Biochimica et Biophysica Acta 1853 (2015) 3043–3052

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

A guide to mechanobiology: Where biology and physics meet☆

Karin A. Jansen [a], Dominique M. Donato [b], Hayri E. Balcioglu [c], Thomas Schmidt [b], Erik H.J. Danen [c], Gijsje H. Koenderink [a,*]

[a] Systems Biophysics Department, FOM Institute AMOLF, Science Park 104, 1098 XG Amsterdam, The Netherlands
[b] Physics of Life Processes, Huygens-Kamerlingh Onnes Laboratory, Leiden University, Niels Bohrweg 2, 2333 CA Leiden, The Netherlands
[c] Faculty of Science, Leiden Academic Center for Drug Research, Toxicology, Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands

ARTICLE INFO

Article history:
Received 29 January 2015
Received in revised form 28 April 2015
Accepted 2 May 2015
Available online 18 May 2015

Keywords:
Mechanosensing
Extracellular matrix
Cytoskeleton
Integrins
2D vs 3D
Mechanotransduction

ABSTRACT

Cells actively sense and process mechanical information that is provided by the extracellular environment to make decisions about growth, motility and differentiation. It is important to understand the underlying mechanisms given that deregulation of the mechanical properties of the extracellular matrix (ECM) is implicated in various diseases, such as cancer and fibrosis. Moreover, matrix mechanics can be exploited to program stem cell differentiation for organ-on-chip and regenerative medicine applications. Mechanobiology is an emerging multidisciplinary field that encompasses cell and developmental biology, bioengineering and biophysics. Here we provide an introductory overview of the key players important to cellular mechanobiology, taking a biophysical perspective and focusing on a comparison between flat versus three dimensional substrates. This article is part of a Special Issue entitled: Mechanobiology.

1. Introduction

Cells in our body actively sense and respond to a variety of mechanical signals. The mechanical stiffness of the surrounding extracellular matrix (ECM) critically determines normal cell function, stem cell differentiation and tissue homeostasis [1,2]. Conversely, abnormal changes in ECM stiffness contribute to the onset and progression of various diseases, such as cancer and fibrosis [3]. Cancer tissues can be up to 10-fold stiffer than healthy tissues, which is correlated with tumor cell survival and enhanced proliferation [3–5]. Additionally, cells often experience forces in the form of shear stress during breathing and blood flow, compression and tension due to muscle contraction. Forces also play a crucial role in regulating tissue morphogenesis in developing embryos [6,7]. The sensitivity of cells to forces and substrate stiffness has been recognized as a powerful tool in tissue engineering, where it can be harnessed to design biomaterials that optimally guide stem cells or resident cells in the patient towards generating a functional replacement tissue. Given its central importance in cell function and human health, mechanobiology has emerged as a new and growing field that attracts researchers from disciplines ranging from cell and developmental biology, to bioengineering, material science and biophysics.

A central element in mechanobiology is cellular 'mechanosensing' (see Box 1). Cells actively probe the rigidity of their extracellular environment by exerting traction forces via transmembrane proteins termed integrins [8]. It is still poorly understood how probing by traction forces allows cells to sense matrix stiffness and how cells transduce this mechanical information into a cellular response. Answering these questions is complicated by the large number of mechanosensors and -transducers that have been identified so far [9]. Prominent examples are paxillin [10], vinculin [11,12], talin [13], p130CAS [14,15], integrins [16,17], the actin cytoskeleton (CSK) [18–20] and mechanosensitive ion channels [21]. It is still unclear how these components work together to regulate mechanosensing. Also, most experimental studies until now were performed with cells cultured on top of two dimensional (2D), and often rigid, substrates, which inadequately mimic most physiological contexts.

Mechanosensing and -transduction are cellular processes that involve both intra- and extracellular components, as illustrated in Fig. 1. The main structural components that contribute are (1) integrins, (2) the ECM and (3) the intracellular CSK. Mechanical forces and biochemical signaling are integrated by various intracellular signaling pathways. In this review, we will provide an overview of the roles of these contributors to cellular mechanobiology. Note that we will not touch upon mechanosensitive ion channels, which are reviewed elsewhere [21], nor will we discuss cell–cell interactions, which also play an important role in mechanosensing [22,23]. We will focus on a comparison between cellular mechanobiology on 2D substrates and inside 3D environments designed to mimic connective tissue. Furthermore, we will comment on the applications of mechanobiology in tissue engineering.

☆ This article is part of a Special Issue entitled: Mechanobiology.
* Corresponding author.

http://dx.doi.org/10.1016/j.bbamcr.2015.05.007
0167-4889/

Box 1 Terms that are often used in the field of mechanobiology.

