

MECHANOTRANSDUCTION AT CELL-MATRIX AND CELL-CELL CONTACTS

Christopher S. Chen,¹ John Tan,¹ and Joe Tien²

¹*Department of Biomedical Engineering, Johns Hopkins School of Medicine,
Baltimore, Maryland 21205; email: cchen@bme.jhu.edu*

²*Department of Biomedical Engineering, Boston University, Boston,
Massachusetts 02215; email: jtien@bu.edu*

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■ **Abstract** Mechanical forces play an important role in the organization, growth, maturation, and function of living tissues. At the cellular level, many of the biological responses to external forces originate at two types of specialized microscale structures: focal adhesions that link cells to their surrounding extracellular matrix and adherens junctions that link adjacent cells. Transmission of forces from outside the cell through cell-matrix and cell-cell contacts appears to control the maturation or disassembly of these adhesions and initiates intracellular signaling cascades that ultimately alter many cellular behaviors. In response to externally applied forces, cells actively rearrange the organization and contractile activity of the cytoskeleton and redistribute their intracellular forces. Recent studies suggest that the localized concentration of these cytoskeletal tensions at adhesions is also a major mediator of mechanical signaling. This review summarizes the role of mechanical forces in the formation, stabilization, and dissociation of focal adhesions and adherens junctions and outlines how integration of signals from these adhesions over the entire cell body affects how a cell responds to its mechanical environment. This review also describes advanced optical, lithographic, and computational techniques for the study of mechanotransduction.

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INTRODUCTION

To perform as single integrated machines, multicellular organisms have evolved complex mechanisms to communicate signals throughout their bodies. One principal mechanism for this global coordination is the transmission of mechanical forces through viscoelastic tissues, cells, and fluids (1–3). Whether externally applied or internally generated, these forces play essential roles in the development of proper mammalian physiology and in the response of an organism to injury: Gravitational compressive forces control the deposition of bone; isotonic tension causes muscles to grow (2, 4). Normal hydrostatic pressure in blood vessels promotes the maturation of vascular smooth muscle, whereas hypertensive pressure causes the walls of arteries to thicken (5). Shear stresses that arise from the flow of blood help to prevent activation of coagulation cascades and maintain endothelium in an anti-inflammatory and antiatherogenic state (6, 7). Contractile forces generated by endothelial cells regulate vascular permeability; those generated by myofibroblasts in a wound reduce the area of granulation tissue over time (8–10).

Although it is clear that common physiological processes expose cells to a variety of mechanical stimuli, how cells sense and integrate these forces at the molecular level to produce coordinated behaviors is an open question. Cellular structures are subjected to stresses of 0.01–0.1 atm *in vivo*, equivalent to 1–10 nN per cell contact. Even small changes in the magnitudes or distribution of these forces may lead to compensatory remodeling of cell-matrix and cell-cell contacts and may initiate a variety of cell behaviors. For instance, a slight increase in time-averaged shear stress in an artery induces proliferation of vascular cells and widening of the artery until the shear stress renormalizes to (nearly) its initial value (11, 12).

Recently, it has become clear that cultured cells *in vitro* possess specific mechanisms to sense and generate mechanical forces and that mechanical signals provide a fundamental means for cells to respond to their microenvironment (13–23); whether the same mechanisms operate *in vivo* within multicellular organisms is currently under intense study (24, 25). Mechanotransduction, or the translation of mechanical signals into biochemical ones that affect cell function, appears to occur primarily at the surface of a cell *in vitro* (Figure 1). In mesenchymal cells that are grown on two- or three-dimensional scaffolds of the extracellular matrix (ECM), mechanotransduction occurs at specialized structures that link cells to the ECM, such as the focal adhesions (FAs) that develop in cells cultured on a rigid substrate (13, 26). In epithelial cells, which are bound to the ECM basally

and surrounded by neighboring cells laterally, mechanotransduction also occurs at cell-cell junctions called adherens junctions (AJs) (27–29). In both types of cells and in blood-borne cells, shear and hydrostatic stresses from surrounding fluid may provide yet another avenue for mechanical signaling (30–32).

Cells adhere to the ECM and to each other through specific classes of transmembrane adhesion receptors (33–35). These receptors bind to ligand extracellularly and provide an anchor to the intracellular cytoskeleton via cytoplasmic scaffolding proteins (29, 36). Linkages between external cellular contacts, adhesion receptors, and cytoskeleton provide a means for bidirectional communication between the inside and outside of a cell. Dynamic changes in adhesions and cytoskeletal systems may thus play a critical role in regulating mechanotransduction (37).

The cellular response to mechanical forces is inherently coupled to the internal organization of the cytoskeleton and to adhesion to surrounding cells and the ECM. Structural cues such as anisotropy or topography of the ECM or location of cell-cell contact can cause a cell to reorient its body, change its shape, or alter its functional state (38–40). Similarly, changes in the shape and internal organization of cells alter how cells adhere to their surroundings and affect their function (41–43). Application or removal of a gross external load from a cell causes the cell to actively adapt its adhesions and cytoskeleton and transduce the altered mechanical environment into biochemical signals (44).

This review focuses on the mechanical and biochemical properties of cell adhesions and how these adhesions might be involved in the ability of cells to transduce structural and mechanical signals into adaptive behaviors. First, we describe recent advances in techniques used for the study of mechanotransduction. Second, we examine the responses of single-cell-ECM and cell-cell adhesions to mechanical force, as well as the integration of mechanical signals over an entire cell, to produce observable behaviors. It is important to note, however, that not all forms of mechanotransduction take place at adhesions. For instance, shear stresses experienced by the endothelium are not only transmitted through cell bodies to basal adhesions but also appear to be directly transduced through receptors located within the apical membrane (31). In some cases, a single protein may act as a mechanosensor. For example, stretch-activated ion channels appear to change conductivity in response to stresses applied to the plasma membrane (45). For further details on non-adhesion-based mechanotransduction, we refer the reader to several recent reviews (31, 46, 47).

TECHNIQUES TO STUDY MECHANOTRANSDUCTION

Studies of mechanotransduction examine how mechanical forces influence intracellular signaling and subsequent behaviors of cells. Typically, these studies perturb the mechanical state of a cell by varying the degree to which a cell is spread (48–52), the rigidity of the substrate to which a cell adheres (22, 53, 54), or the ability of a cell to generate intracellular forces (55). Measurements in these studies

include observation of the assembly (or lack of assembly) of adhesions (19, 56, 57), biochemical analyses of proteins known to interact with adhesion complexes (58, 59), quantification of gross cell behaviors (proliferation, apoptosis, migration, or differentiation) (41, 60–62), and measurements of forces exerted by cells (15, 18, 19, 63).

Early experiments exposed cells to spatially uniform mechanical environments and measured the averaged response of cells to these stimuli. To control the degree of cell spreading, cells were cultured on substrates coated with different densities of ECM proteins (49); the denser the adsorbed layer, the better the cell spread. To control the rigidity of the substrate, cells were cultured on thin flexible sheets of polymer, or in three-dimensional gels of ECM (usually collagen) that were cross-linked for different times (53, 54, 64). To control the ability of a cell to generate contractile forces, cells were cultured in the presence of agents that destabilized or enhanced cytoskeletal filaments throughout the entire cell (55). Analyses of cell adhesions and downstream signaling relied on several standard biological techniques, including immunofluorescence, Western blotting, and assays for proliferation and apoptosis.

