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Feeling Things Out: Bidirectional Signaling of the Cell-ECM Interface, Implications in the Mechanobiology of Cell Spreading, Migration, Proliferation, and Differentiation

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Abstract

Biophysical cues stemming from the extracellular environment are rapidly transduced into discernible chemical messages (mechanotransduction) that direct cellular activities - placing the extracellular matrix (ECM) as a potent regulator of cell behavior. Dynamic reciprocity between the cell and its associated matrix is essential to the maintenance of tissue homeostasis and dysregulation of both ECM mechanical signaling, via pathological ECM turnover, and internal mechanotransduction pathways contribute to disease progression. This review covers the current understandings of the key modes of signaling used by both the cell and ECM to coregulate one another. By taking an outside-in approach, the inherent complexities and regulatory processes at each level of signaling (ECM, plasma membrane, focal adhesion, and cytoplasm) are captured to give a comprehensive picture of the internal and external mechanoregulatory environment. Specific emphasis is placed on the focal adhesion complex which acts as a central hub of mechanical signaling, regulating cell spreading, migration, proliferation, and differentiation. In addition, a wealth of available knowledge on mechanotransduction is curated to generate an integrated signaling network encompassing the central components of the focal adhesion, cytoplasm and nucleus that act in concert to promote durotaxis, proliferation, and differentiation in a stiffness-dependent manner.

Keywords

extracellular matrix; mechanotransduction; migration; proliferation; differentiation

1. Introduction

One of the first reports stressing the importance of the cell-ECM interaction was published in 1968 by Stoker and colleagues.[1] Using both normal and transformed cells, the group showed that cell adhesion to a rigid substratum is necessary to complete mitosis.[1] It is now known that cell adhesion to the ECM is not only essential for proliferation, but also directing growth, apoptosis and differentiation.[2] The extracellular matrix (ECM) is a proteinaceous

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meshwork that provides structural support, establishes tissue architecture, and elicits signals relaying the status of the mechanical environment to adherent cells. As cells establish connections to the ECM, high strength adhesion complexes, or focal adhesions (FAs), form a link between the cytoskeleton and extracellular space. FA formation is central to cellular mechanotransduction, the sensing of biophysical cues and translation of mechanical information into biochemical signals that direct cell behavior. A key question that arises from this intimate relationship between cell and ECM is how – in an organism with tissue stiffness ranging from elastic moduli of ~.1 kPa (fluids, brain, and lung) to well into the GPa range (bone) –cells respond to mechanical stimuli from the extracellular environment to meet the needs of the resident tissue while also regulating the status of the ECM to properly sustain themselves? The answer to this question relies on a proper balance of "responder" signals from the ECM that direct cell behavior (outside-in signaling) and "effector" signals from the cell that modulate mechanical properties and/or constituents of the local ECM (inside-out signaling). Irrespective of signaling directionality, mechanotransduction is essential to achieve proper balance of both outside-in and inside-out signals. When bidirectional signaling between the cell and ECM is perturbed as a result of aging or disease, dysregulated ECM turnover occurs and leads to loss of normal tissue function.[3] For instance, cancer cells develop complex signaling mechanisms that induce cancer-associated fibroblasts (CAFs) to synthesize a rigid tumor stroma that later potentiates the processes of malignant growth and invasion.[4] Meanwhile, cardiac injury can induce hyperactivity of cardiac fibroblasts, leading to excessive ECM deposition in the myocardium and, later, cardiac fibrosis.[5]

Adding to the complexities of mechanotransduction, there is a large body of evidence that implicates mechanical signaling through cell-cell contacts - via machinery within adherens junctions – as a central regulator of collective cellular activities including endothelial cell migration and epithelial morphogenesis.[6] Though outside the scope of this review, it is worth noting that mechanical inputs from cell-cell and cell-ECM contacts synergize to support physiological tissue functions. As an example, the loss of cell-cell mechanosignaling during epithelial-to-mesenchymal transition (EMT) has been postulated to contribute to the metastatic nature of cancer cells by shifting mechanical responses towards cell-ECM signals alone.[7] Thus, complex networks of mechanical signals are generated at contact points between both adherent cells and the ECM. Continuous integration and transduction of physical stimuli stemming from both cell-ECM and cell-cell contacts is essential to maintain proper tissue homeostasis. Focusing hereafter on the cell-ECM interaction, tissue morbidity, injury, or aging can lead to dysregulation of intrinsic mechanotransduction pathways, causing ECM stiffening and pro-fibrotic phenotypes that exacerbate pathogenesis.

In this review we will attempt to coerce the intracellular mechanisms that convert mechanical cues from the cell-ECM connection to biochemical signals, touching on mechanically regulated cell components and pathways well connected to canonical cellular activities. We take an outside-in approach, beginning with a discussion of the ECM and the diverse range of mechanical profiles the tissues of the body acquire to support their function. In addition, we describe how cells utilize a combination of biophysical and biochemical mechanisms to remodel the ECM, touching on how dysregulation of ECM remodeling drives disease progression. We then consider the role of integrins and mechanosensitive

modules of the FA in regulating mechanotransduction. Specifically, we examine the mechanical regulation of cell spreading, migration, proliferation, and differentiation with emphasis on the role the plasma membrane and focal adhesions in recruiting core intracellular signaling pathways to initiate downstream changes in cell phenotype as a result of mechanical stimulus.

2. The Extracellular Matrix: Foundations of Matrix Mechanics and Cell Stiffness Sensing

2.1. Matrix Mechanics: Stiffness (Rigidity) and Non-linear Elasticity

A wide range of material properties contribute to the global mechanical profile of the ECM. Bulk isotropic rigidity, or elastic modulus (E), is one of the best studied mechanical properties of the ECM and is intimately tied to tissue function. Tissues under constant mechanical loading (e.g. bone) exhibit exceedingly high elastic moduli ($E \sim 2.5$ GPa) whereas internal organs that undergo extensive deformation (e.g. lung) can have $E < 500$ Pa. Stiffness, the magnitude of local forces sensed by adherent cells, is largely governed by the protein composition of the ECM with higher concentrations of fibrillar collagens, and in some cases proteoglycans, directly correlating to a stiff (high E) matrix.[8] For instance, proteomic analysis of bone ECM have estimated collagen I constitutes upwards of 90% of the organic phase.[9] As collagens are synthesized and inserted into the matrix, cells organize collagen fibers in various orientations and crosslinked states. Crosslinking by cell-secreted enzymes such as lysyl oxidase (LOX) and lysyl oxidase-like 2 (LOXL2) along with the orientation of collagen fibers regulate collagen packing and, by extension, density of collagen in the ECM. Improper cell-mediated collagen organization is a hallmark of multiple diseases. In breast cancer, increased mammary density and tangential alignment of collagen fibers to the tumor boundary increase stromal stiffness.[10, 11] Though the roles of collagen packing in directing matrix stiffness have been well-characterized, a comprehensive list of parameters governing local matrix rigidity has yet to be defined.

Non-linear elasticity, of the matrix has also received considerable appreciation in recent years. *In vivo,* the non-linearity of the elastic profile of the ECM is largely governed by degree of hydration and is dependent on the presence of cells as demonstrated by the almost perfectly elastic behavior of acellular, bulk ECM.[12] Interestingly, cell-derived strain-stiffening of soft (~200 Pa), fibrous ECM can result in an increase in the local stiffness sensed by neighboring cells, leading to a cell phenotype reminiscent of that observed on gels with E ~ 50 kPa.[13] These results are in agreement with additional studies that have shown cells seeded on soft, non-linear elastic matrices show large cell areas (a hallmark of active mechanotransduction on stiff substrates) equivalent to those observed on stiff, linear elastic matrix.[14, 15] By combining our discussions of non-linear elasticity and Elastic modulus of bulk ECM, the various ECMs of the body can be thought of existing along a 2-D continuum of both bulk elastic modulus and local non-linear rigidity in which proper balance of the two properties establishes unique matrix profiles that guide cell behavior (Figure 1).

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2.2 Traction Force Generation and Cellular Sensing of Matrix Mechanics

Cells detect the rigidity of bound matrix by generating traction forces at adhesion complexes and assessing the resistance of the ECM to deformation. As adhesion complexes mature, the actin cytoskeleton is linked to ECM-bound integrin. Retrograde flow of actin (myosin motor-driven rearward treadmilling) at mature adhesion complexes induces the development of traction forces on the ECM, providing a mechanism of detecting matrix resistance. Traction force dynamics during cell migration are well described by the popular "molecular clutch" mechanism which claims that, when physically linked to adhesion complexes, actin flow is slowed and myosin-motor activity generates rearward traction forces that are used to propel the cell body forward during migration (for a fantastic review of the clutch mechanism see reference 16b).[16] Future studies by Gardel and colleagues would add to the collective understanding of intrinsic clutch behavior through the discovery that F-actin flow rates and traction force generation at FAs exhibit a spatially dependent, biphasic relationship.[17] Rapid F-actin flow at lamellipodial FAs near the leading edge form an inverse relationship with traction force generation whereas lamella-bound, mature FAs display a direct relationship between F-actin flow speed and traction force generation below a critical F-actin speed threshold of ~10 nm/s.[17] Subsequent works that incorporated the aforementioned biphasic relationship into mathematical models of F-actin network dynamics via an assumed "stick-slip" behavior have revealed additional insights into the spatial heterogeneity of F-actin flow in the leading edge and the resulting effects on traction force generation and lamellipodial width.[18]

Concurrent with the discoveries made by Gardel, the mechanically dependent nature of the clutch hypothesis was explained in a model that predicted clutch behavior upon attachment to ECM of varying rigidity.[19] By including ECM stiffness as an added variable to the original clutch theory, Odde and colleagues were able to show that the molecular clutch acts in a mechanosensitive manner with cells generating higher traction forces on stiffer substrates. Further, their model predicted that low rigidity, high compliance substrates exhibit oscillatory "load-and-fail" dynamics wherein tension builds slowly among individual actomyosin clutches, slowing retrograde flow until the tension reaches a critical threshold at which time loss of one clutch engagement (i.e. the ECM-cytoskeleton connection) leads to subsequent disruption of all other actin-clutch interactions. Conversely, high stiffness substrates induce a rapid buildup of tension in engaged clutches that leads to shorter clutch lifetimes and fast actin flow rates. Some predictions made by the stiffness-sensitive clutch model have been validated experimentally. For instance, cells adhered to stiff substratum can have multiple actin stress fibers disrupted with minimal change in cell shape whereas disruption of just a single stress fiber in cells seeded on soft substrates can have drastic impacts on cell behavior likely due to the "load-and-fail" mechanism previously discussed.[20] Recently, there have been growing efforts to extend the observations reported in 2D clutch models to 3D systems. Though still largely unexplored, preliminary studies have revealed that a second cytoskeletal clutch may exist in the 3D environment that cooperates with the previously mentioned 2-D clutch system to elongate stress fibers anchored at FAs, providing migrating cells a mechanism of maintaining consistent attachment to ECM throughout locomotion.[13] Further studies that continue to expand 2D clutch observations

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into three-dimensions will likely lead to exciting discoveries that extend current understandings of the molecular clutch to *in vivo* processes.

2.3 Cell-Mediated Biophysical Remodeling of the ECM

2.3.1 Force-Induced Unfolding of Fibronectin—Forces applied to the ECM can have serious implications on the conformational status of its constituent proteins. One of the most prominent force-sensitive ECM proteins is fibronectin (Fn), a large glycoprotein with a significant role in mediating adhesion, migration, growth, and differentiation (reviewed in reference 21a–d).[21] In the unstressed state, Fn fibers embedded in the ECM assume an unhinged, extended conformation in which type III repeats (FnIII) remain in a closed state.[22] Binding of β1 integrins to the RGD motif within the 10th repeat of FnIII (10FnIII) directs the arrangement of individual Fn fibers into bundled fibrils in a cytoskeletal dependent manner.[23] Specifically, traction forces exerted by cells bound to fibronectin are hypothesized to physically stretch the Fn protein, unfolding and exposing the 1FnIII cryptic site, providing nucleation sites for Fn bundling and fibril formation.[24]

The FnIII repeats possess additional cryptic sites that can elicit unique biological effects upon mechanical unfolding. Molecular simulations of Fn dynamics have proposed force-induced unfolding of the 10FnIII repeat decouples the RGD motif from a PSHRN synergy sequence situated along 9FnIII, decreasing affinity and selectivity of integrin binding partners.[25] Indeed, it has been shown that unfolding of 10FnIII creates a critical increase in distance between the Fn10III RGD and Fn9III PSHRN motifs, leading to lowered α5/α3 affinity while αV integrin binding remains unaltered, suggesting a role of cell-derived forces in autonomically tuning the integrin selectivity of the associated ECM.[26] Such “integrin switching” could induce profound implications in numerous biological processes including differentiation, pathological and physiological angiogenesis, and progression of fibrosis.[27] Recent efforts in the biomaterials community have attempted to engineer synthetic Fn fragments containing the vital 9FnIII and 10FnIII repeats to better exhibit the mechanosensitive properties of natural Fn and offer tuning of the mechanically induced integrin switch observed in vivo. Fn fragments engineered to promote α3/α5β1 binding have been utilized to direct mesenchymal stem cell (MSC) differentiation towards an osteogenic lineage, mediate epithelial to mesenchymal transition, and promote the formation of mature, prominent vasculature.[26, 28, 29] Control of integrin binding specificity will represent an essential design parameter in the engineering of mechanoresponsive biomaterials.

