development. Cilia and primary cilia are microtubule-based structures enveloped in a specialized membrane that originate from the cell body into the extracellular space (Figure 1B). They act as mechanosensors steering the development of organs and tissues, such as kidney, pancreas, liver, cartilage, and bone among others. Although the molecular basis for cilia mechanosensing is still elusive, cilia appear to transduce mechanical stimuli in part by gating polycystin-based ion channels and modulating Sonic Hedgehog and Wnt signaling. The specific mechanosensory roles for cilia have been reviewed in detail elsewhere (Wallingford, 2010; Wallingford and Mitchell, 2011; Bisgrove and Yost, 2006; Berbari et al., 2009).

Thus, one can appreciate that despite the ubiquitous presence of forces driving developmental processes, only a small handful of studies have begun to shed light on these mechanical events. As genetic and biophysical tools continue to mature, we anxiously anticipate a substantial increase in the pace of discovery in this space.

Parting Perspectives

Identifying, characterizing, and understanding how mechanical forces contribute to cell and developmental biology depends on the establishment of tools to both measure and manipulate forces in cells and tissues. Over recent decades, substantial progress has been made in establishing tools, such as methods to measure traction forces, to apply forces to cells, and to modulate ECM stiffness. Although these techniques have enabled much of the current understanding of cellular mechanotransduction, much of the current work is limited to a few cell types in a few contexts, so our appreciation of when and where forces might be dissimilar is still quite limited. In addition to expanding the application of existing techniques to a wider range of biological settings, significant innovation is still needed to build a more powerful toolkit for the field. For example, while forces can be measured for cells cultured on specialized surfaces, they are only beginning to be observed in 3D hydrogels. We still cannot measure forces on natural ECMs such as collagen owing to their complex mechanical properties. In vivo, methods are even more limited and as such the force distribution between cells and tissues in each stage of development is still unknown. To begin to address this question, Brodland and colleagues combined computational finite element analysis with time-lapse movies obtained with two-photon microscopy to estimate forces during *Drosophila* development (Brodland et al., 2010). The finite element method is a general, widely used approach in engineering to model the mechanics of complex structures by examining the mechanics of each individual voxel (finite element). Although done for the first time in a living organism, the combination of measuring deformation with finite element analysis is still an indirect method to estimate force vectors. This shortcoming may be overcome by the development of molecular force sensors. Recently, a calibrated force sensor that undergoes efficient FRET upon deformation has been developed, allowing the measurement of traction forces across vinculin (Grashoff et al., 2010) and α-actinin (Meng and Sachs, 2011) in living cells. When applicable to other force transmitting molecules, this type of force beacons might provide a platform for "visualizing" forces in vitro and in vivo.

In addition to force mapping, the field would benefit from additional techniques to mechanically manipulate cells in vivo. Such techniques could involve the use of caged proteins to control drug release, targeting mechanotransduction mechanisms to specific sites within the cell. Optogenetics approaches involving transgenic expression of light-gated ion channels are also being developed to manipulate myosin contractility. For example, Arrenberg and colleagues modulated the beating rate of a zebrafish heart by emitting light pulses (Arrenberg et al., 2010). A possible approach to circumventing the challenges of in vivo models is to focus more effort on organotypic in vitro models that might mimic in vivo mechanotransduction events. For lung tissue, this biomimetic approach has been successful for gaining insight into nanoparticle uptake of lung epithelial and underlying endothelial cells under cyclic stretch, mirroring alveolar expansion during breathing (Huh et al., 2010). Such models offer the advantages of in vitro accessibility and control, with the potential for capturing more complex tissue behaviors.

In conclusion, experimental evidence is mounting to suggest that forces are present and evolving in virtually every biological setting, whether that setting involves the forces of physical activity, remodeling in multicellular and ECM structures, or

even simple changes in cell-cell and cell-ECM adhesion. These forces also appear to regulate many basic cellular processes from cell adhesion itself to cellular signaling, proliferation, and differentiation. As such, it may be best not to regard mechanics as applicable only to specialized circumstances, but instead as an ever-present physical dimension of cell and developmental biology. Similar to studying biochemical signaling, exploring mechanotransduction relies on the development of enabling technologies. Over the past 30 years, the development of PCR, antibodies, and fluorescent molecules have drastically changed the way scientists investigate biochemical signaling pathways. Now, several laboratories are developing tools such as traction force microscopy, micropost array detectors, patterned substrates and tunable hydrogels to facilitate mechanotransduction research. It will only be a matter of time before derivatives of these tools will be part of a standard biology laboratory tool kit.

ACKNOWLEDGMENTS

This work was supported by NIH (GM60692, EB 00262). J.E. is a postdoctoral fellow of the Research Foundation - Flanders (FWO). X.Y. acknowledges support from the American Heart Association postdoctoral fellowship program.

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