Although the results of these experiments demonstrated a strong correlation between mechanical forces and changes in cell behaviors, these experiments could not prove a causal relationship between the two. The realization that cell-matrix and cell-cell contacts may be the primary sites of mechanotransduction has motivated the development of techniques to control or record the mechanical state of a cell with the spatial resolution of a single cell contact ($\sim 1 \mu\text{m}$). These techniques—coupled with advanced optical methods for probing a cell, such as the use of fluorescent fusion proteins that target to adhesions (56) and the use of correlational microscopy to determine how adhesions evolve over time (56, 57)—have begun to elucidate the causal relationships between mechanical forces and intracellular signaling. Three recent developments have proven to be invaluable in the study of mechanotransduction: (a) measurements of the distribution of mechanical stresses exerted by a cell with subcellular resolution, (b) application of force locally to only a portion of the cell membrane, and (c) confinement of the areas at which a cell can exert mechanical force.

Measurement of Forces Exerted by Cells

Cells exert nanonewton-scale contractile forces against adhesive structures that couple the cell to its external environment (20). In vitro studies of these minute forces have relied on the culture of cells on soft materials such as uniformly cross-linked hydrogels or flexible rubber membranes, where the degree of cross-linking controls mechanical compliance (Figure 2A) (14, 22, 53, 65–67). Initial experiments with thin membranes demonstrated conclusively that cells exerted contractile forces (also referred to as tension or traction forces). Contractile forces generated by cells deformed rubber membranes to form microscopic wrinkles that were easily observed by phase-contrast microscopy (53). Subsequent studies have

focused on dynamic changes in the magnitude and direction of forces exerted by cells and how these forces correlate with the shapes of cells during cytokinesis and cell migration (65, 66).

Quantitation of force exerted by cells on thin membranes consisted of counting the number of wrinkles near cell bodies, measuring the lengths of these wrinkles, and using these values to calculate the total force exerted on the membrane (66). Because membranes deformed grossly in these studies, it was difficult, if not impossible, to calculate the force exerted locally by cells with subcellular resolution. Improvements on this setup led to the development of a method called traction force microscopy to quantify force exerted by cell (Figure 2B) (68). Here, cells are plated onto a membrane that is pre-stressed to prevent wrinkling of its surface. Force that is generated by the cells deforms the membrane only slightly; fiduciary markers, such as spherical beads embedded within the membrane, aid in measurement of local displacements. Because the substrate deforms only within a plane, is basically incompressible (i.e., has a Poisson's ratio of 0.5) and is linearly elastic, the stress field $\mathbf{F}(\mathbf{r})$ and displacement field $\mathbf{u}(\mathbf{r}')$ are related by a Fredholm integral equation of the first kind:

$$u_i(r) = \int dr' G_{ij}(r - r') F_j(r'), \quad (1)$$

where $\mathbf{G}(\mathbf{r} - \mathbf{r}')$ is the tensorial Green's function of the elastic isotropic halfspace and represents the displacement at \mathbf{r} that results from the application of a point force at \mathbf{r}' . For instance, the displacement at \mathbf{r} that results from a single point force at \mathbf{r}' is given by Equations 2 and 3:

$$u_x(r) = G_{xx}(r - r') F_x(r') + G_{xy}(r - r') F_y(r') \quad (2)$$

$$u_y(r) = G_{yx}(r - r') F_x(r') + G_{yy}(r - r') F_y(r') \quad (3)$$

Given a displacement field \mathbf{u} measured from the movement of embedded beads, the challenge in traction force microscopy is to invert Equation 1 to obtain the stress field \mathbf{F} . Because the integral operation is a smoothing operation, inversion does not always lead to a unique solution (71). As with any inversion operation, regularization schemes are often used to provide additional information to restrict the set of possible solutions (72); examples of commonly used guides include the assumption that force can be exerted only at focal adhesions and that only the least complex solution is used (68). The complete description of computational methods to calculate force and the derivation of the Green's function have been well described (73).

The recent use of microfabricated regular arrays of fluorescent particles as markers has allowed better tracking of the deformation field (Figure 2C) (15); algorithms for inversion of Equation 1 have also improved and no longer require the continual use of supercomputers. The computation is still sufficiently intensive, which makes the analysis of forces exerted by large populations of cells impractical. Moreover, because the displacement of discrete markers cannot fully describe the

deformation of a continuous surface, finding a unique solution of force can be achieved only by placing constraints on the deformation field, on the nature of the cellular forces, or on the location of adhesions (67).

These limitations are inherent to the use of flat, continuous substrates where a deformation at one location can propagate to another part. Alternatives to these substrates for measurement of force initially used a microfabricated device containing a horizontally mounted cantilever that deflected along one axis as individual cells migrated across it (Figure 2D) (18). Because the moveable unit in these devices (the cantilever) is mechanically decoupled from its surroundings, lateral deflections of the cantilever result only from local forces exerted on it; measurements of these deflections give a direct value for the local force generated at the cell surface. As originally designed, however, the geometry of the cantilever (with the flexible arm in the same plane as the displacement) restricted the measurement of force to one axis and a few locations (18).

We anticipated that, by altering the design of the device so that the arm of the cantilever did not reside in the same plane as the displacement, it would be possible to place multiple cantilevers in a single device and thus to measure local forces across the entire body of a cell (Figure 2E). In practice, we used lithographic techniques to mold arrays of closely spaced vertical cantilevers (i.e., microneedles or posts) in a silicone elastomer; cells attached and spread across the tips of multiple posts and bent the posts as the cells probed the tips (63). For small deflections, the posts behave like simple springs so that their deflections are directly proportional to the local forces applied by the attached cell. This behavior is described for beams composed of linearly elastic material under pure bending by Equation 4,

$$F = \left(\frac{3EI}{L^3} \right) \delta, \quad (4)$$

where F , E , I , L , and δ are the bending force, Young's modulus, moment of inertia, length, and resulting deflection of the post, respectively (74). Because each post moves independently of its neighbors, its deflection directly reports the direction and magnitude of the local cell-generated force without the need for a priori assumptions.

Application of Localized Forces to Cells

In the previously described methods, the substrate passively responds to forces exerted by the cultured cell. Often, however, it is useful to be able to deliberately expose a cell to external mechanical force and then observe its response (Figure 3). The first studies in this area used stretchable membranes to impart a uniform strain (and, by extrapolation, a uniform stress) to cells (75). As with techniques for the measurement of force, methods for the application of force have gradually evolved beyond the use of spatially homogeneous substrates; recent advances have enabled the exposure of selected portions of a cell to a controlled stress.

