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MECHANOTRANSDUCTION

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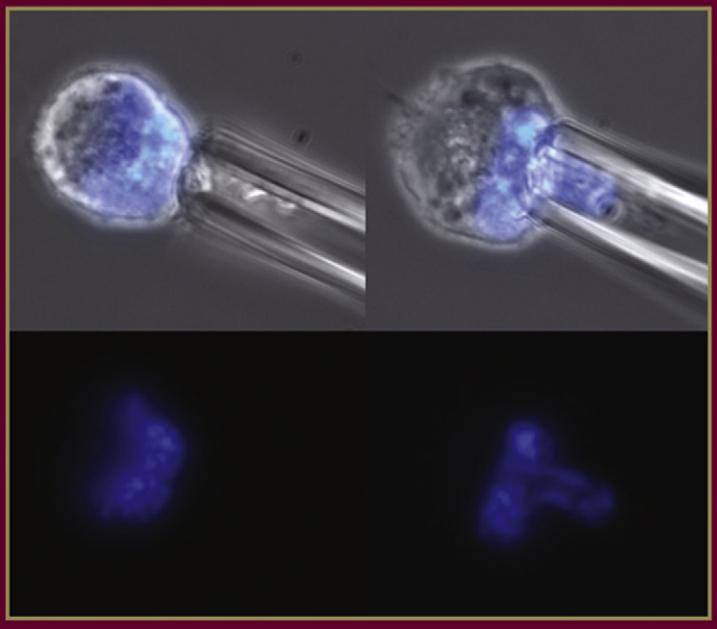

CHAPTER FOUR

Mechanosensation: A Basic Cellular Process

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Contents

Abstract

It has been shown that focal adhesion proteins are crucial for the ability of cells to transmit external forces and to generate cytoskeletal tension. Force transmission over considerable distances and stress focusing at the focal adhesion sites make them prime candidates for mechanosensors. Temporal and spatial changes in the cytoskeletal protein configuration due to mechanical stimulation have been detected and characterized by a wide range of biophysical techniques, including magnetic twisting, magnetic tweezer, traction microscopy, atomic force microscopy, nanoscale particle tracking, and many more. The combination of these techniques will help to understand force transmission and structural remodeling in cells under loading conditions. Force transmission

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and force sensing represent basic biological processes that are crucial for a variety of higher fundamental cell functions including cell division, motility, and differentiation that have implications in medicine and biology.

1. INTRODUCTION

1.1. Historical development

Julius Wolff hypothesized more than 100 years ago that bone adapts under mechanical load by remodeling itself. He proposed that the specific effect on bone structure depends on the duration, magnitude, and rate of loading.[1] As we know today, the remodeling of bone in response to loading is achieved via a cascade of different steps including mechano- and biochemical coupling, signal transmission, and cell response, which are part of the mechanotransduction pathway. Specifically, upon sensing a load, osteocytes regulate bone remodeling either via molecule signaling or direct contact. Osteo-progenitor cells, which may differentiate into osteoblasts or osteoclasts, are regarded as mechanosensors.

Cecil D. Murray laid down in the 1920s a formula that relates the blood vessel radius to the required minimum energy by the organism that is, larger vessels lower the energy required in pumping blood because the pressure drop in the vessels reduces with increasing diameter according to the Hagen–Poiseuille equation.[2] In a seminal study, West *et al.*[3] showed that the allometric scaling relation ultimately leads to the minimization of energy consumption in blood pumps. In a certain sense, frictional forces in the blood vessels therefore dictate the body size of a living being.

Today, Murray's formula has gained increasing use as a biomimetic design tool in the field of mechanosensing/-transduction. It is, for example, applied in the design of minimum mass vascular networks carrying a liquid healing agent to areas of damage in a self-healing material.

1.2. Mechanosensation/-transduction

A typical example of mechanosensing/-transduction can be found in sensory cells of the inner ear.[4–6] Here, so-called "hair" cells transduce the mechanical vibration of the inner ear fluid into an electrical signal that propagates to the brain. Specifically, at the apical surface of hair cells, stereo cilia form bundles, which are able to slide relative to one another when the bundle is pushed one way or the other. An adaption motor that moves

along the internal actin filaments, which are tethered to the ion channel, modulates the tension. When filaments slide relative to each other, a force is generated to the point where filaments are connected to the side of the stereo cilium. This force changes the conformation of a transmembrane protein that acts as an ion channel, causing it to open thus allowing the transient entry of calcium ions. The flux of cations initiates the electrical signal that eventually reaches the brain where it is perceived as sound. Details of force transmission to the ion channel of hair cell excitation are still unknown. This is a classic example of the many ways a cell can physically "feel" its surroundings.

Other mechanisms are only now being explored, including (i) conformational modification of intracellular proteins associated with transmission of external forces to the cell interior, leading to the modulation in reaction rates through a change in binding affinity; (ii) changes in the viscosity of the cell membrane, altering the rate of diffusion of transmembrane proteins and consequently their reaction rates; and (iii) direct transmission of forces to the nucleus, affecting expression of specific genes. These mechanisms are less well understood than mechanosensitive channels, and it is likely that other mechanisms exist that have not yet been identified.[7–11] Although the detailed mechanisms remain undeciphered, the consequences of force applied to cells are well documented.[12–19]

1.3. Effects of extracellular matrix stiffness

There is growing evidence that the link between the mechanical properties of the extracellular environment and cellular decision-making mechanotransduction processes are important. Our current understanding of adhesion-mediated environmental sensing is still fragmentary and several design principles have emerged from experiments. For example, surface chemistry, namely, the presence of diverse matrix proteins, has a strong effect on the selection of specific integrin receptors and, consequently, on the initial assembly of the integrin complexes. The mechanical properties of the extracellular environment play a much more important role in cellular behavior than originally thought. It has been shown that cells (i) more strongly upregulate the cytoskeleton and cell–matrix adhesion on stiffer substrates, and (ii) locomote in favor of stiffer or strained substrates, and that (iii) extracellular mechanical properties and cellular decision-making are connected to the internal force developing at cell–matrix contacts due to cellular actomyosin contractility.[20,21]

