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Review

Focal adhesion regulation of cell behavior

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Abstract

Focal adhesions lie at the convergence of integrin adhesion, signaling and the actin cytoskeleton. Cells modify focal adhesions in response to changes in the molecular composition, two-dimensional (2D) vs. three-dimensional (3D) structure, and physical forces present in their extracellular matrix environment. We consider here how cells use focal adhesions to regulate signaling complexes and integrin function. Furthermore, we examine how this regulation controls complex cellular behaviors in response to matrices of diverse physical and biochemical properties. One event regulated by the physical structure of the ECM is phosphorylation of focal adhesion kinase (FAK) at Y397, which couples FAK to several signaling pathways that regulate cell proliferation, survival, migration, and invasion.

Keywords: Focal adhesion; Focal adhesion kinase; Integrin; Src; 3D matrix; Cell migration; Tubulogenesis

1. Introduction

Focal adhesions are sites where integrin and proteoglycan mediated adhesion links to the actin cytoskeleton. The components of focal adhesions are diverse and include scaffolding molecules, GTPases, and enzymes such as kinases, phosphatases, proteases, and lipases. Several excellent reviews exist on focal adhesion structure and regulation [1–6]. The purpose of this review is to consider mechanisms by which focal adhesions create combinatorial signaling complexes and mediate integrin function to regulate cellular behaviors such as cell migration. Moreover, we will consider the role of focal adhesions as mechano-sensors allowing cells to respond to matrices of diverse physical and molecular properties.

1.1. Defining focal adhesions

Different types of focal adhesions are defined by their subcellular location, size, and composition. For the purpose of this review, we define four different structures: focal complexes, focal adhesions, fibrillar adhesions, and three-dimensional (3D) matrix adhesions. Small focal adhesions, often referred to as focal complexes, at the periphery of spreading or migrating cells are regulated by Rac and Cdc42 [7], and precede larger focal adhesions that are regulated by Rho activity [8–10]. Focal adhesions are found both at the cell periphery and more centrally, associated with the ends of stress fibers in cells cultured on two-dimensional (2D) rigid surfaces. Currently, efforts are underway to define subsets of focal complexes and adhesions [11], but it is not yet entirely clear which components of a focal complex distinguish it from a focal adhesion. Fibrillar adhesions form as an elongation of focal adhesions and specifically contain $\alpha5\beta1$ integrin and tensin [6,12]. While the previous adhesions have been described for cells adhering to rigid 2D surfaces, 3D matrix adhesions have been defined for fibroblasts adhering to 3D cell-derived fibronectin matrices [2,13] and collagen gels [14] and epithelial cells in 3D collagen [15,16].

2. Focal adhesions as signaling complexes

Multiple protein:protein interactions have been defined at focal adhesions. Because most proteins have several potential interacting partners, this allows the cell an opportunity to construct various signaling complexes leading to diverse behaviors. Table 1 shows several focal adhesion molecules

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Table 1
Molecules at focal adhesions regulate various cell behaviors

	Integrin avidity	FA turnover	Cell migration	Mechano-sensing	Proliferation	References
FAK	+/−	+	+	+	+	[34,153,154,186]
Src	+/−	+	+	+	+	[38,151,152]
p130Cas	+/−		+		+	[62,71,187]
Crk	+/−		+			[62,72,81]
Paxillin			+/−			[73,79,81,188]
Talin	+	?		+		[45,92,97]
Vinculin		?	+	+		[133,166]
Mena/VASP		?	−			[136]
PTP-PEST		+	−			[47,51]
Calpain	?	+	+			[124,125]
H-Ras	−	+	+		+	[189–191]
R-Ras	+	−	+/−			[27,70,113]
Cdc42		+	+		+	[7,192,193]
Rac		+	+		+	[7,120,192,194,195]
Rho		−	+/−	+	+	[9,15,140,141,155,157,196]
Rap	+					[112]

Shown here is a table of focal adhesion proteins (top panel) or small GTPases (bottom panel) and their roles in focal adhesion dynamics and different cell behaviors. Legend: +, molecule has known positive role; −, molecule has known negative regulation; +/−, molecule has positive or negative role; ?, role is implicated.

and GTPases and their roles in regulating cell behavior. Although specific binding interactions are well defined, it is not well understood how a cell regulates these combinatorial interactions in a spatial and temporal manner to regulate cell behavior. Nor is it known precisely how changes in matrix composition alter the composition of these signaling complexes.

2.1. Tyrosine phosphorylation at focal adhesions

Tyrosine phosphorylation is one of the key signaling events occurring at focal adhesions. Recent work by Kirchner et al. [17], using YFP-Src-SH2 domains as a live cell probe for tyrosine phosphorylation events, indicates that recruitment of proteins such as FAK, vinculin, and paxillin to focal adhesions precedes significant tyrosine phosphorylation. This suggests that phosphorylation of these molecules and subsequent signaling occur primarily at focal complexes and adhesions. Tyrosine phosphorylation at the focal adhesion creates docking sites for the binding of SH2-containing proteins and regulates the subsequent activation of additional kinases and phosphatases. Two of the major kinases found in focal adhesions are Focal Adhesion Kinase (FAK) and Src, which bind to different partners to regulate focal adhesion dynamics and cell behavior. Although other tyrosine kinases such as Abl, Csk and PYK2, and ser/thr kinases such as ILK, PAK, and PKC are also found in focal adhesions [11], they will not be discussed here.

2.1.1. Focal adhesion kinase

Since its discovery, FAK has emerged as a key signaling component at focal adhesions (reviewed in this issue). FAK is a 125-kDa tyrosine kinase that was first identified as a protein phosphorylated in response to Src transformation and shown to localize to focal adhesions [18]. FAK localizes to focal adhesions via its C-terminal FAT (focal adhesion targeting) domain [19]. This localization is crucial to its signaling function, since FAK mutants that fail to localize to focal adhesions exhibit impaired autophosphorylation and are unable to phosphorylate FAK substrates in response to cell adhesion [20].

In addition to the FAT domain, FAK contains an N-terminal FERM domain and two proline-rich motifs, which allow it to interact with multiple signaling partners. The FERM domain plays an autoinhibitory role that is relieved by its interaction with the β1 integrin cytoplasmic tail [21]. Through the proline-rich motifs, FAK binds to the SH3 domain of p130Cas and the LD2 domain of paxillin [22,23]. Both p130Cas and paxillin are heavily phosphorylated upon integrin stimulation and, in part, this phosphorylation is attributed to FAK [24]. Although it is apparent that FAK is important for phosphorylation of focal adhesion components, kinase dead FAK retains most of FAK's function, suggesting the main role of FAK is as a scaffold rather than as a kinase [25].

