Cell interactions with three-dimensional matrices

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Signaling and other cellular functions differ in three-dimensional compared with two-dimensional systems. Cell adhesion structures can evolve *in vitro* towards *in-vivo*-like adhesions with distinct biological activities. In this review, we examine recent advances in studies of interactions of fibroblasts with collagen gels and fibronectin-containing matrices that mimic *in vivo* three-dimensional microenvironments. These three-dimensional systems are illuminating mechanisms of cell–matrix interactions in living organisms.

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Abbreviations

3D-adhesion	three-dimensional matrix adhesion
3D signaling	signal transduction in 3D matrices
ECM	extracellular matrix
ERK	extracellular-signal-regulated kinase
FAK	focal adhesion kinase
MAPK	mitogen-activated protein kinase
MMP-13	matrix metalloproteinase 13
TGF-β	transforming growth factor-β

Introduction

Research in cell biology frequently depends on tissue culture, yet artificial substrates such as plastic or glass are likely to distort findings by forcing cells to adjust to artificially flat and rigid surfaces [1]. By contrast, the authentic substrate for most cells in living organisms is the extracellular matrix (ECM), which is three-dimensional, complex and dynamic in its molecular composition, and variable in pliability [2,3]. The ECM is organized and sensed by cells via integrins, which are membrane-spanning heterodimeric receptors that mediate communication between the ECM and cells [4•]. Integrins in various types of membrane-associated structures, termed cell–matrix adhesions, transmit information in a bi-directional manner between ECM and cytoplasm [5•]. Cell–matrix adhesions mediate physiological responses regulating cell growth, migration, differentiation, survival, tissue organization and matrix remodeling [6]. The types of cell–matrix adhesions organized by integrins *in vitro* and the signals they transduce are strongly affected by the flat, rigid surfaces of tissue culture dishes; therefore, a closer approximation to *in vivo* environments should be attained by growing cells in three-dimensional gels or matrices.

Culturing cells within such three-dimensional matrices is not a novel idea. In 1972, Elsdale and Bard [1] described a model system for fibroblastic cells in the body using collagen I matrices polymerized *in vitro* to form a three-dimensional fibrous network. These three-dimensional collagen gels induced morphological changes in fibroblasts that partially mimicked connective tissue cells *in vivo* [1]. Collagen gels have been used widely since then for studying cell motility [7] and the roles of the physical state of three-dimensional gels [8]. In cancer research, tumor–stromal interactions are considered important in tumor progression [9], and collagen gels are used as microenvironments to study primary melanoma explants, for example for characterizing the importance of integrins in the process of malignant cell motility [10]. Three-dimensional environments that mimic *in vivo* microenvironments promote normal epithelial cell polarity and differentiation [11] and have been successfully used as model systems for mammary [12] and prostate [13] cancer progression. By identifying the factors controlling the architecture and patterning of cells *in vivo*, it should theoretically be possible to control not only the types and positions of cells in a matrix, but also to present topographical structures, architecture and stimuli mimicking the natural surroundings and regulatory microenvironments of cells *in vivo* [14•,15,16••].

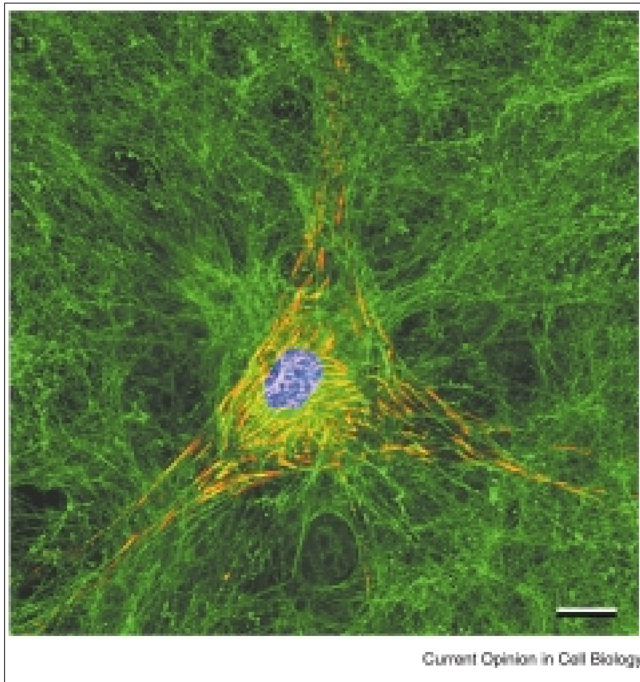
In this review, we focus on cell adhesions formed within three-dimensional matrices. We discuss three-dimensional-dependent signaling events and interdependencies between types of cell adhesions of fibroblastic cells encountering the third dimension *in vivo* and in systems that mimic *in vivo* three-dimensional microenvironments (Figure 1).