In recent years, there has been an increased effort to study the effect of externally applied forces on cells. Several different experimental techniques have been applied for this purpose, which include magnetic twisting cytometry, magnetic/laser optical tweezers, atomic force microscopy (AFM), cell poking, rheology, and micropipettes.[22–27] These studies showed that there is a strong correlation between aggregation of cell–matrix contacts, build-up of forces, and triggering of certain signaling cascades determining physiologically important processes, including cell division, migration, and apoptosis (programmed cell death). In particular, there is a close relation between the proper functioning of cell–matrix contacts and certain diseases, such as cancer.[28,29]

Cells may sense anisotropic mechanical properties of the matrix and orient themselves accordingly. Future experiments should, therefore, focus on the relation between structural versus mechanical cues for cell organization in hydrogels, while modeling is needed to account for the mechanical (in particular, viscoelastic) properties of hydrogels.[30] Some evidence now indicates that integrin-based cell–matrix contacts act as local mechanosensors that change mechanical information about the environment directly into cellular decision-making answers. It has been suggested that upregulation of cell growth due to matrix contacts in a stiffer environment might originate from the fact that it is triggered by a threshold force. A similar viewpoint is that growth of cell–matrix contacts is faster on stiffer substrates. To test this hypothesis, correlation studies of the growth of cell–matrix contacts and cellular organization are needed, for example, studying areas close to substrate boundaries, where cells can amplify the mechanical input provided by boundary-induced strain through active mechanosensing. Quantitative data about the growth behavior of cell–matrix contacts will allow for further refining of models possibly also including modeling of cellular features, such as morphology and force pattern.[31–34]

1.4. Stress generated by external compression/contractility

The signal transduction pathways that are activated in response to mechanical force include many components and elements that are shared by other signaling pathways. For instance, mechanotransduction in cardiomyocytes is particularly complex, in that individual muscle cells both respond to externally applied mechanical forces and generate internal loads that are transmitted to adjacent cells and to the surrounding extracellular matrix (ECM).[35,36]

Cells in a prestressed environment have been reported to orient perpendicularly to the axis of compression/extension.[37] This is regarded as an effect of strain avoidance parallel to this axis. Vice versa, a cell can exert dipole-like forces on its surroundings, which can be mechanically transmitted to far distances. A neighbor cell will, therefore, upregulate its contractile apparatus aiming at an alignment along the same direction. This scenario constitutes a positive mechanical feedback loop for cell alignment. At low cell densities, a common pattern for the organization of elastically interacting cells will therefore be the formation of strings of cells, similar to the case of electric dipoles.[9,38–40]

1.5. Stress generated by cell contractility

Forces exerted by mechanically active cells on the environment are mainly due to actomyosin contractility. The cells involve nonequilibrium processes that are tightly regulated by biochemical events inside the cell. Actomyosin contractility is the basis of cell mechanical activity. However, thus far it has not been possible to reconstitute actomyosin contractility in vesicular systems. This is different for the formation of adhesion plaques, which have been reconstituted with lipid vesicles carrying "sticker" and "repeller" molecules that adhere to ligand-coated substrates. Several theoretical studies have been devoted to the possible mechanisms driving plaque formation, including elastically and entropically induced interactions. Motivated by these experiments with elastic substrates, we investigated whether a similar description can be employed for cells. We asked what kind of information a cell can extract from its environment using its contractile machinery (Fig. 4.1).[18,19]

1.6. Biological relevance of external and internal stress

Various forms of force application, whether transmitted via cell membrane adhesion proteins (e.g., integrins and cadherins) or by the effects of fluid shear stress, transmitted either directly to the cell membrane or via the surface receptors elicit a biological response. Known responses to force can be observed in a matter of seconds, such as in channel activation, but can continue for hours after the initiating event, for example, as changes in gene expression, protein synthesis, or morphology. Many signaling pathways that mediate these cellular responses have been identified and have been extensively reviewed.[5,15,41,42]

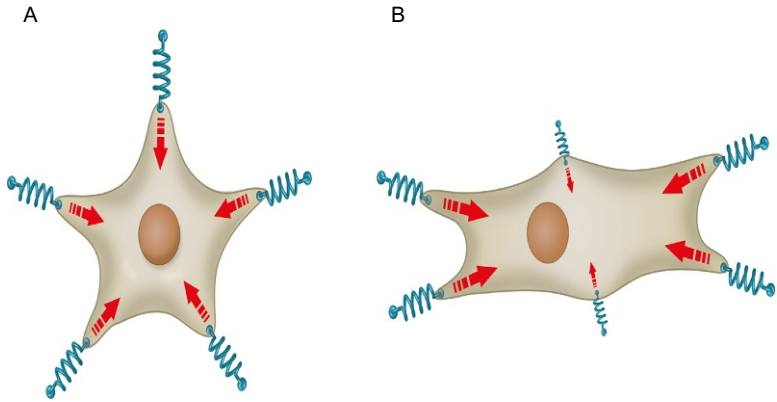

Figure 4.1 An adherent cell actively pulls on its environment through cell–matrix contacts. The cell orients itself in the direction of maximal stiffness of the environment by active mechanosensing. The local elastic environment is represented by linear springs. (A) In an isotropic environment, all spring constants are the same, growth at different contacts is similar, and the cell does not orient. (B) If spring constants are larger in one direction, corresponding contacts outgrow the others and the cell orients in the direction of maximal stiffness of the environment.