Aside from the self-regulation of integrin-binding, cell-derived forces on Fn can also impact the global matrix profile. As forces are applied to Fn, type III repeats become exposed and the conformation of the Fn fiber undergoes extensive elongation. Near maximum elongation a strain-stiffening behavior is observed in which fiber stiffness increases from roughly 50 kPa to 1–2 MPa.[30] Strain-stiffening along the Fn fiber is largely owed to the exposure of cryptic sites which, following unfolding, lead to the formation of high strength cryptic bonds that significantly increase the fiber’s resistance to yield and rupture.[31] Previous work in our lab has shown the monumental impacts Fn’s strain-stiffening behavior on cell phenotype and activity.[14] Fibroblasts seeded onto soft, non-linear elastic (Fn-rich) substrates display cell areas nearly equivalent to those of cells seeded on stiff, linear elastic (Col I-rich) substrates.

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We proposed a mechanism in which adherent fibroblasts apply tensile forces that strain-stiffen the underlying Fn fibrils and subsequently increase local matrix rigidity. By association, neighboring fibroblasts sense this increased matrix rigidity and increase contractility and cytoskeletal remodeling themselves, initiating a positive feedback mechanism that drives active matrix remodeling towards a more rigid profile. Interestingly, fibroblast-induced strain stiffening of the ECM, a hallmark of lung fibrosis, is believed to be enhanced by upregulation and increased activation of the αVβ3 integrin.[14] By applying tensile forces to non-linear elastic matrix, fibroblasts can spatiotemporally regulate the stiffness of the surrounding ECM and mechanically-induce local cells to begin ECM remodeling and turnover.

2.3.2 Physical Remodeling of Collagens, Thrombospondins, and Other Matrix Proteins—

Biophysical remodeling of the ECM is also achieved through collagen remodeling/reorganization and is a commonly observed feature of many cancers including ovarian, thyroid, and prostate.[32] Collagen reorganization is a rather crude process in which cells actively bind collagen fibrils through the β1 integrin subunit and forces generated by actin retrograde flow physically bend and shift collagen fibrils bound at the cell surface.[33] Reorganization of collagen fibers into aligned lattices has a twofold impact on matrix mechanics and cell activity. First, collagen alignment increases collagen packing density and, thus, increases matrix rigidity. Second, aligned collagen fibrils enhance migration potential of adherent cells. The latter of the two consequences is of particular interest in tumorigenesis as fiber alignment in a perpendicular orientation to the tumor interface drastically increases cancer cell invasiveness and, by extension, metastatic potential.[11, 34]

There are many additional ECM constituents whose biophysical remodeling are under intense study. The adhesive glycoprotein thrombospondin is commonly observed in vasculature and isoform thrombospondin-1 mediates cell behavior by sensing shear stresses, though the structural basis for this phenomenon is still poorly understood.[35] In the arterial wall, disturbed flow upregulates thrombospondin-1 expression and leads to wall stiffening through the activation of profibrotic genes and may contribute to the progression of vascular diseases such as atherosclerosis.[36] Conversely, lack of hydrodynamic shear stress on thrombospondin-1 initiates endothelial cell apoptosis through an autocrine loop containing thrombospondin-1 and its cell surface receptor, αVβ3.[35] Tenascin, a small proteoglycan expressed during development and in processes such as wound healing, contains numerous FnIII repeats that give the molecule mechanoresponsive properties. The role of the splice variant tenascin-C in organogenesis has been shown to play a critical regulatory role in controlling neurite outgrowth, motor axon outgrowth, and neuronal differentiation.[37] These studies of tenascin behavior provide further evidence that the extensible FnIII repeats serve an important biological role in ECM proteins by conferring mechanical sensitivity to higher-order protein structures. The discovery of novel ECM protein isoforms containing FnIII repeats, or sequences with similar properties, will be vital to developing a more complete understanding of the matrix response to cell forces and the resulting impacts on cell behavior. We have seen how biophysical remodeling of the ECM by adherent cells drives changes in matrix material properties and that the consequences of such changes can have

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pronounced impacts on cell behavior. We now address the biochemical avenues utilized by cells to actively remodel the ECM.

2.4 Cell-Mediated Biochemical Remodeling of the ECM

2.4.1 Matrix Metalloproteinases (MMPs) and Tissue Inhibitor of Metalloproteinases (TIMPs)—Adherent cells utilize a combination of intracellular and extracellular biochemical mechanisms to dynamically remodel the underlying ECM. A key feature of ECM remodeling is the coordinated turnover of protein constituents through degradation of denatured ECM proteins. Matrix metalloproteinases (MMPs) constitute a family of 23 matrix-associated zinc metalloproteinases with a conserved HEXXHXXGXXH $Zn2+$ binding motif (reviewed in 38a-c).[38] Two subsets of soluble MMPs with well described roles in organizing matrix remodeling are the collagenases and gelatinases. Collagenases target a Gly-Ile/Leu bond in the α-chain of collagen types I, II, and III for cleavage. Numerous Gly-Ile/Leu cleavage sites exist along the collagen triple helix, but proteolytic cleavage appears to be biased towards sites located in regions of the helix that easily unfold.[39] These observations suggest a potential mechanism in which stiff matrices drive increased cell contractile forces which potentiate proteolysis by exposing collagen cleavage sites and reducing steric hinderance between cleavage site and catalytic site of the collagenase, allowing for cancer cell invasion and survival signaling through the exposure of cryptic αVβ3 sites.[40, 41] Upregulation of MMP-mediated degradation of the ECM can have lofty consequences on disease progression by altering integrin binding and potentially regulating mechanoresponsive pathways. MMP-9 mediated degradation of fibronectin creates fragments that upregulate cell surface expression of β6 integrin subunit and promote breast cancer cell invasion.[42] Though lacking a specific mechanism, it is possible that MMP-9 cleaves Fn fibrils to expose cryptic binding sites on Fn fragments that promote migration, as seen with MMP-14 mediated degradation of collagen in cancers.[40] Further, proteolytic cleavage of collagen IV has been shown to expose cryptic sites that switch integrin specificity from α1β1-dependent binding to αVβ3 and drives angiogenesis.[43] Thus, matrix remodeling through MMPs provides a robust mechanism by which adherent cells can regulate the presentations of adhesive ligands in the local microenvironment to direct the behaviors of neighboring cells. Targeting of MMP activity will represent a major focus in the development of cancer therapeutics in the coming years.

Canonically, MMP activity is regulated by the tissue inhibitor of metalloproteinases (TIMP) family of molecules. TIMP expression has been shown to both enhance and repress tumor malignancy and is dependent on tissue type.[44] Elevated expression of TIMPs prevents matrix degradation and causes the build-up of fibrous matrix components such as Fn and collagen, leading to matrix stiffening. In pulmonary fibrosis, TIMP-2 is highly expressed in fibroblastic foci and is believed to protect the fibrotic ECM from collagenolysis.[45] Inhibition of collagen degradation has also been implicated in breast cancer progression as increased stromal collagen in mammary tissues increases tumor formation and cancer cell invasion.[46] Taken together, a paradigm can be described in which the mechanically induced activation of MMPs and TIMPs must be tightly regulated such that temporal remodeling of the ECM proceeds without excessive matrix degradation nor accumulation of fibrous ECM components and preserves native tissue mechanics.

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2.4.2 Matrix Crosslinking Through Lysyl Oxidase (LOX) Family—Other cell-secreted factors target the ECM by chemically modifying matrix constituents to alter the material properties of the bulk ECM. A common mechanism cells utilize to increase ECM stiffness is the crosslinking of fibrillar collagen fibrils into high density, interlaced lattices. Collagen crosslinking *in vivo* is mediated by the activity of the LOX family of copper-dependent amine oxidases. Hydroxylation of lysine residues on the collagen triple helix by LOX family members drive the formation of pyridinoline cross-links between adjacent triple helices.[47] Elevated LOX activity is a common feature of stiffness driven pathologies and has important roles in tumorigenesis and progression of tissue fibrosis.[48] Namely, pathological LOX activity indirectly regulates cell behavior through matrix remodeling. LOX-mediated increases in matrix rigidity produce mechanical signals that induce breast cancer cell invasion through enhanced focal adhesion signaling.[49] Bleomycin-induced mouse models of lung fibrosis show upregulated LOX activity and fibroblast-to-myofibroblast differentiation, providing a role for LOX in recruiting matrix-secreting cells to further stiffen the ECM in both normal wound healing and pathological fibrosis.[50] Further research will be needed to probe the mechanistic basis behind upregulation of LOX signaling and whether LOX activity is dynamically controlled to provide mechanical stimulation to local cells through its cross-linking behavior.

2.4.3 Posttranslational Modifications of Matrix Components—ECM proteins can also be subjected to enzyme-mediated posttranslational modifications (PTMs) that alter the protein's biochemical composition. PTMs elicit a dynamic range of effects on the state of the matrix and have implications in progression of diseases such as rheumatoid arthritis, cancer, and fibrosis.[51–53] Examples of well-studied PTMs include citrullination, nitrosylation, glycosylation, and isomerization.[52] Previous work in our lab has studied the specific impacts of Fn citrullination on integrin-Fn clustering dynamics and the resulting impacts on cell behavior.[53] Briefly, citrullination describes the conversion of arginine residues into citrulline through the enzymatic activity of peptidyl arginine deaminases.[54] Using mass spectrometry, we found 24 different citrullination sites exist within the Fn protein, five of which reside within the RGD-containing FnIII9 and FnIII10 repeats. Citrullination of Fn drives a shift in integrin binding dynamics that reduces $\alpha V\beta 3$ binding and enhances $\alpha 5\beta 1$ interactions. From a mechanotransduction perspective, loss of $\alpha V\beta 3$ binding in favor of $\alpha 5\beta 1$ correlates with increasing ECM rigidity as $\alpha 5\beta 1$-mediated mechanical signaling has higher sensitivity on high stiffness ECM than $\alpha V\beta 3$.[55] Thus, PTM of ECM molecules can synthetically elicit robust cellular mechanoresponses by mimicking a high stiffness environment without changing the mechanical properties of the matrix. Though still in early stages of research, further study of known PTMs within the ECM, combined with the discovery of new PTMs and their associated impacts on cell-ECM interactions and potential downstream changes in cell phenotype, show promise in providing new insights into the role of extracellular protein modifiers in the regulation of mechanotransduction. While many extracellular mechanisms have been shown to modify signaling crosstalk between cells and the ECM, internal pathways can also regulate ECM synthesis through modulation of transcriptional targets.

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2.4.4 Transcriptional and Translational Regulation of Matrix Components—

Matrix material properties play a prominent role in producing signals that enhance or suppress transcription of ECM-related genes. A key element of wound healing, fibroblasts cultured on stiff collagen matrices upregulate expression of MMPs and fibrillar collagens.[56] Elevated transcription of MMPs is also observed in many metastatic cancers due to stromal stiffening by CAFs and contributes to cancer cell invasion and metastasis.[57] Thus, stiff matrix induces a positive feedback loop that drives production of additional matrix components that contribute to further matrix stiffening. The development of a stiffness-associated positive feedback loop may also occur during stiffness-dependent differentiation of MSCs towards osteogenic lineages. Osteogenic induction of MSCs on stiff substrates display upregulated expression of collagen I genes with collagen I gene expression being dependent on α2 integrin subunit expression on the cell surface.[58] Secreted collagens from osteoprogenitors stiffen the underlying matrix, eliciting mechanical signals that direct further collagen expression and drive movement towards an osteogenic lineage.

Following transcription, mRNAs encoding ECM proteins can undergo additional modifications such as splicing to produce numerous isoforms of the original molecule and unique splice variants can enhance or abrogate functions associated with the native protein (reviewed in references 59 and 60).[59, 60] For example, Fn isoforms lacking extra domain A (EDA) protect against the development of fibrosis by preventing fibroblast activation to a myofibroblast phenotype.[61] Presence of the Fn EDA domain is believed to augment both cell spreading and migration, suggesting that EDA binding upregulates mechanotransduction signaling pathways.[62] Interestingly, the EDA domain of Fn is not a target ligand of the α5β1 or αVβ3 integrins that bind RGD motifs in FnIII repeats.[60] Given previous discussions of the roles α5β1 and αVβ3 integrins play in mechanotransduction, the EDA domain of Fn may represent a cell adhesion domain that "blinds" cell stiffness interpretation, causing the cell to default to stiff matrix programming (e.g. high levels of spreading/migration). By this logic, upregulation of EDA^+ Fn fibers would promote focal adhesion growth and subsequent development of stress fibers, manifesting in myofibroblast differentiation. A second possibility that may contribute to the lack of α5β1 or αVβ3 binding within EDA domains is that the Fn EDA region contributes to the instability of nearby FnIII repeats, disrupting the availability of RGD/PSHRN motifs.[63] The specific implications of Fn (and other ECM protein) splice variants on disease progression and cell behavior is an area of ongoing research that promises to yield valuable insight into the roles of pre-modified ECM ligands on cellular mechanical activation.