Mechanobiology: A field at the interface of biology, physics, and bioengineering, which focuses on how cell/tissue mechanics and physical forces influence cell behavior, cell and tissue morphogenesis, and diseases related to these processes.
Mechanosensing (|sensation): The process of a cell sensing mechanical signals provided by its environment.
Mechanotransduction: The process of translating mechanical signals into a cellular response.
Durotaxis: Directed cell motility in response to gradients in substrate rigidity.
Contact guidance: Directed cell migration or orientation based on anisotropy (alignment) of the microenvironment, such as collagen fibers in 3D or micropatterned adhesive lines on a 2D substrate.
Outside-in signaling: Mechanical cues in the environment causing intracellular signaling cascades, which affect cellular processes such as migration, growth, and differentiation.
Inside-out signaling: Intracellular processes affecting the mechanical properties of the environment by exertion of traction forces and secretion/breakdown of ECM material.
Integrin: Heterodimeric transmembrane protein that physically connects the ECM to the CSK and acts as a bidirectional signaling receptor.
Slip-bond: Receptor-ligand interaction whose lifetime is reduced when mechanically loaded.
Catch-bond: Receptor-ligand interaction whose lifetime is enhanced with increasing load to a maximum value, followed by a gradual decrease when the load is further increased.
Cell-matrix adhesion: Cell-ECM connections mediated by clusters of integrin in the plasma membrane. This term includes FAs, focal complexes, focal contacts, fibrillar adhesions, and nascent adhesions.
Adhesome: The collection of more than 150 proteins associated with cell-matrix adhesions that links the ECM and the CSK.
Nascent adhesion: A cell-matrix adhesion during its initial phase of formation. Usually, such an adhesion is significantly smaller and more punctuate than mature FAs. Nascent adhesions are thought to be enriched with FA proteins such as talin and paxillin.
Focal complex: A cell-matrix adhesion that is usually found at the leading edge of migrating cells. Focal complexes can either be nascent adhesions on their way to maturation or simply short-lived ECM-cell contacts. Like nascent adhesions, they are smaller and more punctuate than focal adhesions. They contain a larger subset of adhesome proteins than nascent adhesions, but still a smaller subset than FAs.
Focal adhesion (FA, a.k.a. focal contact): Cell-matrix adhesions that are usually associated with actin stress fibers. They have an elongated form and are found at the front, rear, and periphery of the cell. They are one of the most mature ECM-cell contact types, besides fibrillar adhesions, and are therefore associated with a larger variety of proteins from the adhesome. They are also usually at least twice as large as nascent adhesions or focal complexes.
Fibrillar adhesion: Elongated cell-matrix adhesions that are usually not found in the lamellipodium, but under the nucleus and the lamella behind the lamellipodium. The length of these adhesions is several times larger than that of FAs.
Acto-myosin contractility: Contractile activity of the actin cytoskeleton mediated by non-muscle myosin II-A and II-B motor proteins. Actomyosin contractility is responsible for traction forces exerted on the substrate at cell-matrix adhesions.

2. Contributors to cellular mechanosensing

Integrins play a central role in cellular mechanosensing because they physically connect the CSK to the ECM, typically in clusters termed 'focal adhesions' (FAs). Integrins are transmembrane proteins that are heterodimers of an α and β subunit and are restricted to the metazoa [8]. So far, 24 different heterodimers formed by combinations of 18 different α subunits and 8 β subunits have been identified [8]. Most integrins recognize multiple ligands, which share common binding motifs such as the RGD or LDV motif [24]. The integrin αvβ3 can for instance bind vitronectin, fibronectin and fibrinogen through the RGD-binding motif.

The *extracellular matrix* (ECM) is a complex protein meshwork that forms the scaffold to which cells adhere. It provides mechanical support to cells and tissues, and acts as a reservoir for growth factors, cytokines

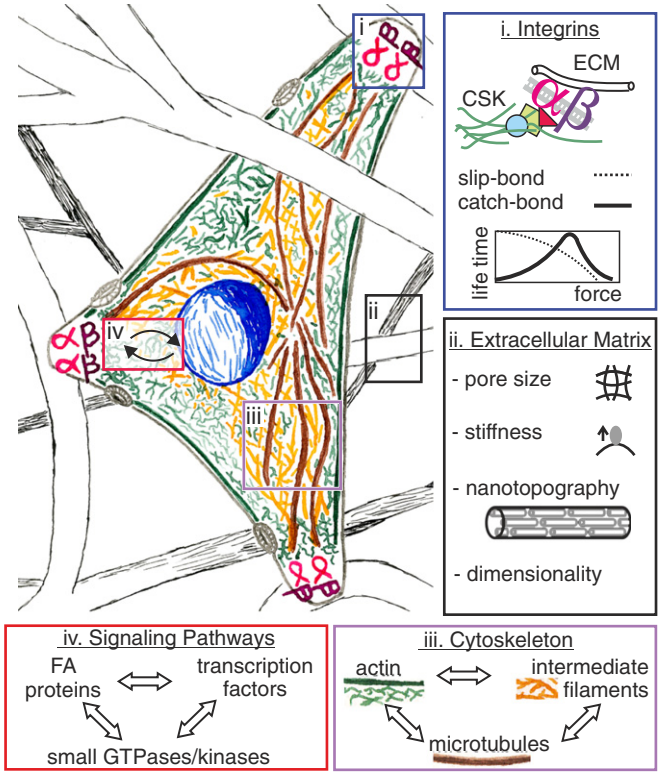

Fig. 1. Schematic showing a cell inside a three dimensional fibrous extracellular network. The boxes indicate the focus areas of this review. (i) Integrins are composed of an α (pink) and β subunit (purple) and are clustered in focal adhesions (FAs) together with other FA proteins (triangle, square and circle). The adhesions connect the extracellular matrix (ECM) and the (actin) cytoskeleton (CSK). Integrins can be classified as slip- or catch-bond adhesion molecules, which differ in their bond lifetime under an applied force. (ii) The ECM provides multiple cues to the cell, specifically pore size, stiffness, nanotopography and dimensionality. (iii) The CSK is composed of actin (green), intermediate filaments (yellow) and microtubules (brown). (iv) Summary of important signaling pathways. Note that we will not discuss mechanosensitive ion channels (gray pores). The cell nucleus is depicted in blue.