The range of stresses (force per area), to which different tissues are naturally exposed, is huge. Cytoskeletal structures are not only responsible for passively providing material strength, but they are also intimately involved in the sensing of external forces and transmitting those forces. How cells respond to mechanical stress depends not only on specific molecular sensors and signaling pathways but also on their internal mechanical properties or rheological parameters. These material properties determine how the cell deforms when subjected to force.[11,43–46] It is assumed that different structures and mechanisms are responsible for mechanical sensing. For example, cartilages typically experience stresses on the order of 20 MPa, and individual chondrocytes alter their expression of glycosaminoglycans and other constituents as they deform in response to such large forces.[47] On the other hand, endothelial cells undergo a wide range of morphological and transcriptional changes in response to shear stresses less than 1 Pa, and neutrophils activate in response to similar or even smaller shear stresses.[48] Not only the magnitude but also the geometry and time course of mechanical perturbations are critical to trigger specific cellular effects. Some tissues, for instance, tendons or skeletal muscle, experience or generate mainly uniaxial forces and deformations, while others, such as the cells lining blood vessels,

normally experience shear stresses due to fluid flow. These cells often respond to changes in stress or to oscillatory stress patterns rather than to a specific magnitude of stress.[49,50]

Many cells, including endothelial blood vessel and epithelial cells in the lung, experience *large-area-dilation* forces, and in these settings, both the magnitude and the temporal characteristics of the force are critical to cell response. For example, in vascular endothelial cells, mechanosensing is believed to control the production of protective ECM,[40,51] whereas in bone, mechanosensing is at the basis of bone repair and adaptive restructuring processes.[52] Osteocytes have been studied *in vitro* after extraction from the bone matrix in parallel plate flow chambers. The sites for mechanosensing might be those where strain is high if some large distortion of the sensing element is required to create a signal, in other words, if the sensor is "soft." On the other hand, the sites for sensing might also be those where stress is focused and where little strain occurs if the sensing element requires a small distortion, or is "hard," and functions by having a relatively high force threshold. To understand the mechanobiology of the cell requires a multiscale biophysics view. Externally applied stresses or traction forces are transmitted through focal adhesion (FA) receptors and are distributed throughout the cell, leading to conformational changes, phosphorylation events, and enzymatic activities. In addition, individual mechanosensing proteins may change their binding affinities.

There are also many examples of mechanotransduction that lead to disease forms: (i) arteriosclerosis, i.e., the hardening and narrowing of the arteries, mainly causing shear flow changes, in which endothelial cells sense the level of stress and regulate their behavior concomitantly;[53] (ii) arthritis, i.e., an inflammation of the joints, in which pressure increases are sensed by resident cells;[54] (iii) asthma, i.e., a common inflammatory disease of the airways, in which epithelial cells react to trans-epithelial pressure;[55] and (iv) polycystic kidney disease, i.e., a cystic genetic disorder with massive enlargement of the kidney.[56] All these processes (i–iv) are mediated by an array of signaling cascades that are started by shear stress.

In cell–matrix and cell–cell adhesions, cluster lifetime is usually much longer than the time scale for changes in loading. At the experimental stage, it would, therefore, be helpful to examine FAs as potential mechanosensors. One intriguing possibility is that force at FAs could lead to mechanical opening of domains in certain FA proteins. This might result in certain signaling events leading to the recruitment of additional bonds. If this information

could be validated in an independent experiment, it would give support to the notion that FAs (or parts of them) are regulated to be close to critical thresholds, a property which is known from certain signaling pathways.

2. FOCAL ADHESIONS

FAs are large, multiprotein complexes that provide a mechanical link between the cytoskeletal contractile machinery and the ECM. FAs exhibit mechanosensitive properties. They self-assemble and elongate upon application of pulling forces and dissociate when these forces are decreased.[57] A thermodynamic model for the mechanosensitivity of FAs has been proposed. Molecular aggregates, subjected to pulling forces, tend to grow in the direction of force application by incorporating additional molecules.[9] It was demonstrated that this principle is consistent with the phenomenology of FA dynamics and that FA protein aggregates exhibit distinct modes of assembly under force and different regimes of FA assembly, including growth, steady state, and disassembly.[58]

A mechanosensitive behavior of FAs is an important component of cells' ability to spread and move along substrates. The basic observation underlying FA mechanosensitivity is that alterations in the mechanical force applied to these adhesion sites, either by the contractile machinery of the cell or after an external perturbation, have a dramatic effect on FA properties. The effect of external forces on cell–matrix contacts is believed that FAs act as mechanosensors, converting force into biochemistry.[59] Today, it is common practice to monitor FAs in real time and in live cells by using fluorescence constructs for one of the many proteins localized in FAs.[60] In many situations, mature adhesions are characterized by additional active processes, which further increase the mechanical load of the FAs, namely, the build-up of so-called stress fibers.[61] The force-generating activity of the molecular motors requires ATP and is activated by signals from the FAs, in particular, by the small GTPases from the *Rho* family. Stress fibers end in FAs, which are elongated in the direction of the stress fibers. In Fig. 4.2, we show an image of a keratinocyte (A) and a schematic representation of the system of FAs and stress fibers (B), which is characteristic for mature cell–matrix adhesion at substrates.[61,62] FAs are usually connected to stress fibers and are elongated in this direction. Recent observations claim a linear correlation between internal force and size of FAs.[63] Since size in turn correlates with signaling, this indicates that FAs could act as mechanosensors, which convert force into signaling. In other studies, it was also shown that FAs act as mechanosensors with regard to