3. Integrin Dynamics and Mechanotransduction

Integrins represent a superfamily of transmembrane proteins that act as adhesion receptors by binding to the extracellular matrix and other cell-surface ligands (review in reference 64a–c).[64] To date, 18 α and eight β subunits have been shown to form 24 distinct integrin heterodimers. Integrin regulation has been well studied in mediating cell migration, survival, proliferation, and differentiation.[65] Cellular adhesion is first reliant upon proper integrin-ECM binding and the formation of stable interactions that later mature into tightly anchored FA sites that anchor cytoskeletal components to the ECM. Once matured, FAs not only

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anchor the cell to its underlying matrix, but also serve as robust mechanical signaling hubs that relay the status of the biophysical environment to cellular control centers.

3.1 Selective Integrin Expression is Directed by Mechanical Stimuli

Mechanical signals from the extracellular environment regulate the specificity of integrin subunit expression. For instance, stimulation by shear stress upregulates the exocytosis of α5 integrin at lipid rafts in endothelial cells and increases in tissue stiffness upregulate β1 integrin presentation.[66, 67] Fibrotic tissues are characterized by increased ECM stiffness and myofibroblast infiltration. Upregulation of α6β1 integrin expression has been reported to enhance myofibroblast invasion and collagen IV degradation, suggesting a feedback loop in which fibrotic ECM drives β1 subunit expression in myofibroblasts and β1 mechanosignaling drives further remodeling and invasion.[67] β1 family integrins are required to effectively respond to stiffness and foster adhesion in fibronectin-rich tissues and upregulation of cell surface expression of β1 integrins may represent a mode by which cells increase their mechanoresponsiveness to environments that require constitutive mechanosensing (e.g. stiff, high rigidity substrates).[68] β1 integrin activity is required for vascular smooth muscle cell (SMC) adhesion to Fn and β1 integrins are found dispersed along the SMC basal surface during early migration.[69] Interestingly, the same study also showed that SMC migration is largely dependent on β3 integrin activity, found localized to the cell periphery throughout migration.[69] Perhaps one of the most important studies describing the distinct roles of stiffness-directed β3/β1 integrin function was a study by Roca-Cusachs and colleagues who showed that, in cells seeded on Fn, α5β1 is primarily responsible for withstanding matrix forces whereas αVβ3 integrins exhibit more robust mechanotransduction and reinforce adhesions by recruiting cytoskeletal components to adhesion sites.[55]

While the previous examples have largely consisted of observations made from studies of mesenchymal cell populations, immune cells also utilize unique integrin modules to support their intrinsic functions *in vivo*. The broad specificity of β2 integrin binding partners have been proposed to assist in preliminary adhesion and migration of circulating polymorphonuclear granulocytes along the endothelial monolayer towards sites of inflammation.[70] Mechanotransduction through β2 family integrins may also represent a central node of the immunological mechanical response as ablation of β2 cell surface expression significantly perturbs both traction force generation and FA force coupling.[71] These discoveries place mechanosignaling through the β2 family integrins as a central regulator of immune cell migration and, indeed, neutrophils lacking β2 cell surface expression fail to infiltrate collagen-free zones at wound centers.[72] β2 integrin adhesion to adsorbed fibrinogen has also been shown to drive upregulation of pro-inflammatory cytokine secretion, suggesting that, aside from mechanical regulation, β2 integrins may also play a role in phenotype modulation through the activation of signaling pathways controlling the inflammatory response.[73] Thus, spatial regulation of distinct integrins represents a powerful regulatory mechanism to control FA dynamics and respond to extrinsic forces during cell migration or ECM stiffening. Along with differential integrin function, cells regulate mechanotransduction and FA activity by aggregating, or clustering, integrins in small domains within the plasma membrane.

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3.2 Integrin Clustering: A Mechanotransduction Amplifier

In order to mediate cell adhesion, individual integrins must not only initiate tight interaction with ECM ligands but also strengthen intermolecular interactions by clustering. Inside-out integrin activation and subsequent global conformational changes in integrin structure lead to a higher affinity state that enables tighter ligand binding, whereas clustering of integrins promotes overall adhesion-ECM avidity and, thus, stabilize FAs.[74] Clustering of integrins also plays a large role in the response to mechanical stimulus. Recruitment and activation of focal adhesion kinase (FAK), a key mediator of mechanotransduction, correlates well with integrin clustering, suggesting that integrin clustering initiates the assembly of mechanotransduction machinery at the FA site.[75] As previously mentioned, force application induces αVβ3 clustering and mobilization of the force-sensor protein talin to focal adhesions to enhance mechanotransduction whereas α5β1 clustering fortifies adhesion strength and modulates cell contractility.[55] Although clustering is critical to the cell's biophysical response system, the exact mechanisms governing integrin clustering are still elusive and multiple elements have been implicated in the process.[76]

Integrin clustering has been shown to be lipid raft dependent.[77] Lipid rafts are plasma membrane domains, rich in cholesterol and sphingolipids, that recruit membrane-associated proteins to the raft region.[78] Aside from regulating integrin affinity and avidity, lipid rafts also recruit/mobilize other FA-associated proteins during adhesion and can therefore manipulate mechanotransduction in a broad manner. Csk binding protein (Cbp), responsible for negatively regulating the activity of the Src family kinases (SFKs), is exclusively localized in lipid rafts and becomes transiently phosphorylated by Fyn, a lipid raft localized SFK, during cell adhesion.[79] The phosphorylated Cbp then recruits Csk to the plasma membrane inner leaflet and induces deactivation of SFK. Thus, Cbp/Csk operate in concert to modulate mechanotransduction at the FA by diminishing SFK activity. These discoveries suggested that microdomains of the plasma membrane can fine-tune integrin-mediated signaling during cell adhesion and migration by differentially mobilizing lipid raft components.

3.2.1 Regulation of Integrin Clustering—Our group has recently reported that Thy-1, a GPI-anchored membrane-bound glycoprotein, also couples integrin αvβ3 with Cbp and Fyn in lipid rafts.[80] This functionality requires cis-interaction between Thy-1 and integrin αvβ3 and is dependent on the lipid raft localization of Thy-1. It has been shown that Thy-1 plays a pivotal role in lipid raft-regulated integrin signaling.[81] By recruiting Fyn and Cbp to αvβ3, Thy-1 could promote fast activation of SFKs and FAK surrounding the pre-clustered integrin nanodomain as well as rapid deactivation of these kinases through the Cbp-Csk pathway. Therefore, Thy-1 offers mechano-sensitivity to cells and loss of Thy-1 expression could contribute to the progression of fibrosis. Chemokine receptors also play a part in integrin clustering. CAFs have shown strong capability to induce clustering of β1 integrin on gastric cancer cell surfaces and promote tumor invasion and progression, potentially through β1-mediated mechanotransduction and ECM remodeling.[82] Clustering is induced by CAF-mediated activation of CXCL12/CXCR4 on the cancer cells and treatment with an anti CXCL12 agent disrupted chemokine receptor signaling and reduced cancer cell-CAF interaction, leading to suppressed tumor progression. A similar

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phenomenon has been identified in prostate cancer, in which invasive growth of cancer cells is correlated to CXCL16/CXCR6 or CXCL13/CXCR5 induced clustering of integrin αvβ3.[83]

The glycocalyx is another central regulator of integrin clustering. On the cell surface, glycocalyx constitutes a layer of plasma membrane-bound macromolecules consisting of hyaluronan, glycoproteins, sulfated proteoglycans, and adhesion receptors. Glycocalyx, especially endothelial glycocalyx, has long been recognized as an important player in mechanotransduction.[84] Models describing the behavior of integrins inside glycocalyx predict that the thickness and rigidity of glycocalyx can affect integrin clustering depending on integrin affinity and conformational state.[85] The model also suggests that more robust integrin clustering occurs on rigid matrix with high ligand density. The same group later discovered that bulky cell surface glycoproteins in glycocalyx can funnel active integrins into premature clustering sites and promote integrin activation in an actomyosin-independent manner.[86] Dynamically, a recent study found that in the presence of glycocalyx, the binding kinetics of nascent and mature focal adhesions is in good agreement with the Monte Carlo simulation (see below for an explanation of the Monte Carlo simulation). The study also indicated that higher thickness and rigidity of glycocalyx decreases binding affinity at the nascent adhesion site but does not affect mature adhesions, effectively remobilizing integrins from new, unstable adhesions into mature adhesions.[87] Over the past half century, research into focal adhesion biology has provided a myriad of insights into its constituents, functions, and roles in pathology. We now know that the focal adhesion is not only an anchoring structure for directing stable adherence to the ECM, but it is also a key response element in the cell interpretation of ECM-derived signals.

4. Mechanosensing at the Focal Adhesion Complex

4.1 Formation and Maturation of Adhesion Complexes

Nascent adhesions rapidly form following ECM-integrin binding and mark the smallest adhesion structures on the cell with diameters measured at less than 1 μm.[88] Nascent adhesions have been troublesome to fully characterize but are known to exist in a transient, unstable state of maturation or disassembly. Over time, maturing nascent adhesions develop into large, organized FAs. FAs consist of highly specialized protein aggregates responsible for interpreting and responding to mechanical stimuli at the cell-matrix interface. The major classes of proteins found at focal adhesions include cytoskeletal components, mechanosensing enzymes, scaffolding proteins, and regulatory proteins. Proper FA formation first requires the recruitment of mechanotransduction machinery and cytoskeletal components to the adhesion complex. Localization of kindlin-2, FAK, F-actin, and myosin II have all been observed as being necessary for complete FA maturation.[75, 89] These results suggest dual roles for signaling molecules and cytoskeletal components in regulating focal adhesion maturation dynamics with myosin II forming late linkages between the adhesion complex and cytoskeleton while FAK, talin, and kindlin-2 promote recruitment of focal adhesion scaffolding and signaling proteins. The mechanotransduction components of the focal adhesion each serve unique roles in deciphering mechanical signals and are discussed in depth below.

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4.2 Cytoskeletal Recruitment and Adhesion Growth: Role of the Talin-Vinculin Complex

4.2.1 Talin—Two classes of mechanotransduction proteins coexist at the FA complex, proteins that change conformation in response to mechanical stimulus (mechanosensors) and proteins that recruit downstream effectors in response to mechanical stimulus (mechanoeffectors). Among the mechanosensors, talin is arguably the most critical for proper FA maturation and mechanotransduction. Talin is a mechanosensing scaffolding protein comprised of a globular head domain that binds β-integrins and a rod domain that possesses numerous vinculin binding sites.[90] Application of force across the talin protein is believed to "activate" the mechanoresponsive properties of talin by exposing cryptic vinculin binding sites within the extensible talin rod domain and mediating the formation of the F-actin vinculin complex.[91] Further, talin binding active integrin subunits has been shown to elicit ECM binding in adjacent integrins, initiating a positive feedback loop in which integrin clustering drives recruitment of talin to the adhesion site and, by extension, additional linkage to the actin cytoskeleton.[92] This mechanism provides a fundamental role for talin in force-mediated assembly of the mechanotransduction complex wherein mechanical stimulus activates talin and drives coupling of adhesion to actin assembly at maturing adhesions.

Talin-induced coupling of the cytoskeleton to the integrin complex is highly advantageous in modulating cell processes such as contractility, spreading and migration, but dysregulation of talin signaling can drive pathology and has been linked to aggressive forms of oral and prostate cancers.[93] In the context of pathogenesis, improper talin regulation can have a two-fold impact on cell behavior. Overexpression of talin enhances cytoskeletal linkage and mechanotransduction leading to upregulated growth, invasion, and protection from anoikis, likely through increased cell contractility and/or talin-induced activation of FAK.[94] In contrast, it has been suggested that talin cleavage by cathepsin H reduces talin residency times at FAs and leads to increased migration.[95] The latter may represent a mechanism by which cancer cells are able to upregulate FA turnover and avoid long-term cytoskeletal anchoring to migrate across the stiff, stromal ECM. Thus, it is clear proper balance of talin activity is required to establish stable focal adhesions while also preventing aberrant cell contractility and elevated mechanical signaling.