and proteolytic enzymes. There are two broad classes of ECM: basement membrane and connective tissue. Basement membranes are thin structures that provide a two-dimensional (2D) substrate onto which polarized cells such as epithelial and endothelial cells adhere. Its main components are laminin, collagen IV, nidogen and heparan sulfate proteoglycans [25]. In contrast, connective tissues provide a fibrous 3D scaffold whose structural components are mainly fibrillar collagens (mostly type I and II, mixed with III and/or V), proteoglycans and glycosaminoglycans [26]. The diameter and organization of the collagen fibers are tailored to the biomechanical function of each tissue. The fibrils are, for example, thick and aligned in stiff tissues like tendon to ensure tensile strength, whereas they are thin and organized in meshworks in the cornea to ensure optical transparency. Proteoglycans and glycosaminoglycans are hydrophilic macromolecules forming a background matrix for the collagen fibers, which facilitate water retention and influence cell migration and ECM deposition [27]. The ECM also contains nonstructural components that modulate cell-ECM interactions, such as thrombospondin 1 and tenascins [28]. Under influence of force, ECM proteins could also act as a mechanotransducer by exposing cryptic sites and growth factors [29]. During wound healing, cells encounter a provisional ECM that forms as a result of blood clotting. This matrix consists of a scaffold of fibers made of the plasma proteins fibrin and fibronectin. Due to their biocompatibility and physiological scaffold role, both collagen and fibrin are popular biomaterials for *in vitro* studies and tissue engineering. However, it is important to emphasize that such simplified matrices do not mimic the full tissue-specific context (in terms of architecture and chemical composition) that is offered by the *in vivo* ECM. The architecture, composition and stiffness of the *in vivo* ECM is further subject to changes during disease progression and aging. Since cells are sensitive to all of these extracellular cues, the ECM is increasingly recognized as an active player and potential therapeutic target in diseases such as fibrosis, artherosclerosis and cancer [5,30–33].

The *cytoskeleton* (CSK) is a space-filling network of protein filaments that enables cells to maintain their shape and mechanical strength [34]. The CSK enables cells to withstand external forces, while at the same time being dynamic and self-deforming. The mammalian CSK comprises three types of protein filaments: actin, microtubules (MTs) and intermediate filaments (IFs). Actin and MTs are polar filaments with two structurally distinct ends, which are capable of generating pushing and pulling forces by coupling polymerization to nucleotide hydrolysis. In contrast, IFs are nonpolar and more stable. All three filaments can be classified as semiflexible polymers: they remain straight under the influence of thermal fluctuations over a length scale that is comparable to their 'persistence length'. This characteristic length scale is much longer for MTs (a few mm) than for IFs (0.5 μm) and actin (10 μm). As a result, actin and IFs are generally considered to provide the main source of cell stiffness, whereas the more rigid MTs may provide resistance to compression forces [35]. Purified networks of actin and IFs increase their stiffness under the influence of force. In other words, these networks strain-stiffen in response to mechanical shear or stretch [36–38]. This phenomenon allows cells to actively stiffen their actin cytoskeleton on hard substrates by contraction with myosin motors [1]. Moreover, strain-stiffening of IFs is thought to prevent excess deformation of cells and epithelial tissues [37,38].

3. Role of the ECM in mechanobiology

In this section, we will focus on the influence of physical cues provided by the ECM on cell behavior. Cells embedded in 3D interstitial matrices are influenced by various factors that are difficult to decompose, such as global (i.e. macroscopic) and local (i.e. fiber) stiffness, matrix topography, the porosity and the dimensionality. Below we will review experimental studies that have sought to disentangle these factors using biomimetic 3D ECM matrices or 2D substrates.

3.1. ECM stiffness

It is now well recognized that cells cultured on top of a 2D substrate actively sense and respond to its stiffness [1,39–41]. Many fundamental aspects of cell behavior are mechanosensitive, including adhesion, spreading, migration, gene expression and cell-cell interactions [40,42–46]. Substrate stiffness can also regulate stem cell differentiation and compete with biochemical cues [1]. Recent experiments with stem cells on photodegradable substrates showed that stem cells even remember the mechanical history of their environment [47].

Studies of cells on 2D substrates are usually performed with nonadhesive polyacrylamide (PAA) or polydimethylsiloxane (PDMS) coated with ECM proteins or ligands such as RGD peptides. Surface coupling should be chosen with care, since the distance between tethering points can influence cell fate [48] and cells can pull ligands from the surface if they are anchored too weakly [49]. The thickness of the gels should also be chosen with care, because cells can feel the stiff underlying glass/plastic substrate if the gel is too thin [50,51]. Systematic studies showed that cells on top of PAA gels can sense over a distance of a few tens of microns [50,51]. However, this length scale can be increased to ~200 μm for fibrous networks of collagen [52] and fibrin [53]. The long range of force transmission in these ECM networks has been variously ascribed to strain-stiffening under the influence of cellular traction forces [53] or to the fibrous nature of the ECM [54]. The second explanation is supported by finite-element modeling of the transmission of traction forces in collagen [54] and fibrin [55] networks. These simulations show that cell tractions are concentrated in the relatively stiff ECM fibers, thus propagating farther than in a homogeneous elastic medium even if this elastic medium strain-stiffens [54]. Finite-element modeling and analytical theory showed that cell-induced alignment of collagen fibers further contributes to making force transmission anomalously long-ranged [56].

