

Chapter 2

Substrate Elasticity as a Probe to Measure Mechanosensing at Cell-Cell and Cell-Matrix Junctions

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This chapter is part of Section I: Mechanisms of Cell Adhesion and Mechanotransduction

Abstract In vivo, most cells are mechanically and chemically connected to other cells or to a variety of polymeric networks generically called the extracellular matrix (ECM). Adhesive contacts are formed by distinct classes of transmembrane protein complexes that have specific binding sites for extracellular targets on one side of the membrane and cytoplasmic domains that engage specific elements of the cytoskeleton and signal transduction systems. Engagement of cell-cell or cell-matrix contact both initiates and depends on mechanical signaling from inside and outside the cell, but also depends on the forces generated at the cell-cell or cell-ECM junction. This chapter will summarize some recent studies of mechanotransduction at cell adhesion sites and present examples of the interplay between cell-cell or cell-matrix contacts in fibroblasts, endothelial cells, cardiac myocytes, T lymphocytes and other cell types.

2.1 Introduction

With a few exceptions such as erythrocytes, nearly all cells in multicellular organisms are bound either to other cells or to extracellular matrices. Even single cell organisms such as yeast and bacteria form contacts between soft inner membranes and stiffer outer coats, or to the cells they invade. Such contacts are not only adhesive, they are also focal points for concentrating signaling proteins and lipids and help organize the architecture of the plasma membrane and the cytoskeleton. At the center of such contacts is one of several large classes of transmembrane proteins, bound at the outside to the matrix or to another cell and at the inside to a number of cytosolic proteins that bind either cytoskeletal elements or signal transduction intermediates (Fig. 2.1). Not all signaling that initiates at adhesion sites is triggered by ligation of the transmembrane receptors alone, and in recent

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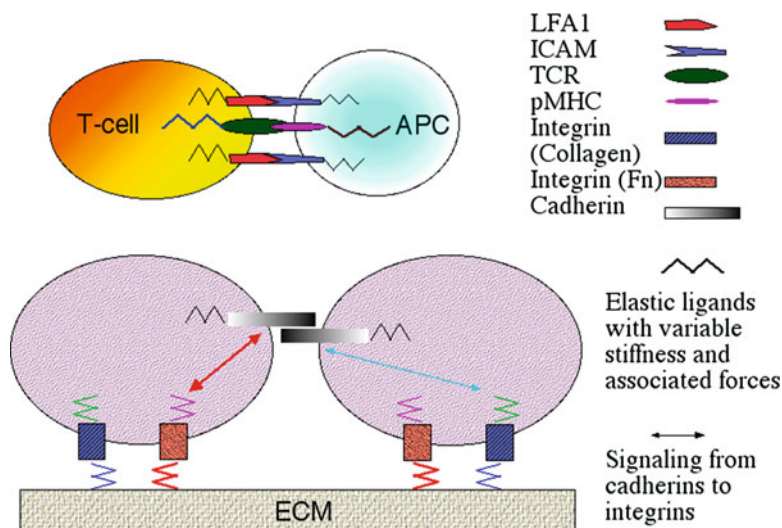


Fig. 2.1 Cell-cell and cell-ECM adhesion complexes. Various transmembrane proteins with extracellular domains specific for ligands expressed either at cell membranes or the extracellular matrix are linked to elastic elements with different mechanical properties depicted as springs with different stiffness

years it has become clear that forces generated at or applied to sites of cell-cell or cell-matrix contacts have important effects on cell structure and function on both the single cell [1, 2] and whole tissue level [3]. Transmitting force within the cell and transducing force to biochemical reactions that engage intracellular signaling pathways are important aspects of mechanobiology, and recognition of external force or of resistance to internally generated force has been termed mechanosensing. This chapter will emphasize one aspect of mechanosensing, the response of cells to the stiffness of the material to which they adhere. Mechanosensing through cell-cell and cell-matrix adhesion complexes are distinct processes that are mediated by distinct subsets of proteins and respond to different levels of force, displacement, or stiffness. A few example of mechanosensing will be discussed in an effort to suggest unifying themes that might reveal the mechanism of these processes.