4.2.2 Vinculin—Vinculin is a well-studied mechanoeffector first found colocalized with F-actin at developing FAs.[96] Structurally, the vinculin protein is comprised of four head domains (D1-D4) and a tail domain (D5) that exhibit strong intramolecular interactions in an inactive conformation.[97] Upon phosphorylation or mechanical unfolding, the head and tail domains separate to expose cryptic binding sites for talin, α-actinin, paxillin, and F-actin.[98, 99] These structural observations give credence to the theory that vinculin operates as a "mechanical clutch", mediating force transfer between the cell-ECM interface and the cytoskeleton.[100] Thus, vinculin is a primary responder in mobilizing cytoskeletal components to fortify FAs in response to mechanical stimulus. Mechanistically, it has been suggested that increased extrinsic forces on the focal adhesion, namely as a result of ECM stiffening, promote vinculin-induced assembly of a highly stable talin-vinculin-actin complex that can exert greater cytoskeletal force on the ECM.[101] Indeed, vinculin association is required for the stabilization of FAs in response to tensile stresses and

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recruitment of vinculin to the FA proceeds in a force-dependent manner.[102] These results imply vinculin is both recruited to and maintained at FAs subjected to high forces, promoting force transfer to the cytoskeleton and agree with observations that high ECM rigidity drives increased talin-vinculin residency times and decreased turnover rates at FAs.[103–105] Interestingly, the talin-vinculin complex tightly associates with the β1 subunit tail and may provide a rationale behind the role of β1 integrins in the response to ECM stiffness wherein β1 integrins are primarily responsible for growing adhesions.[55, 106]

4.3 Mobilization of Signaling Machinery: the FAK-Src Complex

4.3.1 Focal Adhesion Kinase (FAK)—FAK is a 125-kDa protein tyrosine kinase found localized to adhesion sites. Functional evaluations of FAK activity have identified FAK's roles as a scaffolding protein and highly active protein kinase responsible for regulating mechanotransduction as well as FA turnover dynamics. FAK is activated by autophosphorylation and exerts its signaling functions through phosphorylation of downstream targets such as paxillin and Src family kinases.[107] The numerous signaling pathways regulated by FAK's primary targets make the kinase one of the most prominent mechanoeffectors of the FA complex and a major initiator of mechanical signaling. The recruitment of FAK to the focal adhesion and its subsequent activation have been proposed to be both force and chemically driven, though lacking a formal mechanism. Chemically, integrin clustering has been suggested to trigger production of phosphatidylinositol 4,5-bisphosphate (PIP2) which recruits FAK and subsequently triggers FAK autophosphorylation at Tyr397.[108] Mechanically, increasing matrix rigidity has been shown to increase FAK recruitment and activation through upregulated myosin II contraction.[109] In both cases, FAK is actively recruited to growing adhesions and may be correlated to an increased requirement for supervised mechanotransduction at mature adhesion complexes.

Interestingly, FAK's role in controlling FA growth dynamics becomes antagonistic in mature FAs as FAK autophosphorylation at Tyr-397 and/or Tyr-576, are required for FA disassembly and loss of FAK leads to limited migration and increased FA size, though a holistic mechanism is still lacking.[110, 111] This function places FAK as a critical component in the control of cell migration and improper FAK regulation, specifically through elevated FAK activity, has been well linked to the enhanced migratory properties of cancer cells.[112] There is evidence that mechanical stimuli work to enhance FAK signaling in cancer, suggesting a link between mechanically induced FAK activation and pathology. Stiffening of the tumor stroma by LOX-mediated crosslinking has been shown to direct FAK phosphorylation leading to an invasive phenotype in colorectal cancer.[113] Though no formal mechanism has been suggested, stiffness-driven FAK activation may initiate the recruitment of binding partner paxillin and subsequent downstream signaling through the mitogen-activated kinase (MAPK) pathway, leading to pro-migratory gene expression.[114] Thus, the FAK mechanical activation pathway may be a critical process in stiffness-driven pathologies and targeting of FAK activation or its downstream effectors represents a promising therapeutic target for the treatment of cancer.[115]

4.3.2 Src and the SFK family—Among its numerous binding partners, FAK is most tightly associated with the SFK members Src, Fyn, and Yes which complex with FAK to

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form robust signaling hubs at the FA. As with FAK, the SFKs are essential for modulating FA disassembly and loss of Src activity inhibits migration and cell spreading, implying both FAK and Src are necessary but not sufficient for proper FA turnover.[111, 116] Classically, FAK-Src complex formation was found to occur through integrin-mediated FAK activation which primes FAK's Src recognizing domain for binding.[117] However, an additional mechanism of FAK-Src activation has been discovered in which clustering of β3 integrins activates and stabilizes Src at the plasma membrane.[118] An interesting detail in the report by Arias-Salgado and colleagues is the specificity of β3 integrin in mediating Src activation. As discussed previously, mechanotransduction is generally stronger through the β3-family integrins compared to β1-family integrins. Src mobilization may confer a state of rapid FA assembly and FAK-Src mediated disassembly at the β3 integrin, forming the less stable cell-ECM linkages Roca-Cusachs and colleagues observed while studying αVβ3-Fn binding dynamics.[55] This feature would be advantageous in priming cells for rapid migration in processes such as wound healing or germ layer formation, but dysregulation could also be catastrophic in the progression of pathology as aberrant β3 integrin expression (specifically αVβ3) has been well correlated to metastatic phenotypes.[119] While β3/FAK/Src signaling may represent a key mechanism of cancer cell migration, β1/FAK/Src signaling has proven to be an equally vital component to oncogenesis. Hirata et al. reported CAF stiffening of the melanoma stroma primes the β1/FAK/Src signaling axis, leading to enhanced cell survival and drug resistance.[120] Thus, the downstream effects of FAK/Src signaling appear to be integrin-dependent and the coordinated regulation of FAK/Src signals is a key component of cancer progression. Once bound and activated, FAK-Src targets numerous FA-associated proteins for phosphorylation, of particular interest in the maturing FA are the adaptors paxillin and p130Cas (Cas) (discussed in the following section).

Taken together with the previous discussion, the FAK-Src and talin-vinculin signaling axes appear to exhibit distinct roles in maintaining FAs. Talin-vinculin signaling is responsible for fortifying the FA-cytoskeleton linkage in response to mechanical stresses through interaction with F-actin, eliciting stronger cell contractility and anchorage. Meanwhile, FAK-Src signals are essential for promoting FA disassembly and coordinating cell motility. This suggests a paradigm in which increasing stress on the adhesion complex directs a stable talin-vinculin interaction and induces integrin recruitment and adhesion growth to better distribute mechanical stresses. Indeed, these claims are given credence by the discovery that talin knockout has no impact on initial cell spreading, but talin knockout cells fail to establish maintained adhesion and spreading.[121] Given that protrusion formation is a FAK-Src-Rac mediated process, these results suggest that initial adhesion formation and cell spreading is FAK-Src dependent, but sustained adhesions are regulated by talin-vinculin activity. Thus, although intimately linked, FAK-Src and talin-vinculin signaling are responsible for modulating different modes of the mechanoresponse element at FAs and coordinated regulation of both pathways is essential for orchestrating FA turnover dynamics.

4.4 Paxillin and p130Cas: Central Mediators of FAK-Src Signaling

4.4.1 Paxillin—Cytoskeletal linkage to the FA can also be mediated by the scaffolding protein paxillin. Structural analyses of paxillin have shown the protein is comprised of binding domains for a number of FA-associated proteins including FAK, vinculin, and

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actopaxin.[122] Unlike talin, paxillin's activity and residency time at FAs are unaffected by substrate stiffness or force application.[104,105] Rather, paxillin activation is regulated by kinase activity and, upon phosphorylation by FAK-Src at Try31 and Tyr118, initiates the molecule's adaptor activity and exposes binding domains for the effector kinase and/or docking sites for other signaling components such as Crk, Csk, and Src.[109, 123] Given Csk's role in negatively regulating Src activity, paxillin's adaptor function may serve to tightly control Src signaling by positioning negative regulators in close proximity to the active signaling complex. It is worth noting that paxillin phosphorylation has been linked to mechanical strain, but this coincided with FAK phosphorylation and may represent a correlative rather than causal relationship wherein mechanically sensitive FAK is activated and subsequently activates paxillin downstream.[124] Thus, it is possible that paxillin itself is not mechanically sensitive but rather FAK's mechanosensing properties directly impact paxillin's activation status. Paxillin recruitment to the FA was shown to be ubiquitous on stiff substrates, but dependent on cytoskeletal contractility when cultured on low rigidity substrates, suggesting that actomyosin contractility, along with FAK, are major regulators of paxillin activity below a critical stiffness threshold.[105]

Once active within the FA, paxillin binds the rod domain of vinculin and provides an alternative route of linking the actin cytoskeleton to FA complexes.[125] Though paxillin and talin both bind vinculin and, by association, actin, the linkage complexes exhibit unique function and provide a mechanism of spatiotemporal regulation of vinculin activity. Case et al. discovered that inactive vinculin is recruited to paxillin and is spatially situated at the lower (cytoplasm-proximal) portion of the FA and mechanical activation induces vinculin to localize to more membrane-proximal regions, encouraging talin binding during focal adhesion maturation.[126] Thus, given paxillin's role in mechanical signaling, paxillin-bound vinculin may represent the mechanical signaling module of cytoskeletal linkage that can rapidly rearrange or disassemble the actin linkage at the focal adhesion in response to extrinsic forces or intrinsic migration signals. This ideology is supported by reports that the paxillin-vinculin interaction exists transiently compared to talin-vinculin.[98] Further, paxillin recruitment of vinculin is likely a key initiator of tension-mediated FA maturation and activated vinculin localizes with talin to fortify the maturing FA. Taken together, talin-vinculin complexes promote long-term, stable connections between the FA and cytoskeleton to properly anchor the cell during tension-mediated FA maturation while paxillin and vinculin form transient mechanotransduction complexes that regulate actin dynamics during changes in cell shape, such as leading edge protrusion formation.[127]

4.4.2 p130Cas—The paxillin scaffold has also been suggested to target Cas, a major mechanosensory protein and a main target of SFK signaling, to the growing FA.[128] Cas is linked to the paxillin scaffold through the adaptor protein Csk.[129] Numerous studies have given Cas a regulatory role in migration, survival, and invasion.[130] Similar to talin, the structure of the Cas protein features an extensible substrate domain that, upon force application, promotes Cas phosphorylation by Src making Cas a canonical mechanosensor.[129, 131] Studies by Braniš and colleagues eloquently utilized Cas mutants to elucidate the roles of distinct Cas domains in regulating various aspects of Cas behavior.[132] They reported that deletion of the FAK-binding, N-terminus SH3 domain and C-terminus Cas-

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family homology (CCH) domains prevent localization of Cas to developing focal adhesions, confirming previous studies that the C-terminal binding domain is necessary for Src interaction and recruitment of Cas.[133] Further, mechanical stress is correlated with longer Cas residency times at FAs, a phenomenon also observed in talin.[104, 105] Thus, increased residency times of primary mechanosensors at the FA may suggest that an elevated flux of mechanical cues elicits a higher demand for signaling mediators, causing slower turnover rates at the benefit of enhanced mechanotransduction.

We have seen that multiple members of the focal adhesion act in concert to transduce mechanical signals at the integrin-ECM interface to downstream effectors. ECM stiffening (or similar processes that lead to the sensing of a high stiffness environment) drives the recruitment and activation of the mechanosensor talin at the maturing FA, leading to vinculin association and linkage to the actin cytoskeleton (Figure 2). Concurrently, FAK and Src recruit molecular adaptors that assemble numerous mechanotransduction components at the FA in addition to providing alternative routes of vinculin/F-actin activation. By linking integrin force sensing to the cytoskeleton and cytoplasmic signaling pathways, the components of the FA orchestrate the formation of a complex signaling hub that actively maintains mechanical homeostasis and simultaneously regulates multiple cell processes. We now address how mechanical signaling from FA components direct mechanotransduction through the cytoplasm to control cell behavior.

5. Signaling Through the Cytoplasm

Mechanical stimulus is now known to actively regulate a wide range of cell functions outside of adhesion dynamics. The focal adhesion complex carries out mechanotransduction by actively recruiting a number of canonical signaling pathways to effectively transduce biophysical cues into coordinated signals that instruct migration, proliferation, survival, and differentiation (reviewed in reference 134a–d).[134] Keeping within the scope of this review, we highlight the role of the mechanical microenvironment and the coordinated actions of FA components, the actin cytoskeleton, and canonical cytoplasmic signaling cascades in controlling cell behaviors. Specifically, we describe how it is the complex interplay of FA and cytoskeletal dynamics that properly orchestrate cell spreading, motility, differentiation and proliferation with emphasis on the roles of the core mechanotransduction complexes at the FA.

5. 1 Cell Morphology is Mediated by the Mechanical Microenvironment

As cells adhere to substrate, rapid changes in FA component activity and cytoskeletal dynamics cause pronounced changes in cell morphology, intracellular contractility, and cell stiffness in a mechanically dependent manner.[135, 136] Classically, increases in substrate rigidity are met with increases in cell area, contractility and stiffness in conjunction with the formation of thick, organized F-actin stress fibers. This phenomenon was first characterized in a study by Yeung and colleagues who found that fibroblasts seeded on high stiffness Fn-coated substrates exhibit substantial increases in cell spreading above a critical substrate stiffness threshold between 3 to 9 kPa.[131] Spreading is accompanied by a concurrent increase in cytoskeletal contractility as a result of stress fiber formation and similar trends

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have been observed in epithelial and cancer cells.[137, 138] Furthermore, between substrate stiffnesses of 1–5 kPa, fibroblast stiffness (elastic modulus) has been found to be nearly equal to the stiffness of the surrounding environment, suggesting that physiologically-relevant (soft tissue) stiffnesses direct fibroblasts to internally mirror the mechanical properties of the extracellular environment.[136] Indeed, in a separate study, substrate mechanics were shown to dominate cell stiffness changes at soft tissue rigidities.[139] At pathological soft tissue stiffnesses (>5 kPa), cell stiffness plateaus at a maximum value and the actin cytoskeleton begins to form dense stress fibers, suggesting the cell utilizes secondary mechanisms to strengthen itself after stiffness saturation is reached.[136] Taken together, these findings point to a complex interplay between substrate mechanics, the actin cytoskeleton, and cell geometry wherein increasing substrate rigidity and changes in cell shape coregulate cell stiffening.[139] At very high stiffnesses, cell area is maximized, and the actin cytoskeleton exhausts all resources to further stiffen the cell, leading to the formation of compensatory, high strength stress fibers to withstand additional extrinsic forces.

