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CRITICAL REVIEW

Biophysical regulation of tumor cell invasion: moving beyond matrix stiffness†

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Invasion of cancer cells into the extracellular matrix (ECM) is a key step in tumor infiltration and metastasis. While the strong influence of ECM stiffness in governing tumor cell migration has been well established in two-dimensional culture paradigms, investigation of this parameter in three-dimensional (3D) ECMs has proven considerably more challenging, in part because perturbations that change 3D ECM stiffness often concurrently change microscale matrix parameters that critically regulate cell migration, such as pore size, fiber architecture, and local material deformability. Here we review the potential importance of these parameters in the context of tumor cell migration in 3D ECMs. We begin by discussing biophysical mechanisms of cell motility in 3D ECMs, with an emphasis on the cell-matrix mechanical interactions that underlie this process and key signatures of mesenchymal and amoeboid modes of motility. We then consider microscale matrix physical properties that are particularly relevant to 3D culture and would be expected to regulate motility, including matrix microstructure and nonlinear elasticity. We also discuss how changes in 3D matrix properties might be expected to trigger transitions in subcellular mechanisms, which in turn contribute to mesenchymal-amoeboid transition (MAT) by imposing restrictions on 3D motility. We expect that the field will gain valuable insight into invasion and metastasis by deepening its understanding of microscale, biophysical interactions between tumor cells and matrix elements and by creating new 3D scaffolds that permit orthogonal manipulation of specific matrix parameters.

1. Introduction

Tumor cells implement a variety of migration and invasion strategies to infiltrate their primary tissue and metastasize to distant sites, and these depend on and are defined by specific biophysical interactions between tumor cells and their extracellular matrix (ECM).[1–7] As the field has sought to decompose the complex inputs present in the biophysical microenvironment, the mechanical elasticity (stiffness) of the ECM has emerged as a particularly powerful regulator of the behavior of tumor cells.[1] For example, hallmarks of malignant transformation in mammary epithelial cells can be induced in culture simply by manipulating ECM stiffness,[4] and changes in ECM stiffness can strongly influence the migration and proliferation rate of malignant brain tumor cells.[3] Moreover, changes in sensitivity to ECM stiffness can correlate with malignant transformation, as evidenced by the finding that

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Insight, innovation, integration

Extracellular matrix (ECM) stiffness is now recognized to strongly regulate tumor cell motility in 2D culture, yet comparatively little is known about the importance of this regulation in tissue-like 3D cultures. This may derive from the difficulty of varying stiffness in 3D ECMs without changing other microstructural and micromechanical parameters that affect motility. The importance of these scaffold parameters is somewhat underappreciated, and our review offers a concise introduction to these concepts for cell and cancer biologists and materials scientists. Our understanding of how these parameters control motility has been facilitated by sophisticated materials fabrication and imaging technologies. We argue that additional advances in tuning microscale biophysical properties of 3D ECMs will be critical to progress in this field.

Ras-transformed fibroblasts can proliferate on highly compliant ECMs whereas untransformed fibroblasts cannot.[6] In neuroblastoma, the expression of prognostic markers (*N*-Myc) and sensitivity to clinical differentiation agents (retinoic acid) are strongly sensitive to ECM stiffness.[2] Importantly, these studies have been made possible by the development of two-dimensional (2D) culture paradigms that permit orthogonal control of ECM stiffness and biochemical ligand density,[5,7] which have concurrently led to a broader appreciation of ECM stiffness as a key regulator of the behavior of stem cells[8] and differentiated tissue cells.[9]