Signal transduction within three-dimensional systems

In vivo adhesion events occur three-dimensionally, where cells attach to surrounding three-dimensional mesh-like fibers rather than to two-dimensional coated surfaces. Comparisons between signal transduction in three-dimensional matrices (3D signaling) with signaling events on two-dimensional coated surfaces reveal some striking differences. For example, a major discrepancy can be observed in three-dimensional compared with two-dimensional signaling of the non-receptor focal adhesion kinase (FAK). Auto-phosphorylation levels of FAK are down-regulated in steady-state three-dimensional cultures compared with two-dimensional controls for both three-dimensional collagen gels [17] and cell-derived three-dimensional matrices, even while phosphorylation levels of proteins such as paxillin are unchanged [16••]. In both systems, concomitant phosphorylation of downstream mitogen-activated protein kinase (MAPK) is enhanced [16••,17], suggesting independence from the FAK-regulated pathway.

Remodeling of three-dimensional matrices in chronic ulcers leads to elevated expression of matrix metalloproteinase 13 (MMP-13) mRNA. A similar induction of this

Figure 1



The 'three-dimensional matrix adhesion' formed by cells in three-dimensional matrices. Human fibroblast 3D-adhesions containing α_5 integrins (orange and yellow) interact with a fibronectin-based matrix produced by mouse NIH-3T3 fibroblasts (green). The cell nucleus is blue. Bar = 10 μm.

metalloproteinase is observed *in vitro* in three-dimensional collagen gels compared with conventional two-dimensional cell cultures [18]. The induction of MMP-13 expression is attributed to contact of fibroblasts with the three-dimensional collagen gel, leading to activation of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins and coordinated stimulation of three distinct classes of MAPKs (i.e. ERK1/2, JNK and p38) [19]. In collagen gels, $\alpha_1\beta_1$ integrin has also been implicated in 3D signaling resulting in regulation of gel contraction, fibroblast migration but not attachment [20], and inhibition of collagen I accumulation [21].