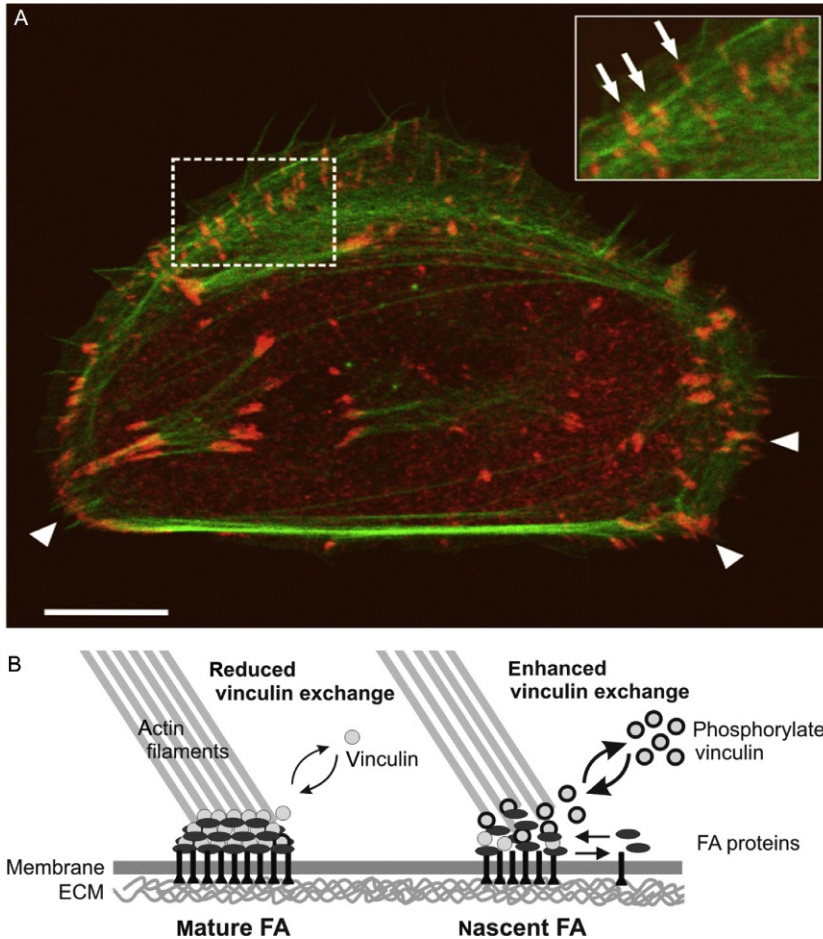

Figure 4.2 (A) Fluorescent staining of a migrating keratinocyte (actin = green; vinculin = red). Nascent adhesions develop in the lamella behind the leading edge and exhibit an elongated shape (inset, arrows). The lamella contains a loose actin meshwork consisting of small bundles that are mostly oriented parallel to the leading edge and perpendicular to focal adhesions (inset). Mature focal adhesions are mainly located at the rear of the cell and are typically bigger than nascent adhesions (arrowheads). Scale bar = 10 µm. (B) Model for vinculin exchange-dependent adhesion maturation. In nascent FAs (right), mainly phosphorylated vinculin is present and bound with high exchange dynamics within the complex. In mature FAs (left), there are stable structures that can resist strong tractions.[61] *With permission from Wiley Press.*

external force, that is, after quantitative analysis of elastic extracellular substrates.[51] Further quantitative evaluation of adhesion experiments should be done to better understand the detailed mechanism of the mechanosensor at FAs.

2.1. Mechanotransduction/-signaling

The process of mechanotransduction refers to cellular mechanisms by which load-bearing cells sense physical forces, transduce the forces into biochemical signals, and generate appropriate responses leading to alterations in cellular structure and function. The signal transduction pathways that are activated in response to mechanical forces include many unique components as well as elements shared by other signaling pathways. Mechanotransduction in both atrial and ventricular cardiomyocytes, for instance, not only regulates the *beat-to-beat* cardiac performance but also profoundly affects the proliferation, differentiation, growth, and survival of the cellular components that comprise the human myocardium. Intrinsically generated and externally applied mechanical forces are transmitted bi-directionally to internally situated sarcomeres of the rod-shaped cardiomyocytes. Attachment is clearly one way in which costameres and FAs contribute to mechanotransduction. There is also evidence that mechanical forces (generated by passive stretch and active tension development of cardiomyocytes) are "sensed" by costameres and FA complexes and transduced into biochemical signals, leading to sarcomeric assembly and altered gene expression. Understanding the cellular and molecular basis for mechanotransduction is, therefore, central to our overall understanding of cardiac structure and function in the normal and diseased heart.[64]

2.2. Focal adhesion proteins

2.2.1 Vinculin

Vinculin is one of the major proteins of the submembrane plaque of FAs. It can be tagged with green-fluorescent protein (GFP) at its amino terminal. GFP-vinculin localizes and marks FAs with very high optical quality. One intriguing possibility is that force at FAs leads to mechanical opening-up of vinculin domains. Recent computer analyses have shown that subtle conformational changes may lead to protein activation.[65–67] Vinculin has binding sites on talin, normally buried within a five-helix bundle in the talin rod domain that can rotate out of the helix core when force is applied. Once degraded, this domain becomes accessible for binding. In the case of talin, it is likely that unfolding is not required, since the structure of the bound complex is consistent with a simple helix exchange from talin to vinculin. An alternative mechanism has also been proposed by other authors,[68] involving a breakdown of the rod domain in order to expose the vinculin-binding site. Vinculin residing in a "closed" conformation

can be mechanically triggered to reveal cryptic binding sites. Similarly, small conformational changes may also change the binding affinity or enzyme activity. For example, when protein binding occurs through hydrophobic site interactions, a conformational change could modify this function and potentially disrupt it totally.

Force transmission from the ECM to the cell interior occurs through a chain of proteins, located in the FA sites, that comprise an integrin–ECM protein bond (with fibronectin, vitronectin, and others), integrin-associated proteins on the intracellular side (paxillin, talin, vinculin, and others), and proteins linking the FA complex to the cytoskeleton.[63] In human foreskin fibroblasts, for instance, it has been shown that the amount of tension generated by a FA correlates directly with the FA size and with the amount of fluorescence of the FA adaptor protein GFP-vinculin.[63]

2.2.2 Zyxin

In other studies, the fluorescence intensity of GFP-zyxin, another FA adaptor protein, has been compared with the traction forces exerted by FAs in migrating fish fibroblasts. In contrast to the analysis with vinculin, the fluorescence intensity of GFP-zyxin in FAs demonstrated an inverse correlation with the generated traction stress in the respective FAs.[69,70] Using a 2D GFP-β3-integrin marker, the two examples of the changing intensities of the FA markers, GFP-vinculin and GFP-zyxin, showed dramatically that FAs are complex structures that require multiple functional parameters to describe their behavior, such as fluorescence intensity, traction forces, and FA mobility (also termed "*sliding*").