5.1.1 Rho GTPases and Regulation of the Actin Cytoskeleton at the Leading Edge—The Rho family GTPases heavily contribute to remodeling the actin cytoskeleton in response to stimulus, and multiple family members have been implicated in both cell spreading and cell migration (Figure 3). Cell spreading/migration is induced at the plasma membrane by coordinated actin polymerization that generates filopodial and lamellipodial protrusions at the leading edge. Protrusion formation is spatiotemporally regulated by a variety of pathways, but is heavily reliant upon the activity of Rho family members Cdc42 and Rac to produce functional filopodia and lamellipodia, respectively.[140] Cdc42 is a downstream target of paxillin signaling and is believed to stimulate the polymerization of filopodial actin filaments independent of Rac. Filopodial actin polymerization is engaged when Cdc42 forms a complex with IRSp53 (or BAIAP2) and mammalian-enabled (Mena) protein, whose concentration is tightly correlated to filopodial growth.[141] In addition, neural Wiskott-Aldrich syndrome protein (N-WASP) and WASP-family verprolin-homologous protein 2 (WAVE2) have also been shown to regulate actin polymerization in a Cdc42-dependent manner.[142] WAVE and WASP family proteins canonically mediate actin polymerization through activation of Arp2/3, however, filopodia have been shown to form in the absence of WAVE and WASP.[143] Given that Arp2/3 requires intact actin filaments to form new nucleation points, WASP/WAVE activity is more likely involved in lamellipodia formation rather than *de novo* actin polymerization seen in filopodia. These considerations suggest that the aforementioned Cdc42-IRSp53-Mena pathway is a vital effector of filopodia formation.

Cdc42 also plays a central role in protrusion formation by stimulating the activation of Rac. Active Rac can signal to numerous actin binding proteins to control protrusion extension at the leading edge. Classically, lamellipodia formation is directed by Rac signaling to WASP which recruits Arp2/3 to stimulate actin polymerization along previously formed filaments.[144] Additions to mature filaments lead to the characteristic branched actin networks within lamellipodia. As Rac stimulates Arp2/3-mediated actin polymerization it concurrently induces a secondary pathway that leads to cofilin inactivation through LIM kinase 1 (LIMK-1).[145] Loss of cofilin, a potent initiator of actin depolymerization, stabilizes Arp-

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mediated actin nucleation within the growing lamellipodia and enhances protrusion formation. As lamellipodia develop along the spreading cell, components of the adhesome are recruited to the protrusion and begin forming adhesion complexes at the leading edge. Rac is believed to mediate part of the integrin recruitment process as vascular endothelial cells seeded on fibrinogen upregulate αVβ3 (the integrin receptor for fibrinogen) recruitment to lamellipodia, suggesting that high-affinity integrins are recruited to the cell edge in a coordinated manner that optimizes adhesion dynamics.[146] Though yet to be proven, it will be interesting to see if a similar phenomenon exists with other integrins. In conclusion, the combined activity of Cdc42 and Rac at the leading edge cooperatively regulate actin dynamics to generate filopodial projections and lamellipodial branched networks that promote cell spreading/migration.

5.1.2 Regulation of Cell Spreading and Rho GTPases by Focal Adhesion Signaling—Mechanical regulation of Rho GTPase activation and the accompanying impacts on actin dynamics are exerted through the activity of numerous FA components including FAK, Paxillin, and Cas, making the Rho GTPases key mediators of mechanotransduction signaling to the cytoskeleton. Rho GTPases are activated in a stiffness-dependent manner and the expression levels of Rac and RhoA have been shown to increase proportionally with increasing substrate stiffness.[147] Upstream, FAK activation appears to be the key driver of mechanical Rho activation as re-expression of FAK in FAK–/– cells induces cell spreading.[148] To date, two distinct, FAK-associated pathways have been described to converge on Rac. First, FAK/Src activates and complexes with Cas, leading to Crk recruitment and subsequent activation of the Dock180-engulfment and cell motility (DOCK180/ELMO) protein complex. Activated Dock180/ELMO, acting as a RhoGEF, stimulates the activation of Rac pointing to Dock180/ELMO as an alternative pathway to Rac activation independent of Cdc42 activity.[149] In the case of a high stiffness substrate, upregulation of FAK activity would lead to slower Cas turnover rates at the focal adhesion, driving downstream Rac activity through Cas-Crk-Dock180/ELMO signaling, fostering protrusion formation.

In addition to Cas, paxillin has ties to Rac-activation and paxillin mutants incapable of binding FAK show markedly lower spreading.[150] In response to mechanical activation, paxillin’s adaptor function is initiated by FAK/Src. Activated paxillin can then bind a number of Rho GTPase regulatory molecules through complex formation with paxillin kinase linker (PKL) and Crk.[151] As with Cas, paxillin bound Crk can recruit Dock180/ELMO for Rac activation whereas PKL complexes with p21 activated kinase (PAK), PAK-interacting exchange factor (PIX) and Nck.[149, 152] PIX, acting as a Rho GEF, is a central mediator of Cdc42/Rac1 activation and loss of the Rac-PIX interaction leads to significantly reduced cell spreading.[153] PAK is a downstream target of Rac signaling that targets extracellular signal-regulated kinase (ERK) activation and subsequent inhibition of cdGAP (a Rac1/Cdc42 GAP).[154, 155] Protein-protein interactions between PIX and PAK are imperative for proper signaling at the leading edge and perturbation of PIX/PAK complex formation inhibits lamellipodia formation.[156] An important consequence of the PIX/Rac/PAK signaling axis is activation of LIMK-1 which drives cofilin inhibition in the growing protrusion.[157] Interestingly, the same Paxillin-PKL-PIX-PAK-Nck complex has

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been implicated in downregulating cell spreading as overexpression of a paxillin mutant with perturbed PKL binding domains led to uncontrolled protrusion formation and sustained Rac-activation, suggesting that PKL-PIX-PAK can act as both a Rho GEF and GAP and tight regulation of the complex may be instrumental in coordinating directional migration.[151, 158] Further research will be necessary to be better understand spatiotemporal control mechanisms governing the PKL-PIX-PAK complex and the effects unique protein-protein interactions within the complex impart on Rho activity. Nevertheless, it is clear that mechanical regulation tightly controls the underlying activity of the Rho GTPases and their downstream effects on protrusion formation.

To conclude our discussion on stiffness-activated cell spreading, we focus on a central mediator of RhoA activity found localized to the FA, p190RhoGAP. RhoA activation is essential for the formation of dense actin stress fibers at mature adhesion complexes and RhoA expression is enhanced in many types of metastatic cancers.[159] During nascent adhesion formation, Src activates p190RhoGAP which leads to suppressed RhoA activity at the maturing FA.[160] By suppressing RhoA, it is believed that actin polymerization occurs at developing protrusions without actomyosin bundling and stress fiber formation, removing the burden of internal contractility on protrusion growth. Suppression of actomyosin contractility would be highly favorable at the leading edge and, indeed, protrusion formation cannot occur when p190RhoGAP is inhibited.[160] Crosstalk between RhoA and p190RhoGAP activity is also essential in the lamella as p190RhoGAP activity must be downregulated for RhoA-mediated stress fiber formation in maturing FAs. Talin may exert a temporal control mechanism that leads to inhibition of p190RhoGAP given that loss of talin prevents ECM-cytoskeletal linkage but has no impacts on cell spreading.[121]

Taken together, the spatiotemporal activities of the Rho family GTPases RhoA, Rac, and Cdc42 are tightly regulated during protrusion formation and coordinated inhibition of RhoA along with concurrent activation of Rac/Cdc42 at the cell boundary drive protrusion formation. Over time, RhoA GEFs and Rac/Cdc42 GAPs are recruited to the newly formed lamella to suppress actin polymerization and promote stress fiber formation, leading to adhesion growth and maturation in a spatiotemporally dependent manner. Mechanical regulation is imparted on the Rho GTPases through the integrated activity of the FA components FAK/Src, paxillin, and Cas, all of whom exhibit enhanced activity when matrix stiffness is increased, making the mechanical microenvironment and FA mechanosensors essential in directing stiffness-mediated cell spreading. Given that cell spreading is an isotropic form of cell migration, *per se*, many of the Rho GTPase mechanisms previously discussed also hold true in our following discussion of stiffness directed cell migration.

5.2 Durotaxis and Haptotaxis: ECM and Mechanically Motivated Cell Migration

Cell migration is a ubiquitous process central to the formation and upkeep of tissues. During migration, highly coordinated FA turnover propels the cell body forward as adhesion complexes are disassembled at the rear of the migrating cell, matured in the lamella, and reassembled at the leading edge. Numerous physiological and pathological processes rely on intracellular response systems that direct cell migration towards gradients of extrinsic stimuli. To date, many unique sources of signals have been shown to induce directed

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migration. Chemokines (chemotaxis) recruit neutrophils to sites of inflammation; ECM organization (haptotaxis) promotes cancer cell migration along gradients of laminins, fibronectin, and collagen IV; electrical fields (electrotaxis) exist endogenously during tissue regeneration and have been clinically applied to guide neuron migration; and gradients of stiffness (durotaxis) along substrates trigger MSC migration and differentiation.[161, 162] Mechanotransduction through the FA complex is a highly conserved feature that regulates all modes of directed migration and seminal studies have shown that FA instructed migration is largely regulated by FAK/Src signaling activities.[163] Keeping within the scope of this review, we analyze the role of mechanotransduction components in the command of haptotaxis and durotaxis, two directed migration processes intimately tied to the cell-matrix interface.

5.2.1 Haptotaxis—Haptotaxis is strongly influenced by the cell's integrin profile, and crosstalk between specific integrins generate signals that coordinate the direction of migration. MSCs exhibit haptotaxis on matrices coated with vitronectin (Vn), Fn, and collagen I, implicating a potential mode of collective cell migration throughout germ layer formation and axis specification during embryogenesis.[164] Interestingly, haptotactic migration appears to occur via signaling through unique integrin subunits. Keratinocyte haptotaxis across Fn-coated substrates was found to be dependent on αVβ6 activity whereas α5β1 integrins bound to Fn strictly mediated cell spreading and unsupervised migration.[165] In contrast, α3β1 signaling controls keratinocyte migration across laminin-5 and FAK-induced activation of ERK at β1 focal adhesions is believed to drive haptotaxis in mouse embryonic fibroblasts on Fn.[166, 167] These seemingly conflicting results suggest that the β1 integrins regulate different modes of migration in a ligand and cell-specific manner. Given that immature (undifferentiated) cells exhibit restricted integrin expression profiles, cell-specific haptotaxis may be coupled to the integrin repertoire available to the cell in its present state. In macrophages, crosstalk between various integrins have been shown to coregulate both haptotaxis and chemotaxis. Abshire et al. reported that macrophage haptotaxis is dependent on pro-migratory signals stemming from FAK activity at α5β1, and α5β1 signaling potentiates downstream activation of integrin α4β1, a central component of chemotactic signaling.[168] Thus, downstream activities of the haptotactic program may also prime other migratory modes for activation in cell types that respond to multiple forms of directed migration.

Integrins can also negatively regulate migration by generating inhibitory signals that promote immobilization. For instance, signals from α6β4, a major integrin component of hemidesmosomes, inhibits α3β1-mediated haptotaxis of keratinocytes seeded on laminin-5 and may indicate that a coordinated interplay of pro-migratory and anti-migratory signals stemming from distinct integrin families regulates matrix-directed migration.[166] Sources of pro- and anti-migratory signals may arise from the signaling activities of the various mechanosensors targeted by FAK during mechanical activation. For example, overexpression of paxillin downregulates migration whereas overexpression of Cas augments migration.[169] Cas has been shown to upregulate migration through interaction with substrate Crk and downstream activation of Rac, perhaps through the activation of the Dock180/ELMO Rho GEF previously discussed.[170] Overexpression of active paxillin may

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downregulate migration by enhancing vinculin recruitment and adhesion growth.[109] However, the nature of paxillin's regulation is more complex as it was discovered that paxillin can also recruit PIX and PAK to promote FA disassembly at the trailing edge of the cell through PAK-mediated phosphorylation of paxillin, a pro-migratory process.[171] Perhaps paxillin's regulatory role is controlled by upstream activators that expose migration-associated binding sites in a spatially dependent manner. This would allow paxillin to provide anti-migratory signals within the lamella, leading to vinculin recruitment, cytoskeletal linkage, and adhesion growth. In contrast, at the trailing edge, paxillin signaling through pro-migratory modules enhances the rate of FA disassembly and recycling.