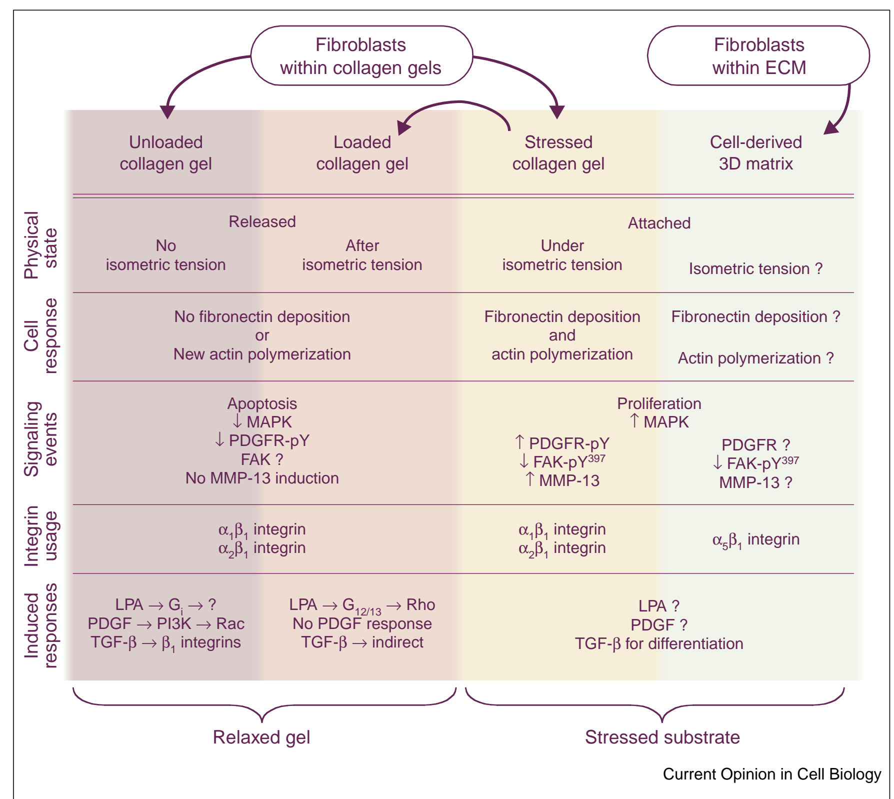
Collagen gels that remain attached to a culture dish after polymerization are considered mechanically 'stressed' gels. Cells develop isometric tension within these stressed gels, and subsequently when these gels are released — for example, by detaching from the dish — they contract and are termed 'relaxed and loaded' collagen gels. However, if collagen gels are released from the dish before the cells within the gels develop intrinsic tension, the contracting gel is termed 'relaxed and unloaded' (Figure 2). The fibroblast–collagen-gel contraction model provides a unique way to assess the role of isometric tension developed within stressed three-dimensional collagen gels, as well as to study the mechanisms that regulate wound contraction [8]. Fibronectin deposition and actin polymerization are dependent on the isometric-tension state of a three-dimensional substrate [22].

3D signaling also varies depending on the physical state of the three-dimensional substrate. Fibroblasts in attached, stressed collagen matrices proliferate, whereas cells in loaded or unloaded relaxed matrices become quiescent [23]. Interactions between fibroblasts and collagen gels lead to a decreased level of receptor auto-phosphorylation stimulated by platelet-derived growth factor within mechanically relaxed (loaded and unloaded) gels, which results in downregulation of cell proliferation (Figure 2; [8,24]). Cell quiescence in relaxed collagen gels may be explained by decreased signaling through the ERK pathway [25], although the absence of isometric tension in the cells cannot account for the ERK changes [23]. Specific ligands such as lysophosphatidic acid (LPA [8]) and transforming growth factor-β (TGF-β [26•]) act through distinct signaling pathways, depending on the loading state of contracting collagen gels. In turn, cellular sensing of the physical state of a three-dimensional microenvironment is regulated by mechanoregulatory growth factors (TGF-β_1 and TGF-β_3), which regulate the expression of specific integrins [27•]. Fibroblast-driven contraction of loaded collagen gels results in Ca^{2+} influx, which upregulates cAMP and phosphatidic acid through phospholipase D activation [28].

Taken together, the observations discussed above not only underscore differences between two-dimensional and 3D signaling, but also show that the physical state of a given three-dimensional substrate can affect signaling. The mechanisms may involve several factors, including three-dimensionality and different modes of presenting ECM fibrils to cells. While these factors still need experimental evaluation, the above studies indicate that matrix tension can regulate 3D signaling (Figure 2). Stressed collagen gels actively contract after being released from the dish [8], and fibronectin fibrils shrink up to fourfold after severing their attachment to cells [29]. This isometric tension might be involved in presenting the matrix proteins in a favorable conformation for specific integrin binding (i.e. preferential integrin usage), thus influencing signaling. In fact, cells become dependent on a particular integrin receptor when plated in collagen gels [10,27•] or in cell-derived three-dimensional fibronectin matrices [16••].