2.2 Mechanosensing by Cell-Matrix Adhesions

The importance of the mechanical properties of the extracellular matrix (ECM) for cell morphology [4], motility [5] and differentiation [6] has been known for decades and recently re-emphasized as an essential control parameter in vivo distinct from and coordinated with biochemical signaling.

Most cells adhere to extracellular matrices or to cell culture substrates using one or more of a large class of integrins, which are heterodimeric transmembrane complexes with variable affinity and specificity to ECM proteins such as collagen,

fibronectin and laminin. Some integrins bind cell surface proteins such as ICAMs to mediate cell-cell contacts that differ in structure and function from those formed by cadherins. Integrins are heterodimers consisting of an alpha and a beta sub-unit and the ability of an integrin dimer to bind a particular protein depends on the composition of the dimer. For instance $\alpha 1 \beta 2$ dimers primarily bind collagen whereas $\alpha v \beta 3$ dimers bind multiple proteins such as fibronectin containing the amino acid sequence RGD. In addition to having different extracellular binding partners, different integrins engage different cytoskeletal proteins and trigger different signaling pathways (Fig. 2.1).

Many of the first studies demonstrating the effects of substrate stiffness on the phenotype of cells bound by integrins to deformable surfaces were done using cultured fibroblast or epithelial cell lines [7], but also early passage primary cells such as vascular smooth muscle cells [8] and mesenchymal stem cells [9]. The results of early studies are summarized in recent reviews [10–12]. As the mechanical responses of more cell types have been studied, one pattern that emerges is that changes in properties such as spread area, cytoskeletal structure, proliferation, differentiation [13–15], or cell stiffness [16, 17] do not necessarily change linearly or even monotonically with substrate stiffness. Rather, these responses saturate at different substrate stiffnesses or show maxima or minima at intermediate stiffnesses that approximate those of the tissue from which the cells were derived [18]. For example, neonatal rat heart cells exhibit optimal morphology and function when cultured on collagen-coated gels with intermediate stiffness (10–20 kPa) that approximates the stiffness of adult rat myocardium [19] and neonatal rat ventricular myocytes also produce a more nearly native phenotype when the substrate stiffness is within this intermediate range [20–22].

Cell motility is also reported to reach a maximum at intermediate substrate stiffness in a study of neutrophil migration on gels coated with fibronectin [23], and in several cases cell motility is directed to regions of increased substrate stiffness, as shown in studies using gels with stiffness gradients [24–26].

In many cases the trends observed with increasing stiffness in 2D systems are also observed in 3D systems of the same elastic modulus such as the differential growth of neurons and astrocytes grown in fibrin [27, 28] or the stiffening of endothelial cells grown in collagen gels [29], but in other cases cell responses to simple linear elastic gels like polyacrylamide that are coated with adhesion proteins are different from those of the same cell type bound to or within a 3D network made of the same protein [30]. Differences in morphology of cells bound in 2D or 3D are also strongly affected by formation of dorsal cell-ECM adhesions. A rapid, substrate stiffness-dependent transition from a well-spread, flat morphology to an elongated bipolar or stellate morphology closer to the structure in vivo occurs when a second ECM surface is placed on top of fibroblasts initially cultured in 2D [31, 32].

Stiffness responses can depend strongly on the nature of the adhesive ligand and therefore the type of integrin that engages the substrate. For example, fibroblasts show a much stronger stiffness-dependent morphology when spread on substrates coated with both collagen I and collagen V than compared to surfaces coated with collagen I alone [33]. Melanoma cells increase spread area with increasing substrate