5.2.2 Durotaxis—Durotaxis describes directed cell migration up towards regions of increasing substrate stiffness. Studies into the role of the substrate in initiating durotaxis have shown that a combination of gradient strength (gradient slope), along with absolute stiffness, coregulate migration of both MSCs and vascular smooth muscle cells.[172, 173] Cells sense matrix stiffness and direct migration towards regions of highest rigidities via intracellular traction force sensing.[173] In an eloquent study performed by Plotnikov and colleagues, it was shown that fluctuations in traction forces through the FA components FAK, paxillin, and vinculin are required for proper durotaxis.[174] Further, the authors proposed a mechanism in which fluctuations in traction forces at focal adhesions allow the cell to sample the elasticity of the surrounding ECM and move towards regions that resist traction force-induced deformations to the greatest degree (signifying the most rigid region of the ECM).[174] FAK/paxillin/vinculin activity may be implicated in this process because, at adhesion complexes situated along the stiffest regions of ECM, traction forces will generate the most intracellular tension, forming an intrinsic mechanical sensor at the plasma membrane. The resulting initialization of mechanotransduction programs at the stiffest regions of ECM would then lead to downstream vinculin recruitment through paxillin activation and formation of the FAK-paxillin-vinculin signaling complex, effectively signifying the membrane domains experiencing the greatest extrinsic forces. Further, spatial control of adhesion maturation or disassembly at the leading edge may be skewed towards maturation at adhesion sites situated on the stiffest substrates as tensile force triggers adhesion maturation through myosin II contractility. Thus, nascent adhesions formed on regions of substrate with the highest rigidity will generate the most tension, while simultaneously being preferentially matured over adhesions generating lower tension, leading to directed migration. This proposed mechanism is reconciled by the observation that stiffness dependent activation of Cas by Src leads to the generation of intracellular tension and concurrent adhesion growth.[175] FAK may also be an essential upstream effector of cytoskeletal remodeling at the leading edge of the migrating cell. FAK/ERK signaling has been shown to inhibit cdGAP activity, leading to enhanced protrusion formation and migration along stiff surfaces.[155] Durotaxis is also an important feature in the progression of diseases with fibrotic signatures. During pulmonary fibrogenesis, formation of stiff foci in lung tissues initiates fibroblast migration towards the lesion, leading to expression of α-smooth muscle actin, a marker of myofibroblast activity and a pro-matrix secretory phenotype.[176]

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5.3 Proliferation/Cell Cycle Regulation by Mechanotransduction

Cell proliferation is tightly regulated by a combination of intrinsic and extrinsic signals that coordinate both initiation of, and progression through, the cell cycle. For instance, the receptor for hyaluronic acid (HA), CD44, has been shown to induce a proliferative response in glioma cells seeded on soft substrates in the presence of HA to a similar degree as glioma cells seeded on stiff substrates lacking HA.[177] Further, matrix stiffness alone has been shown to upregulate proliferation rates in non-malignant mammary epithelial cells independent of matrix composition or architecture, suggesting specific receptor-ligand binding events and matrix mechanics alone act redundantly to trigger proliferative responses given the correct cellular inputs.[178] From a mechanotransduction perspective, components of the focal adhesion constantly interpret complex mechanical signals that dictate whether the proliferation requirements of anchorage-dependence and contact inhibition are met (reviewed in reference 179).[179]

5.3.1 FAK Signaling and Control of Proliferation—FAK was the first FA component found to modulate proliferation when Gilmore and Romer fortuitously discovered that inhibition of FAK in human and mouse cells resulted in markedly lower proliferation rates.[180] We now know that FAK can bidirectionally regulate progression through G1/S checkpoint of the cell cycle and the directionality of FAK's regulation is activation state dependent as overexpression of FAK stimulates proliferation and inactive FAK arrests cell growth.[181, 182] By extension, matrix stiffening can induce FAK activation and may represent a mechanism of hyperproliferation in cancer cells.[113] Both hepatocellular carcinoma (HC) and glioblastoma cells have been shown to assume a pro-proliferative phenotype in response to increased matrix stiffness.[183] In HC cells, it was proposed that mechanically regulated proliferation signals are transduced through β1-integrin/FAK signaling, a mechanism also implicated in the regulation of proliferation in embryonic stem cells.[184]

FAK's control of cell proliferation is executed through a complex array of signaling systems that converge on activation of the MAPK/ERK signaling network. The role of MAPK as a downstream effector of FAK signaling was first reported in a series of studies that showed stretch induced FAK activation led to ERK1/2 and p38 MAPK activation and loss of FAK signaling abrogated MAPK-activation as well as stretch associated increases in proliferation.[185] Tissue-stiffening and subsequent changes in cytoskeletal tension have now been shown to mediate ERK1/2 phosphorylation in both keratinocytes and endothelial cells.[186] Within the FA complex, it is known that FAK/Src activation primes FAK for interaction with Grb2 which serves as an adaptor, linking the Ras/MAPK cascade to FAK signaling.[187] Interestingly, fibroblasts lacking N-Ras have reduced migratory potential and proliferation rates but show increased expression of collagen I and Fn perhaps exhibiting a crosstalk between matrix secretion and cell growth pathways.[188] FAK/Src can also stimulate proliferation independent of Grb2 by activating the Cas SH2 domain which leads to the binding of the adaptor Nck and downstream ERK activation through the Ras-ERK pathway.[189] Given the stiffness-dependent nature of Cas activation, the latter mechanism may represent a signaling mode wherein mechanical stimuli activate mechanosensing FA components and drive the upregulation of proliferation signals through ERK

phosphorylation. Regardless, Grb2 and Nck-mediated activation of ERK appear to be complementary pathways in regulating proliferation as loss of either Grb2 or Cas downregulates proliferation while dual knockout prevents proliferation almost entirely.[190] Along with the previously described FAK/Ras/MAPK signaling axis, FAK activation in response to increased matrix stiffness has also been shown to upregulate Cyclin D1 expression through a Rac-dependent mechanism, perhaps suggesting that Rac and Ras proliferative signals are redundant pathways that converge on proliferative nuclear targets.[191]

Upon activation via Ras or Rac signaling, mechanically induced ERK translocates to the nucleus where it is believed to target Cyclin D1, a regulator of cell cycle progression from the G1 to S-phases, making FAK signaling particularly relevant at the onset of mitosis.[192] Cyclin D1 activity may also be upregulated by elevated matrix stiffnesses as it has been reported that culture on stiff substrates enhances Cyclin D1-dependent Rb phosphorylation and S-phase cell cycle entry.[191] The activity of nuclear proteins responsible for regulating proliferation may also be related to spatial availability (or lack thereof). Recently, it was shown that, activation of stretch channels drives the cytoplasmic localization of the cell cycle inhibitor p27Kip1, promoting progression into the S phase of the cell cycle.[193] A similar phenomenon was also observed in multicellular spheroid cultures wherein compressive stresses placed on the spheroid promoted nuclear p27Kip1 activity and proliferation arrest in late G1 phase.[194]

5.3.2 Rho GTPase Activity and Proliferation—In addition to FAK, Rho family GTPases have strong ties in linking mechanical signals to cell proliferation.[179] Rho activity is increased with increasing substrate stiffness and has been tied to enhanced proliferation in both endothelial cells and numerous cancers.[195] Though yet to be thoroughly described, proliferation signals from Rho, as a result of a high stiffness environment, may be regulated by FAK activity as inactive FAK inhibits Rho-associated increases in proliferation in endothelial cells, perhaps due to a lack of ERK activation and cdGAP inhibition.[182] The Rho family exerts its control of cell proliferation by activating multiple MAPK pathway members including ERK, p38 MAPK, and Jun N-terminal kinase (JNK).[196] Further, Rho can transcriptionally regulate proliferation in response to mechanical cues through downstream activation of myocardin-related family of transcription factors (MRTFs). MRTF is tightly coupled to cytoskeletal activation as inactive MRTF proteins are sequestered within the cytoplasm by G-actin. Upon recruitment of G-actin during actin polymerization, MRTF is freed and can localize to the nucleus where it serves as a coactivator of transcription with Rho-activated serum response factor (SRF).[197] Once localized in the nucleus, MRTF-SRF targets a number of genes including c-fos which induces transcription of the G1 cyclins.[198] Thus, mechanical activation of Rho drives recruitment of G-actin to sites of actin polymerization, freeing MRTF and allowing for coordinated transcription of genes regulating cell cycle progression into the S-phase. Mechanotransduction through the FA and mobilization of the actin cytoskeleton synergistically drive the induction of proliferative signals.

The role of matrix stiffness on control of MRTF activation appears to be essential in promoting contact-mediated proliferation and metastasis. Forced overexpression of MRTF

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overcomes the requirement of stiffness-mediated G-actin recruitment and leads to protection from anoikis in mammary epithelial cells, regardless of matrix stiffness, implying that mechanical cues strictly regulate MRTF activation, not endogenous MRTF signaling functions.[199] Further, mouse models of peritoneal fibrosis have shown that lysophosphatidic acid (LPA) signaling at sites of tissue injury induces MRTF nuclear activation and upregulation of connective tissue growth factor (CTGF) expression in mesothelial cells.[200] Mesothelial CTGF then acts as a profibrotic mitogen, stimulating proliferation and collagen secretion in local fibroblasts.[200] Upregulated matrix secretion leads to ECM stiffening and primes the mechanical environment with additional biophysical cues that further enhance proliferation and fibrotic progression, creating a pathological positive feedback loop. Taken together, a combination of biochemical and cytoskeletal components are mechanical regulators at the FA, linking forces at the cell-ECM interface into a downstream proliferative response. Proper function of the mechanically sensitive proliferation pathway is required for regulating the proliferation requirements of anchorage-dependence and contact-inhibition. Upregulated activation or expression of FAK, ERK pathway members, and/or the Rho-actin-MRTF axis are responsible for loss of proliferation control mechanisms that underly pathological hyperproliferation.[199, 201]

5.3.3 External Cues and Control of Proliferation—Recent efforts in the fields of mechanobiology have also aimed at elucidating internal mechanisms used by cells to overcome the naturally occuring spatial confinments that restrict cell growth and, by extension, proliferation. An elegant study performed by Nam *et al.* found that mechanical environments exhibiting rapid stress relaxation are significantly more conducive to proliferation than matrix with slow rates of stress relaxation.[193] The ability of a constrained cell to overcome confinement is derived from the generation of protrusive extracellular forces derived from cytoskeletal structures including the interpolar spindle and cytokinetic ring, suggesting the deformation capacity of the ECM is a central factor in overcoming spatial restrictions.[202] The role of spindle associated proteins, however, may not only be withheld to spatial confinement as a recent study showed stiff matrices can upregulate the expression of spindle pole body component 25 (*SPC25*), leading to Cyclin B1 upregulation and enhanced proliferation.[203] It will be exciting to see the insights new research into the role of extracellular confinement, ECM stress relaxation, and spindle protein regulation play in the mechanical regulation of proliferation.

5.4 Mechanotransduction as a Key Regulator of Cell Fate

Stem cells within the embryo (ESCs) and adult tissues (ASCs) are maintained in tightly regulated microenvironments (niches) that maintain stemness until proper stimuli initiates migration out of the niche and cell fate commitment. In vivo, stem cell differentiation is tightly regulated by a combination of mechanical and chemical cues, making the development of culture systems that properly instruct stem cell behavior a major hurdle in the development of biomaterials for stem cell applications.[204] To date, a number of microenvironmental cues including matrix stiffness, growth factor presentation, ECM composition, hypoxia, cell-cell cues (e.g. Notch), and stiffness gradients within the ECM have been shown to influence cell fate decisions.[29, 162, 205, 206]

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5.4.1 Matrix Mechanics Trigger Mechanically Sensitive Differentiation Programs—The importance of the ECM, and its associated mechanical properties, in regulating differentiation was first truly appreciated by the greater scientific community when Engler et al. showed that differentiation of MSCs can be directed strictly by mechanical cues and the inhibition of myosin II prevents stiffness-dependent lineage specification.[207] Underlying Engler's findings was an independent study done two years prior that reported cell shape directed stem cell fate decisions through actomyosin-derived contractility and subsequent RhoA/ROCK signaling.[208] Here, it was demonstrated that high stiffness substrates activate Rho/ROCK activity in MSCs to direct differentiation towards an osteoblast lineage whereas low stiffness substrates induce basal levels of Rho/ROCK activity and favor adipogenesis.[208] Rho/ROCK activity not only directs stem cell lineage commitment, but also has roles in germ layer migration and axis formation during embryogenesis, making Rho/ROCK signaling and cytoskeletal organization a key intermediate of mechanically mediated stem cell differentiation.[209]

Along with Rho/ROCK, mechanotransduction through integrins and FA components is responsible for directing differentiation responses. In bone marrow MSCs, Du et al. showed that soft substrates significantly enhanced caveolin-1-mediated internalization of β1 integrin subunits and neural differentiation could be attenuated through the inhibition of β1 internalization.[210] Mechanistically, it was found that ECM elasticity potentiates neurogenesis by downregulating the pro-osteogenic BMP/SMAD pathway through β1 internalization.[210] Along with downregulating BMP/SMAD activity, β1 signaling through FAK/ERK is necessary for neurite formation and outgrowth following differentiation from MSCs as well as directing differentiation and survival of pancreatic islet cells on collagen matrices.[211] Further, loss of β1 expression in ESCs prevents keratinocyte differentiation and downregulates α2 surface expression.[212] Interestingly, on stiff matrices, α2 integrin expression is upregulated and α2-derived signaling has been reported to mediate osteogenesis through a ROCK-FAK-ERK1/2 axis.[58] Talin may be a primary regulator of β1 integrin activity in undifferentiated SCs. Loss of talin downregulates β1 expression and significantly restricts cell fate commitment in ESCs plated on Fn substrates.[213] Thus, the mechanical microenvironment can bias stem cell differentiation towards specific lineages by regulating the integrin profile on the cell surface. Integrin-specific pathways and the associated components of the FA may be responsible for activating lineage-specific programs early in the differentiation process. For example, ESCs seeded on Vn and Fn promote differentiation to definitive endoderm through αV and α5 signaling, respectively.[214] Further, osteogenic differentiation is favored on high stiffness, collagen I-coated scaffolds.[215] Given that α2β1 is a potent collagen I-binding integrin, talin-induced β1 expression may upregulate α2 surface expression, a requirement for proper induction of the osteogenic differentiation program, in response to high stiffness mechanical cues. This would induce a positive feedback loop leading to upregulated collagen secretion and subsequent increases in α2 expression in a collagen-dependent manner. Indeed, adhesion of MSCs to collagen-I scaffolds potentiates osteogenesis and increasing collagen density has been shown to accelerate osteogenic differentiation rates.[206, 216]