In addition, fibroblasts can interact with different collagen-recognition sequences, depending on the topographical organization of the substrate [30]. Moreover, stretching of the fibronectin molecule not only exposes cryptic self-polymerization sites necessary for matrix assembly [5•], but it can also regulate the exposure of different integrin recognition sites. Steered molecular dynamic simulations predict that stretching of fibronectin will reduce binding of $\alpha_5\beta_1$ integrin to RGD and synergy PHSRN sites [31], leaving the 70 kDa amino-terminal fibronectin integrin-binding site [32] in a more favorable position for binding to this integrin. Interestingly, binding of cells to two-dimensional surfaces coated with this (70 kDa) portion of the fibronectin molecule promotes cell motility but suppresses

Figure 2



Properties of experimental systems mimicking *in vivo* three-dimensional microenvironments and the cellular responses. The composition and physical state of matrices trigger distinct cellular responses. Although different in composition, collagen gels in a certain physical state and cell-derived three-dimensional matrices appear to evoke similar cellular responses, signaling events, and other ligand-induced responses. These events occur regardless of the type of integrin receptor used by cells to interact with the matrix ('Integrin usage'). Instead, they depend primarily on the physical properties of the ECM ('Physical state'). Question marks indicate that further direct comparisons are still needed. $G_{12/13}$, the $G\alpha_{12/13}$ class subunit of heterotrimeric G proteins; G_i, the $G\alpha_t$ class subunit of heterotrimeric G proteins; LPA, lysophosphatidic acid; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PI3K, phosphoinositide 3-kinase.

FAK and paxillin phosphorylation [32], suggesting possible differential signaling depending on which molecular domain is exposed during formation of a three-dimensional matrix. Future research using cell culture models should help in addressing these potential molecular mechanisms.

Maturation model: from focal complexes to three-dimensional matrix adhesions

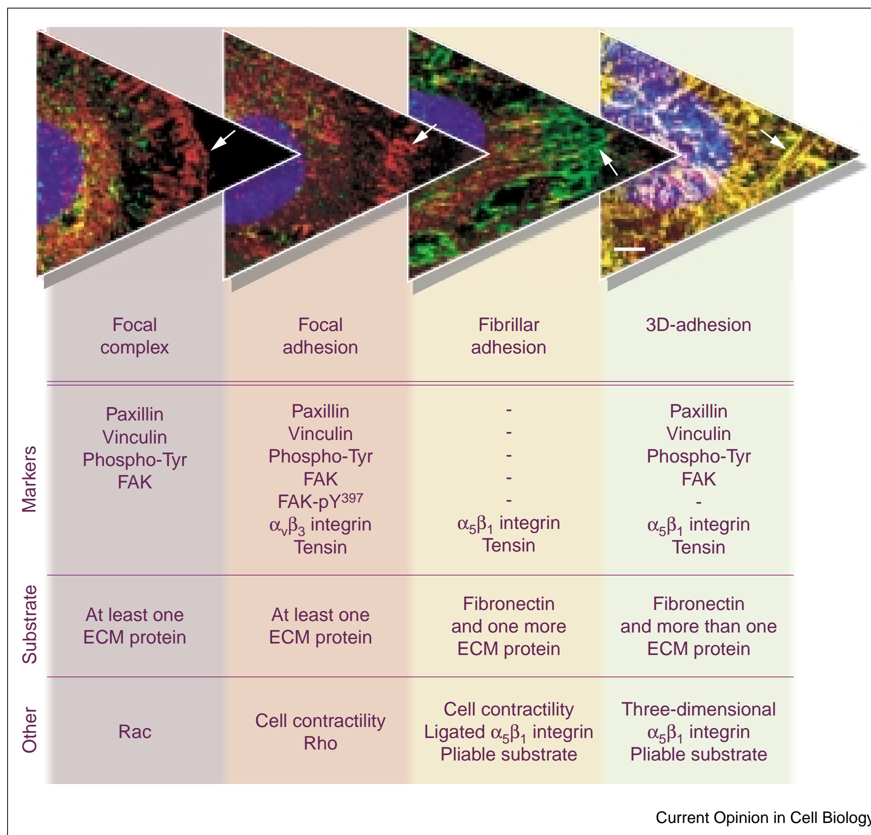
Several recent publications identify *in vitro* evolution or maturation of cell–matrix adhesions [5•,16••,33,34]. A summary of proposed relationships between four different types of cell–matrix adhesions is illustrated in Figure 3. The very first encounter of a cell with its surrounding substrate (even before integrin engagement) is regulated by cell-surface-associated hyaluronan [35].