Several key tyrosine residues become phosphorylated upon FAK activation. FAK is activated via autophosphorylation at tyrosine 397 (Y397) that is initiated by integrin engagement with its ligand [26]. Clustering of FAK into focal adhesions enhances this autophosphorylation, since positive regulators of focal adhesion formation also enhance FAK phosphorylation at Y397 [27]. When phosphorylated, Y397 becomes a binding site for the tyrosine kinase Src, which phosphorylates FAK at Y576 and Y577 to further activate FAK kinase activity [28]. Src also phosphorylates Y861 and Y925, creating docking sites for other SH2 domain-bearing molecules, such as Grb2, which links FAK to activation of Ras and the MAPK pathway [29–31].

FAK phosphorylation via Src causes FAK to be excluded from focal adhesions [32]. This is contradictory to the

observation that FAK phosphorylation increases under conditions associated with enhanced focal adhesion formation [27]. However, these differences can be explained by considering which site is phosphorylated. FAK Y397 phosphorylation increases under conditions in which FAK is clustered via integrins or other experimental means [33]. In contrast, phosphorylation of FAK at Y925, found within the FAT targeting domain, causes FAK loss from focal adhesions [32]. Based on these observations, it is likely that an individual FAK molecule dynamically cycles in and out of focal adhesions as different sites become sequentially phosphorylated. A role for FAK in the dynamics of focal adhesions is supported by the observation that FAK (−/−) cells have enhanced focal adhesions and impaired migration [34].

FAK also plays a role in activating small GTPases by directly binding and phosphorylating their exchange factors. This is significant because GTPases modulate focal adhesion formation. FAK binds to p190RhoGEF directly via a sequence within the FAT domain, and co-expression of the two molecules results in enhancement of their phosphorylation as well as GTP loading of Rho [35]. In addition to p190RhoGEF, Trio, an exchange factor for Rho family GTPases, binds to FAK at the cell periphery and enhances the phosphorylation and exchange activity of Trio [36]. In reverse, overexpression of Trio enhances the kinase activity of FAK, which may suggest a role for Trio in focal adhesion dynamics.

2.1.2. Src

Src tyrosine kinase is closely linked to FAK in signaling events at focal adhesions. Src is activated upon binding of its SH2 domain to FAK pY397, removing an autoinhibitory intramolecular interaction [37]. Upon activation, Src phosphorylates several components of focal complexes including FAK, p130Cas and paxillin (reviewed in Ref. [38]). Src seems to be a key kinase in these events since Src knockout cells show low levels of total tyrosine phosphorylation [39]. Src has multiple means to bind potential substrates, as it also interacts with the proline-rich regions of FAK, p130Cas and paxillin through its SH3 domain. This binding may stabilize the interactions with these molecules during their phosphorylation by Src [40].

It is becoming clear that proper targeting of Src to focal adhesions is crucial for its regulation and function [41]. Src binds several components of the focal adhesion including FAK and p130Cas, interactions that are necessary for Src localization to focal adhesions, and for enhancing its enzymatic activity [40]. Recently, Src family kinases have been shown to bind directly to β integrin cytoplasmic domains, providing a mechanism for the activation of Src by integrins [42]. The catalytic activity of Src is dispensable for Src localization to focal adhesions but it is crucial in mediating its effects on cell behavior and morphology [41,43]. Src transformation is associated with decreased adhesion, consistent with an important role for Src in regulating focal adhesion turnover, as discussed below and reviewed by Frame et al. [38]. Mechanisms by which Src cause the disassembly of focal adhesions are emerging, as Src phosphorylation of β integrin subunits, FAK, and PIPKIγ regulates binding of these proteins to each other and within the focal adhesion [32,44,45] (see further discussion below).

2.1.3. PTP-PEST

Tyrosine dephosphorylation is as important as tyrosine phosphorylation in regulating the signaling events in focal adhesions [46]. A major phosphatase found at focal adhesions is PTP-PEST. PTP-PEST (−/−) cells have increased focal adhesions and decreased cell migration [47]. Overexpression of PTP-PEST results in a significant decrease in p130Cas phosphorylation, failure of p130Cas redistribution to the leading edge of migrating cells, as well as reduced association levels between p130Cas and its binding partners [48]. In addition to the interaction via the catalytic domain, PTP-PEST binds to the SH3 domain of p130Cas and the C-terminal LIM domains of paxillin [49,50]. These noncatalytic domain interactions stabilize the interaction between PTP-PEST with p130Cas and paxillin, as well as bring the phosphatase in closer proximity to other components of focal adhesions [49].

Both overexpression and targeted deletion of PTP-PEST inhibit cell migration [47,48]. This suggests that a proper balance in phosphorylation of focal adhesion components is a key regulator of this process. It was recently demonstrated that the effects of PTP-PEST on cell migration are mediated in part by the small GTPase Rac [51]. PTP-PEST localizes to the leading edge of migrating cells and its overexpression suppresses activation of Rac in response to integrin activation [51].

2.2. Signaling scaffolds found at focal adhesions

In addition to tyrosine kinases and phosphatases, focal adhesions contain several adaptor proteins such as p130Cas, paxillin and Crk. These molecules function as signaling scaffolds for the components of focal adhesions, allowing them to complex in a manner that brings together kinases and substrates, and leads to changes in cell morphology and behavior. Activation of kinase and exchange factors often occurs through these scaffolds at focal adhesions. This represents a useful way to achieve spatial activation of kinases and small GTPases at sites of focal adhesions.

2.2.1. p130Cas

One of the key scaffolding molecules at the focal complexes is p130Cas. p130Cas was first identified as a hyperphosphorylated protein in cells transformed by v-Src and v-Crk and later was shown to associate with both cellular Src and Crk in a tyrosine phosphorylation-dependent manner [52–54]. Indeed, p130Cas is required for Src-induced transformation [55].

p130Cas is a large adaptor molecule that contains an SH3 domain followed by a proline region, a substrate domain

composed of 15 YXXP motifs, a serine-rich domain, and a carboxy-terminal domain containing the consensus sequence for binding of the Src SH2 domain. p130Cas localizes to focal adhesions via its SH3 domain, most likely through association with FAK, as well as through the YDYV motif, which binds Src [56,57]. p130Cas phosphorylation is crucial for its localization to membrane-associated fractions and its association with other signaling molecules [53]. Phosphorylation of the p130Cas substrate domain has been attributed mainly to Src, while FAK can phoshorylate the YDYVHL Src-binding motif in the C-terminus of p130Cas [24,58]. Once phosphorylated, the YXXP motifs become docking sites for several molecules containing SH2 domains, including Crk, Nck and PTP-PEST [49,50,53,59]. Because p130Cas can bind to several different partners, these interactions can lead to diverse cell behaviors (Fig. 1).