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5.4.2 The ECM Cooperates with Local Soluble Factors to Control Cell Fate—

The ECM can also coordinate with available growth factors to direct the activation of specific differentiation programs. In the presence of transforming growth factor-beta (TGF-β), increased collagen crosslinking correlates with enhanced chondrogenesis.[217] Interestingly, a separate study showed that MSCs seeded on stiff collagen matrices favor SMC differentiation in the presence of TGF-β while soft substrates bias towards chondrogenesis and adipogenesis, suggesting that not only ECM composition, but also ECM organization is an important regulator of stem cell fate decisions.[218] In the same study, Park and colleagues also noted that suppression of cell adhesion strength on stiff substrates initiated the same differentiation patterns observed on soft substrates.[218] The impacts of cell adhesion on lineage commitment may be correlated to vinculin residency times at mature adhesions. Mature adhesions recruit vinculin to link the integrin complex to the actin cytoskeleton, leading to enhanced adhesion strength and, in MSCs plated on stiff substrates, vinculin was shown to suppress adipocyte differentiation by promoting nuclear localization of transcriptional coactivator with a PDZ-binding motif (TAZ).[219] TAZ, along with co-activator yes-associated protein (YAP), are key regulators of high-stiffness differentiation programs. As substrate stiffness increases YAP/TAZ become activated and translocate to the nucleus where they modulate transcription of numerous genes (reviewed in reference 220).[220] In MSCs seeded on stiff substrates, YAP/TAZ have been shown to be necessary for osteogenic differentiation and inhibition of YAP/TAZ signaling abrogates osteogenesis, independent of substrate rigidity.[221] In total, the mechanical microenvironment provides a number of cues that influence cell fate decisions. Signaling through the cytoskeleton, focal adhesion, and mechanically sensitive transcription factors tightly coordinate the activity of differentiation programs by interpreting the state of the ECM and its associated mechanical properties.

6. Conclusions and Future Perspectives

The field of mechanobiology has made great strides in delineating the mechanisms that control the cell’s ability to sense and respond to the local mechanical environment. In this review, we have highlighted the role bidirectional signaling between adherent cells and the ECM play in instructing cell function. Through proper balance of outside-in and inside-out signals, the cell and its substrate coregulate one another to maintain tissue homeostasis by controlling intracellular mechanotransduction (outside-in) and extracellular matrix mechanics (inside-out). Biophysical signals interpreted by FA mechanosensors are integrated into complex downstream signaling networks that are effectors of many cell processes including migration, proliferation, and differentiation (Figure 4). Dysregulation of mechanical signaling pathways have profound implications in driving progression of diseases with perturbed ECM mechanics and aberrant ECM turnover. Specifically, pathologies that induce ECM stiffening lead to upregulated signaling flux through mechanotransduction complexes whose downstream effectors enhance several well-described disease features including migration, invasion, proliferation, and further ECM stiffening. Though the role of the adhesome in controlling downstream cell activities by responding to biophysical cues is no longer questioned, many mechanisms underlying the inherent behaviors of adhesion complexes remain largely unknown.

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Many new aspects of focal adhesion biology and the mechanotransduction response network have been under intense study in recent years and will require further attention to complete our understanding of the adhesome. New multiplex imaging approaches that provide high spatiotemporal resolution will undoubtedly be a critical tool for studying the cytoplasmic regulation of FA mechanical adaptors (e.g. paxillin, Cas, etc.), and the activities of their associated binding partners, in response to mechanical cues.[222] A specific application for multiplexed microscopy techniques may be necessary in the study of the paxillin-PKL-PIX-PAK scaffold where, as previously mentioned, undiscovered changes in scaffold geometry or spatiotemporal activation of scaffold-bound proteins are hypothesized to mediate the Rho GEF/GAP activities of the complex during protrusion formation. Further, a comprehensive understanding of the spatial control of FA dynamics within distinct spatial domains during cell migration would likely prove useful in predicting migratory potential in cells during physiological and pathological processes.

In addition to microscopy tools, the engineering of novel biomaterial systems to recapitulate cell-ECM interactions will be essential in both basic and translational science. 3D material constructs will provide researchers with an added dimension of complexity that, while potentially burdensome, will be quite useful in bridging conclusions drawn from *in vitro,* 2D culture systems to observations made *in vivo.* Marrying new developments in biomaterials and mechanobiology will likely bring about a new class of material systems that utilize ECM constituents or other force-responsive fibers to produce synthetic microenvironments, allowing for tunable systems to better study mechanotransduction *in vitro.*[223] Indeed, it will be exciting to witness how the biomaterials field integrates discoveries in mechanobiology into novel material systems that not only mimic the biochemical microenvironment, but also recapitulate the biophysical cues cells use to coordinate their actions in vivo.

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Appendix

Glossary of Terms

Term	Acronym/Abbreviation
Cancer associated fibroblast	CAF
Csk binding protein	Cbp
Elastic modulus	E
Embryonic stem cell	ESC
Extra domain A	EDA
Extracellular matrix	ECM
Extracellular signal-related kinase	ERK
Fibronectin	Fn

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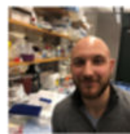
Term	Acronym/Abbreviation
Fibronectin type III repeat	FnIII
Focal adhesion	FA
Focal adhesion kinase	FAK
Jun N-terminal kinase	JNK
LIM kinase-1	LIMK1
Lysyl oxidase	LOX
Mammalian enabled	MENA
Matrix metalloproteinase	MMP
Mesenchymal stem cell	MSC
Mitogen-activated protein kinase	MAPK
Myocardin-related transcription factor	MRTF
Neural Wiskott-Aldrich syndrome protein	N-WASP
p130Cas	Cas
p21 activated kinase	PAK
PAK-interacting exchange factor	PIX
Paxillin kinase linker	PKL
Posttranslational modification	PTM
Serum response factor	SRF
Smooth muscle cell	SMC
Src family kinase	SFK
Tissue inhibitor of metalloproteinases	TIMP
Transcriptional coactivator with a PDZ binding motif	TAZ
Vitronectin	Vn
WASP-family verprolin-homologous protein	WAVE
Yes-associated protein	YAP

Biographies



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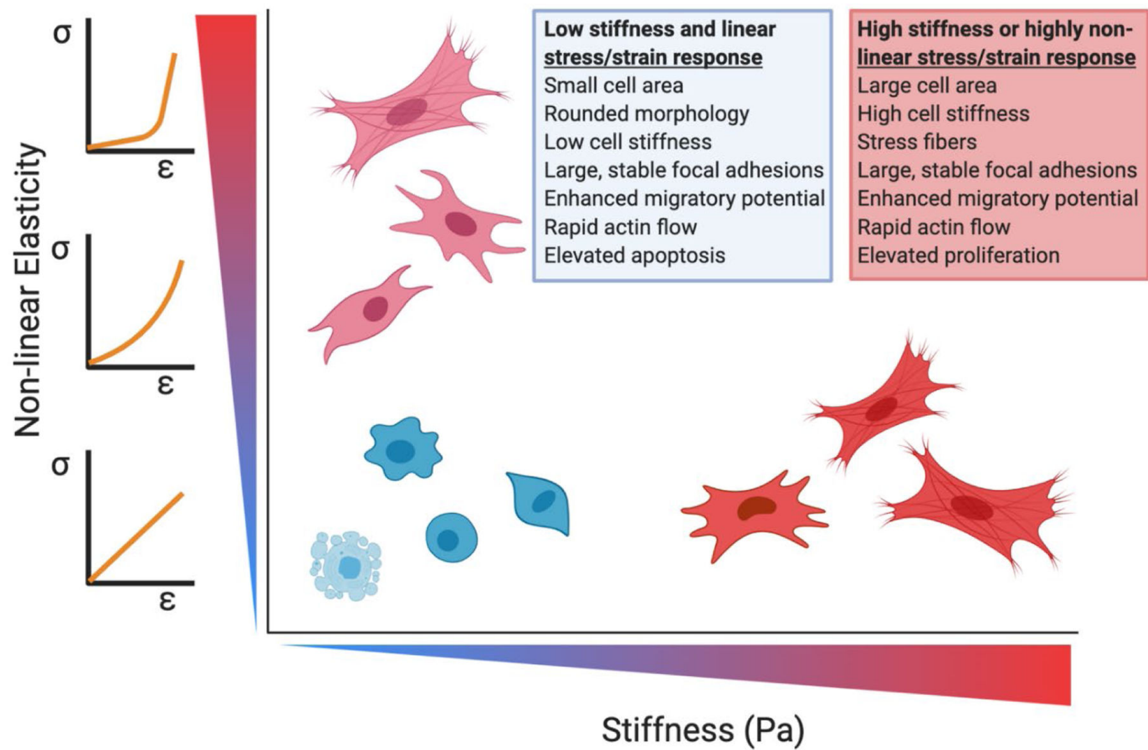

Figure 1.

ECM mechanics drive observable changes in cell behavior. Material properties of the ECM, specifically Young's modulus (stiffness) and non-linear elasticity (local strain-stiffening of the ECM), are critically important for coordinating cell behavior and phenotype. Increasing matrix non-linear elasticity is associated with similar cell phenotypes to those commonly observed on high elasticity substrates. In the cases of both highly non-linear elastic/low stiffness and linear elastic/high stiffness matrices, mammalian cells assume an elongated, well spread morphology accompanied by the formation of dense F-actin stress fibers that cooperate with myosin II motors to increase intracellular stiffness by generating contractile forces. Conversely, linear elastic/low stiffness substrates confer a rounded morphology with small cell areas and the absence stress fibers.

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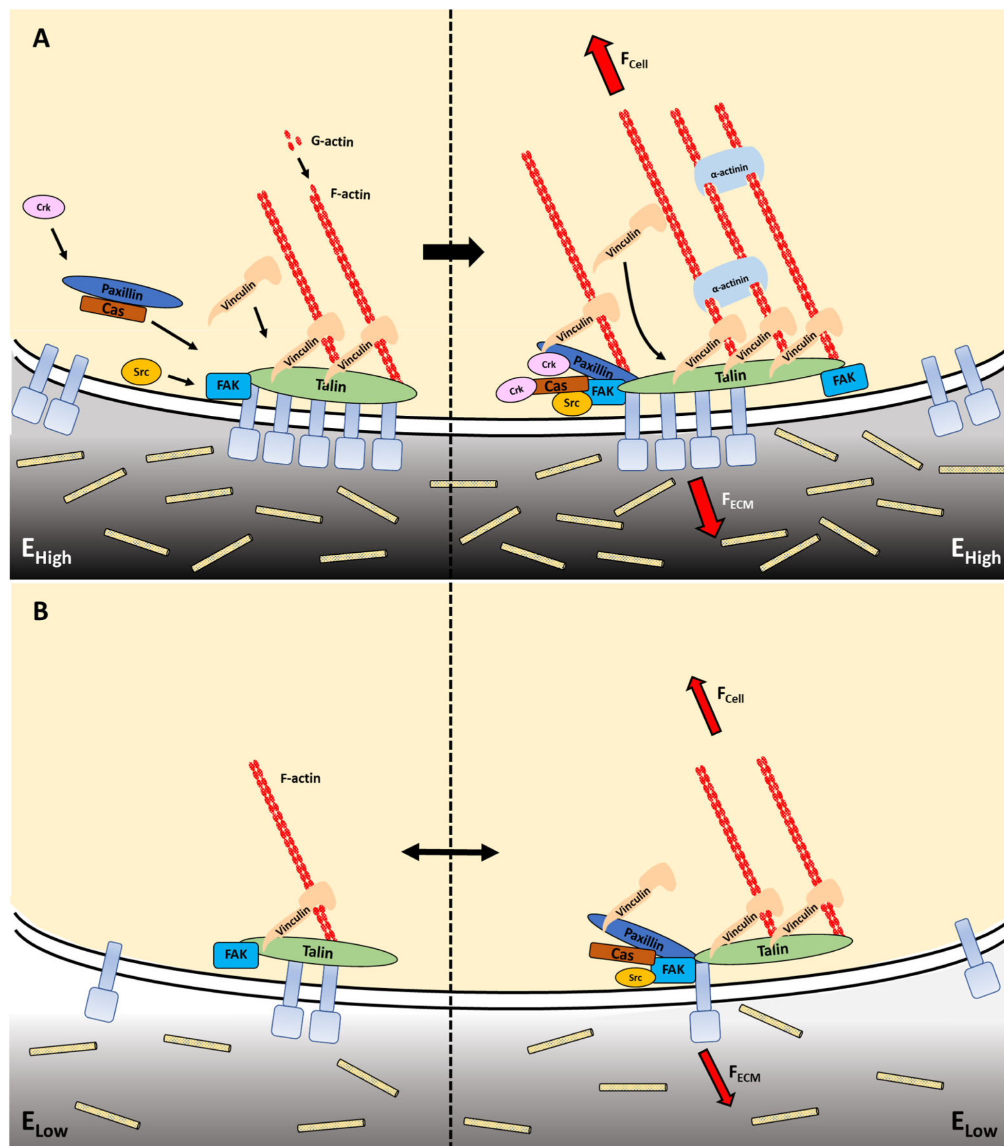