The demonstrated relevance of ECM stiffness to tumor biology in 2D culture has spurred an intense effort to understand the potential regulatory importance of this cue in three-dimensional (3D) culture paradigms, which more closely resemble the *in vivo* microenvironment. However, these efforts have been complicated by the experimental reality that it is extremely challenging to manipulate ECM stiffness in 3D culture independently of other parameters that are likely to influence cell migration. For example, perhaps the simplest way to vary ECM stiffness in 3D matrices is to change the protein density of the matrix; however, this concurrently changes ligand density and may also potentially alter other microstructural properties, such as pore size and fiber architecture, which independently influence the invasive phenotype.[10–16] For this reason, the relative contributions of these microstructural ECM properties to individual cellular mechanisms and their cumulative role in dictating various modes of cell motility are not yet completely understood. Closely related to this issue is the fact that 3D motility may rely on significantly different subcellular mechanisms than 2D motility, as cells embedded within 3D matrices must contend with a microenvironment in which productive motility often requires the removal of steric barriers, extensive matrix remodeling, or both. Consistent with this picture, much recent work has highlighted direct relationships between ECM 3D microstructural properties, cell motility, and underlying molecular events.[12,15–19] Nonetheless, a much deeper understanding is needed of the biophysical mechanisms through which cells invade 3D ECMs and how these mechanisms relate to specific biophysical properties of the ECM that include but are not limited to bulk stiffness.

In this review, we explore properties of 3D ECMs that are likely to regulate the invasive behavior of tumor cells, as well as the machinery through which cells interface with these properties. We first discuss the role that bulk matrix properties play in dictating cell migration phenotypes in 2D and 3D ECMs, followed by a summary of key cellular mechanisms that drive cell migration in the 3D environment. Then, we explore the roles of matrix microstructural properties in controlling cell motility mechanisms and modes of tumor cell invasion. In addition to considering effects of matrix stiffness, we argue that the field should take a broad view in approaching biophysical microenvironmental regulation of cell invasion, and take into account microstructural features such as pore size, fiber morphology and non-linear deformability of the matrix network. These parameters also have important implications for the design of material scaffolds for *in vitro* modeling of tumor invasion.

2. Extracellular matrix regulation of cell motility: 3D is different from 2D

Cell motility in 2D culture can be conceptualized as a sequential process that involves protrusion of the leading edge of the cell, formation, stabilization, and maturation of cell-ECM adhesions, contraction of the cell body, and rupture and retraction of the trailing edge, which collectively yield forward translocation of the cell.[20,21] The dependence of cell motility on the biophysical properties of the ECM may be parsed in terms of each step of the migration process: namely, localized and dynamic formation and breakage of cell-ECM adhesions, generation of traction forces, and the activation of force-dependent signaling pathways.[3,7,15,21–23]

Given that cell migration is a physically integrated process that involves exchange of mechanical force between the cell

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and the substrate, it seems almost obvious in retrospect that cell motility is exquisitely sensitive to matrix physical properties, including ligand density and stiffness.[3,9,24–26] A variety of studies in both normal and tumor cells have revealed that increasing ECM stiffness induces a phenotype that includes stabilization of cell-ECM adhesions, activation of actomyosin contractility, and increased spreading area, whereas highly compliant ECMs yield weak cell-ECM adhesion, cell rounding, and abrogation of motility.[3,7–9,26] Gradients in stiffness can even drive migration (durotaxis), implying that cells are capable of continuously sampling stiffness on a length scale much smaller than their own length and tuning their behavior accordingly.[27–30] While the details of this rigidity sensing remain incompletely understood, cells are believed to utilize mechanosensory molecules located in cell-matrix adhesion complexes,[25,31] which in turn are capable of initiating signaling cascades that can alter the strength and turnover of adhesions, assembly of cytoskeletal structures, and generation of traction forces.

Conceptual translation of stiffness regulation of cell motility from 2D ECMs to 3D ECMs has proven challenging, in part because the bulk stiffness of a fibrous material reflects the composite effects of many microscopic properties including fiber density, fiber strength, degree of cross-linking, filament length and constitutive deformability of the scaffold. This is fundamentally unlike the 2D ECM scaffolds commonly used to study stiffness effects, in which key governing parameters can be effectively captured by "bulk" measurements of material samples that are much larger than a single cell. For example, the elastic modulus (stiffness) of a 2D hydrogel matrix can be obtained from macroscopic measurements such as shear and extensional rheometry,[3,32–34] and these are often in good agreement with cell-scale measurements such as atomic force microscopy.[34–36] In contrast, 3D ECMs are often fibrous in nature (*e.g.*, collagen, fibrin)[37] and are therefore highly structured on the length scale of a single cell, thereby rendering the bulk measurements considerably less predictive of cell behavior. Ultimately, one would expect that cells sense and respond to these local microscale cues rather than the global properties of the material, and this notion is supported by recent studies showing that cells embedded in collagen matrices tend to limit matrix compaction to a zone that extends only tens of micrometres from the cell surface.[38] Even in non-fibrous matrices, changes in dimensionality can alter the presentation of ECM molecules, which in turn can affect ECM-dependent cell migration. For example, MV3 melanoma cells cultured on hyaluronic acid (HA) coated 2D substrates migrate faster as the concentration of HA is increased, whereas migration speed is independent of HA concentration when MV3 cells are embedded in 3D collagen-HA matrices.[39]