2.2.2. Crk/CrkL

One such p130Cas-binding molecule is the adaptor protein Crk, which was first identified as a viral oncogene, and is associated with transformation and enhanced migration [60,61]. Crk interacts with phosphorylated p130Cas via its SH2 domain. This interaction is crucial for the effects that p130Cas has on cell transformation, morphology, and migration [62]. Crk phosphorylation on tyrosine 221 is important for the localization of Crk to the membrane as well as for Rac activation [63].

The Crk–p130Cas complex results in activation of a novel exchange factor, Dock180 [64–66]. Dock180 is not a classic exchange factor, since it does not have the tandem Dbl-homology (DH)/pleckstrin-homology (PH) domains that are usually found in exchange factors for the Rho family of GTPases [67]. Rather, Dock180 acquires its activity by binding to Crk and ELMO, and only when assembled in this complex does it activate the small GTPase, Rac [68,69]. Rac activation via this mechanism further enhances p130Cas phosphorylation, as well as the Crk–Cas association, suggesting a positive feedback mechanism that promotes increased cell migration [64].

p130Cas and Crk also contribute to the activation of the Ras family GTPases by complexing with a more conventional exchange factor, C3G. C3G binds via its proline-rich motifs to the SH3 domains of 130Cas and Crk, which targets the exchange factor to focal adhesions. C3G activates Rap1 and R-Ras, both of which regulate inside-out integrin activation [70–72].

We propose that molecules such as p130Cas and Crk can be thought of as components of multi-subunit enzymes, intrinsically necessary for exchange factor or kinase activity in vivo by complexing with and activating molecules such as DOCK180/ELMO. Such a strategy would allow a cell to create a combinatorial approach to assembling enzymatic function. It is likely that other novel enzymatic activities will emerge, as we understand the regulation and composition of these complexes.

Remaining questions are how cells regulate the association of p130Cas/Crk with DOCK180/ELMO vs. with C3G, whether these associations are mutually exclusive, and whether there is regulation in a spatial or temporal sequence. These questions are particularly relevant as the functional outcome of activating DOCK180/ELMO, leading to Rac-mediated cell protrusion, differs from the functional out-

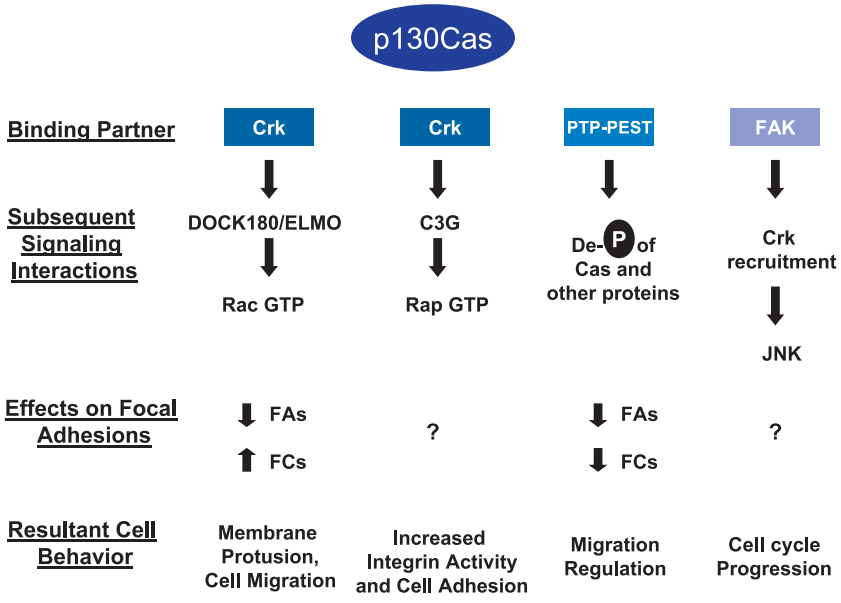

Fig. 1. Different protein:protein interactions at the focal adhesion can mediate several diverse, and often opposing, cell processes. In some cases, one protein may bind to different partners to modulate several different behaviors. An example shown here is p130Cas. p130Cas can bind to several different proteins, including Crk, PTP-PEST, and FAK. The subsequent signaling interactions have different effects on focal adhesions and the resulting cell behavior. It is unknown what regulates the binding of p130Cas to one protein vs. another protein. FA, focal adhesion; FC, focal complex. Note that this model is not inclusive of all p130Cas interactions.

come of C3G, leading to enhanced cell adhesion. In this way, it is clear that the various signaling complexes assembled at focal adhesions are important regulators of cell behavior (Fig. 1).

2.2.3. Paxillin

In addition to p130Cas, paxillin plays a pivotal role as a scaffold at focal adhesions. Paxillin has a proline-rich motif that binds to the Src SH3 domain, five LD motifs that are responsible for its interactions with several proteins including FAK and PKL, as well as four C-terminal LIM domains with which paxillin attaches to the cell membrane and interacts with the phosphatase PTP-PEST [73]. Paxillin can also be recruited to adhesive complexes by binding directly to β1 integrin cytoplasmic tails, as well as to the α4 cytoplasmic tail [74].

Like p130Cas, tyrosine phosphorylation of paxillin through a FAK/Src complex is important for focal adhesion formation and for function of paxillin as a docking molecule at focal adhesions [75–78]. The key tyrosine phosphorylation motifs in paxillin are Tyr31 and Tyr118, which can bind to various molecules such as Crk [77,79]. The interaction between paxillin and Crk is necessary for the localization of paxillin to focal adhesions and for effects on cell migration [80]. Depending on cell type and association with Crk, paxillin phosphorylation has been linked both to increased and decreased cell migration [75,79,81]. Paxillin can also bind to the α4 integrin subunit, and this interaction inhibits α4β1-dependent cell migration [82,83].

Paxillin also contributes to the activation of small GTPases, as it activates Rac via a complex with its exchange factor, β-PIX, Crk and GIT [80]. GIT2/PKL binds paxillin and β-PIX to form a multiprotein complex. Overexpression studies show that Crk recruits this complex to focal adhesions via its SH2 and the N-terminal SH3 domains, which leads to elevated levels of active Rac and enhanced migration [80].

3. Regulation of integrin function through focal adhesions

3.1. Integrin-cytoplasmic linkages

Several structural components link integrin cytoplasmic tails to the actin cytoskeleton, including paxillin (discussed above), talin, α-actinin, and filamin. α-Actinin binds to integrin cytoplasmic tails, and plays a crucial role in the maintenance of integrin-actin linkages and focal adhesions [84,85]. α-Actinin is found in newly forming focal complexes [86], suggesting its importance in focal adhesion formation and regulation. Moreover, α-actinin is associated with force-dependent adhesion strengthening [87], a process that involves integrin clustering. Another protein that plays a role in establishing integrin-cytoplasmic linkages is filamin. Filamin binds to the cytoplasmic tail of β1A and β7 [88–90]. Filamin binding inhibits cell migration [90], providing another example of how specific integrin-cytoskeletal linkages can regulate cell behavior. Filamin is also implicated in the sensing of mechanical forces [91] and adhesion strengthening [92]. We will not review here the complexities of the structural components with regard to integrin and actin binding as several comprehensive reviews exist already [93–95], but rather consider regulation of a few components relative to focal adhesions and the outcome for integrin function and cell phenotype.