Unclear is whether cells sense their environment by applying a constant stress (i.e. force) and reading out the strain (i.e. deformation) or vice versa. Theoretical models suggest that cells may readjust their contractile activity and CSK organization to maintain either an optimal strain or an optimal stress [57]. Experiments with elastic micropost array substrates indicated that epithelial cells and fibroblasts maintain a constant substrate strain [58,40,59]. However, recent measurements of traction forces for fibroblasts on PAA gels with a wider range of Young's moduli (6 to 110 kPa) suggest that cells switch from maintaining a constant strain on soft gels (Young's modulus below 20 kPa) to maintaining a constant stress on stiffer substrates [49]. It was proposed that the cells increasingly align their actin stress fibers to sustain a constant substrate strain as the substrate stiffness increases. At substrate rigidities above 20 kPa, the maximal contractile force that the aligned actomyosin units can generate would reach a limit. This interpretation is supported by a study of substrate-dependent stress fiber alignment [60] and a model representing the cell as a prestrained elastic disk attached to an elastic substrate via molecular bonds [61]. It is still unclear how these findings translate to the situation of a cell embedded in a 3D fibrous matrix. One study of fibroblasts inside porous collagen-glycosaminoglycan (GAG) matrices suggests that cells maintain a constant traction stress [62].

Unlike synthetic PAA and PDMS hydrogels, whose stiffness is constant up to large strains, networks of fibrin and collagen strain-stiffen as soon as the strain reaches values of a few percent [38,63,64]. It has been proposed that this nonlinear elastic response strongly influences cellular behavior based on studies of fibroblasts and stem cells cultured on top of thick fibrin biopolymer gels, which revealed that cell spreading was independent of the linear elastic modulus of the gels and similar to spreading on stiff PAA gels [53]. Apparently, the cells sense a stiff environment because they actively stiffen the fibrin network by exerting traction forces. Atomic force microscopy (AFM) nanoindentation as well as macroscopic shear rheology showed that cells cultured inside or on top of fibrin gels indeed cause network stiffening [65,53]. In case of collagen gels, there is also evidence that buildup of stresses

originating from cellular traction forces affect fibroblast morphology and motility [66]. Cell-induced ECM stiffening may play an important role in diseases, such as cancer and fibrosis, where it can provide a positive feedback that enhances cell contractility [33].

In summary, there is overwhelming evidence that mechanical properties (linear and nonlinear) of the substrate or ECM play an important role in determining cell fate. It is still an open question to what extent a cell embedded inside a 3D fibrous ECM matrix senses the stiffness of the overall network (i.e. *global* stiffness), as on 2D substrates, or the *local* stiffness, i.e. the resistance of individual ECM fibers to bending and stretching. Furthermore, recent studies of cells on 2D hydrogels varying in their viscous but not their elastic modulus showed that cell differentiation is also sensitive to the viscous modulus [67,68]. Moreover, cell spreading on soft substrates was shown to be strongly enhanced when the substrate (an ionically crosslinked alginate hydrogel) exhibited stress relaxation, an effect that could be recapitulated using a stochastic lattice spring model [69]. It was proposed that stress relaxation in the substrate may facilitate cell spreading by allowing cells to cluster ECM ligands. Viscous effects are indeed likely to be important since cellular time scales of traction force generation can be slower than the time scales at which the mechanical properties of cell substrates are generally measured [70].

3.2. Nanotopography

Structural components such as collagen [71], fibrin [72] and fibronectin [73] form hierarchically structured fibers that are radically different from the surface presented by standard 2D hydrogels. However, developments in nanotechnology and micropatterning have allowed for more advanced 2D substrates with controlled topography and adhesion areas that mimic tissue morphologies [74–76]. When the surface is patterned with nanoridges, cells align parallel to the nanoridges and migrate along them, in a process known as 'contact guidance' [74–77]. Furthermore, it was shown that cells can distinguish differences in height of a few nanometers [77–79] and can cling onto adhesion regions as small as 8 nm [80]. Cells are also sensitive to the distance between adhesion islands, as demonstrated by studies with ordered patterns of RGD-coated nanoparticles [81,82]. Furthermore, disorder in the position of small adhesion islands can optimize cell differentiation [83]. Nanotopography is therefore a powerful design parameter in tissue engineering, as illustrated by a recent study showing that a controlled nanotopography enhances bone formation around tooth implants [84].

3.3. Pore size

In vitro studies of cells embedded inside reconstituted networks composed of collagen or fibrin have shown that cell spreading and migration is hampered when the mesh size becomes smaller than the size of the nucleus [85,65,86]. The critical mesh size where cell migration is affected depends on the ability of the cells to degrade the matrix with proteolytic enzymes and on the deformability of the nucleus, which is governed by lamins [87,88]. The pore size of collagen and fibrin networks can be controlled by tuning the protein concentration and polymerization temperature [89,90,86,88]. However, these variations also affect the global and local (fiber) stiffness and network structure. The influence of pore size on cell behavior can be studied in isolation by using microfabricated channels [91] or synthetic polymer gels [92,93]. These studies revealed that pore size controls migration speed [91,92] and stem cell fate by controlling cell shape [93].