The change in dimensionality of the ECM is reflected in differences in how individual components of the cell motility machinery function in 3D *vs.* 2D, although the mechanistic origins of these differences are still being discovered.[40] One recent study showed that nonmuscle myosin IIB plays a crucial role in generating tractional forces that power the movement of collagen fibers at the leading edge of fibroblasts in 3D matrices; however, on 2D collagen substrates, myosin IIB is centrally located and its abrogation does not affect cell migration.[41] Another recent study found that inhibition of myosin II ATPase activity slightly increased the migration speed of fibroblasts in 2D but reduced migration speed in 3D.[40] Persistent cell migration in 2D requires crosstalk between microtubules and myosin II activity in which non-muscle myosin IIA promotes microtubule instability and prevents polymerized microtubules from accumulating in lamellae and driving ruffling.[42] Conversely, in either 1D or 3D culture, inhibition of microtubules produces protrusions in multiple directions and reduces migration speed overall.[40] Finally, the activation level of Rac GTPase, which is traditionally thought to promote protrusion and adhesion dynamics, has been observed to be lower for cells cultured in 3D than in 2D. Interestingly, suppression of Rac in 2D reduces cell spreading and gives rise to a polarized cell morphology and uniaxial migration phenotype reminiscent of that observed in 3D.[43]

On 2D substrates, lamellipodia frequently exhibit a flat morphology, cell-ECM adhesions are discrete and plaque-like, intracellular contractile forces are generated by thick stress fiber bundles, and cells often adopt a highly spread morphology. In contrast, the behavior of individual mechanisms of cells in 3D ECMs changes based on their adopted mode of motility—mesenchymal, amoeboid or other hybrid modes. In these different modes, cell morphology varies from elongated to spindle-shaped to ellipsoid. In addition, cell migration through dense 3D matrices also often requires remodeling of the matrix by pericellular proteolysis to create gaps for cell invasion. The dramatic structural differences between 2D and 3D matrices and the different mechanisms that cells invoke to locomote in each topology can give rise to opposing correlations between stiffness and motility in 2D *vs.* 3D.[3,16]

Differences in migration in 2D and 3D matrices are strongly reflected in the molecular systems through which cells engage the ECM. While the architecture of cell-ECM adhesions on 2D substrates has become increasingly well characterized, the morphology and taxonomy of adhesions in 3D and their specific roles in different modes of cell migration is a crucial part of ongoing debates about matrix regulation of cell adhesion and migration.[44–46] One study showed that on 2D fibronectin matrices, fibroblasts incorporate both α5β1 and αvβ3 integrins into fibrillar and focal adhesions, respectively; however, in 3D matrices, α5β1 localizes to adhesions whereas αvβ3 does not.[46] Another recent study showed that unlike in 2D, aggregated adhesions in human fibrosarcoma cells embedded in 3D collagen matrices are small and short-lived, and focal adhesion proteins occupy diffuse distributions; here, their main role appears to be the regulation of adhesive protrusions that in turn drive cell motility.[45] A third study revealed that the architecture of cell-ECM adhesions depends strongly on the ECM material, with distinct types of cell-ECM adhesions detected in fibroblasts seeded on four different ECMs (cell-derived matrix, hydrogels of collagen type I, fibrin, and basement membrane extract).[44]

The ability of the cell to sense dimensionality, as evident in differences in cell morphology and structure of cell-ECM adhesions between 2D and 3D, has been experimentally related to the ECM stiffness encountered by the cell at the microscale.[45,46] Tellingly, when 3D matrices are stiffened by

chemical crosslinking, cell-matrix adhesions come to resemble the integrin-based focal adhesions seen on 2D substrates.[46] These findings suggest that cell's ability to sense stiffness and dimensionality might go hand in hand, and that mechanical cues play a complex and vital role in dictating cell migration strategies in 3D environments.