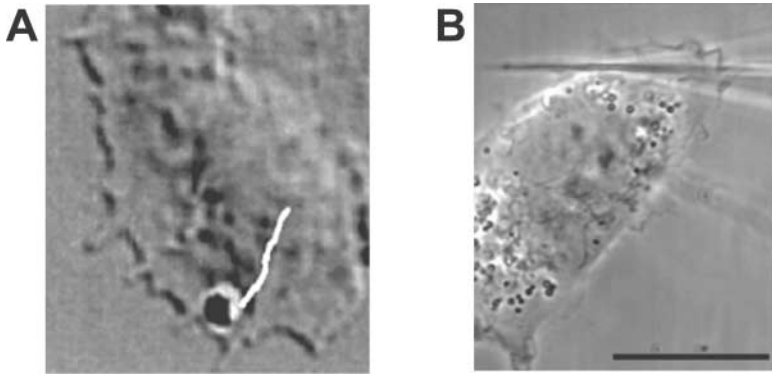


Figure 3 Techniques to apply external forces to cells: (A) Movement of optically trapped microbeads on the cell surface measures both the externally imposed force and the reaction generated by the cell. Reproduced with permission from Reference 20. (B) Deformation of the cell membrane by a micropipette imparts a gross force to the area of contact. Reproduced with permission from Reference 16.

Many of these advances rely on the use of micrometer-sized beads that can be manipulated by an external electromagnetic field (Figure 3A) (20, 21, 76). In these studies, beads are coated with ECM proteins and are allowed to passively settle onto cells or are placed directly on the cell membrane. Application of a magnetic field to an attached paramagnetic bead forces the bead to align in the direction of the magnetic field; this technique generates a twisting stress that rotates the cell membrane (21). Application of a non-uniform electromagnetic field, such as that resulting from focusing light through an optical lens, to an attached bead whose index of refraction differs from that of its surroundings attracts the bead to the regions of highest light intensity; this technique (known as optical tweezers) generates a lateral stress that pulls and pushes the cell membrane (77). Equations to describe the force exerted on a bead in these magnetic setups or optical tweezers are well known and lead to straightforward calculations of force exerted on attached beads. Because these techniques are compatible with optical microscopy, it is possible to simultaneously exert force on the bead and observe whether the bead can escape from its magnetic alignment or optical trap. The degrees to which beads resist their applied forces can thus give a direct measurement of the force exerted by the cell on the beads in response to the force exerted on the cell by the beads.

Other techniques to impart a localized force to cells use the controlled movement of an attached micropipette across the surface of a cell (Figure 3B) (16, 78). These techniques may consist of (a) translating a rigid tip a defined distance and using simplifying assumptions of the mechanical properties of the cell to calculate the applied force (16), (b) translating a flexible cantilever across the cell and calculating the applied force from Equation 4, and (c) translating a rigid tip against the underlying substrate of a cell to increase or decrease the local stiffness

of a gel or membrane (78). Here, the determination of exerted force is not as exact as with magnetic or optical forces, but these tip-based methods are often favored because they are easily implemented with nothing more than a translation stage and optical microscope.

Confinement of Cell-Matrix and Cell-Cell Contacts

A complementary set of techniques controls where cells can exert force with sub-cellular resolution (Figure 4). These techniques all rely on the use of nonadhesive surfaces to restrict the regions to which a cell can adhere and exert force (79). In early studies, cells were cultured on thin films of palladium that were patterned by evaporation through a fine metal mask (mesh size $\sim 100 \mu\text{m}$) on a nonadhesive substrate (51, 52). Cells were restricted to the islands of metal and adjusted their shapes to the shapes of the islands. Subsequently, many strategies to create more complex patterns by using lithographic printing techniques have been developed (79a, 79b), one of which involves contact printing of small organothiols onto a thin film of gold defines adhesive and nonadhesive regions (Figure 4A) (41, 80–82). Micro-contact printing of these features is not limited to discrete adhesive islands (in contrast to surfaces made by evaporation through a mask) and can be readily applied to non-planar substrates (81). Cells cultured on these patterned surfaces adhere selectively to the adhesive regions; because the stamp used for printing is made by microlithography, the definition of adhesive surfaces takes place with sub 100-nm

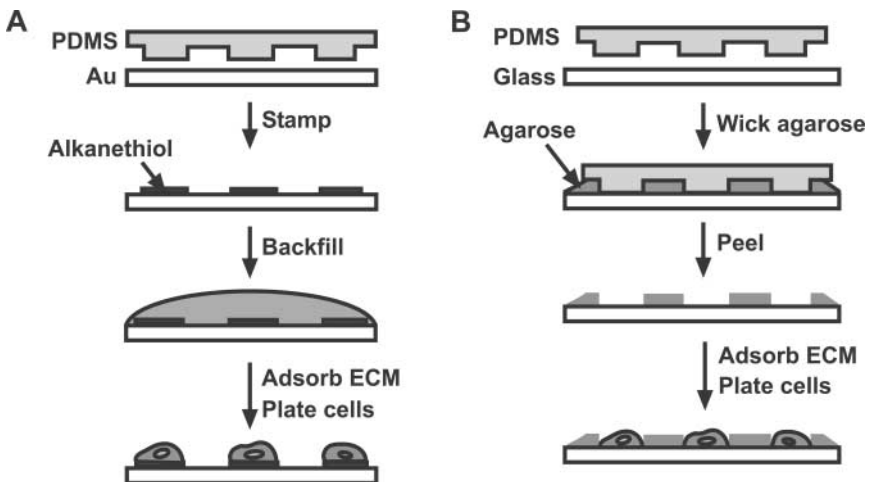


Figure 4 Techniques to confine the locations of cell-matrix and cell-cell contacts: (A) Micro-contact printing of adhesive and nonadhesive regions on a flat surface controls the locations of cell-matrix contacts. (B) Micromolding of a nonadhesive gel further confines the locations of cell-cell contacts. The gel in (B), which is several micrometers thick, physically restricts the formation of cell-cell contacts, whereas the nanometer-thick printed regions in (A) do not.

resolution and allows a single cell to attach to multiple, discrete micrometer-scale patches.

To control where cells can contact neighboring cells, we have also used lithographic techniques to mold thin nonadhesive gels of agarose into microscale meshes (Figure 4B) (60, 83). The molded agarose forms walls that block cells from making contact with their neighbors. Cells cultured through a suitably patterned mesh thus can adhere to the ECM and other cells in defined locations.

The development of techniques to measure force exerted by cells, to expose cells to defined forces, and to localize where cells exert force, all with subcellular resolution, has led to recent breakthroughs in the study of mechanotransduction at cell-ECM and cell-cell contacts. We anticipate that the development of techniques with even finer (nm scale) spatial resolution and techniques that allow rapid dynamic changes in the forces exerted at an adhesion will uncover new insights into mechanical signaling in cells.