3.1.1. Talin

Talin, a key component of focal adhesions, has emerged as an important regulator of inside-out integrin activation [96–98]. Talin binds, through its FERM domain, to the conserved NPXY motifs found in several integrin β tails [97]. This interaction can be regulated by phosphorylation of the β integrin subunit at NPXY by Src, which results in loss of integrin binding to the talin head [44,45,99,100].

Talin plays a role in focal adhesion formation, as talin (−/−) cells have minimal and delayed focal adhesion formation [92,101]. This may be due to the mislocalization of vinculin, as talin induces conformational changes in vinculin causing it to be targeted to focal adhesions [102]. Talin is also necessary for the recruitment of paxillin to adhesion sites, but is not necessary for adhesion-induced phosphorylation of FAK and Src [92]. Finally, talin is required for the initial weak link between small clusters of integrins and the cytoskeleton [103], and for the reinforcement of integrin linkages to the cytoskeleton induced when cells encounter mechanical forces [92], again suggesting that talin plays a role in focal adhesion formation.

A more recently described mechanism by which talin regulates focal adhesion formation is through its association with PIPKIγ. Talin binds and activates specific splice isoforms of phosphatidylinositol phosphate kinase type Iγ (PIPKIγ) [104,105]. The association between talin and PIPKIγ is targeted to focal adhesions where production of PtdInsP2 is thought to be increased at integrin clusters [104,105]. The localized enhancement of PtsInsP2 at focal adhesions can feedback to regulate talin, vinculin and other focal adhesion proteins, which may contribute to signaling events regulating focal adhesion formation.

New evidence suggests that Src regulates the association of an isoform of PIP 5-kinase, PIPKIγ661, with talin [45]. In this case, the effect of Src on PIPKIγ661 is exactly the opposite as its effect on integrin, as phosphorylation of PIPKIγ661 at Y644 enhances the association of PIPKIγ661 661 with talin [45]. Because integrins and PIPKIγ bind to the same site on talin [106], the enhanced affinity of phospho-PIPKIγ661 for talin allows it to compete with β1 integrin tails for binding of talin [45]. Thus, these data suggest that Src may regulate focal adhesions by modulating the binding of talin to PIPKIγ661 vs. β-integrin tails (Fig. 2). Moreover, these data are consistent with a role for PIPKIγ661 in focal adhesion dynamics. The regulation of talin binding to integ-

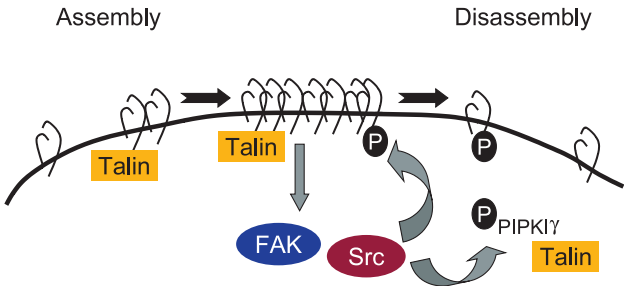

Fig. 2. The assembly and disassembly of focal adhesions is important for several cellular processes, including cell motility. Several molecular mechanisms can regulate focal adhesion dynamics. Talin binding to the β1 integrin cytoplasmic tail promotes focal adhesion clustering and assembly. This clustering activates FAK, which then binds and activates Src. Src can phosphorylate both the β1 integrin cytoplasmic tail and PIPK1γ. Phosphorylated PIPK1γ binds to talin so it cannot bind to the integrin. This, and the phosphorylation of β1, leads to focal adhesion disassembly.

rin vs. PIPKIγ661 allows the cell to alter the combinatorial signaling complexes and thereby regulate cell behavior.

3.2. Regulation of integrin avidity by clustering into focal adhesions

The composition of the matrix controls the recruitment of specific integrins into focal adhesions, suggesting that the conformational changes associated with ligand binding help to regulate integrin cytoplasmic linkages. Both ligand binding and clustering are necessary for full integrin function and the recruitment of several focal adhesion-associated proteins [107,108]. When integrins are clustered with antibodies without ligand binding, or bind ligand without clustering, then minimal tyrosine phosphorylation and complex recruitment occurs [107]. Integrin cytoplasmic domain alpha subunit mutations that inhibit clustering of integrins decrease cell adhesion [109], whereas transmembrane mutations that induce clustering enhance adhesion and FAK phosphorylation [110]. These data suggest that integrin function requires clustering and resultant avidity changes.

Integrin binding to the ECM alone is not sufficient to induce integrin clustering and focal complex formation. Rather, signaling events driven from the inside of the cell are required [10]. Notably, the finding that Rho-mediated contractility drives focal adhesion formation from the "inside-out" led to the theory that focal adhesion formation is a bidirectional event that requires signaling events in addition to integrin–ligand binding [9]. As discussed below, changes in local integrin avidity and adhesion strength affect membrane protrusion and cell migration, and may be involved in the response to 3D matrices.

3.3. R-Ras regulates focal adhesions and integrin function

Another important regulator of integrin function and focal adhesion formation is R-Ras, a member of the Ras family of small GTPases. R-Ras regulates integrin affinity and avidity to enhance cell adhesion [70,111]. Activation of R-Ras enhances focal adhesion formation and promotes the clustering of α2β1 integrins on collagen [27], further supporting the hypothesis that the clustering of integrins in focal adhesions is a positive regulator of integrin avidity. In addition, R-Ras enhances phosphorylation of FAK and p130Cas [27]. Although this enhancement is in part due to enhanced ligand binding, R-Ras can also signal to FAK and p130Cas by a novel pathway independent of Src and PI3K, unlike normal integrin signaling [27]. Consistent with this observation, R-Ras localizes to focal adhesions, providing proximity to focal adhesion components for specific regulation of signaling events by R-Ras [112]. In light of its effects on integrin avidity and focal adhesions, it is of interest that R-Ras regulates cell migration [113], membrane protrusion (Wozniak and Keely, unpublished observations), and epithelial tubulogenesis [113].