3. Mechanisms for cell invasion in 3D matrices

Despite the many similarities between cellular mechanisms responsible for cell migration on 2D surfaces and cancer cell invasion inside 3D ECMs, the choreography of motility and its dependence on specific matrix parameters differ dramatically between these two topologies. Before discussing biophysical properties of 3D ECMs that might regulate motility, we first briefly review the cellular and subcellular events that underlie motility in these matrices and their functional differences from 2D cell migration, which will enable us to better rationalize the relationships between cellular mechanisms and ECM properties.

The mechanisms used by tumor cells to migrate and invade the surrounding tissue share many similarities with those at play in cell migration during tissue development, homeostasis, and wound healing. Despite these commonalities, individual subcellular mechanisms often vary between specific cell types and matrix conditions, which has given rise to a large diversity of observed modes of motility.[40,47,48] That said, two of the most common modes of single cell migration in 3D are: (1) mesenchymal migration, which is characterized by an elongated fibroblast-like morphology, highly condensed cell-matrix adhesions, and formation of contractile actomyosin bundles reminiscent of stress fibers observed in 2D culture,[39,49] and (2) amoeboid migration, which is characterized by an ellipsoid shape, formation of bleb-like protrusions, restriction of actomyosin contractility to the cell cortex, and transient, punctate adhesions with the ECM. As an entry point to exploring the differences between these modes, we first summarize key mechanisms that make up the motile machinery of the cell in 3D (Fig. 1).

Cell migration in steps

Protrusions at the leading edge. Polarization of the cell body into an elongated shape[50] and extensions at the leading edge that probe the ECM are commonly regarded as first steps in cell motility. While these protrusions vary in morphology and size in different contexts, they all develop from highly regulated actin polymerization.[51] Whereas on 2D ECMs, cells form a flat, continuous lamellipodium that defines the leading edge,[20] leading edges in 3D ECMs show significant diversity, from thin filopodia to cylindrical pseudopodia.[52] In some cases, leading edge structures extended by the cell into 3D tissue dynamically interact with the surrounding environment and probe biomechanical properties of the matrix fibers.[39,53,54] Cells inside 3D fibrillar matrices can also extend pseudopodial protrusions that form adhesions with the ECM fibers along the cell body and drive cell motility. Cell protrusions vary in size and shape according to dimensionality and microstructure of their extracellular environment, but in many cases they are indispensable for cell motility. For example, rabbit corneal fibroblasts in 3D collagen I matrices simultaneously extend pseudopodial protrusions and form focal adhesions along collagen fibers, while actomyosin contractile forces induce forward propulsion of the cell body by constricting and compressing fibers. Pharmacological inhibition of actin polymerization causes disassembly of pseudopodial extensions and adhesions between cell and collagen fibers, and relaxation of the compressed ECM.[55] Similarly, inhibition of actin polymerization causes complete collapse of the migration machinery in glioma cells cultured on 2D ECMs.[3] Thus, the origin of motility is directly connected with cell polarization and actomyosin-based protrusions.

Cell-ECM adhesions. As protrusions emerge and come in contact with the surrounding matrix, they engage ECM fibers *via* transmembrane adhesion receptors of the integrin family.[56,57] Nascent adhesions form as soon as the integrins come into contact with matrix ligands and recruit intracellular adhesion proteins to develop the adhesion cluster into small focal complexes. Different ECM proteins (*e.g.* fibronectin, laminin, vitronectin and fibrillar collagen) are recognized by different integrin pairs (*e.g.* $\alpha2\beta1$, $\alpha5\beta1$, $\alpha6\beta1$), which activate, cluster and begin to recruit a variety of structural and signaling components to the growing adhesion.[39,46,58] Adhesions formed by cells embedded in 3D matrices vary in shape and size depending on the specific matrix conditions and mode of cell motility being utilized.[11,46,59] For example, adhesions formed between fibroblasts and collagen fibers in 3D matrices have been shown to critically depend on $\alpha_5\beta_1$ integrin, with associated adhesive plaques attaining an elongated and fibrillar shape distinct in shape from focal adhesions observed on 2D surfaces.[46] In mesenchymal motility, mature adhesions actively engage with the ECM fibers, support contractile forces generated by the cell, and remodel the matrix.[39,49] Conversely, in amoeboid motility, small and short-lived transient adhesions regulate adhesive protrusion dynamics [45] and provide traction for the cell body.[47,48]