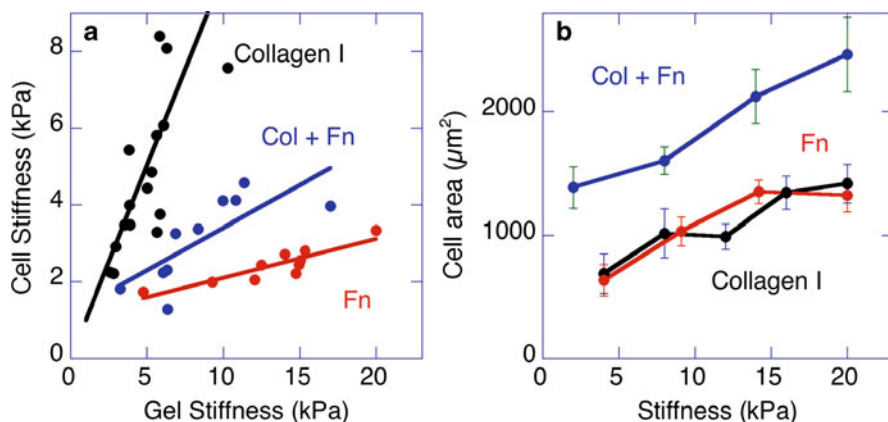


Fig. 2.2 Stiffness-dependent cell spreading and stiffening depends on the type of adhesion complex. Cellular stiffness measured by AFM (a) and adherent area (b) of A7 melanoma cells cultured for 24 h on polyacrylamide gels laminated with collagen I or fibronectin or a mixture of collagen I and fibronectin. Both proteins were added as saturating concentrations to the gels using methods described in [65]

stiffness whether the substrates are coated with collagen I or fibronectin, but only change their own stiffness to match that of the substrate when they adhere through collagen receptors [16]. Stiffening of cells bound by collagen receptors requires the function of the actin crosslinker filamin A, but spreading of cells bound by fibronectin receptors is evidently mediated by other cytoskeletal linkers [29, 34]. The specificity of different integrins and different integrin ligands in mechanotransduction from the ECM to the cell is illustrated in Fig. 2.2, where the stiffening and spreading of filamin A-expressing A7 human melanoma cells is compared on substrates with different stiffnesses that are coated with either fibronectin or collagen I. Stiffening (Fig. 2.2a) but not spreading (Fig. 2.2b) of A7 melanoma cells depends very strongly on whether integrins specific for fibronectin or collagen are engaged. When A7 cells are plated on gels coated with saturating amounts of either Fn or collagen I, they spread to approximately the same extent, but the cells on collagen I are much stiffer than those adherent to Fn. When both Fn and collagen I are present, allowing both $\beta 1$ and $\beta 3$ integrins to bind, adherent area increases, but cell stiffness reaches a value intermediate between those on Fn or collagen I alone.

Specificity in the mechanotransduction through different integrins has been observed in various other contexts. Using a spinning disk assay to apply shear stresses to cells, it has been shown that the residues flanking the RGD sequence influence how strongly the cells adhere to the substrate [35] and that adhesivity increases with cell spread area and time of adhesion [36]. When challenged by shear stress caused by fluid flow, osteoblasts adhere more strongly to fibronectin coated substrates than to vitronectin coated surfaces and least strongly to collagen coated substrates [37].

Quantitative measurements of the forces exerted or resisted by single clusters of integrins bound to specific ligands have emerged from studies using atomic force microscopy. In one study, a fibronectin coated polystyrene bead was attached to a cantilever and brought in contact with a smooth muscle cells for 2, 5 or 8 min and then withdrawn. Three characteristic release forces of 40, 55 and 80 pN were measured, and adhesion could be blocked by adding antibodies blocking the function of $\alpha 5 \beta 1$ integrins prior to adhesion [38].

In another study CHO cells were allowed to spread on an AFM cantilever and the cell-coated tip was used to probe a collagen-I coated substrate. In this study the time allowed for adhesion was varied from 200 ms to 10 min. The 200 ms time point was used to measure a force of 50 pN for binding of a single integrin that is not reinforced by other adhesion proteins or the actin cytoskeleton. Allowing the cells to adhere for an extended time increased the release force to 500 pN [39]. Surprisingly this larger adhesion force was not affected by inhibition of actomyosin contraction and appears to result from integrin clustering and formation of large adhesion plaques.