When cells begin to spread and form lamellae, small (1 µm) dot-like structures appear at the edge of lamellipodia [5•]. These small structures, termed focal complexes, are the first well-defined integrin-containing adhesive structures formed by the advancing lamellipodium. Focal complexes are believed to be precursors of focal adhesions, the best-characterized and most commonly observed *in vitro* cell–matrix adhesions [5•,34]. Focal adhesions anchor bundles of actin filaments and mediate strong adhesion to the substrate [5•,34,36]. Once a focal adhesion is stable and mature, it serves as an anchoring point from which engaged and activated $\alpha_5\beta_1$ integrins translocate centripetally on lamellae towards the cell body [5•,37•]. This translocation drives the formation of a different type of cell–matrix adhesion, the fibrillar adhesions. These structures are believed to apply tension to fibronectin, exposing cryptic sites for polymerization and thereby facilitating fibronectin fibrillogenesis in a tensin-dependent manner [37•,38•].

Ongoing fibrillogenesis leads to accumulation of a thick matrix, which presents a new three-dimensional environment for the cells. This process occurs spontaneously in confluent fibroblastic cell cultures such as human foreskin fibroblasts, NIH-3T3 cell cultures [39], and lung fibroblasts, or in a TGF-β-dependent manner in differentiating myo-fibroblasts [33]. As the three-dimensional fibronectin meshwork accumulates, cells adapt by substituting adherence to it rather than to the artificial flat and rigid surface of the tissue culture dish. This adaptation is marked by the appearance of a new type of cell–matrix adhesion — three-dimensional matrix adhesions (3D-adhesions) — which possess a distinct composition and phosphorylation pattern [16••].

Under *in vitro* tissue culture conditions, the different types of cell–matrix adhesions appear consecutively and in an interrelated manner. For example, inhibition of Rho or cell contractility prevents the transition of focal complexes into focal adhesions [34], and inhibition of $\alpha_5\beta_1$ integrin translocation out of focal adhesions abolishes the formation of fibrillar adhesions [37•]. This interconversion of

Figure 3



Maturation model illustrating the relationships and evolution of cell–matrix adhesions *in vitro*. The arrowhead-shaped images point in the direction of generation, maturation or interconversion from the focal complex to the 3D-adhesion. Each type of adhesion is distinguished by a characteristic morphology (see white arrow in each image) and specific molecular components ('Markers'), which include paxillin (red) and α_5 integrin subunit (green). 3D-adhesions are characterized by overlapping localization of paxillin and α_5 integrin (yellow). Different types of adhesion have differing requirements for integrin ligands ('Substrate'). Other factors involved in triggering the formation of a particular type of adhesion are indicated. Bar = 2 µm.

cell–matrix adhesions *in vitro* suggests the existence of a related maturation process *in vivo* for producing 3D-adhesions. The *in vitro* maturation process may represent a means for cultured cells to improve their microenvironment by bringing it closer to *in vivo* conditions.

The lengthy process of adhesion maturation can be bypassed experimentally by plating cells directly into isolated, cell-free matrices produced from cell- or tissue-derived three-dimensional matrices. Interestingly, 3D-adhesions are formed quite rapidly in cells within these matrices, indicating that essential information is retained in the three-dimensional matrices even after removal of the cells that formed them. In addition, cell attachment, proliferation and migration are enhanced compared with a variety of two-dimensional substrates [16••]. The cell-derived three-dimensional matrix provides a new, molecularly complex three-dimensional system for studies of cell–matrix interactions that can take full advantage of traditional *in vitro* tissue culture (Figure 1).