Actomyosin contractility. The cellular cytoskeleton generates traction against the ECM through contractile forces created by actomyosin bundles and transmitted to the ECM *via* cell-ECM adhesions. Actin filament alignment and bundling are facilitated by cross-linking proteins, such as α-actinin and myosin,[60] and their coupling to maturing focal contacts is mediated by adapter proteins such as zyxin and talin.[61] Actomyosin complexes primarily exist within the cortical actin network beneath the plasma membrane and as larger contractile actomyosin bundles in the cytoplasm. Actomyosin contractility, generated by the relative sliding between non-muscle myosin II and actin filaments, is responsible for the largest tensile forces exerted by the cell. For example, in glioma cells, pharmacological inhibition of the Rho/ROCK/myosin II pathway curtails stress fiber contractility, releases intracellular tension, reduces the ability of these cells to sense ECM stiffness, and as a result slows down cell migration on stiff ECMs while rescuing motility on soft ECMs.[3] In mesenchymal motility, in addition to propulsively "pulling" the cell forward during motility, these forces can facilitate remodeling of matrix components to create contact guidance

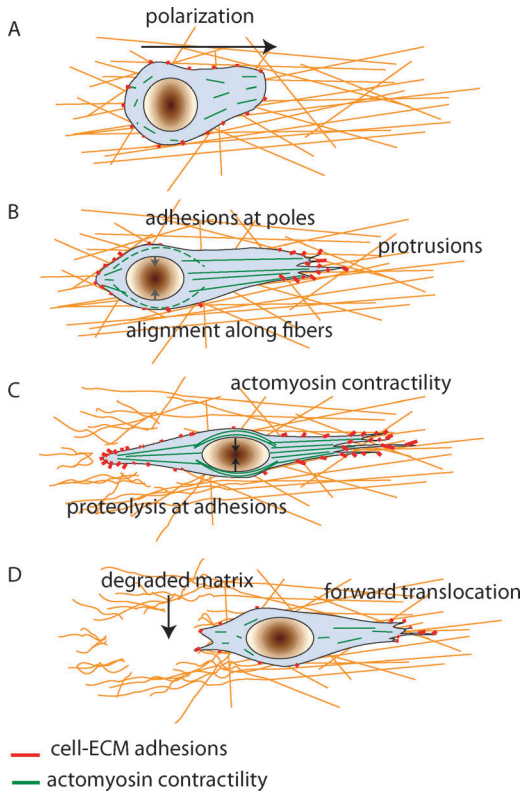

Fig. 1 Cell migration in steps. (A) A cell interacts with the ECM and polarizes in one direction. (B) Extended protrusions at the leading edge probe the surrounding ECM fibers and form cell-ECM adhesions. (C) Further polarization and strengthening of adhesions is accompanied by a rise in actomyosin contractility and proteolytic degradation of ECM fibers at cell-ECM junctions. (D) Retraction of the trailing edge is followed by forward translocation of the cell and completion of the migration cycle.

cues.[62] In amoeboid motility, hydrostatic pressure generated by the actin cortex extrude the cell through preexisting matrix pores. These forces are particularly indispensible when the nucleus, which is much stiffer than the rest of cell, is wider than the matrix pore and must be compressed for translocation to continue. Notably, malignant brain tumor cells express myosin at significantly higher levels than normal endogenous brain cells, and their invasive behavior can be suppressed by reducing myosin expression.[17]