2.2.3 Talin

Another candidate for force sensing is talin, one of the FA major components, which undergoes a regulated conformational change upon its interaction with vinculin.[59,71] Talin is essential for early FA reinforcement under force that leads to the recruitment of vinculin, which stabilizes FAs. Both talin and vinculin can exist in closed and open conformations, a fact which might point in the direction of a *mechanosensor* function at FAs.[72] The incubation of cells in serum-free medium greatly reduced the size and intensity of GFP-paxillin spots at cell edges, whereas the "pipette" shift induced formation of typical focal contacts elongated in the direction of pulling.[73] Force-induced formation of focal contacts was accompanied by the recruitment of talin, vinculin, paxillin, and actin and the elongation in interference reflection microscopic images. These results indicate that focal contacts induced

by external force have the same structural characteristics as those produced by cells during spreading and locomotion.[73] The force-driven opening of membrane ionic channels may contribute to their mechanosensing. Talin and vinculin might also act as nucleators[74,75] for the actin cytoskeleton, thus locally modulating the effects of the small GTPases *Rac* and *Rho*.

2.2.4 Paxillin, Pyk2

When extracellular tension is reduced, FA sites lose the ability to recruit paxillin and detach from the relaxed substrate, suggesting that continuous generation of intracellular tension (and hence high-density FAs) is required to maintain mechanical signaling. Force-induced changes at adhesion sites visualized in live cells by expression of GFP-paxillin were similar to those visualized by GFP-vinculin fluorescence. Adaptor proteins that are stacked on top of each other within the actin backbone of FAs, such as vinculin and paxillin, represented markers of the FA volume.

Although Pyk2 is predominantly localized to the cytoplasm, a minor component of the enzyme colocalized with paxillin in FAs of cultured neonatal rat ventricular myocytes. Pyk2, like focal adhesion kinase (FAK),[76–78] acts as an important scaffolding protein and transduces signals from G-protein-coupled receptors to downstream MAPK signaling pathways depending on which signaling kinase and adaptor protein binds to the phosphorylated enzyme.[79] Pyk2 has also been shown to link a variety of stressful stimuli, including Ca^{2+} overload, UV irradiation, and tumor necrosis factor treatment to MAPK activation in several cell types. Hirotani *et al.*[80] demonstrated that Pyk2 is an essential signaling component in endothelin- and phenylephrine-induced cardiomyocyte hypertrophy, perhaps acting via the Ca^{2+}- and/or PKC-dependent activation of Rac1.

2.2.5 p130Cas

Comparing stretched to nonstretched cells, FA proteins bind preferably to stretched cytoskeletal networks. Binding of vinculin, p130Cas, and PKB/Akt to actin were all found to be enhanced under stretching. Phosphorylation of p130Cas is achieved by nonreceptor tyrosine kinase (*SRC*) substrate.[81–84] It is interesting to note that the actin cytoskeleton also features crosstalk to the microtubule system when stretched.[85]

2.2.6 Focal adhesion kinase

It has recently been demonstrated that the mechanically stretched, Triton-resistant cytoskeleton of fibroblasts engages signaling molecules such as

paxillin and FAK, to stretched FAs.[86] These signaling molecules are recruited to FAs and not to the actin cytoskeleton extended between them. This is of particular importance because it suggests that the mechanical distortion of FAs itself is at the origin of mechanical signaling. However, it has to be shown that the mechanical distortion of integrin receptors or the specific adaptor proteins such as FAK or paxillin, which extend between integrins and the actin cytoskeleton, is involved in mechanical sensing.

Although physical concepts, such as force and elasticity, are essential to understanding active mechanosensing at FAs, the biochemical aspects of these systems are equally important and far from understood. FAK is a protein tyrosine kinase, which has been shown to be a key component of mechanosensing at FAs. It is activated by integrin ligation and one of its main downstream targets is the small GTPase *Rac*, which leads to reorganization of the actin cytoskeleton into an isotropic network structure. At the same time, FAK-activation downregulates another small GTPase, *Rho*, mainly through activation of p190*Rho*GAP.[87] *Rho* promotes the reorganization of the actin cytoskeleton into stress fibers and it often has an antagonistic role to *Rac*.[88] Both small GTPases belong to the *Rho* family and are also activated by pathways involved in cell survival (epidermal growth factor) and lysophosphatidic acid in the cases of *Rac* and *Rho*, respectively). These schemes focus on important downstream targets of integrin signaling to the actin cytoskeleton.

2.3. Force transduction at focal adhesions

Forces have been measured in resting fibroblasts, where intracellular tension gives rise to stresses in FAs of the cells adherent to flexible two-dimensional substrates.[63,89] The contractile forces are associated with intracellular molecular motors of the myosin family. Heidemann *et al.*[90] failed to observe this phenomenon in living fibroblasts, when they applied various mechanical disturbances to the cell surface through integrin receptors. They found that such disturbances produced only local deformations. However, the authors did not confirm the formation of FAs at the points of application of external loading, which is essential for load transfer between cell surface and the interior cytoskeleton.[44] Thus, their results remain controversial. The majority of data, however, indicate in cells, when a force is applied through integrin receptors at the cell surface, FAs are formed at the site of force application. Intermediate filaments appear to be important contributors to cell

contractility and prestress.[91] They serve as molecular "guy wires" that facilitate transfer of mechanical loads between the cell surface and the nucleus and stabilize microtubules. These observations provide evidence in support of the cellular tensegrity model.[92,93]