2.3 Mechanosensing Through Cell-Cell Adhesions

Mechanical control of cell-cell contacts is mediated by homophilic binds between cadherins or the immunoglobulin superfamily glycoprotein N-CAM or by heterophilic contacts between I-CAM and integrins such as LFA1 [40]. The importance of cell mechanics for the function of cell-cell contact through cadherins was demonstrated by studies showing how differences in effective surface tensions, mediated in part by cadherin-cadherin binding energy, can drive cell sorting [41, 42]. The resistance of cell-cell contacts to disruption by force, especially for cadherins, has been fairly extensively studied (e.g. [43]). Cadherins are clearly implicated in mechanosensing, for example by transmitting signals from the plasma membrane to distant sites within the cell [44] and by reacting to applied force by increasing local cell adhesion size [45], but mechanical signaling through cell-cell contacts or the response of these contacts to differences in stiffness are relatively understudied compared to analogous studies of cell-ECM adhesions.

At the neuromuscular junction in *Drosophila*, vesicle clustering at the presynaptic terminal depends on mechanical tension within the axons that leads to stress at the interface connecting the neuron to the muscle [46]. When this junction is mechanically broken, vesicle movements are randomized, but not prevented, and the axonal cytoskeleton is disrupted. Application of nN forces to the tip of the severed axon restores both cytoskeletal structure and anterograde bias to vesicle movements [46].

T lymphocyte activation has also been shown to be strongly dependent on force applied to the bond between the lymphocyte T-cell receptor complex (TCR) and the MHC-agonist peptide complex at the surface of an antigen-presenting cell [47, 48]. ICAM-LFA1 bonds and occupation of the TCR with an agonist peptide are each necessary but not sufficient for T-cell activation. The combination of both cell-cell contacts, along with force production by the lymphocyte on the

antigen-presenting cell surface is hypothesized to be required for full activation of the T-cell response. The mechanical resistance provided by an antigen-presenting cell, or a fixed substrate, to the activated TCR might be essential to prevent inappropriate triggering of T-cells by soluble ligands.

Quantitative measurements of the forces applied at sites of binding to cadherins and the dependence of cadherin signaling to the cytoskeleton on mechanical compliance are beginning to be elucidated using micropost and soft gel systems originally developed to examine cell-substrate effects. C2 myogenic cells cultured on polydimethylsiloxane (PDMS) microposts coated with an FC-N-cadherin construct that efficiently binds cellular N-cadherin developed stresses similar to those generated by the same cells bound to the posts through fibronectin receptors [49]. As the stiffness of N-cadherin-coated substrates increases, the size of the cell's cadherin adhesion also increases as does the magnitude of force generation [50]. As a result, the morphology of the cell ranges from well spread on stiff substrates (~ 100 kPa) to poorly spread and rounded on soft substrates (~ 10 Pa) regardless of whether the substrates were PDMS pillars or polyacrylamide gels [50].

Similar effects are also seen when neonatal rat cardiomyocytes are cultured on N-cadherin coated gels [20], although in this case there is a pronounced optimal stiffness at which these cells spontaneously elongate and form myofibrils, as seen in Fig. 2.3. On soft N-cadherin-coated polyacrylamide gels (shear modulus $G \sim 100$ Pa) neonatal ventricular cardiomyocytes exhibit a rounded morphology,