Proteolysis—matrix degradation and remodeling. In addition to structurally coupling the cytoskeleton and ECM, cell-ECM adhesions can also facilitate the local activation and secretion of proteolytic enzymes, notably matrix metalloproteases (MMPs), which degrade the neighbouring ECM fibers and remove barriers to cell migration. There is a vast diversity of MMPs defined by their ECM substrate specificity and intracellular localization. For example, matrix metalloproteinase-1 (MT1-MMP) preferentially localizes to β1 and αvβ3 integrins and is capable of cleaving collagen I and II, fibronectin, vitronectin, laminin, fibrin, and proteoglycans.[63,64] Cells can escape the restraints of a dense network of ECM fibers by using a combination of adhesion and proteolysis, *e.g.* by displacing fibers as the cortical actin network expands, forming adhesions, and cleaving ECM fibers to generate sufficient space for the cell to move forward. As the cell moves forward and degrades the matrix along the way, it leaves a path behind that can serve as a migration track for subsequent invasive cells.[18,62,65] For example, HT-1080 fibrosarcoma and MDA-MB-231 breast cancer cells polarize along collagen fibers and generate force at the leading edge of the cell without interfering with the proteolytic degradation of the matrix in the rear, and systematically arrange the collagen fibers into microtracks that guide cell movement.[62] This proteolysis step in cell invasion was long assumed to be necessary for 3D migration; however, landmark studies of migration in the presence of protease inhibitors revealed that cells can adopt alternative, "proteolysis-independent" migration strategies to compensate for an inability to generate paths by matrix degradation. Under these conditions, the cell converts to a rounded shape and adopts an amoeboid mode of motility with propulsive squeezing of the cell body through existing matrix pores.[59] This phenomenon, termed as mesenchymal-amoeboid-transition (MAT), is revisited in greater detail below. Proteolytic migration allows directionally persistent migration of the cell with reduced cell body deformation and physical stress, and simultaneous alignment and bundling of the ECM fibers.[66]

Modes of migration—Mesenchymal-Amoeboid-Transition (MAT)

As discussed earlier, the invasion phenotypes adopted by cells through 3D matrices are broadly defined in terms of two modes—mesenchymal or amoeboid (Fig. 2A and B). In the mesenchymal mode, cells wider than the matrix pore size degrade the matrix *via* proteolytic and force-based matrix remodeling, thus migrating in a "path-generating" manner, while amoeboid cells squeeze through preexisting pores in a protease-independent fashion in a "path-finding" mode.[59,65] Despite the stark mechanistic contrasts between these modes, under some circumstances cancer cells can convert their mode from mesenchymal to amoeboid, which is commonly referred to as mesenchymal-amoeboid-transision (MAT).[11] Such transitions can be triggered *in vitro* through a variety of interventions, including inhibition of proteolysis or integrin based cell-ECM adhesion,[59,67–70] controlled inhibition of Rho and Rac signaling,[70,71] or manipulation of the ECM microstructure.[16,59,68,72] Cells undergoing mesenchymal motility polarize along matrix fibers, form stable adhesions at the poles of the elongated cell, and migrate in a cyclic fashion in which actomyosin bundles exert traction force and rupture the adhesions at the trailing edge of the cell. The spindle-shaped morphology in mesenchymal migration allows axial alignment of forces, directionally persistent propulsion of the cell, and proteolytic matrix degradation in the path of migration (Fig. 2A).[11,14,73] The inhibition of proteolysis or integrin-dependent adhesion can be compensated by a weak- or non-adhesive amoeboid mode of migration in which the cell adopts a rounded morphology and changes its shape by generating hydrostatic pressure at the cell cortex, thus forcibly extruding processes through available spaces in the porous matrix and eventually deforming the cell body (Fig. 2B).[48,59]