The fibrous nature of the ECM limits the availability of binding sites for cells. There have been several studies using model (synthetic) 3D matrices where the ligand density was varied independently of the network stiffness and pore size [94,95,92]. These studies suggest an increase in cell spreading and migration speed with increased ligand density. This is in contrast to 2D studies, where an optimum in both parameters is observed at intermediate ligand densities [42,43]. However, it should be noted that the pore size of the synthetic 3D gels was in the nm-range, which is outside the physiologically relevant size regime. The thickness of the fibers in the ECM limits the size of FAs [96]. However, it was shown that cells can bend and reorient the fibers to increase the adhesion area.

We finally note that the ECM pore size can also affect cell behavior in tissues by influencing the permeability and hence interstitual flow. Fluid pressure in tissues was shown to affect cell migration and the distribution of vinculin, actin and α-actinin [97].

3.4. Dimensionality

When considering a cell inside a 3D fibrous ECM, it is unclear what is the effective dimensionality that the cell perceives. If the cell encounters a single fiber, the environment is perhaps effectively 1D. Indeed, the cell migration speed on thin micropatterned lines of ligands on a 2D substrate was shown to be comparable to the migration speed inside 3D cell-derived matrices, suggesting that the 1D situation is relevant *in vivo*, at least in certain contexts [98]. However, when the collagen fibers are thick due to bundling, as in dermal tissue, sarcoma cells were shown to behave as if on a 2D environment [99]. Cells embedded in reconstituted collagen networks, which consist of thinner collagen fibrils, usually interact with multiple fibers and may therefore sense a more 3D environment. The cells typically adopt a spherical or spindle-like shape instead of the flat 'pancake' shape seen on (rigid) 2D substrates [100,101]. These characteristic cell shapes are recovered when cells are sandwiched between two flat substrates, suggesting that simultaneous adhesion of the ventral and dorsal sides of the cell contributes to the 3D phenotype [102].

4. Integrins

Integrins are bi-directional signaling receptors. Intracellular proteins bind to the tail region of integrins, thus causing conformational changes in the head region that increases the affinity for its extracellular ligands (*inside-out signaling*). Vice versa, ligand binding triggers conformational changes that activate intracellular signaling cascades (*outside-in signaling*). Ligand binding additionally promotes integrin clustering, which is essential for cell spreading [81]. Integrins recognize specific motifs in the ECM and also respond to physical ECM properties. In this section, we will briefly review the molecular features of integrin mechanosensing and compare the role of integrins in 2D and 3D environments.

4.1. Molecular basis of integrin mechanosensing

Single-molecule force spectroscopy measurements using AFM or optical tweezers have shown that mechanical loading can directly influence the lifetime of integrin-ECM bonds. Some integrins, such as αIIbβ3, exhibit slip-bond behavior characterized by a decreased lifetime with increasing load [103], whereas others, such as α5β1, exhibit catch-bond behavior characterized by an increased lifetime with increasing load [104,105]. Catch-bond behavior is a common response for many adhesion molecules [106]. Theoretical modeling has shown that catch-bond clusters can in principle act as autonomous mechanosensors [16,17]. However, the relative importance of this mechanism compared to that of other putative mechanosensors involved in connecting integrins to the nucleus and the CSK is unresolved [44].

The spatial distribution of extracellular ligands has been shown to play a role in stem cell behavior [107], lineage determination [108] and the cellular response to an applied force [109]. Clustering of integrins to form FA complexes requires a certain minimum ligand density. Various studies based on nanopatterned surfaces showed that the maximum distance between ligands where FA complexes can still form is about 80 nm [81,82,110–112]. Force measurements performed on single integrin-RGD pairs showed that the force per integrin

increases with reduced ligand spacing. This is somewhat counterintuitive, since one would expect load-sharing to lower the force per integrin. Perhaps the existence of a threshold ligand density to induce integrin clustering and enhance actomyosin contractility explains this observation [113].

4.2. Role of integrins in 2D versus 3D environments

Studies of cells on 2D substrates have shown that different integrins binding to the same ECM protein can lead to different phenotypes. Cells adhering to fibronectin substrates through αvβ3 versus α5β1 integrins, for instance, differ in traction force generation [114–116], binding dynamics [117], actin CSK remodeling under influence of cyclic strain [116] and adhesion [117,118]. These integrins activate different intracellular signaling cascades [115,119] and interchanging the ligand binding domains reverses the signaling phenotype [120,121]. Similarly, expression of αvβ6 integrins in the presence or absence of α5β1 changes traction force generation [17]. Different splice variants of α6β1 with distinct cytoplasmic domains also give rise to different phenotypes due to the two distinct cytoplasmic domains [122]. Thus cells can regulate their mechanosensitivity by modifying their integrin expression profile.

In 3D environments, integrins are required for the fibrillogenesis of various ECM proteins [123,124]. Most research on mechanobiology in 3D matrices focused on integrins with the β1 subunit, which binds most ECM proteins including collagen [24]. The β1-integrins, in combination with alterations in matrix stiffness, have been shown to promote tumor progression [31,5,33]. However, β1-integrins also appear to suppress tumor metastasis in some contexts [125,126]. Inhibition or deletion of the β1-integrins can induce metastasis via upregulated TGF-β signaling and increased expression of αV integrins has been implicated in this process [127,128]. Interestingly, several αV integrins can bind and activate the latent TGF-β complex, which is an integral component of the ECM. For integrin αVβ6 it has been demonstrated that traction forces that are transduced from the actin CSK, through integrins, alter the conformation of the integrin-bound latent TGF-β complex, thereby supporting TGF-β activation [129].