As indicated above, C3G is an exchange factor for Rap1 and R-Ras that is activated through Crk and p130Cas [71,72]. Like R-Ras, Rap1 is also a positive regulator of integrin adhesion and signaling events [114]. C3G is required for formation of integrin β1 and paxillin positive focal adhesions since C3G-deficient fibroblasts do not form these structures [115]. C3G plays a role in focal adhesion formation and becomes activated and phosphorylated upon cell adhesion [116,117], which supports the idea of a bidirectional relationship between integrin function and focal adhesions.

4. Focal adhesion dynamics and the regulation of polarized cell migration

4.1. Assembly of focal adhesions

A particular difficulty in understanding focal adhesions is that most work gives a static picture of their components, and elucidating the dynamics of various components at the focal adhesion has been more difficult. For migration to occur, a cell must extend a protrusion to make an initial contact with the ECM. Some of these initial Rac-dependent contacts develop into Rho-dependent focal adhesions that stabilize the cell during migration. Recent advances in imaging individual molecules in real time allow a spatial and temporal dissection of the events regulating focal adhesion formation and turnover.

At the leading edge of a migrating cell, membrane protrusion is stabilized by small adhesive foci that initially contain paxillin followed by α-actinin [86,118]. Initial foci of adhesion form just posterior to the actin network, and grow into focal complexes abundant in tyrosine phosphorylation, and containing integrin, talin, paxillin, vinculin and FAK [86,119]. Subsequently, zyxin and tensin are recruited to these complexes as they remodel into focal adhesions, which stabilize the protrusion [119]. These dynamics are

likely controlled by small GTPases, as it is believed that Rac is activated during early events of membrane protrusion and is necessary for focal complex formation, while Rho becomes activated later and is necessary for the maturation of nascent focal complexes to larger focal adhesions to stabilize the cell during cell migration [120–122] (Fig. 3).

4.2. Focal adhesion turnover/disassembly

A cell must be able to continuously remodel focal complexes into focal adhesions, and vice versa, to migrate. Src and FAK are both important regulators of focal adhesion turnover. Src generally causes a reduction of focal adhesions and decreased cell adhesion (reviewed in Ref. [123]), suggesting that tyrosine phosphorylation of focal adhesion components by Src causes focal adhesion turnover. FAK −/− fibroblasts have larger, more stable focal adhesions and lose random migration [34], suggesting a role for FAK in focal adhesion turnover. This may be due to a loss of Src at focal adhesions [25,59] However, even in the absence of FAK, recent data suggest that Src could target to focal adhesions through direct binding to β integrin subunits [42]. Src subsequently phosphorylates numerous proteins, including integrin cytoplasmic domains [44] and FAK at Y925 [32], promoting the turnover of focal adhesions. Thus, a complex picture of precise temporal and spatial regulation emerges (Fig. 2), in which integrins and FAK cluster at focal adhesions, and assemble several molecules including Src, which in turn causes the disassembly of the adhesion complex. This allows the cell dynamic temporal and spatial regulation of focal adhesion signaling events in order to regulate cell migration.

Focal adhesion turnover also occurs through the calcium-dependent protease, calpain. Calpain is important for both focal adhesion remodeling and cell migration [124–126]. Calpain cleaves several proteins found within focal adhesions, including talin, paxillin, FAK, Src, α-actinin, and tensin [127–131]. Because calpain is targeted to focal adhesions through an interaction with FAK [132], this may represent one mechanism by which FAK contributes to focal adhesion turnover. Moreover, calpain cleavage of FAK is enhanced by Src [132]. Inhibition of calpain results in large, stable focal adhesions and diminished turnover of zyxin and vinculin from these focal adhesions [125]. These focal adhesion dynamics are necessary for the translocation of focal adhesions towards the cell center, as well as the release of the trailing edge of the cell [125].

4.3. Regulation of molecules found both at focal adhesions and at lamellipodia

Several proteins are found at both the lamellipodium and in focal adhesions. Thus, it is possible that their relocalization is a key event that regulates signaling complexes and the progression from forward membrane protrusion to stable focal adhesions. For example, the localization of vinculin at the lamellipodium is regulated temporally during cell spreading, and this correlates to vinculin's ability to bind

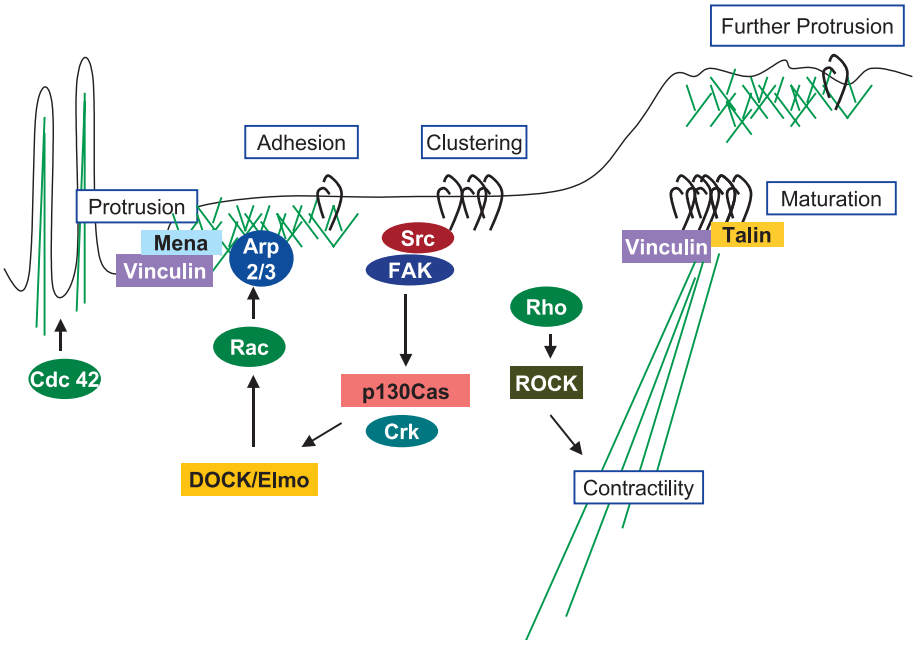

Fig. 3. Spatial events occurring at the leading edge of a migrating cell. Cdc42 and Rac, through regulation of actin dynamics, contribute to forward protrusion stabilized by initial adhesions. Integrin clustering strengthens these adhesions and leads to signaling via Src and FAK, activating Cas/Crk. Rho-mediated contractility through ROCK assembles stress fibers and leads to further strengthening and maturation into focal adhesions. Dynamic regulation of the entire process allows further protrusion and forward movement of the cell.

to Arp2/3 [133]. Arp 2/3 regulates the branching of actin networks and is important for forward protrusion [134]. Because vinculin mutants defective in Arp2/3 binding inhibit lamellipodial protrusion [133], it is possible that vinculin localization to focal adhesions sequesters it away from Arp2/3, which will inhibit lamellipodial protrusion.