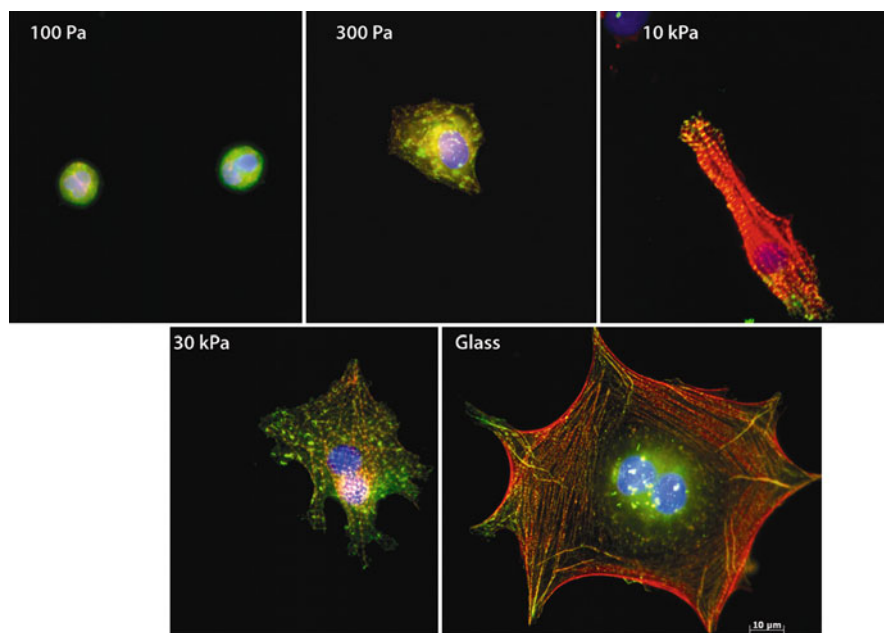


Fig. 2.3 Single cardiomyocytes on N-Cadherin-coated substrates of varying stiffness display differential morphology

with poor sarcomere organization. At physiological stiffness ($G \sim 5, 10$ kPa) cells exhibit striated F-actin and sarcomeric alpha actinin staining and a high aspect ratio. On stiff surfaces ($G \sim 30$ kPa, glass) cells exhibit prominent F-actin filaments devoid of striations and a polygonal shape.

The optimal stiffness resembles that of the native tissue and is similar to that first reported for myotubes using gels coated with ECM proteins [18]. Although the qualitative effects of cell-cell and cell-ECM adhesions are similar using substrates of different stiffness, there are significant quantitative differences that likely reflect the different signaling through cadherins and integrins, and the different spatial distributions of these transmembrane proteins in the native tissue.

2.4 Crosstalk Between Cell-Matrix and Cell-Cell Mechanical Signaling

Signaling, adhesion, and stiffness [51] at cell-cell and cell-ECM junctions are accomplished by different mechanisms and sets of proteins, but the two systems have significant influence on each other [52]. For example, when endothelial cells are cultured on ECM protein-coated gels, their morphologies depend very strongly on substrate stiffness as long as the cells are subconfluent. However, when the cells also make cell-cell contacts, actin bundles resembling stress fibers can form in cells that touch each other but not on single cells on soft substrates, as shown in Fig. 2.4. When endothelial cells become confluent, their gross morphology is no longer apparently dependent on the stiffness of the substrate beneath them [53].

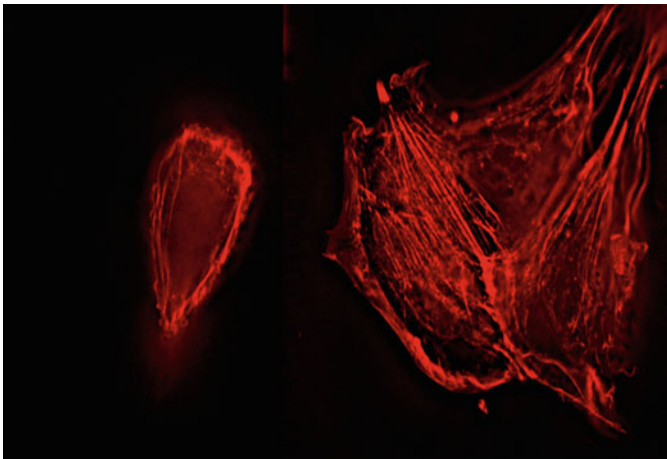


Fig. 2.4 Redistribution of actin cytoskeleton when cells on soft substrates make cell-cell contact. NIH 3T3 fibroblasts on soft gels (180 Pa) with F-actin stained by rhodamine phalloidin. The isolated fibroblast (*left*) appears to have no stress fibers. When the fibroblasts are able to make cell-cell contact (*right*), stress fibers form