Advances in 3D traction force microscopy [130,131] in combination with FRET-based molecular force sensors [132] are necessary tools to elucidate the mechanisms of integrin-mediated mechanosensing in 3D matrices. Microscopic characterization of the size, morphology and dynamics of cell-matrix adhesions within 3D matrices is technically challenging [133,134]. In reconstituted collagen networks, FAs generally appear to be smaller than on (rigid) 2D substrates [133,135]. However, in acellular porcine epithelium, which presents cells with thicker collagen bundles, sarcoma cells were shown to exhibit similar FA size and dynamics as on 2D substrates [99].

5. Signaling pathways

Integrins recruit more than 150 proteins to the cell-ECM interaction sites, which are referred to as the adhesome. The adhesome includes FA adapter proteins, shuttling proteins and kinases that influence gene transcription as well as the CSK [136]. We provide a brief overview of the main signaling pathways below.

5.1. Mechanosensitive FA proteins

Prominent examples of mechanosensitive proteins in the adhesome are talin [13], vinculin [132], and p130Cas [14]. In its unstretched form, talin's cryptic sites are hidden and vinculin cannot bind, but actomyosin contraction opens up talin and recruits vinculin [13]. Using a FRET-based molecular force sensor, the force threshold for vinculin recruitment was shown to be 2.5 pN [132]. Studies of vinculin-knockout cells and vinculin mutants unable to bind p130Cas have shown that vinculin is necessary for p130Cas activation in response to changes in substrate rigidity [137]. p130Cas has a central substrate domain that is intrinsically disordered and can be stretched with AFM or magnetic tweezers [138,139]. Vinculin likely anchors p130Cas into FAs, to allow stretching of the central substrate domain [14]. Stretching can make tyrosine motifs accessible to Src kinases for phosphorylation, which are known to influence FA formation and actin dynamics [140]. In other words, p130Cas transduces cellular traction forces, due to tyrosine phosphorylation motifs that are exposed, and hereby changes actin dynamics and FA formation further downstream. Only recently, studies of p130Cas have been extended to substrates with variable stiffness such as PAA gels [137] and PDMS micropillar arrays [15]. A more extensive review on the functions of talin, vinculin and p130Cas can be found elsewhere [141].

Paxillin, zyxin and Hic-5, which are among the LIM domain proteins, have also been identified as being mechanosensitive [142]. Zyxin recruits the proteins Ena (Enabled) and VASP (Vasodilator-stimulated phosphoprotein) to FAs and to cell-cell contacts, where they promote F-actin polymerization [143,144]. Both zyxin and paxillin contribute to stress fiber repair, a critical process for maintaining the tensional balance within adherent cells [145]. When actin stress fibers were severed by laser ablation or damaged by mechanical strain, zyxin re-located to the newly exposed barbed ends of actin filaments at the damaged sites [146]. Interestingly, LIM proteins exhibit divergent responses to a mechanical strain. While Hic-5 and zyxin localize to stress fibers when cells cultured on 2D substrates are exposed to cyclic stretch, paxillin does not [142]. Even though cells in 3D matrices do not show similar stress fibers as on 2D substrates, zyxin and paxillin do localize at the end of protrusions that are reminiscent of FAs in cells migrating through a network of polycaprolactone fibers [96] and paxillin plays a critical role in 2D and 3D cell migration [147].

5.2. Rho GTPases

Following kinase-mediated phosphorylation, for example of p130Cas, many FA proteins promote Rho GTPase activity. Three members of the Rho family of small GTPases are of particular interest in the context of mechanosensing: RhoA, Rac and Cdc42. Rac and Cdc42 are primarily linked to actin polymerization at the leading edge in lamellipodia (and filopodia in the case of Cdc42). RhoA is mainly associated with the activation of actomyosin contractility, together with ROCK (Rho-associated, coiled-coil containing kinase). Rho GTPases are regulated via GEFs (guanine exchange factors) and GAPs (GTPase activating proteins) [148]. While Rho GTPases are usually considered in relation to actin, there is growing evidence that they are also coupled to IFs [149] and MTs [150].

Most studies of Rho GTPase activity have been performed using 2D cell cultures, but there are some studies in the context of 3D migration of tumor cells in collagen matrices. Rho-mediated actomyosin contractility was shown to be necessary for mammary cancer cells to orient collagen matrix perpendicular to the tumor boundary. These fibers then promote cell invasion by contact guidance [151]. Interestingly, if the collagen matrix was artificially pre-aligned, the Rho/ROCK/MLC pathway became dispensable for invasion. ROCK also contributes to stem cell differentiation: expressing a constitutively active form of ROCK in hMSCs cultured in a 3D hydrogel was shown to induce a switch from adipogenesis (soft tissue fate) to osteogenesis (stiff tissue fate) [131], whereas ROCK inhibition with Y-27632 reduced osteogenesis.

RhoA and Rac are targeted to the plasma membrane in a mechanoresponsive manner, as demonstrated by cyclic stretch experiments with aortic smooth muscle cells on a PDMS membrane [152]. Interestingly, targeting was also microtubule-dependent. Similar stretching experiments performed on monolayers of endothelial cells that mimic the lung epithelium identified Rho GTPases as key regulators of tissue homeostasis [153], which is crucial in the lung endothelium that constantly experiences cyclic stretch.