MECHANOTRANSDUCTION AT CELL-MATRIX CONTACTS

Structure of Cell-Matrix Adhesions

Recent studies have shown that many distinct types of adhesions exist between cells and the ECM; these adhesions differ in size, shape, and biochemical composition and probably differ in function as well (84). The best-characterized and largest of these structures is the focal adhesion (FA; also known as a focal contact) (13). These adhesions are transient in nature, and form in many types of cells that are cultured on a substrate coated with ECM. All adherent cells bind to the ECM through integrins—transmembrane receptors that bind to specific motifs on numerous ECM proteins (85). The binding of integrins to the ECM causes them to cluster and leads to the recruitment of a battery of cytoplasmic signaling and structural proteins to form FAs at the site of integrin clustering (13, 86, 87). Numerous structural proteins (e.g., vinculin, talin, α -actinin, and paxillin) act as scaffolding proteins that strengthen cell adhesion by anchoring FAs to the actin cytoskeleton (13, 86). It is thought that these proteins are able to provide this anchoring function because some of these proteins bind actin filaments directly, whereas others contain domains that can bind to actin-binding proteins. When these proteins coalesce with clusters of integrins, they likely bind to each other through a multitude of interactions to link the integrins to the actin cytoskeleton. Other types of cell-matrix adhesions that have recently been identified—focal complexes, fibrillar adhesions and three-dimensional matrix adhesions—are structurally similar to FAs but differ subtly in composition and morphology (24, 56, 88).

Forces that Regulate Assembly of Focal Adhesions

The distinguishing features of the four different cell-matrix adhesions (FAs, focal complexes, fibrillar adhesions, and three-dimensional matrix adhesions) suggest a central role for mechanical forces in their development. Focal complexes

are found within minutes after suspended cells are placed in contact with an ECM-coated surface (88). These complexes are small, punctate, and exist primarily at the periphery of a spreading cell or at the leading edge of a migrating one (88, 89). Slightly proximal to these focal complexes are the larger FAs. Observations made with time-lapse fluorescence microscopy suggest that focal complexes are one of the precursors of FAs. In cells that express genetically engineered FA proteins linked to green fluorescent protein, focal complexes gradually move centripetally inward from the boundary of a cell, elongate, and evolve into FAs as more adhesion proteins and actin are recruited to the adhesion (56).

The maturation of these complexes into FAs appears to require the application of mechanical stress to the adhesions (15, 90). Early studies demonstrated that altering the activity of several biochemical signaling pathways, particularly those that involve myosin light chain kinase (MLCK) and the small GTPase Rho, regulated both the contractility of the actin cytoskeleton and the formation of FAs (14, 90). Specifically, the number of FAs present in a cell correlated strongly with the contractility of the cytoskeleton. However, these studies did not determine whether it was the biochemical activity of these signals, or the resultant mechanical stresses, that caused FAs to form.

Two different approaches that do not require tampering with endogenous signaling pathways also suggest that mechanical forces have a direct role in the formation of FAs. In the first approach, investigators used flexible substrates that deform when attached cells contract to measure the local distribution of forces that are generated by cells at their adhesions (Figure 3A) (15, 63, 67). Studies using this approach have demonstrated that the size of individual focal adhesions (as determined by fluorescence microscopy) correlates loosely with the measured local forces. [Intriguingly, at a subset of smaller adhesions ($<1 \mu\text{m}^2$ in area), cells exerted significant traction force that does not correlate with adhesion size.] This study also provided strong evidence that forces and focal adhesions correlate in un-manipulated cells, whose signaling pathways remain intact; nevertheless, cause and effect were not distinguished.

In the second approach, investigators applied external forces directly to adhesions (Figure 3B) (16, 19, 20, 91). Applying force to FAs from externally bound beads or pipettes led to assembly of components into FAs (16). This force-dependent assembly of FAs resulted in increased strength of adhesion; the strength of adhesion increased with the amount of force applied (20). Taken together, these studies suggest that intracellular tension against initial cell-ECM contacts is necessary for these contacts to develop into FAs. These studies have clearly shown that the application of force causes FAs to increase in size, to stabilize, and to strengthen their coupling to the cell (presumably through the actin cytoskeleton). Externally applied mechanical forces can replace the activation of Rho-associated kinase (ROCK), a downstream effector of Rho that regulates cell contractility, but cannot replace the function of mDia1, an effector of Rho that allows actin to polymerize (16).

These findings collectively suggest that maturation of FAs requires either tensile forces generated by actin-myosin contraction of the anchoring actin filaments

or external loading of the bound ECM. In addition, these findings outline a three-stage process for the formation of FAs in cultured cells: Occupation of integrins by ligand initiates condensation of FA proteins, clustering of integrins by multivalent ECM recruits further components of FAs, and exertion of tensile forces through integrins to the ECM provides a signal to mature the adhesions.

Fibrillar adhesions appear to be a separate form of FAs and manifest as streaks that lie centripetal to FAs (56, 57). Time-lapse recordings have shown that fibrillar adhesions quickly slide centripetally from FAs to the central region beneath the cell; these adhesions appear to originate from the proximal ends of FAs (56). Fibrillar adhesions appear to track along actin stress fibers that anchor into the originating FA and move at speeds comparable to the treadmilling rate of actin. These adhesions contain slightly different mixtures of components and integrin subtypes than do FAs and are enriched in fibronectin and $\alpha_5\beta_1$ integrin (57). Culture of cells on ECM that is chemically cross-linked onto a rigid substrate greatly reduces the formation of fibrillar adhesions and favors the formation of FAs instead. These results suggest that FAs form when the cell can exert large stresses against an ECM, whereas fibrillar adhesions form when the ECM can support only smaller contractile forces. Whether this hypothesis is correct awaits the use of optical tweezers or compliant substrates to determine the effect of force on the formation of fibrillar adhesions.

Adhesions to a three-dimensional matrix appear to be a distinct class of structures that form only when cells are cultured on naturally assembled ECM fibrils (versus a flat adsorbed layer of ECM). This three-dimensional ECM typically consists of a fibrous composite of collagen, proteoglycans, elastin, and adhesive glycoproteins (92). It is usually oriented, highly porous, and heavily cross-linked. The three-dimensional matrix adhesions appear to have a composition distinct from FAs, fibrillar adhesions, and focal complexes; in particular, they lack phosphorylated focal adhesion kinase (24). What remains unclear is whether these matrix adhesions form as a result of the difference in matrix architecture, mechanical compliance, or ligand presentation, such as might result from the hydration state of the matrix. When the compliant three-dimensional ECM is either compressed into a sheet or chemically cross-linked to produce a rigid three-dimensional ECM, the distribution of adhesion proteins changes. These results suggest that mechanical forces again may play a key role in the formation of these adhesions; they also suggest that the architecture of the ECM—whether flat or a porous volume—may control the localization of proteins to these adhesions.

Several aspects of the cell-matrix adhesions that are likely relevant for cell mechanics remain unresolved. First, it is not clear how the compositions of adhesions differ between cells or between adhesions in a single cell, and how a particular composition determines downstream signaling. No stoichiometric relationships have been determined in defining the composition of these adhesions, and, given the numerous proteins that are involved, it is uncertain whether specific stoichiometries should even exist in these structures. In fact, a wide range of compositions may provide similar functions to the different types of matrix adhesions (84). In vitro several of these FA proteins appear to be redundant for normal cell adhesion

and function. In vivo, however, deletion of any of these proteins in mice is lethal, suggesting that at different times during development there are periods where each of these scaffolding proteins is critical for adhesive function (93).