In some cases activation of one class of contacts enhances formation of the other, and in other cases signaling between cell-cell and cell-ECM contacts appears to be antagonistic. For example, engagement of integrins increases the strength of E-cadherin junctions in cultured fibroblasts and carcinoma cell lines by a mechanism that involves the Src kinase [54]. Cell-cell contacts between epithelial cells can also increase the force applied to cell-ECM contacts, as observed when clusters of MDCK cells cultured on fibronectin-coated microposts apply greater traction force than individual cells even though only one cell is pulling on an individual post at a time [55]. Similarly, mesodermal cells from the developing *Xenopus laevis* embryos apply stronger tractions (25 kPa) when in small clusters (4–7 cells) than individual cells (18 kPa) [56].

On the other hand, spreading of epithelial cells on artificial substrates appears to depend on a competition between cell-cell and cell-substrate adhesion [42]. Engagement of E-cadherin decreases lamellipodial protrusions at adjacent sites of integrin ligation in epithelial cells [57]. In this case, the difference between integrin and cadherin signaling for protrusion of the leading edge of cells would control the directionality and persistence of cell migration [57]. In confluent epithelial sheets, force magnitudes and directions are more complex than can be inferred from analysis of local forces applied by single cells. Forces are distributed throughout a moving sheet of cells and not just at the leading edge. Cells throughout the sheet exhibit both pushing and pulling forces [58]. Coordinated motions on length scales much larger than a single cell and maintained over long times are observed to depend strongly on the stiffness of the substrate, even when the overall cell morphologies are similar [59].

Cross-talk between integrins and cadherins is likely to be essential for coordinated movements of cell sheets such as endothelial and epithelial surfaces to enable the intact sheet to move across an ECM while maintaining constant intact cell-cell boundaries [60]. Stiffness cues might be particularly important for development of vasculature and other multicellular structures, as suggested by the finding that traction forces and the structure of multicellular arrays of endothelial cells depended strongly on substrate stiffness, with soft substrates leading to lower traction forces but more robust formation of branching networks of cells that could develop into discrete tubes rather than flat sheets [61, 62].

The stiffness of the substrate and presumably the ECM also affects the crosstalk between cell-cell and cell-ECM signaling. When embryonic fibroblasts were cultured on collagen coated polyacrylamide gels of two stiffnesses 2.7 kPa (soft) and 7.7 kPa (stiff), cells grew on soft gels initially as isolated cells that aggregated and grew as expanding clusters, whereas on stiff gels cells remained scattered, begin to spread and eventually form branching clusters [63]. Whether cells remain clustered or scatter on substrates also depends on the type of ECM ligand and therefore the class of integrins. For example, epithelial cells adhering to fibronectin or laminin maintain intercellular adhesions while in contrast, if these cells attach to collagen, the intercellular junctions dissipate and the cells disperse [64], suggesting that dominance between cell-cell and cell-ECM signaling depends on both chemical and mechanical signaling.

2.5 Conclusions

Mechanosensing, as determined by the cell's reaction to forces applied at its surface and to the resistance that its surroundings exert on the forces the cell generates internally, has recently regained interest as an essential component of tissue formation and function. Cells contact each other and their extracellular matrices through numerous transmembrane protein complexes that link the cell interior and engage signaling pathways in highly distinct ways. Not all adhesion sites are equally responsive to forces, and the mechanical stresses generated at any particular site depend strongly on the mechanical properties of the system, but also on the vast array of other signals the cell receives from both chemical and mechanical stimuli at other sites. The development of new soft materials and imaging methods has now revealed that both cell-ECM and cell-cell adhesion receptors are mechanically sensitive and that one system can strongly influence the mechanical response of the other. One challenge for the future is to identify not only the proteins and signals involved in mechanosensing but also the physical principles that enable cells to measure stiffness and force with the precision required to use these inputs to control their fate.

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