Another molecule found both at leading edges and in focal adhesions is Mena/VASP, which regulates cell protrusion and migration by controlling actin filament branching at the leading edge [135,136]. Mena/VASP binds to vinculin [137]. A role for Mena/VASP in focal adhesions is not yet clear, as removal of Mena/VASP from focal adhesions has no obvious effect on cell adhesion [136]. Despite this, adhesion regulates Mena/VASP phosphorylation [138], which affects the ability of Mena/VASP proteins to bind actin, SH3 domains [139], and the tyrosine kinase, Abl [138]. As for vinculin, a possible model for the regulation of membrane protrusion could include the sequestration of Mena/VASP into focal adhesions away from the leading edge of the cell.

If, indeed, focal adhesions sequester certain molecules, then the formation of large focal adhesions might be expected to inhibit membrane protrusion. Several pieces of data suggest that this may be the case. Large, more dense focal adhesions are associated with the lateral and more rearward region of the cell [122], regions where the cell spatially restricts membrane protrusion. Moreover, signaling events that induce large focal adhesions such as the activation of Rho [140,141] or the activation of R-Ras (Wozniak and Keely, unpublished observations) inhibit membrane protrusion (Fig. 4). In contrast, events leading to turnover of large focal adhesions into smaller focal complexes are associated with an increase in protrusion and cell migration, such as PDGF treatment [142], EGF treatment [143], Rac activation [120], or src activation [38]. Focal complexes at the leading edge are more stable, have fewer integrin subunits, and transmit propulsion forces better than more rearward adhesions [122,144], confirming spatial differences in adhesion function. In addition, experimental conditions that promote stronger adhesion such as altered integrin expression, altered integrin activation, or altered substratum concentration decrease cell migration in a bimodal manner [145–148].

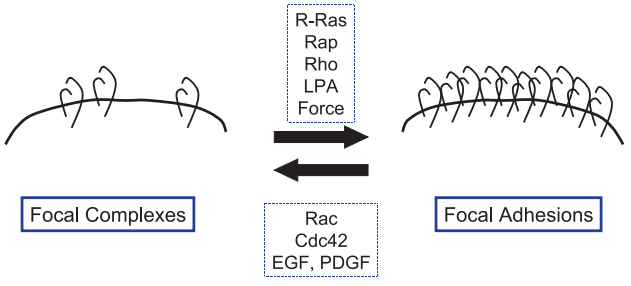

Fig. 4. A cell forms several distinct cell:matrix interactions, two of which are focal complexes and focal adhesions (these are detailed in the text). In several processes, such as cell migration, a cell must continuously remodel focal complexes into focal adhesions, and vice versa. This diagram shows several regulators that remodel focal complexes into focal adhesions and the disassembly of focal adhesions into focal complexes.

5. The role of force in focal adhesion formation and regulation

Physical and mechanical forces are necessary for several cellular processes, including tissue development and morphogenesis [149]. Differentiated cells exist in a constant state of isometric tension, which is an exertion by the cells of a force equal to that of their local matrix environment [150]. This tension prevents cell shortening and changes in cellular architecture that would disrupt normal tissue organization [1,149,150]. Mechanisms by which cells respond to mechanical forces are just beginning to be elucidated. Focal adhesions are likely involved as mechano-sensors, as many of the molecules implicated in mediating signal transduction in response to mechanical stimuli are found at focal adhesions, including Src [151,152], FAK [153,154], Rho [15,155,156], and talin [92].

Not only do focal adhesions transmit external mechanical signals, but external force can regulate the types of focal adhesions that cells form [3]. Because we propose that focal adhesions are key regulators of cell behavior and phenotype, it is important to explore the role of force in focal adhesion formation and regulation. In this review, force will be discussed in two ways, intracellular and extracellular. Intracellular force refers to the force that the cell exerts on its substrate, which is generated by cellular contractility. Extracellular force refers to the force exerted on a cell by the surrounding matrix or additional external stimulus.

5.1. Intracellular Rho-generated force and focal adhesion formation

Rho has emerged as a key regulator of focal adhesion formation and regulation. The ability of Rho to promote focal adhesion formation and stress fiber formation is dependent on its ability to generate contractile forces [9,157]. Contractile force is regulated by myosin light chain phosphorylation, which increases myosin ATPase activity to allow contraction through actin and myosin interactions. Rho activation of its effector, Rho Kinase (ROCK), enhances myosin light chain phosphorylation both by inactivation of myosin light chain phosphatase [158] and direct phosphorylation of myosin light chain [159,160]. The bundling of actin filaments into stress fibers clusters integrins, leading to focal adhesion formation [9].

In vitro experiments demonstrate that ROCK can mediate the contraction of isolated stress fibers [161]. Because both MLCK and ROCK can phosphorylate myosin light chain, this study also examined the ways in which MLCK and ROCK regulate stress fiber contraction. They found that contraction downstream of MLCK is rapid and more exten-

sive compared to ROCK-mediated contraction [161]. From this, they propose that MLCK generates rapid contraction in vivo, while ROCK maintains the sustained contraction in cells, resulting in isometric tension. Therefore, Rho and ROCK-generated contractility may be regulated in such a way as to maintain isometric tension. We propose that the isometric tension generated by Rho and ROCK-mediated contractility regulates focal adhesion formation in vivo, which will likely regulate downstream signaling events and cell behavior.

5.2. Experimental demonstrations of the role of external force in focal adhesion formation

The mechanism by which cells respond to external force has been studied using several different experimental manipulations, which demonstrate that integrin-mediated signaling events transform mechanical forces into biochemical responses. One demonstration of this was performed by Wang and Ingber [162]. In this study, mechanical force was applied to several different types of cell receptors using a magnetic twisting device. Interestingly, only mechanical stress applied to integrins (the β1 integrin, in this case) causes a force-dependent cell stiffening response and focal adhesion formation [162]. Force applied to other receptors does not cause this cellular response, suggesting that only integrin receptors sense mechanical forces and translate these signals to the cell.

Other lines of evidence support this hypothesis. An elegant study by Choquet et al. [163] used optical tweezers to physically hold fibronectin-coated beads attached to the cell through integrins in order to demonstrate that the strength and rigidity of the link between the integrin and cytoskeleton increases proportionally to the amount of restraining force placed on the bead. This work provides a basis for later experiments demonstrating that the physical rigidity of the ECM can regulate focal adhesion formation [155,164–168].

Force also regulates focal adhesion maturation and structure, as well as playing a key role in migration. Several reviews have covered the regulation of cell migration by force [169,170], which will not be further discussed in this review. The role of force in focal adhesion assembly has been studied using several techniques. Balaban et al. [166] simultaneously measured cell displacements and focal adhesions (visualized by vinculin:GFP) to demonstrate that increased force is correlated with increased focal adhesion formation. Other studies have also shown that mechanical force applied to cells serves to increase focal adhesion formation and tyrosine phosphorylation [171–173]. Force also plays a role in the maturation of focal adhesions, as focal complexes develop into larger focal adhesions when force is applied to the cell through ECM-coated beads [155,168].