The composition of these adhesions has been determined predominantly by two distinct approaches: (a) the cells can be labeled with immunofluorescent probes and fluorescence microscopy used to detect the distribution of specific proteins in the adhesions, or (b) the adherent cells can be permeabilized to remove most cellular proteins and the remaining adhesions solubilized for electrophoretic separation and detection by Western blot. With immunostaining, the distinctions between different classes of adhesions have been made by the presence or absence of specific integrins or FA proteins within the structures and by the morphology of these adhesions. These methods have not yet determined whether these adhesions are indeed distinct subtypes or, instead, vary along a continuum in adhesion composition and properties. However, the complexity and compaction of proteins in these adhesions raise the possibility that fluorescent markers may not be able to penetrate adhesions to label targeted proteins adequately. Methods to selectively collect a particular class of adhesions for biochemical analysis would greatly aid in resolving these issues.

Second, it is not yet clear if there is a particular organization to these adhesive structures. Apart from knowing that integrins exist at the plasma membrane and that actin is linked to the cytoplasmic domains of FAs, the internal structure of these adhesions is basically unknown. From a comprehensive list of positive protein-protein interactions and a list of proteins present at an adhesion, it may be possible to guess the three-dimensional arrangement present at the adhesion. The use of fluorescence resonance energy transfer (FRET) techniques to determine the spacings between proteins may prove useful here (94).

Third, the mechanical properties of these adhesions remain unclear. Given the large number of interconnected protein-protein interactions that arise during adhesion formation, one expects that much of the mechanical stability arises from binding energies between these scaffolding proteins. Because many of the binding energies between proteins have not yet been determined, it is difficult to estimate the theoretical strength of these adhesion sites, let alone the relative contribution of each protein-protein interaction to the strength of these adhesions.

Fourth, it is not clear how the different compositions or mechanical properties of cell adhesions lead to changes in biochemical signaling. Because the molecular definition of these adhesions remains vague, it has been difficult to uncover how specific signaling events arise.

Forces that Regulate Signaling at Focal Adhesions

In all adherent cells, adhesion is needed for proper metabolism, protein synthesis, and survival (95, 96). Although it is well-known that signaling through integrins regulates the formation of FAs (13), only recently has it become evident that the mechanical and structural changes associated with maturation of adhesions in

turn affect signaling (58, 97). Thus cell-matrix adhesions not only play a physical role in organizing cells into tissues but also provide an important biochemical role in the regulation of many cellular processes.

Many signaling proteins (e.g., src, FAK, Ras) localize within cell-matrix adhesions (84, 87). Because these signaling proteins function in cascades initiated by growth factors, their concentration at adhesions suggests that adhesions may act to coordinate integrin and growth factor signaling (96). For example, it is known that most adherent cells will undergo apoptosis quickly in the absence of either adhesion or soluble growth factors and will proliferate only in the presence of both signals (98). In fact, growth factor receptors themselves are thought to localize to FAs. Because specific integrins within these adhesions appear to interact with specific growth factor receptors (99, 100), these adhesions may enable cells to generate unique responses when exposed to particular combinations of ECM and growth factors. For example, signaling through basic fibroblast growth factor receptor in endothelial cells requires ligation of the integrin $\alpha_v\beta_3$, whereas signaling through vascular endothelial growth factor receptor uses $\alpha_v\beta_5$; because $\alpha_v\beta_3$ and $\alpha_v\beta_5$ bind to different classes of ECM proteins, the response of endothelial cells to a given growth factor depends on its underlying ECM (101, 102). Thus adhesions provide a physical structure that allows many important biochemical signals to initiate fundamental changes in cell behavior. Because these adhesions continually remodel in response to changes in the composition, architecture, and mechanical properties of the cell-matrix interface, they appear to be a central mechanism by which cells can change behavior in response to structural and mechanical cues.

Increasing mechanical stress at FAs by increasing intracellular tension or applying extracellular forces leads to important changes in signaling at FAs through focal adhesion kinase (FAK) and its downstream partners (17, 97). FAK appears to be the earliest marker of FA signaling whose activity changes with tension. Recent studies show that applying mechanical stretch can increase FAK phosphorylation, sustain the activation of the mitogen-activated protein kinase ERK, and cause cells to proliferate (103, 104). The activation of FAK by autophosphorylation of tyrosine397 is required for ERK activation and cyclin-dependent progression through G₁ (105). Kinase-dead mutations in FAK appear to abrogate ERK activation and cell proliferation caused by mechanical forces (106, 107); expression of constitutively active FAK, on the other hand, can transform cells so that they proliferate in suspension (108). These findings imply that increases in cytoskeletal tension may be transduced into a proliferative signal in FAs, possibly through changes in FAK activity and that this signal represents a crucial component of adhesion-dependent growth in cells. The hypothesized link between tension and proliferation might explain why agonists that increase cytoskeletal tension, such as thrombin or angiotensin II, cause smooth muscle cells to proliferate (109–111).

Because FAs do not form without the generation of intracellular tension, it has been difficult to determine whether the pro-proliferative effects of tension are mediated by the presence of FAs or whether tension has additional direct effects on the activity of signaling proteins. Recent results have provided tantalizing evidence

that tension may modulate the activity of proteins in a recruitment-independent manner. Application of mechanical stretch to permeabilized cells, i.e., depleted of additional cytoplasmic proteins that can localize to FAs, can itself result in phosphorylation of paxillin, FAK, and Cas (17). This result implies that stretch may possibly alter the conformation and thus the activity of molecules that have already been recruited to FAs. Changes in activity that result from changes in conformation are well known for the FA proteins vinculin and src (112); although tension-generated changes in cell signaling have previously been ascribed to binding of soluble factors to FA proteins, the demonstration that some of these changes can take place in the absence of soluble factors suggests that additional mechanisms for altering biochemical activity operate in adherent cells.

Integrated Forces Alter Cell Behavior

The response of a cell to changes in its mechanical environment depends on the integration of signals transduced at numerous distinct FAs; *in vitro* a cell typically forms numerous adhesions to surrounding ECM. How these signals from each adhesion are coordinated over the entire cell to drive cell behavior collectively must be considered in the context in which mechanical tension is generated throughout the cell. The ability of a cell to generate tension depends partly on the architecture of its anchoring cytoskeleton; cytoskeletal structure, in turn, varies with the global placement of adhesions. This interplay has led to the hypothesis that cell shape—as controlled by the location of cell-matrix adhesions—may regulate cell behavior through changes in intracellular tension and downstream signaling at FAs (37).

It has been known for some time that ligation of integrins alone is insufficient to support cell proliferation. Folkman & Moscona (48) first observed that cells proliferated only when allowed to spread and flatten against a solid substrate, suggesting that cell morphology may provide a regulatory signal for cell function. Studies later showed that the projected area of the cell (cell spreading) can be directly controlled by the density of immobilized ECM; increased cell spreading led to increased proliferation (113). Because ECM coating density not only affects cell spreading but also integrin activation, it remained controversial whether the effects of increased cell area merely reflected increased integrin activation or were separate from it (114–116).