5.3. Integrin-mediated regulation of gene transcription

Integrin-mediated mechanosensing feeds into cell fate decisions by activating various downstream signaling cascades connected to gene expression [136]. One of the most widely studied pathways involves the mitogen-activated protein kinase (MAPK) family. The MAPK pathway is an evolutionarily conserved signaling mode that controls cell proliferation, survival and differentiation. It involves three protein families: the extracellular signal-regulated kinase (ERK) family, the p38 kinase family and the c-Jun N-terminal kinase (JNK) family. Activation of receptor tyrosine kinases (RTK) causes activation of ERK, which can subsequently phosphorylate nuclear substrates that in turn enhance or suppress gene transcription. The MAPK family has been established as a key regulator of the mechanoresponse of osteoblasts and osteoprogenitor cells [154]. Cyclic stretch or shear flows can cause activation of members of the MAPK family, which enhances osteoblast proliferation and differentiation [155]. Similar effects are seen for vascular smooth muscle cells and endothelial cells [153]. However, it is still an open question how these observations, mostly made for cells on rigid substrates, translate to more physiologically relevant environments, especially given that MAPK signaling is known to be dependent on substrate stiffness [154]. Vinculin stretching was recently shown to initiate stiffness-sensitive mitogen-activated protein kinase 1 (MAPK1) signaling in hMSCs, causing differentiation to a muscle phenotype [156].

A second network that links integrin-mediated mechanosensing to gene transcription is the Hippo network, which functions as a tumor-suppressor pathway in vertebrates [157,158]. Its central components are the transcriptional co-activators YAP1 (Yes-Activated Protein) and TAZ (transcriptional co-activator with PDZ-binding motif). YAP/TAZ binds to transcription factor partners, driving a transcriptional program that specifies cell growth, proliferation and cell fate decisions. In cells cultured on 2D hydrogels or micropillar substrates, YAP increasingly relocates from the cytoplasm to the nucleus when the substrate stiffness is increased [159,160]. Cell stretching can likewise cause YAP relocation to the nucleus [161,162]. It has been suggested that YAP/TAZ respond to substrate stiffness by sensing contractile actin networks, since YAP activation is dependent on myosin contractility and is therefore enhanced on stiffer substrates [163]. In addition, the actin-binding proteins Diaphanous and Cofilin [161,159] and the Rho GTPases Rac and Cdc42 have been implicated in YAP/TAZ activation [164,165]. YAP/TAZ activation on a rigid substrate promotes osteogenic differentiation of mesenchymal stem cells, whereas silencing YAP/TAZ favors the adipocyte fate regardless of substrate stiffness [159].

A third family of co-activators of gene transcription is provided by myocardin and the related transcription factors MRTF-A and MRTF-B, which mediate transcriptional regulation of the Serum Response Factor (SRF) [166]. Rigidity-dependent signaling through MRTF-A involves direct binding of MRTFA-A to the actin CSK. By binding and sequestering actin monomers, MRTFA-A prevents actin polymerization [167]. Mechanical stress exerted by actomyosin contractility (on rigid substrates) or exerted externally promotes actin polymerization and thus releases MRTF-A, which is then able to move into the nucleus and activate SRF. For fibroblasts, pulling on β1 integrins using collagen-coated beads held in magnetic tweezers causes nuclear translocation of MRTF-A, resulting in transcriptional activation of smooth muscle actin and differentiation to myofibroblasts [168].

6. The cytoskeleton (CSK)

In this section we will summarize recent findings reporting the contributions of actin, MTs and IFs to cellular mechanosensing. Although the emphasis has mostly been on the role of the actin CSK, which is responsible for traction force generation [19], there is growing evidence that crosstalk between all three cytoskeletal systems is important [169–171].

6.1. Actin

Cells in 2D culture typically show stress fibers, which are contractile bundles of actin and myosin II [19]. There are several different classes of stress fibers [19,18]. Ventral stress fibers usually span almost the entire cell length and are anchored at both ends to FAs. Dorsal stress fibers are shorter and only connected to a FA at one end. Transverse arcs are present in the leading edge during cell migration and are not associated with FAs. The newly discovered actin-cap stress fibers span over, and are anchored to, the cell nucleus (see Fig. 2). The degree of actin crosslinking and bundling increases with increasing substrate stiffness. For fibroblasts, this allows cells to adapt their stiffness to that of the substrate [172].

Actomyosin contractility helps to mature nascent FAs into larger and mature FAs [173] and reinforces actin anchoring via talin and vinculin [174]. In motile cells, nascent adhesions form in the lamellipodium without myosin II activity. However, without actin-myosin contractility, these adhesions will not mature and will instead turn over rapidly [173]. Actin polymerization is crucial for the formation of nascent adhesions. The forces exerted on nascent adhesions is set by the speed of actin retrograde flow. However, in FAs that are anchored to stress fibers this correlation no longer holds [173].

In reconstituted 3D ECM networks, stress fibers tend to be fewer and thinner compared to 2D, and localized near the cell membrane [101]. However, similar to cells in 2D, the formation of stress fibers is dependent on matrix stiffness [175]. For stem cells, stiffer matrices result in a higher actin concentration near the cell cortex [100]. In 3D collagen gels under dynamic compression, actin protrusions are correlated to matrix remodeling [176].

6.2. Microtubules (MTs)

Despite the well-known roles of MTs in cell polarity and migration [177], their role in mechanosensation has received relatively little attention.