These findings were challenged by the observation that nascent focal adhesions generate the most force on their substrate during cell migration [174]. These studies were done by mapping traction stress and following focal adhesion dynamics using zyxin:GFP [174]. However, this discrepancy seems to be resolved by Tan et al. [157] in which cellular forces were measured and focal adhesions characterized in cells that were plated onto microfabricated ECM-coated posts. Using this method, increased force was correlated to increased focal adhesion formation only for adhesions greater than 1 μm^2 [157]. Adhesions smaller than this did not show any correlation, which may help explain why previous studies showed conflicting results when looking at the correlation between force and focal adhesions. The smaller complexes, which Beningo et al. [174] found to generate the largest amount of traction force, were likely smaller than 1 μm^2 [175].

6. Focal adhesions in three dimensions (3D matrix adhesions)

Although focal adhesions are well-characterized structures, the majority of these studies have investigated tissue culture cells plated on a 2D ECM-coated surface. This is very different from the physiological environment cells normally encounter in two main ways. First, many cell types are not in contact with just one ECM component. Fibroblasts, for example, contact connective tissue while epithelial cells maintain contact with basement membrane, both of which include several different ECM proteins. Thus, different integrins may be activated, resulting in the integration of multiple signaling pathways in vivo. Second, plating tissue culture cells on an ECM-coated surface, usually a coverslip or Petri dish, imposes a biophysical environment that differs from physiological conditions. This leads to a key question: do focal adhesions exist in vivo, or are they simply tissue culture artifacts? Recent work has used in vivo tissue and in vitro 3D matrices to examine if and how focal adhesions form in a more relevant environment [13,15,156]. These studies show that cells do form 3D matrix adhesions that are not the same as their 2D counterparts, suggesting that these different focal adhesions will alter signaling events to regulate cell behavior and phenotype [1,2].

6.1. The use of 3D matrices for studying cell behavior

In vivo, fibroblasts and epithelial cells have very different morphologies and functions. Fibroblasts organize themselves into a dendritic network or differentiate into myofibroblasts and are necessary for the synthesis and maintenance of connective tissue [176]. Epithelial cells are differentiated and organized into tissue. However, under cell culture conditions, fibroblast and epithelial cell lines behave similarly—they migrate and proliferate, do not take on organized structures, and do not differentiate. This alone demonstrates that studying cell behavior in 2D, although

very useful, is unlikely a reliable recapitulation of cell behavior in vivo.

The culture of fibroblasts in 3D collagen gels has been beneficial for studying how cells interact with the chemical and physical signals given by their surrounding matrix. When fibroblasts are cultured in a floating collagen gel (that is, a gel that is detached from the sides and bottom of the dish so that it floats in medium), the fibroblasts become quiescent and take on a dendritic shape similar to that seen in resting connective tissue [156]. The specifics of these studies are well explained in a recent review [177]. Interestingly, fibroblasts in fixed gels (in which they are attached to the dish) continue proliferating and do not form organized structures [178,179]. However, it is unknown why fibroblasts behave differently when cultured on a 2D substrate compared to a 3D floating or a 3D restrained collagen gel.

Another 3D model system is the culture of epithelial cells in collagen gels or in basement membrane (Matrigel). These pioneering studies were first done in mammary epithelial cells. It was shown that mammary epithelial cells differentiate to produce milk proteins only in a floating 3D collagen gel or Matrigel [180,181]. However, the same question remains: why does this occur? Recent work has revealed that both fibroblasts and mammary epithelial cells form focal adhesions in 3D matrices. We propose that the focal adhesions formed in both fibroblasts and epithelial cells in 2D vs. 3D environments are fundamentally different, which influences cell signaling to direct behavior and phenotype.

6.2. 3D matrix adhesions

Although it is important to study focal adhesions in more relevant 3D environments, investigations of focal adhesions in 2D are not without purpose. It is suggested that 2D focal adhesions are an exaggerated version of 3D matrix adhesions [2]. Therefore, understanding focal adhesions in 2D serves as a point of comparison when studying 3D adhesions.

One of the first demonstrations of focal adhesion formation in physiological 3D matrices was performed by Cukierman et al. [13]. This elegant study advanced the field of focal adhesions by characterizing in vivo focal adhesions from embryonic mouse mesenchymal cells [13]. To study 3D adhesions further, fibroblasts were cultured on a fibroblast cell-derived matrix, which is then depleted of fibroblasts [13]. It should be noted that in this system, cells are plated onto a 3D cell-derived matrix, rather than being embedded into the matrix, as was the case for the 3D matrices described above.

While the fibroblasts form focal adhesions on the 3D cell-derived matrix, these adhesions differ from the focal adhesions formed by fibroblasts cultured on a fibronectin-coated coverslip. Notably, α5 integrin and paxillin colocalize in adhesions for cells adherent to 3D, but not 2D, FN matrix [13]. This is significant because on a 2D substrate, α5 integrin localizes to fibrillar adhesions, adhesions that generate fibronectin fibrils, and paxillin localizes to focal adhesions. The fact that these do not segregate to separate structures in 3D suggests that cells encountering a 3D matrix do not make the same type of adhesions as they do on a rigid 2D matrix.

Another interesting difference between fibroblasts plated on fibronectin or cultured in a cell derived matrix is that cells in the 3D matrix lose phosphorylation of FAK at its autophosphorylation site, Y397 [13]. Phosphorylation is also lost in in vivo matrix adhesions [13]. Thus, 3D focal adhesions appear to be distinct from 2D focal adhesions and were therefore termed "3D matrix adhesions" to separate them from their 2D counterparts [13]. The detailed components of fibrillar, focal, and 3D matrix adhesions have been reviewed elsewhere [2,6].

Coincident with a change in focal adhesion structure is a change in cellular phenotype and behavior. Fibroblasts cultured in a 3D cell derived matrix take on a spindle shaped morphology, similar to in vivo fibroblast morphology. These cells also increase proliferation, migration, and adhesion compared to fibroblasts cultured on a 2D flattened cell-derived matrix or on fibronectin alone [13]. It has been proposed that focal adhesions may act as mechano-sensors [3] and this study suggests that focal adhesions formed under different biophysical environments transmit different signals to the cell, leading to different cell behaviors.