To generate substrates that could decouple cell spreading from ECM coating density, we developed a micropatterning technique to synthesize surfaces that contained micrometer-scale ECM-coated islands surrounded by nonadhesive regions (Figure 4A). Cells cultured on these substrates attached and spread to fill the patterned islands (41). Cells that were plated on smaller islands coated with saturating densities of the ECM protein fibronectin were smaller in size and proliferated less (41, 117). Similar studies have shown that changes in cell spreading can also affect the differentiated function of cells (43, 118). Thus it appears that cell spreading independently regulates cells through a mechanism that is distinct from direct integrin ligation (117). Given that cell shape and cytoskeletal architecture are linked

and that cytoskeletal architecture in part drives the organization of FAs, these studies strongly suggest that signaling through matrix adhesions transduces the effects of changes in cell shape.

Cell spreading appears to modulate proliferation by regulating levels of tension within the actin cytoskeleton. Blocking actomyosin tension production in spread cells by inhibiting MLCK or by disrupting the actin cytoskeleton arrests cell cycle progression in late G₁, as if cells were not spread (119). Direct measurements of the traction forces exerted by cells have shown that increased cell spreading increases the magnitude of tensile forces that cells exert against their adhesions (21, 63). These forces appear to be regulated in part by the Rho signaling pathway, which plays a major role in the generation of stress fibers and intracellular tension in adherent cells (90, 120).

Spreading-induced changes in cytoskeletal tension may regulate cell proliferation through changes in signaling through Rho. Microinjection of constitutively activated RhoA promotes DNA synthesis, whereas C3 exoenzyme, which ADP-ribosylates and inactivates Rho, blocks DNA synthesis (121, 122). One Rho effector, Rho kinase (ROCK), is involved in the generation of cytoskeletal tension. Inhibition of ROCK with the compound Y-27632 or with expression of dominant-negative ROCK suppresses the mitogen-induced DNA synthesis of vascular cells *in vitro* and *in vivo* (123–125). ROCK directly phosphorylates both smooth muscle and nonmuscle myosin II regulatory light chain (MLC) and MLC phosphatase to synergistically increase MLC phosphorylation and myosin II contractility (126–128). Blocking Rho, ROCK, MLCK, or myosin activity leads to the same phenotype of cell cycle arrest; because MLCK acts to increase cell tension, it is thought that Rho acts through tension as well to exert its effect on cell proliferation (129).

Changes in cytoskeletal tension may be translated into appropriate proliferative signals by the interplay between cell shape, Rho, and FAs. First, inhibition of Rho greatly reduces the formation of FAs (14, 90). Second, cell spreading increases the total area of FAs quantity in a tension-dependent manner (130). Third, the activation of FAK reduces the activation of Rho (58). Because cells in culture are constantly exposed to agonists, such as lysophosphatidic acid (LPA), that activate Rho, cell shape may act in culture as a master switch to control the area of FAs, activation of Rho, generation of tension, and progression through the cell cycle.

A similar model may apply to the control of cell migration, this time through a different Rho family GTPase, Rac. Here, the data are less conclusive than for the role of Rho and tension in proliferation. Cells migrate, in part, by polarizing the distribution of force that they exert against their adhesions (131). Activation of Rac leads to the formation of lamellipodia, which are commonly observed at the leading edge of migrating cells and appear essential for migration (88). Moreover, activated Rac leads to the formation of focal complexes between cells and their substrate, which further recruit and activate Rac at the membrane. Cortically oriented tension (i.e., parallel to the cell periphery) decreases the activation of Rac and reduces the extension of lamellipodia, whereas radial tension has the opposite effect (132, 133). Taken together, these findings support a model in which the coordinated directions

of mechanical tension—which is needed for persistent migration—control the local activation of Rac through focal complexes. This activation may result in extension or retraction of lamellipodia, which leads to further formation of adhesions and redirection of contractile forces.

In contrast to cell proliferation, cell migration appears to be favored at intermediate levels of spreading. In fact, the speed of migration exhibits a unimodal dependence on the degree of cell-ECM contact (134). This variation can be mimicked by culturing cells on substrates of varying stiffnesses. Cells migrate most on substrates of intermediate stiffness and may be induced to migrate in a specific direction by local stiffening of the substrate with a rigid tip (62). These effects may be regulated through FAK because FAK-null cells do not respond to local variations in the rigidity of the substrate and migrate less than do cells with wild-type FAK (78). How signals from FAK regulate the activation of Rac is unclear, although the inhibition of Rho by active FAK suggests that FAK may enhance signaling through Rac by inhibiting Rho and thus changing the magnitudes or directions of cell tension. Mechanical forces may thus provide a link between cell proliferation and migration through the assembly of FAs and focal complexes and through regulation of FAK and Rho family GTPases.

MECHANOTRANSDUCTION AT CELL-CELL CONTACTS

Cell-cell adhesions, similar to cell-matrix adhesions, are emerging as important players in mechanotransduction. Distinct types of cell-cell adhesions include AJs, tight junctions, and gap junctions, which are mediated by cadherins, occludins, and connexins, respectively (135–137). In some cases, especially with blood-borne cells, intercellular adhesions may be mediated by integrins (138). Among these different cell-cell adhesions, the AJ is perhaps one of the most important for transmitting mechanical signals directly to the actin cytoskeleton. The homotypic engagement of cadherins, a family of transmembrane Ca^{2+} -dependent adhesion molecules, initiates the formation of AJs and recruits scaffolding proteins that anchor the actin cytoskeleton (139).

Similarities Between Focal Adhesions and Adherens Junctions

Focal adhesions and AJs exhibit many striking similarities (Figure 1). First, both consist of dense clusters of transmembrane receptors that attach the cell to the external environment. Second, both provide a highly dynamic and responsive mechanical link to the actin cytoskeleton. Third, the architecture of both FAs and AJs consists of a large number of signaling and structural molecules that cluster at the junction through multiple, redundant protein-protein interactions. Several of these components, including α -actinin, Arp2/3, zyxin, moesin, and vinculin, are shared by both types of adhesions (140).

These similarities have motivated the idea that, as in FAs, mechanical force regulates the formation of AJs and their downstream signaling. In contrast to FAs,

however, where the role of cytoskeletal tension in regulating adhesion formation is clear, the response of AJs to tension remains controversial. For instance, increasing Rho GTPase RhoA-mediated cytoskeletal tension can change the morphology of AJs and increase the permeability of cell-cell junctions (141, 142), whereas increasing sphingosine-1-phosphate-mediated cytoskeletal tension appears to stabilize AJs and decrease permeability (143). Similarly, it has been reported that disruption of the cytoskeleton can disturb AJs, leading to loss of cadherins from cell-cell junctions (144); however, we have found that pharmacologically disrupting the cytoskeleton may have no effect on the localization of cadherin (60). Some of these differences may be attributed to differences in experimental conditions and in the particular cell types studied, but no consistent picture of the interaction between mechanical forces and AJs has emerged. Not surprisingly, experiments to determine the effect of tension on AJs often lead to simultaneous changes at FAs that complicate interpretation of data.

Does Force Regulate Assembly and Signaling at Adherens Junctions?

The ability to quantitatively measure force at cell-cell junctions may help reconcile some of the findings and shed light on the role of tension at cell-cell junctions. Although the existence of endogenous tension at cell-cell contacts has not been demonstrated, cadherin-based adhesions are clearly capable of supporting large forces. Suspended cells expressing E-cadherin will adhere to each other and require >100 nN of force to separate them (W.A. Thomas, personal communication). Likewise, the compaction of epithelial layers during morphogenesis generates substantial compressive force (145, 146).