On 2D substrates, MTs do not appear to influence the degree of cell spreading [177]. In contrast, MTs are crucial for cell spreading in 3D collagen networks [177]. In the context of 2D cell migration, MTs promote FA turnover, preventing the FAs to become so large that migration is hampered. The exact mechanism of this regulation is still poorly understood [178]. MTs may be required for delivery of a 'relaxing factor' by kinesin motors [179], or for increased FA turnover via endocytosis [180]. Other studies have shown a paxillin-dependent pathway in regulating MT depolymerization [181,147]. Crosslinking of growing MTs to actin stress fibers is required to guide the MTs to FA sites [182,183].

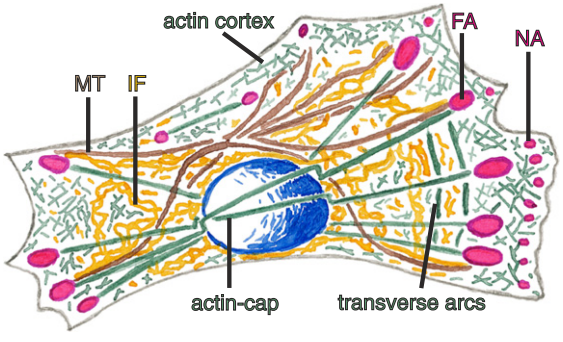

Fig. 2. Schematic of a cell on top of a (stiff) two dimensional substrate. Focal adhesions (FAs, pink) tend to be larger than for cells inside fibrous 3D networks. Actin forms different sets of stress fibers, as indicated. FAs are connected to actin stress fibers, and some can also connect to microtubules (MT) and intermediate filaments (IFs). Newly formed FAs (nascent adhesions, NAs) are not connected to stress fibers. The NAs can mature into larger FAs upon actomyosin contraction. The cell nucleus is depicted in blue and the cell membrane in gray.

MTs also influence FAs by regulating traction forces via crosstalk with the actomyosin machinery. Both for fibroblasts on 2D substrates [184,185] and inside 3D collagen gels [186,187], MT depolymerization causes increased traction forces and thereby FA maturation [188]. Interestingly, this effect was not seen for metastatic breast cancer cells [189]. Inside collagen gels, increasing the matrix stiffness by increasing the collagen concentration triggered MT depolymerization, which enhanced actomyosin contractility by releasing GEF-H1, which activates RhoA [190]. A difficulty with this assay is that changing the collagen concentration changes not only the matrix stiffness, but also its pore size and the ligand density [90,191]. Recently a different assay was reported, where endothelial cells were cultured in collagen networks of fixed density, attached to PAA gels of varying stiffness [192]. In this case, increasing the PAA gel stiffness did not affect the growth persistence of the MTs.

6.3. Intermediate filaments (IFs)

IFs form a large family of proteins that can be classified into five different types based on their self-assembly behavior [193]. Here we will focus on vimentin, which is important in mesenchymal cells like fibroblasts [194]. Through plectins, IFs can interact with actin and MTs [195], as well as with integrins containing the β3 subunit [196]. Also, vimentin directly links α6 integrins with the cell nucleus via plectin and nesprin [197], although the function of this is unclear. In fibroblasts on 2D substrates, the association of vimentin with integrins increases the lifetime of FAs [198,199] and enhances traction forces [200]. FA-binding of vimentin requires an intact MT network [199,200]. Intriguingly, vimentin knockout mice only show defects under conditions of stress. They, for instance, exhibit reduced dilation of arteries in response to shear flow [194]. At the single-cell level, vimentin responds to shear flow [198]. Vimentin knockout mice also exhibit impaired wound healing, which can be traced back to impaired fibroblast migration [149,201]. This migration defect was recently linked to reduced actomyosin contractility [149]. Vimentin increases cell stiffness and can protect the cell against compressive loads [202]. In 3D collagen gels, the vimentin and MT network persists after dynamic compression, while the actin forms local patches to remodel the ECM [176]. Intriguingly, on 2D substrates, the solubility of vimentin depends on the underlying substrate stiffness [171], which may contribute to stiffness adaption of cells to their substrate. In 3D matrices, vimentin-deficient fibroblasts have a dendritic morphology and they make less cell-cell contacts than wild type cells [201]. However, the implications for mechanosensing in 3D are still unknown.

7. Moving forward from 2D to 3D

During the past few decades we have learned a lot about the molecular and physical principles of cellular mechanosensitivity from 2D cell culture studies. There is overwhelming evidence that cell fate critically depends on the stiffness of the substrate, which is therefore an important design parameter in tissue engineering. First studies of cells in reconstituted 3D collagen and fibrin matrices indicate that many results carry over from the 2D to the 3D context. However, the ECM pore size, nanotopography, the thickness and mechanics of the constituent fibers influence cell behavior in complex ways. A key challenge for future research is to design physiologically relevant assays that can unravel these effects. Another key question is, what are the mechanisms by which viscous and nonlinear mechanical properties of the matrix influence cell behavior? On the molecular side, it will be interesting to understand the influence of integrin composition. By changing the fractions of catch- and slip-bond integrins, cells may modulate their sensitivity to forces and matrix mechanics. Finally, there is growing evidence that the actin CSK, that is generally considered to be the main player for mechanosensing, is coordinated with MTs and IFs. Comparatively little is currently known about the roles of MTs and IFs in mechanosensing, especially in a 3D context.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

The authors like to thank Jeroen S. van Zon for helpful feedback. This work is part of the research program of the Foundation for Fundamental Research on Matter (FOM) (projectnr. 09VP07), which is financially supported by the Netherlands Organisation for Scientific Research (NWO). This work is further supported by NanoNextNL, a micro and nanotechnology program of the Dutch Government and 130 partners.

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