Other researchers have proposed conformational changes in intracellular proteins along the force-transmission pathway, connecting the ECM with the cytoskeleton through FAs as the main mechanotransduction mechanism.[94–96] In particular, the hypothesis that links mechanotransduction phenomena to mechanically induced alterations in the molecular conformation of proteins has been gaining increasing support. Stresses transmitted through adhesion receptors and distributed throughout the cell could cause conformational changes in individual force-transmitting proteins, any of which would be a candidate for force transduction into biochemical signals. The process by which changes in protein conformation give rise to protein clustering at FAs or initiate intracellular signaling, however, remains largely unknown.[97]

Consistent is the notion that FAs act as mechanical sensors of stress. Since FAs are membrane-attached anchoring points for the actin cytoskeleton, recent observations are consistent with a role for FAs in this process. For instance, in FAs of eukaryotic cells, transmembrane receptors of the integrin family and a large set of adaptor proteins form a physical link between the extracellular substrate and the actin cytoskeleton. During cell migration, nascent FAs within filopodia and lamellipodia make the initial exploratory contacts with the cellular environment, whereas maturing FAs pull the cell forward against the resistance of "sliding" FAs at the cell rear (Fig. 4.2).[61] Experimental approaches are available for analyzing the dynamics and interior structure of FAs. Analyzing FA dynamics using green-fluorescent protein-linked integrin led researchers to propose that the actomyosin-controlled density and turnover of integrins in FAs is used to sense the elasticity and spacing of extracellular ligands, regulating cell migration by mechanically transduced signaling.[98]

During recent years our knowledge about FAs and their role in cell spreading, migration, and survival has increased vastly. The ever-increasing number of proteins found to participate in FAs makes them one of the most complex protein aggregates formed in a cell. FAs fulfill mechanical and sensing functions that involve reversible anchorage of the actin cytoskeleton to the ECM during migration, monitoring intracellular or extracellular tension. Understanding the molecular mechanisms that account for these distinct functions of FAs is a major challenge.[62]

Eukaryotic cells have differently sized and shaped cell–substrate adhesion sites. In fibroblasts, the FAs are commonly referred to as focal complexes, focal contacts, and fibrillar adhesions. Many attempts have been made to classify FAs using descriptive features such as shape, size, cellular location, GTPase dependency, and protein composition. Unfortunately, some of these characteristics vary depending on the environment of the cells. The use of functional criteria to classify FAs according to their physiological role, for example, sensing the environment or providing mechanical support, give new definitions to distinguish focal complexes from focal contacts.

The recent use of chimeras comprising GFP attached to various FA proteins has made important contributions to our understanding of FAs. Owing to the stoichiometric fusion of GFP to FA proteins, such GFP chimeras can be used not only as markers for cellular attachment sites but also to provide dynamic and quantitative information about the composition of FAs. One of the emerging ideas from these studies is that FAs are mechanical transducing devices with a mechanical sensor function. The topology of FAs and their sensing ability for elasticity and spacing of extracellular ligands has been reviewed by Walcott *et al.*[99] Their model is based on the quantitative analysis of GFP-tagged FA proteins associated with the two-dimensional plane of the plasma membrane, providing dynamic insight into the interior structure of FAs.

In FAs, the actin cytoskeleton is linked through various adaptor proteins to heterodimeric receptors of the integrin family. In contrast to the analysis of GFP-vinculin, for instance, the fluorescence intensity of GFP-zyxin in FAs demonstrated an inverse correlation with the generated traction stress in the respective FAs. We are, therefore, led to believe that the different FA markers used in studies have distinct functions and are recruited by signals that might not originate from mechanical forces applied to FAs. Due to the complexity and multiple functions of FAs, it is very difficult to assign a specific cause to changes in the fluorescence intensities of any particular GFP marker. Hence, we opt for 2D GFP markers for quantitative analysis of tension-dependent changes in FA structure. These markers should serve a mechanical function and should be a part of the physical link between the ECM and the actin cytoskeleton.

It was shown that when a 2D GFP-β3-integrin marker is used to study FAs, the respective fluorescence intensity correlates directly with the packing density of this particular integrin in each FA. The analysis of GFP-β3-integrin in five different FAs of migrating melanoblasts revealed many important features: (i) FAs can be classified into low-density and high-density forms; (ii) FA density can change dramatically with time; (iii) high- and low-density contacts

are located in different cellular compartments; and (iv) only high-density FAs show mobility (sliding). The value of this complex information can be further extrapolated taking into account that low-density FAs form in response to the activity of the GTPases *Rac1* and *Cdc42* and high-density FAs form in a manner dependent on the GTPase *RhoA* and actomyosin contraction. This implies that, at least for β3-integrins, myosin-dependent actin cytoskeleton contraction is at the origin of the formation of high-density FAs. Owing to this mechanical link, either density changes in the actin backbone of FAs or changes in the spacing of extracellular ligands (e.g., induced by extracellular tension) will mechanically distort the link between integrins and actin-bound adaptor proteins.[100,101]

How do cells measure or sense the physical constraints of their environment? It is possible that mechanical sensing occurs inside FAs, considering that the sensing organelles of cells, namely, the filopodia and lamellipodia have low-density FAs that form in a *Rac1*- or *Cdc42*-dependent manner. It has been observed that, on soft substrates, FAs retract, whereas they are reinforced and maintained on a rigid surface, anchoring the cell for forward motion. In addition, brushing against a moving lamellipodium with a microneedle induces the maturation of lamellipodial focal complexes (low-density FAs) into focal contacts (high-density FAs). This maturation of FAs in response to extracellularly applied tension depends on *RhoA* activation and its downstream target Diaphanous (mDia).[102] Because mDia acts as an actin polymerization factor, the observed increase in size and density of FAs could be linked to increased amounts of polymerized actin.