The localization of components to AJs may be driven by tension. One of these components, vinculin, binds various cytoskeletal proteins, including actin, α -actinin, talin, paxillin, VASP, and protein kinase C (147). Vinculin plays a key role in coupling FAs to the cytoskeleton, and the amount of vinculin localized to FAs correlates with the amount of local tension (15, 63). Whether vinculin in AJs plays a similar role, however, is unknown. Another component of cell-cell junctions is β -catenin, which binds along with several adaptor proteins to the cytoplasmic domains of cadherins (148). When localized to AJs, β -catenin plays a structural role in linking cadherin to the actin cytoskeleton. When phosphorylated on tyrosine, however, β -catenin can dissociate from AJs and translocate to the nucleus, where it interacts with the TCF/LEF family of transcription factors to regulate the expression of genes such as *cyclin D* and *c-myc* that regulate cell cycle progression (149–151). Tension at cell-cell junctions may play a role in stabilizing AJs and controlling downstream proliferative signaling pathways by regulating the fraction of β -catenin that is bound to AJs or freely diffusing in the cytoplasm.

Although direct evidence for the effects of mechanical force on AJs remains elusive, evidence for the effects of cadherin engagement on cytoskeletal organization and cell function is accumulating. Perhaps the clearest indication of the regulation

of cell structure by cadherins lies in the requirement for expression of cadherins to define apical and basolateral domains in the formation of differentiated epithelial monolayers (27, 152). It has also been shown that cadherin-mediated cell-cell contact decreases cell-ECM adhesion. For example, increasing the density of cells on a substrate leads to increased intercellular contact and decreased cell spreading on the substrate, a phenomenon described as contact inhibition of cell spreading (60, 83, 153). Many studies have also suggested that cadherin engagement inhibits proliferation or contact inhibition of proliferation: For example, exogenous expression of VE-cadherin in Chinese hamster ovary cells results in decreased growth rates, whereas blocking the function of VE-cadherin leads to increased proliferation (154). Similarly, transformed cells that lack a variety of components of AJs exhibit higher rates of proliferation than when they are transfected with N- or E-cadherin (155, 156). Recent evidence suggests that the engagement of cadherins blocks proliferation by increasing p27kip1 levels and inhibiting cell cycle progression into S-phase (157).

We have used a lithographic method to demonstrate that, when preventing contact inhibition of cell spreading by holding cell-matrix contact constant, cadherin-mediated cell-cell contact no longer inhibits proliferation but instead acts as a stimulatory signal for cell proliferation in vascular cells (60, 83). Moreover, this signal requires intact signaling through MLCK, the Rho pathway, and PI3-kinase. In cells that are allowed to spread freely in this system, cadherin-mediated inhibition of cell spreading is restored and proliferation is arrested. These results imply that AJs can generate two opposing signals that affect cell proliferation: an inhibitory signal involving effects on cell spreading and a stimulatory signal through cell-cell contact-mediated increases in intracellular tension. In most previous experiments with mass cultures, the antiproliferative effects of reduced cell spreading (and reduced tension at FAs) presumably overrode the effects of additional tension generated at AJs and resulted in lower proliferative rates. When cell spreading is held constant, however, the effect of AJ-mediated tension is to increase proliferation.

Recent studies indirectly suggest that cadherins can stimulate such tension by increasing Rho activity. Cadherin engagement appears to sequester a Rho inhibitor, p120 catenin (158, 159). Cadherin engagement also initiates signaling of Rac, a Rho GTPase that causes membrane ruffling (160). In all, while little data exist to provide a clear model of how mechanical forces might alter the structure or signaling of AJs, the engagement of cadherins appears to have dramatic effects on cytoskeletal organization and tension. Thus AJs are at least indirectly involved in modulating mechanotransduction, if not direct sites for such mechanochemical signaling.

Crosstalk Between Focal Adhesions and Adherens Junctions

Our results and those of others have consistently emphasized a link between signaling at FAs and AJs in controlling cell behavior. An emerging principle is that the formation of FAs and AJs results in antagonistic behaviors. For instance, increasing

cell-cell adhesion mechanically competes with and hence decreases cell-substrate adhesion (161, 162). Also, adhesion of ECM- or cadherin-coated beads to the cell surface leads to selective reinforcement of FAs and AJs, respectively (163). Thus the extent to which this interplay results from the redistribution of cytoskeletal tension to FAs and AJs or from the release or sequestration of soluble factors that interact with both types of adhesions is unknown.

FUTURE DIRECTIONS

Much work still needs to be undertaken to create a coherent consistent theory of mechanotransduction at cell-matrix and cell-cell contacts. Unsolved questions include the following: First, and most importantly, what is the nature of the mechanosensor in cells? Is it a stretch-sensitive protein (or group of proteins) that can signal only when its conformation changes? Is it the lateral clustering of membrane proteins at adhesions that occurs when these proteins experience a force parallel to the cell membrane? We believe that multiple proteins are responsible for mechanosensing in a cell. For example, stretch of a chain of proteins may alter all of their conformations and thereby alter their ability to bind, recruit, or modify other cytoplasmic proteins.

Second, how is the shape of a cell determined? In our experiments with culturing cells on microscale islands, we have consistently observed that cells will spread to fill an island if the island is small, but will not if the island is too large. Does the stiffness of the substrate determine when the cell “knows” to stop spreading? Does a cell keep spreading until its adhesions can no longer support the contractile forces exerted on them? Previous results have suggested that whether a cell is under isometric or isotonic tension plays a role in determining if it can spread farther (164). The inhibitory effect of FAK on Rho implies that there is a negative feedback loop that acts to stabilize intracellular tension. Might a cell spread only until the “correct” tension is reached? The majority of our experience with cell shape involves culture on a flat surface; how do we investigate these effects when cells are embedded in three-dimensional scaffolds?

Third, how do the different types of FAs, AJs, ECM, integrins, and cell-cell adhesion molecules interact? In addition, how do these different combinations manifest in different types of cells (transformed versus normal, human versus nonhuman)? Variations in results owing to the use of different cells or differently prepared substrates have rarely been addressed and are often rationalized as investigator-specific anomalies. Whereas the effects of different types of ECM on cell behavior are well known, other types of differences may play a larger role than previously suspected: Swiss 3T3 mouse fibroblasts, a cell type commonly used in studies of mechanotransduction, are favored because they form particularly large FAs. Removing serum from the culture media of these cells causes FAs to disassemble quickly, but this effect is not seen for some other cell types. To what extent, then, are data specific to a particular type of cell? Likewise, differences in how ECM proteins are adsorbed may lead to unexpected changes in the conformation

of adsorbed proteins that can influence the clustering of cell adhesion molecules (165). For instance, adsorbed cadherin fusion proteins do not elicit the same behaviors as native cadherins embedded in a fluid lipid membrane (C.M. Nelson, J. Tien & C.S. Chen, unpublished results).