Figure 2.
Substrate stiffness directs focal adhesion dynamics. Matrix mechanics influence cellular activities through the formation and dissolution of focal adhesions. Components of the focal adhesion form robust signaling hubs that integrate mechanical cues from their associated integrins into downstream biochemical signals. **A.** Stiff ECM induces the formation of large, stable focal adhesions. Integrins densely clustered at the growing adhesion recruit FAK and talin (left). FAK activation leads to the recruitment of many components of the mechanotransductive apparatus including Paxillin, Cas, and Src. In parallel, stiff ECM activates the mechanosensory domains of talin, driving the recruitment of vinculin to formulate cytoskeletal-ECM linkage. Force sensing through the cytoskeleton further enhances cytoskeletal linkages and positively regulates signaling complex formation. Once properly matured the actin cytoskeleton becomes intimately engaged with the focal adhesion complex (right). Actin modifiers such as α-actinin crosslink adjacent F-actin fibers situated

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near the adhesome. Mature focal adhesion complexes remain in a dynamic state of reorganizing. Vinculin molecules are recruited and removed to meet the anchoring needs of the cell. The scaffolding protein paxillin forms additional linkages with vinculin and F-actin that coordinate numerous aspects of mechanotransduction. **B.** Lower rigidity matrices present with unstable, transient adhesion complexes. Components talin or paxillin (not shown) form very few linkages to the cytoplasm and core signaling molecules rapidly associate and dissociate from the adhesome. Low rigidity force sensing occurs through vinculin/F-actin complexes, but vinculin residency times are limited which prevents the formation of well-organized stress fibers (left). Growing internal forces do drive adhesion complex growth (right) and recruitment of mechanotransductive signaling members, but these forces soon after fail and return the complex to its basal, nascent state. Lack of sustained mechanotransduction prevents the spreading behaviors observed on stiff ECM.

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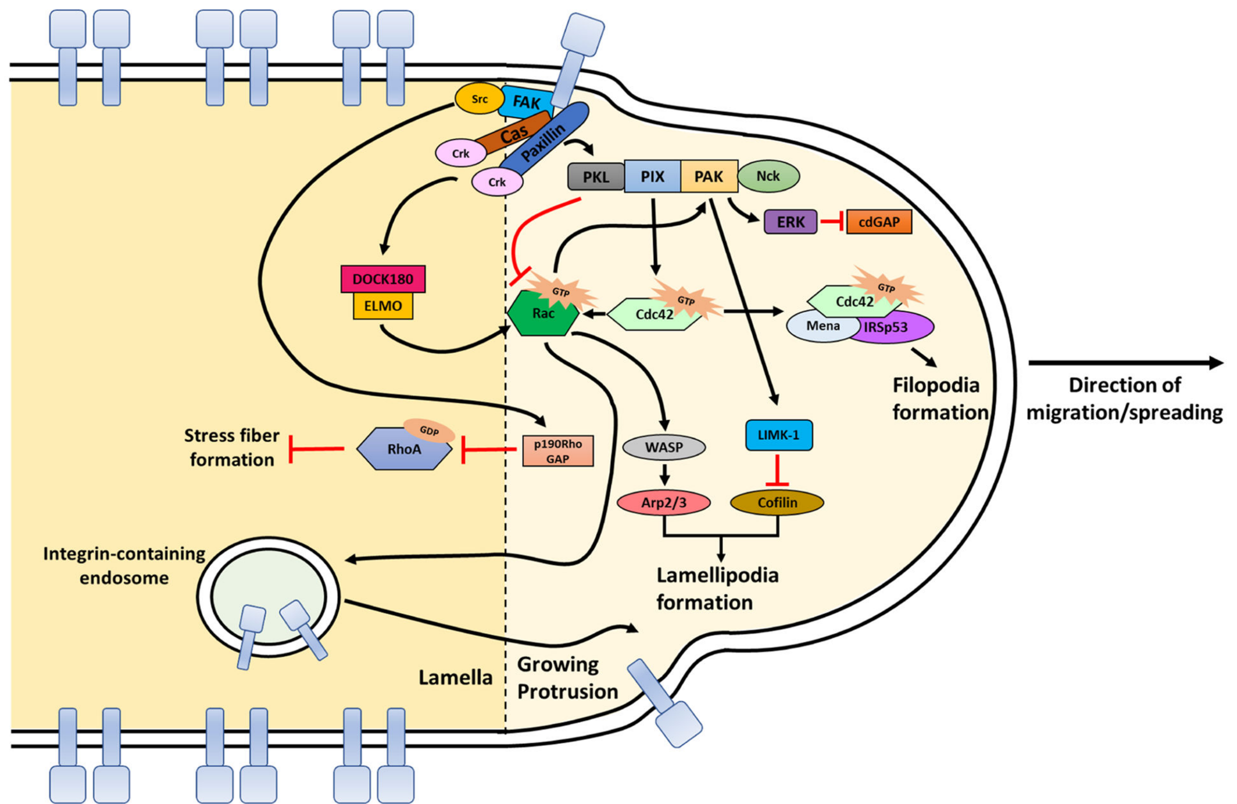

Figure 3.

Cell spreading is regulated by the force-responsive properties of FA components and downstream Rho GTPase regulation. Complex regulation through the focal adhesion coordinates the activity of Rho family GTPases responsible for initiating actin polymerization and protrusion formation. Paxillin acts as a central regulator of protrusion growth and recruits the PKL-PIX-PAK-Nck complex to the focal adhesion. PIX, a Rho GEF, activates Cdc42 which subsequently activates Rac and complexes with Mena and IRSp53 to generate filopodial projections. Paxillin also interacts with the proto-oncogene Crk to activate to stimulate Rac activation through the Dock180/ELMO Rho GEF complex. Rac activity is responsible for stimulating actin branching in the lamellipodial formation through the activation of the Arp2/3 complex and may assist in the recruitment of integrins to the leading edge. PAK, when complexed with PIX, is also activated by Rac and promotes sustained actin polymerization within the protrusion by activating the cofilin inhibitor LIMK-1. Further, PAK can interact with the Ras/MAPK cascade (not shown) to downregulate cdGAP activity and maintain activation of Cdc42/Rac. PKL can also act as a Rho GAP to downregulate Rac activity, making the paxillin-PKL-PIX-PAK-Nck complex a dynamic regulator of the actin cytoskeleton during cell spreading and migration. The FAK/Src complex also contributes to cytoskeletal reorganization by activating p190RhoGAP which inhibits RhoA-mediated stress fiber formation within the protrusion.

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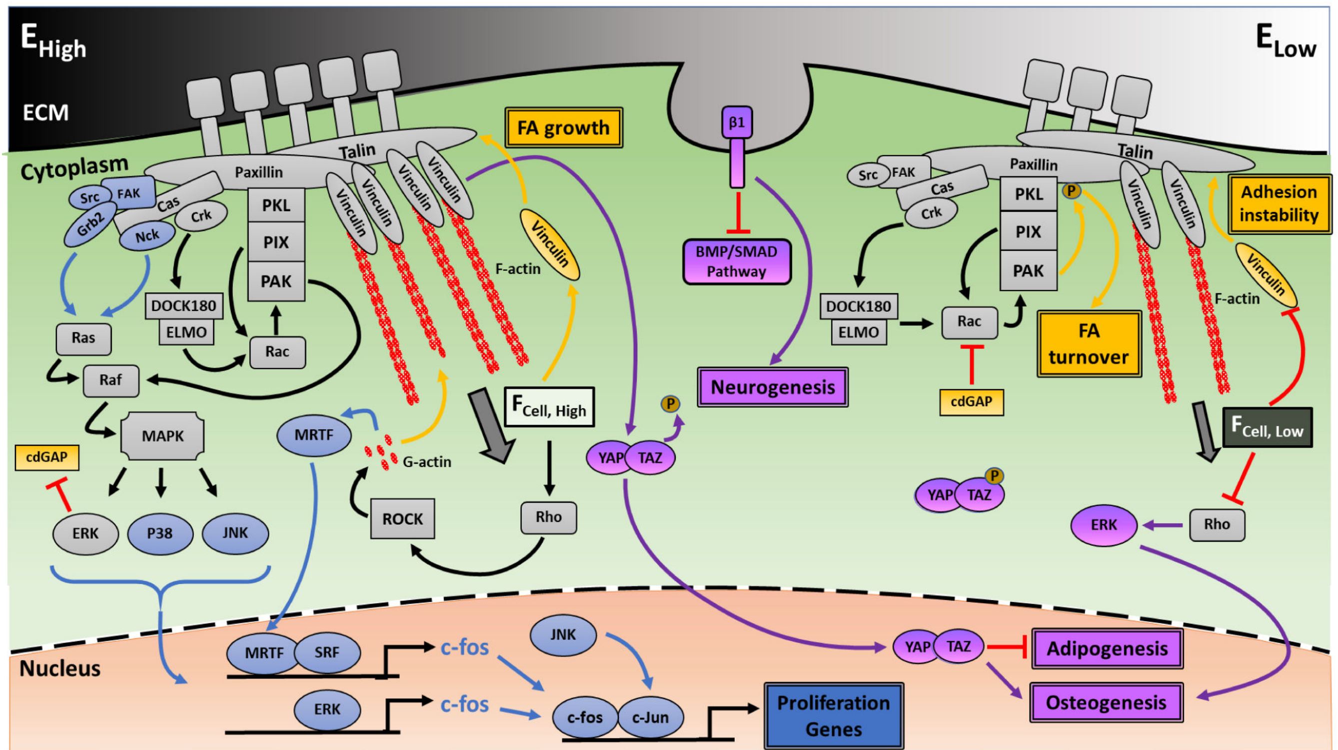

Figure 4.

Mechanotransduction is a central regulator of numerous signaling pathways and cell behaviors. The components of mechanical signaling cascades are multifunctional regulators of downstream changes in cell behavior (grey). Mechanical stimulus can activate pathways that orchestrate the processes of durotaxis (yellow), proliferation (blue), and differentiation (purple). Durotaxis is directed by actin-based traction force generation at the focal adhesion. At regions of high stiffness (left) larger magnitude forces are sensed by the cell and lead to adhesion growth through the recruitment of additional vinculin molecules. Concurrently, force-induced activation of Rho/ROCK stimulates polymerization along new and growing F-actin stress fibers. MAPK signaling through ERK also promotes migration by inhibiting cdGAP to maintain Cdc42 (not shown) and Rac activation at the leading edge. Meanwhile, integrins adhered to low rigidity regions of the ECM (right) experience much weaker traction forces than their counterparts, preventing mobilization of vinculin to the adhesion and subsequent loss of adhesion stability. Rac-mediated activation of PAK leads to the phosphorylation of paxillin at a PAK-associated catalytic site and focal adhesion disassembly. Adhesion instability coupled with disassembly signals act in concert to facilitate focal adhesion turnover at the trailing edge and/or regions of the lamella attached to softer matrices. Mechanically regulated proliferation signals are transitioned from the focal adhesion to the cytoplasm by the FAK-Src-Grb2 and Cas-Nck complexes. Both FAK-Src-Grb2 and Cas-Nck can independently mediate activation of Ras/Raf, leading to MAPK cascade recruitment and nuclear localization of the transcription factors ERK, p38, and JNK. In addition, high magnitude traction forces induce G-actin recruitment to the focal adhesion and the nuclear localization of active MRTF. Nuclear ERK and MRTF (acting with cofactor SRF) can each induce c-fos transcription. Nuclear JNK also contributes to the transcription

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of pro-proliferative genes by activating transcription factor c-Jun which acts in concert with c-fos to drive transcription of genes related to cell cycle progression (e.g. Cyclin D1). The mechanotransduction apparatus is also a central regulator of cell fate decisions. On high stiffness ECM, vinculin molecules are rapidly recruited to growing adhesions and maintain prolonged residency times compared to adhesion complexes on soft surfaces. Vinculin mediates the dephosphorylation and activation of transcriptional coactivators YAP and TAZ. Dephosphorylated YAP/TAZ readily localizes to the nucleus and begins transcribing genes associated with osteogenesis while simultaneously inhibiting gene networks responsible for adipogenesis. In contrast, adhesions situated on lower rigidity ECM do not actively recruit vinculin, preventing YAP/TAZ dephosphorylation and nuclear entry to favor adipogenic differentiation programs. Low stiffness environments also inhibit force-mediated activation of the Rho-ROCK-FAK-ERK signaling axis (not shown) that has been tied to osteogenic lineage commitment. In ESCs, soft ECM is also believed to induce internalization of β1 integrins which subsequently inhibit BMP/SMAD signaling and potentiate neurogenesis.