Different fates of low- and high-density FAs with respect to the elasticity of the substrates have been demonstrated. The absence of mechanical signaling on soft substrates is due to the lack of physical distortion during the contraction of FAs. Different densities of FAs can also be extrapolated from the spacing of extracellular ligands. When cells rapidly spread and attach, they were unable to form focal contacts (high-density FAs) and stress fibers on this substrate. Attempts to examine the proteins of adhesion sites, which are believed to be responsible for surface sensing, have thus far focused primarily on the organization of FAs and related structures. The complexity of the ECM and the uncertainty that surrounds the state of exposure and reactivity of the adhesion mediating domains make it difficult to define the sensing mechanisms. Cellular interactions with such surfaces indicate the need to develop synthetic adhesive surfaces with well-defined structures. The physiological significance of space sensing and the mechanisms whereby the cells measure the particular interligand distance remains unclear.[62]

2.4. Protein crosstalk

Crosstalk between the actin cytoskeleton and the mechanoresponsive matrix sensing machinery clearly plays a crucial role in all types of integrin-mediated adhesions.[57] Thus, existing experimental data on mechanosensing in FAs are considerably more detailed than those on the sensory function of any other type of adhesion. It is worth noting that other types of integrin adhesions, such as podosomes and invadopodia, are also mechanosensitive. Thus, mechanical crosstalk between integrins and the actin cytoskeleton is a key feature of environmental sensing. The major features of the actin–integrin feedback network, as it is presently understood, have been discussed.[62,103] How these diverse molecular mechanosensing devices are indeed integrated into a single mechanosensing module remains a major challenge. Thermodynamic considerations suggest that the application of stretching force to an aggregate of protein subunits should promote the growth of the aggregate in the direction of force, irrespective of any conformational changes in the subunits.[104] FA mechanosensors might also be regarded as a network of tightly interconnected molecular mechanosensing units that operate in a coordinated fashion in response to mechanical forces. These forces might be applied externally and are usually transmitted by the actin cytoskeleton, thereby rendering the formation and maturation of FAs actin-dependent.

2.5. Cell signaling pathways

Mechanotransduction is viewed as a force-induced process initiating biochemical responses (e.g., changing binding affinity, altering phosphorylation state, and/or conformation change) and initiating signal pathways leading to gene expression, protein synthesis, and cellular phenotype change. Activation is started by mechanical stress via second messengers and gene expression. Other forms of mechanotransduction can be (i) stretch-activated ion channel activation, (ii) membrane mechanotransduction (via G-proteins and G-protein-coupled receptors), and various other proteins that connect to FAs/adherence junctions and the cytoskeleton. Shear stress on the membrane can influence the conformation of transmembrane proteins (stress in tension or bending) that leads to, for example, activation of MAPK, *Rac*, *Rho*, et cetera, or (iii) constrains autocrine signaling, for instance, stresses applied to a layer of airway epithelial cells grown on a porous membrane results in changes in gene expression, signaling, and ERK phosphorylation in lungs.[35]

2.6. Translation of information gathered at focal adhesions

Recent developments have shown how cellular forces are measured at the level of single FAs using a novel soft substrate technique. A correlation exists between force and size of FAs.[105] The mutual regulation of force and aggregation cannot proceed without limits, and recent work suggests that the upper bounds are set by the action of microtubules inserting into mature FAs and delivering some kind of stop signal.[100] In particular, cells can learn about the mechanical properties of their environment by monitoring the build-up of force at FAs while pulling on it (active mechanosensing). It has been shown that cells react in a typical way to the elastic properties of their environment, a phenomenon which has been termed durotaxis. Most cell types upregulate their cytoskeleton and their cell–matrix contacts on stiffer substrates, and locomote in favor of stiff or strained regions.[106] In principle, there are many different physical mechanisms that might be at work as mechanosensor at FAs. In fact, another recent study has shown that the aggregation response at FAs under force persists even for permeabilized cells without any plasma membrane.[107]

We have explored how cells behave in a soft environment.[19] It has been suggested that force-induced unbinding of fibronectin on the extracellular side and force-induced unbinding of certain cytoplasmic plaque proteins in FAs (e.g., vinculin) might be involved in mechanosensory processes for tissue cells.[51] At this stage, additional experimental evidence would be very helpful in modeling the mechanosensor at FAs.[103] However, one intriguing possibility is that force at FAs may lead to mechanical opening-up of domains in certain FA proteins such as talin, vinculin, and p130Cas.[59,82,84] In particular, it has been shown that application of external force leads to growth of FAs and, therefore, to strong signaling activity. Protein aggregation has been observed in mature FAs under internally generated force.[61,108]

The dynamics of FAs is also the subject of much current research.[109] Anchorage-dependent cells constantly assemble and disassemble FAs, thereby probing the mechanical properties of their environment. Initial FAs are local processes based on integrin clustering.

2.7. Focal adherence junctions

Cell–matrix adhesions are provided by large FAs, which can contain up to 100 integrin-mediated bonds, while an important part of cell–cell adhesions (focal adherence junctions (FAJs)) are provided by similarly large clusters of

cadherin-mediated bonds.[110] Molecular bonds in a cluster can be arranged and loaded in different ways, including in parallel and serial ways. When cells are experimentally probed, the situation is further complicated by relaxation processes in the viscoelastic parts of the cell that act as force transducers.[111] So far the interaction of a force dipole with the boundary has been considered. In an elastic sphere containing many cells, one could separate the contributions to the effective stiffness into a contribution from the boundary-induced field (i.e., a cell-surface interaction)[9,112] and a contribution from the elastic fields of other cells embedded in the sphere (i.e., a cell–cell interaction term). Further work has to be carried out to understand the complex manner of this interaction.

2.8. Measuring mechanotransduction/-sensation

2.8.1 Flow chambers and cone and plate rheometers

In vascular endothelial cells, mechanosensing is believed to control the production of the protective ECM,[113] whereas in bone, mechanosensing is at the basis of bone repair and adaptive restructuring processes.[52] Osteocytes have been studied *in vitro* after extraction from the bone matrix in parallel plate flow chambers. Flow chambers are commonly used to study the adhesion of leukocytes to endothelium-like substrates. For diluted ligands, one usually observes first order dissociation kinetics, which traditionally has been interpreted as the signature of single-molecule events. Although recent results now point to a more complicated situation involving multiple bonds, flow chambers with diluted ligands can indeed be used to study single-molecule unbinding.