Fourth, is the only purpose of cytoskeletal tension to drive the maturation of adhesions and to enhance adhesion-based signaling? The force generated by cells can not only alter intracellular structures but also had been shown to alter the structure and organization of the extracellular matrix (166, 167). Although we and others have focused nearly exclusively on examining the effects of tension generated by the actin-myosin cytoskeleton, other cytoskeletal systems such as microtubules and intermediate filaments play a part in determining the mechanical properties of a cell (1). It seems reasonable to expect that these less-studied systems may also affect mechanical signaling in cells (albeit indirectly, because complexes do not appear to mechanically couple these other filaments to adhesions). One hypothesis claims that microtubules act as elements to resist compressive forces in cells; depolymerization of microtubules should thus increase cytoskeletal tension, and mimic the effect of enhancing cell contractility, as suggested by some recent studies (168, 169). Transmission of mechanical force may also alter the shape of the cell nucleus and thereby regulate the nuclear import of transcription factors (170, 171). These forces may even direct the organization of chromatin and physically alter the accessibility of genes to transcriptional complexes (172). However, attempts to test these hypotheses have been limited by the lack of direct methods to measure intracellular forces.

In all, there are numerous opportunities for researchers to advance the study of mechanotransduction. Given that many breakthroughs have occurred hand-in-hand with the development of new tools to observe or to perturb cells, we expect that new methods to analyze cells in three dimensions with submicrometer resolution and over fine timescales should open new avenues of investigation in this area.

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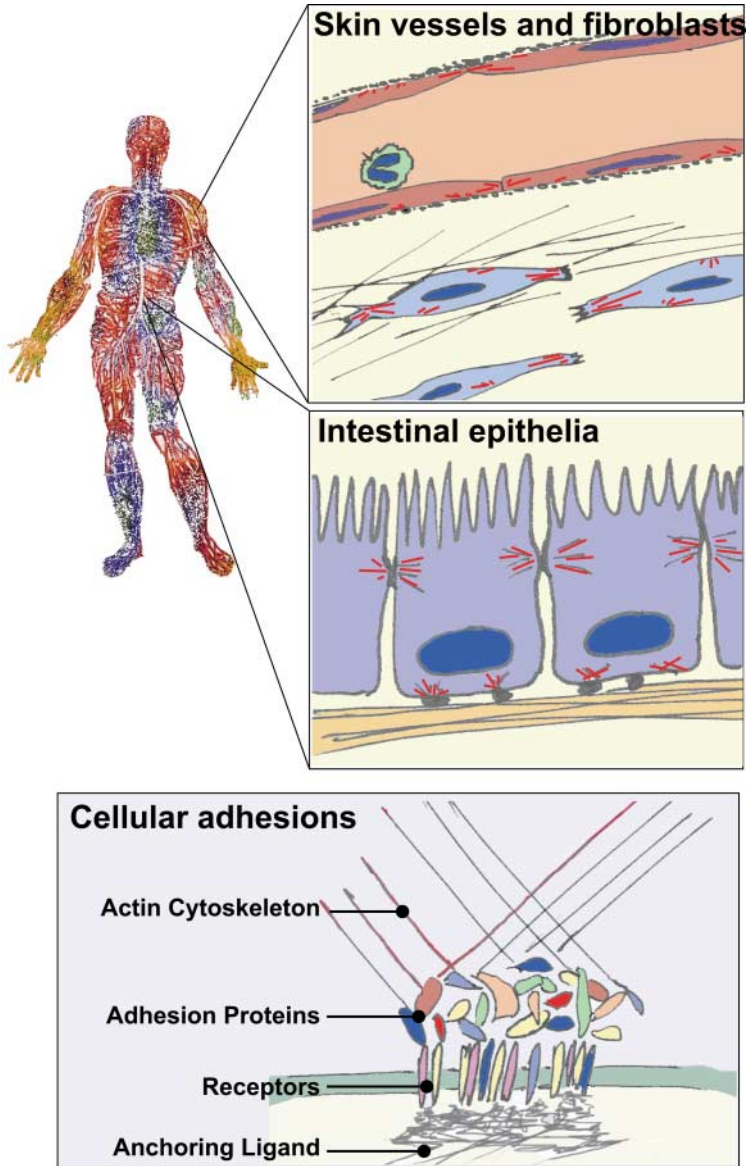


Figure 1 Transmission of mechanical forces to a cell may occur at cell-matrix and cell-cell adhesions. Mechanotransduction at mesenchymal cells relies primarily on cell-matrix contacts, whereas that in epithelial cells makes use of both cell-matrix and cell-cell contacts. All cells may be exposed to shear and hydrostatic stresses from surrounding fluid.

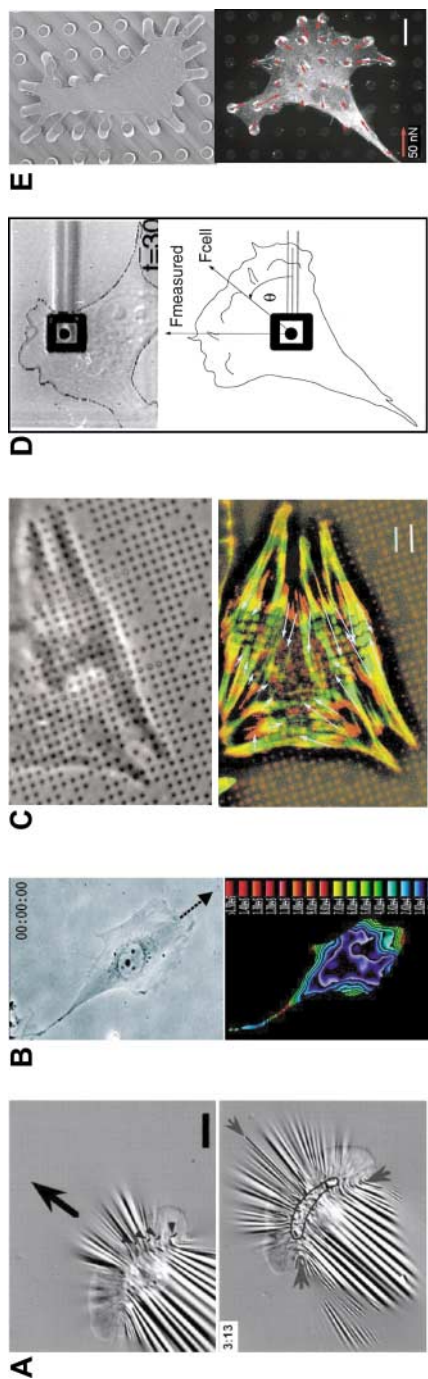


Figure 2 Techniques for the measurement of forces exerted by cells: (A) Contraction of a thin membrane by cells generates microscopic wrinkles in the membrane. Reproduced with permission from Reference 69. (B, C) Translation of beads or patterned markers embedded in an elastic surface yields a stress map of the entire cell body. Reproduced with permission from References 17 and 70. (D) Bending of a cantilever measures the force generated at a single area under a cell. Reproduced with permission from Reference 18. (E) Bending of an array of posts measures force generated at multiple locations across the cell body. Reproduced with permission from Reference 63.



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