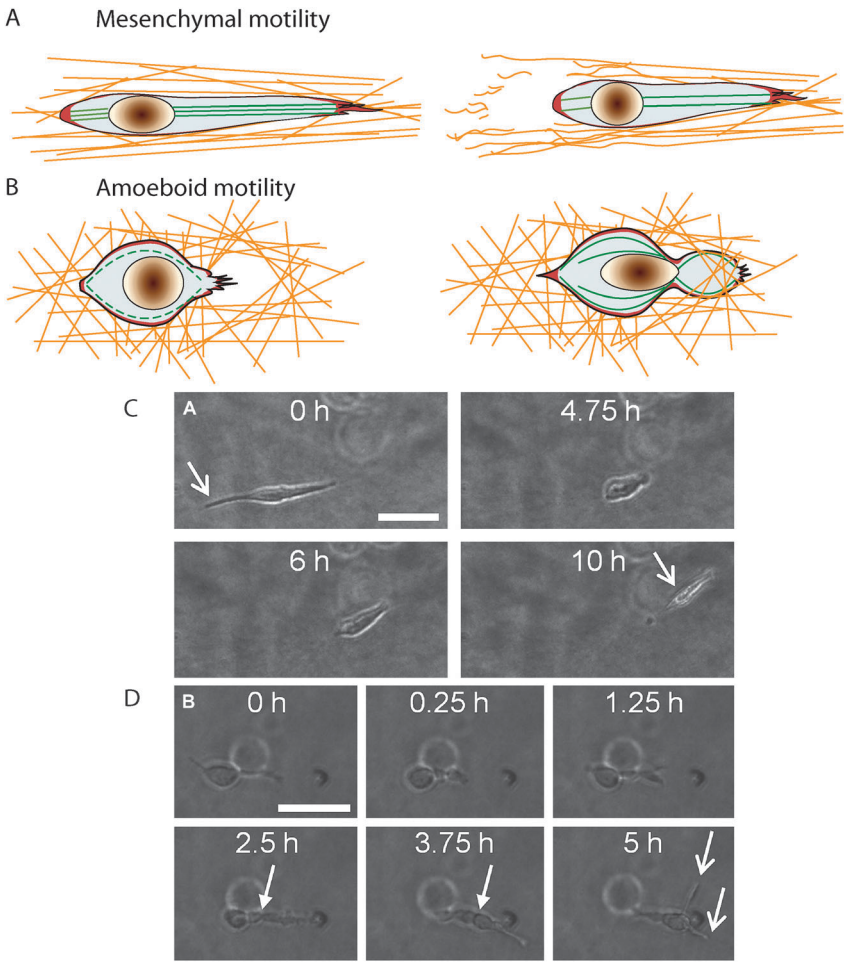

Fig. 2 Mesenchymal-amoeboid-transition due to change in ECM properties. (A) Mesenchymal mode of motility with elongated spindle-shaped morphology, focal adhesions at the poles and enhanced stress fiber contractility. (B) Amoeboid mode of motility with blebby protrusions, rounded ellipsoid morphology and cortical contractility. (C) U373-MG human glioma cells in pure collagen gels (0.5 mg ml^{-1}) have elongated morphology (arrows) and adopt a mesenchymal mode of motility. (D) Cells embedded in agarose-rich gels (0.5 mg ml^{-1} collagen with 0.25% agarose) exhibit a more rounded morphology and other characteristics of the amoeboid mode of motility, such as the presence of constriction rings (solid arrows) and active probing of the ECM by multiple migration paths at the leading edge (open arrows).[16] (C) and (D) Reproduced with permission from Elsevier.[16]

Upon abrogation of pericellular proteolysis by protease inhibitors, highly invasive fibrosarcoma (HT-1080) and breast cancer (MDA-MB-231) cells stop structural remodeling of collagen fibers, align their cell body along preformed fiber strands and migrate through existing pores with fibrillar walls.[59] Thus, MAT may be regarded as an adaptive strategy for continued cell invasion in the setting of impaired matrix degradation and remodeling, in the sense that the cell modulates its shape to suit the constraints of existing matrix microarchitecture.[59,72,74] Similar transition mechanisms can be triggered by changes in the biophysical properties of the ECM, such as when the porosity and fiber mobility of a weak collagen I gel is reduced by addition of agarose, which the cell cannot enzymatically degrade (Fig. 2C and D).[16]