3D matrix adhesions have also been observed in epithelial cells cultured in 3D collagen gels. MDCK cells cultured in a collagen gel down-regulate focal adhesion proteins compared to cells plated on 2D collagen [16]. In mammary epithelial cells, differentiated structures are formed in a 3D collagen gel only if the gel is floating in media, and not if the gel is left attached to the dish or if the cells are cultured on 2D collagen [15,180,182]. Breast epithelial cells in an attached collagen gel form small, punctate 3D matrix adhesions that include FAK phosphorylated at Y397, whereas breast cells in a floating 3D matrix do not form these matrix adhesions and do not localize phospho-FAK to adhesion structures [15]. Thus, the localization of FAK phosphorylated at Y397 to matrix adhesions corresponds to the disruption of normal differentiated morphology in a collagen gel [15]. FAK phosphorylated at this site is also absent from in vivo 3D matrix adhesions in the mesenchymal cells from a mouse embryo [13], suggesting a general phenomenon by which cells in more relevant, 3D environments regulate FAK localization and phosphorylation at Y397. A simple change in FAK phosphorylation at Y397 can be expected to have profound changes in the responses of cells to the ECM, as phosphorylation at this site links to several signaling pathways that regulate cell proliferation, survival migration, and invasion (Fig. 5).

6.3. Focal adhesions as mechano-sensors: the role of FAK

A mechano-sensor is a molecule or molecules that detect external mechanical signals and convert them to

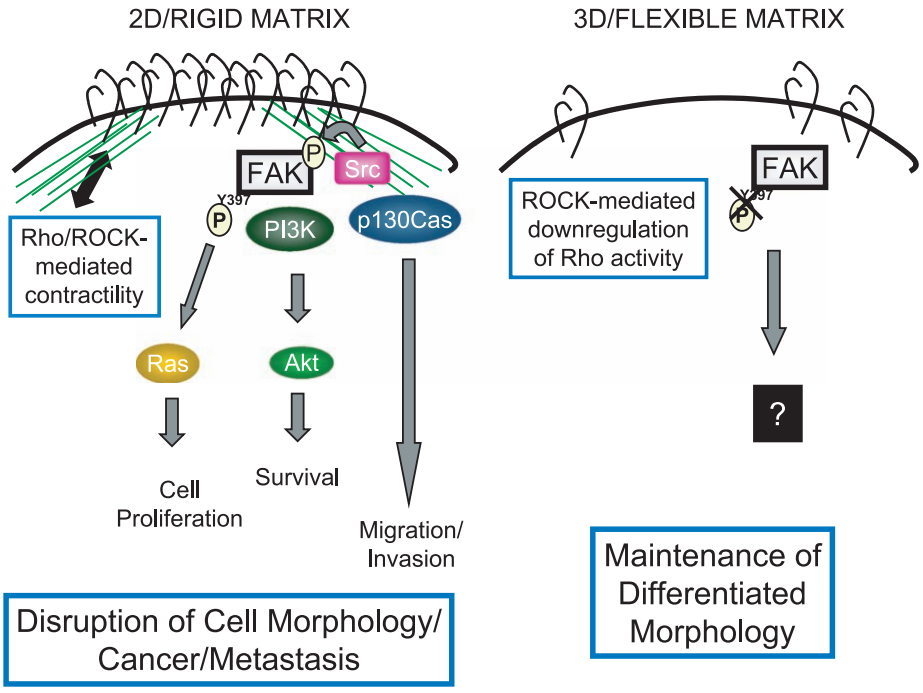

Fig. 5. Cells regulate focal adhesion dynamics in response to matrices composed of different biophysical properties. Cells cultures on a rigid 2D matrix behave differently than cells cultured in a 3D flexible matrix. We propose that focal adhesions are key regulators of this response to ECM rigidity. The phosphorylation of FAK at Y397 is regulated by ECM rigidity and here we show how this modification may alter signaling events leading to cell behaviors that will disrupt normal cell morphology. Several of these behaviors, such as cell proliferation, survival, and migration, will likely contribute to cancer and metastasis. It is possible that cells in a 3D, or more flexible, matrix down-regulate phosphorylation of FAK in order to down-regulate these pathways which would disrupt normal cell function. We propose that other cells will use similar types of focal adhesion regulation in the maintenance of differentiated tissue.

biochemical signals inside the cell. It is known that sensory cells can detect force, usually through ion channels [183], but the mechanisms by which nonsensory cells detect force are not well understood. Focal adhesions have been proposed to be mechano-sensors because they provide a link between the ECM and the cytoskeleton and because they respond to external force, as explained above. This is an important concept because cells in 3D matrices are not receiving the same physical signals as those on more rigid 2D matrices.

FAK has been implicated in mechanosensing by the finding that FAK-null cells cannot detect differences in ECM rigidity [154]. Normal cells will migrate preferentially on rigid substrates; FAK-null cells do not show this preference [154]. The phosphorylation of FAK at its autophosphorylation site, Y397, seems to be involved in the mechanosensing response because FAK is phosphorylated when mechanical strain is applied to smooth muscle and endothelial cells [153,184]. Therefore, it is consistent that FAK is regulated in both fibroblasts [13] and epithelial cells [15] in 3D matrices since these matrices are less rigid than 2D matrices.

6.4. Potential in vivo relevance of 3D matrix adhesions

In vitro, focal adhesions can modulate cell behavior and phenotype. We hypothesize that this may also occur in vivo. We propose that, in vivo, cells will form 3D matrix adhesions in order to modulate cell behavior, which will preferentially contain, exclude, or allow the phosphorylation of certain molecules. This would lead to downstream signaling pathways that regulate gene transcription, cell morphology, and transformation. A candidate molecule to be regulated in this manner is FAK. As described above (Section 2.1.1), because FAK phosphorylation at Y397 links to cell migration, survival, and proliferation, our hypothesis is that the recruitment of phospho-FAK into matrix adhesions in vivo alters cellular signaling to regulate consequent cell behavior.

Differences in cellular responses to matrix density and rigidity attest to the importance of biophysical, and not only biochemical, signaling. Furthermore, several diseases and pathologic conditions can change the mechanical properties of the matrix, such as the increased matrix deposition that is observed during wound healing, or dysplasia. In addition, some pathological conditions may have a component that is regulated by the mechanical properties of the ECM. This may be the case in women with dense breast tissue, containing increased collagen and fibronectin deposition in the stroma, who have an increased risk of breast cancer [185]. In each of these cases, the mechanical properties could alter in vivo matrix adhesion formation, which will change cellular signaling and subsequent behavior.

7. Summary

We propose here that cells respond to matrices of diverse biochemical and biophysical properties by using the focal adhesion as a combinatorial site for creating different signaling complexes. Therefore, the exact composition of a given focal adhesion will regulate cellular behaviors such as adhesion, migration, proliferation, and differentiation. Future studies aimed at understanding the composition and function of focal adhesions in 3D environments will help us better understand the behavior of cells in vivo under normal and pathological conditions.

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