2.8.2 Magnetic and optical traps

These methods are called nonphysiological. Exactly how the ECM–integrin–cytoskeletal complex senses mechanical stimuli remains somewhat of a mystery. Seminal observations[24,27,114] using a magnetic tweezer and twisting device to transfer force directly from integrins to the local cytoskeleton suggest that mechanical deformation of one or more FA protein is the proximal step in an intracellular signaling cascade that leads to global cytoskeletal rearrangements and mechanotransduction at multiple, distant sites within the cell.

2.8.3 Atomic force microscopy and biomembrane force probe

The mechanical opening-up of biomolecular bonds has become a subject of extensive research during the last decade, both experimentally and

theoretically. The main experimental techniques in this field are AFM and the biomembrane force probe (BFP). In AFM experiments, bonds are attached to sharp tips mounted on soft cantilevers, which are moved on a piezo stage. In BFP experiments, bonds are attached, for example, to red blood cells, which are controlled by micropipette aspiration.[115] The main theoretical approaches in this field are Kramer's theory (which describes thermally assisted escape over a transition state barrier) and steered molecular dynamics (atomic level simulations with force fields and an externally applied force). The field of single-molecule force spectroscopy was opened up by seminal AFM experiments conducted by Hermann Gaub's group[23,116] as well as AFM measurements by Matthias Rief,[117,118] who reported for the first time the mechanically induced unbinding of single biotin–streptavidin bonds, with a binding strength of 140 pN. In an experimental context, binding strength usually means the most frequent rupture force in the spectrum of rupture forces measured in different experiments. The mechanotransduction (i.e., force-transmission pathway via FAs, cell–cell contacts, or cytoskeleton-associated proteins occurs at the single-molecule scale.

2.8.4 Cell stretcher

Experiments can be performed using uniaxial or biaxial strain, as well as oscillatory or static stretch in 2D and 3D possible with cells embedded in gels. However, only a few days of stretching are possible and long-term remodeling or even disease progression cannot be observed. On top of this, the response to strain is complex. Comparing, for instance, stretched to non-stretched cells, proteins bind preferably to stretched cytoskeletal networks: Binding of paxillin, vinculin, FA kinase p130Cas, and PKB/Akt are all enhanced during stretch.[84,119]

2.8.5 Hydrostatic pressure

Physical forces encountered by living cells include membrane stretch, gain and loss of adhesion as well as compression due to an increase in pressure. It is conceivable that different mechanosensors are required to sense transverse versus longitudinal stretch, perhaps accounting for differential signaling and cellular phenotype resulting from pressure versus volume overload.[120]

2.8.6 Stretch-activated ion channels

Ion channels play a central role for mechanotransduction in the sensory systems, but the situation at FAs is very different, since speed of response is not an issue at FAs under force.[121] Moreover, studies have shown that the

durotactic response is suppressed when stretch-activated ion channels are blocked with gadolinium. These studies provide an appealing mechanism for signal transduction for mechanically active cells in soft media. However, they are also unspecific and cells might not be able to distinguish between different sources. On the other hand, additional information channels, such as soluble ligands, will certainly supplement elastic signals.[122]

3. CONCLUSIONS

In this review, I have addressed the issue of environmental sensing by cells. I propose that a comprehensive understanding of adhesion-mediated signaling requires the precise characterization of both the sensed surface and the sensory machinery of the cell. In recent years, remarkable progress has been made in both areas. Surface nano-engineering has opened up new possibilities for the systematic modulation of individual surface features, such as surface chemistry, ligand spacing, geometry, and surface rigidity. In parallel, novel techniques of gene modulation enable the selective removal, overexpression, and mutation of individual genes. These effects on the cellular response of the sensory machinery can then be assessed. Although our current understanding of adhesion-mediated environmental sensing is still incomplete, several design principles have emerged from experiments. It seems, for example, that surface chemistry, namely, the presence of diverse matrix proteins, has a strong effect on the selection of specific integrin receptors and consequently on the initial assembly of the integrin nanocomplexes. Indeed, differential activation of integrin's (e.g., $\alpha5\beta1$ integrin compared with $\alpha v\beta3$ integrin) can result in major differences in both the initiation and the progression of the adhesion process.[123] Furthermore, a growing body of evidence implicates mechanical force as central to the regulation of nearly every stage of FA assembly, from the actin polymerization-dependent assembly of the first visible, nascent adhesions, to the myosin-dependent growth and maturation of FAs.[124,125] A deeper understanding of the ongoing interplay between molecular surface design and genetic modulation of the adhesion machinery is likely to reveal the nature of the mechanisms that underlie the sensitivity of living cells to both the chemical and physical characteristics of the surfaces to which they adhere.

FAs are large, multiprotein complexes that provide a mechanical link between the cytoskeletal contractile machinery and the ECM.[46,126,127] FAs exhibit mechanosensitive properties. They self-assemble and elongate

upon application of pulling forces and dissociate when these forces are decreased. A thermodynamic model for the mechanosensitivity of FAs, according to which a molecular aggregate, subjected to pulling forces, tends to grow in the direction of force application (by incorporating additional molecules), has been proposed. This principle is consistent with the phenomenology of FA dynamics by considering a one-dimensional protein aggregate subjected to pulling forces and anchored to the substrate.[128] Depending on the force level, force distribution along the aggregate is predicted to exhibit distinct modes of assembly that are largely consistent with the experimentally observed FA behavior.

In all, the mechanosensitive behavior of FAs is an important component of the cell's ability to spread and move along substrates.[57,62,101] The basic observation underlying FA mechanosensitivity is that alterations in the mechanical force applied to these adhesion sites, either by the contractile machinery of the cell or after external perturbation, have a dramatic effect on FA properties and cellular behavior and should be further elucidated.[129]

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