The *in vivo* quest: cell–matrix interactions in three dimensions

The ultimate 'reality test' for *in vitro* findings is always *in vivo*. Recent studies provide correlative support for *in vitro* concepts about two-dimensional and three-dimensional adhesions. The molecular components of cell–matrix adhesions formed *in vitro* on flat surfaces have been characterized extensively [5•]. For instance, paxillin is a well-characterized multidomain scaffolding protein [40,41] that localizes to focal complexes, focal adhesions and 3D-adhesions, but is deficient in fibronectin-containing fibrillar adhesions (Figure 3). Recent reports present evidence for links between paxillin and fibronectin *in vivo* [16••,42], as well as between paxillin and $\alpha_5\beta_1$ integrin [16••,43]. In addition, TGF-β, which induces matrix deposition, also enhances the production of paxillin [44]. Another example is vinculin, which localizes to the same structures as paxillin (Figure 3) and is found *in vivo* in fibrillar structures containing ECM proteins [16••,45]. The $\alpha_2\beta_1$ integrin serves as an additional example, because it contributes significantly to cell adhesion to fibrillar collagen *in vivo* [46] and may represent a good candidate for an integrin transmembrane linker in 3D-adhesions in natural collagen-based matrices. In *Xenopus* development, lack of fibronectin fibrils correlates with a loss of deep cell polarity [47], emphasizing the importance of fibronectin organization and cell interactions with three-dimensional matrix at very early stages of development *in vivo*. Taken together, the above *in vivo* observations support *in vitro* findings on cell–matrix adhesion in three dimensions.

Conclusions and future directions

Cell–matrix adhesions depend on the differentiation state of cells, their physical location and the local forces sensed

by the cells. One of the main challenges to understanding cell–matrix adhesions is the enormous diversity and complexity of the *in vivo* microenvironments that cells encounter. Consequently, conclusions from one system may not always be true for another.

The differences observed in two-dimensional versus 3D signaling studies emphasize the importance of evaluating three-dimensionality and *in vivo* physiological relevance when drawing conclusions about signal transduction pathways and cell responses in cell culture systems (see Update). Moreover, the physical state of a three-dimensional matrix has also proven to be important in modifying cell responses to a specific signaling molecule [8,26•,27•,31,32].

A maturation or evolution process for fibroblast adhesions *in vitro* (Figure 3) ultimately generates adhesions comparable to 3D-adhesions *in vivo*, at least in embryonic mesenchymal tissue [16••]. However, other tissues *in vivo* may have other types of 3D-adhesions, which may differ in the type of integrin or other ECM receptor and in the cytoskeletal anchoring proteins.

The two-dimensional adhesions observed in cell culture may represent exaggerated stages of dynamic *in vivo* structures in a specific molecular and physical environment. For example, because *in vivo* matrices are dynamic and must constantly regenerate, local anchoring and fibrillogenesis should be needed. Moreover, although focal adhesions appear to be relatively rare *in vivo*, small dot-like structures containing the focal adhesion integrin $\alpha_v\beta_3$ and phosphorylated FAK [16••] may fulfil anchorage functions in three-dimensional systems and *in vivo*.

Two very different types of ECM are the fibrous mesh-like stroma of connective tissue surrounding fibroblastic cells and the basement membrane underlying epithelial cells and separating them from connective tissue [2]. Transient loss of basement membrane in pre-malignant epithelium is an early event associated with tumor progression [48]. This loss facilitates interactions between epithelial cells and stroma. In fact, the majority of tumor-derived myoepithelial cells are deficient in their ability to impart polarity because of aberrant synthesis and matrix deposition [49]. These and other complex alterations in tumor–stromal interactions in cancer progression may be excellent candidates for study using *in vitro* three-dimensional matrix systems.

Traditional tissue culture has provided many insights into cell–matrix adhesions and their regulation by cell type, cell interactions, soluble factors, substrates, and the physical state of a substrate. Understanding the structure, function and maturation steps of these structures can help to illuminate cell–matrix interactions *in vivo*. We are only just beginning to uncover the marvelously multifaceted interactions that cells encounter within three-dimensional extracellular matrices *in vivo*.

Update

Two recent studies have imaged directly the dynamic behavior of lymphocytes in the natural three-dimensional environment of intact lymph nodes [50,51]. Both describe differences in cell behavior when compared with *in vitro* systems. Two-photon real-time fluorescence confocal imaging permits visualization of pre-labeled lymphocytes deep within lymph nodes; their three-dimensional movement appears random and significantly faster [50] than previously reported *in vitro* measurements. In a complementary study utilizing real-time conventional confocal imaging, pre-labeled T cells and dendritic cells were found to display very long-lasting associations within the lymph node [51]. These prolonged cell–cell contacts subsequently initiate lymphocyte activation, and they contrast with the previously described sequential, brief contacts observed in collagen gels *in vitro*.

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