Mechanotransductive signaling

Although much remains unclear about how ECM stiffness promotes transformation and invasion, an emerging paradigm argues that ECM stiffness controls integrin activation and clustering, recruitment of focal adhesion proteins to the cell-ECM interface, and activation of key mechanosensory proteins (*e.g.* Rho GTPases) that can both trigger broader signaling cascades and reinforce adhesions through mechano-chemical feedback.[75] The signaling pathways involved in the steps of the cell migration cycle discussed earlier are inter-coupled, and so elucidating the connectivity and regulatory logic of each pathway remains a challenge. To take one example, the actin-rich protrusions in the mesenchymal phenotype result from activation of Rac GTPase and recruitment of WAVE2 and Arp2/3 complexes[21,76] that nucleate actin filaments, and profilin, which promotes actin polymerization.[77] Conversely, pseudopodial protrusions in amoeboid migration result from waves of actin polymerization over the entire actin cortex that are triggered by the activation of chemo-attractant receptors in the cell membrane.[78,79] The actin-rich protrusions interact with the surrounding ECM and form adhesions *via* integrins and other adhesion receptors,

thus establishing mechanical communication between the actin cytoskeleton and ECM.[56,80–82] In these adhesions, integrins and the actin cytoskeleton are bridged through the action of talin, focal adhesion kinase (FAK), tensin and other actin-binding proteins (α-actinin, paxilin, vinculin).[39,46,58,83] The actomyosin contractility in mesenchymal mode of migration is largely generated by nonmuscle myosin II, which is in turn activated by the Rho GTPase/Rho-associated kinase (ROCK) pathway,[84–86] while amoeboid migration has been associated with myosin contractility activated by myosin light-chain kinase (MLCK).[87–89] The advancing cell body simultaneously degrades the surrounding matrix by surface matrix metalloproteases proteases (*e.g.* MMP1/2) recruited towards the cell-ECM junctions by integrins and other adhesion receptors.[59,65,90,91] However, this proteolytic activity is absent in amoeboid migration where cells squeeze through the matrix pores in an largely adhesion- and proteolysis-independent manner.[48]

4. Matrix microstructure

As the preceding discussion makes clear, the mode of motility in 3D ECMs is closely tied to the microstructural details of the matrix itself, and changes to these details can fundamentally alter how the cell navigates the matrix. We now discuss some of these microstructural parameters and how each is believed to regulate cell motility.

Pore size

One of the most important themes from the previous discussion is that cell migration in 3D is heavily influenced by the microstructure of the matrix, and *in vitro* studies have demonstrated that matrix manipulations that reduce pore size often create steric barriers that slow motility.[15] To overcome the structural barriers posed by the 3D network of fibers, cells exploit protease-dependent mechanisms discussed earlier or undergo amoeboid motility to squeeze through available pores.[16,59,68,71,92,93] The ability of the cell to squeeze through dense matrices in a protease-independent manner is limited by a critical pore size, which would be expected be around the diameter of a polarized cell.[15,94–96] In many contexts, cancer cells have been observed to mobilize actomyosin contractility to deform the cell body and the nucleus to physically displace collagen fibers and clear a path for the invading cell to follow.[16,59,72] For example, as described earlier, below a critical pore size, glioma cells must recruit nonmuscle myosin II to deform the nucleus and negotiate the narrow pores, and indeed invasive glioma cells express nonmuscle myosin II at levels that are significantly higher than normal neurons and astrocytes.[17]

Several strategies have been developed to vary the microstructure of reconstituted collagen I matrices, with the goal of investigating how these matrix parameters regulate cell migration. Collagen microstructure can be tuned within modest ranges of fiber density, pore size and bulk modulus simply by changing the collagen concentration, pH, and gelation temperature.[97–99] For gels in which pore size is controlled by gelation temperature, invasive speed correlates much more closely with pore size than with either stiffness or collagen density.[99] Among other microstructural variations, covalent cross-linking of collagen matrices, which renders matrix pores much less deformable, hampers the ability of cancer cells to squeeze through narrow pores in a protease-independent manner.[68] In a collagen-agarose gel system, specific biophysical properties of the ECM, such as bulk stiffness and pore size, can be varied by adding agarose while keeping the collagen content constant.[16] Addition of agarose in this system reduces pore size, which severely restricts motility in spite of the fact that agarose also increases stiffness. In other words, the steric barriers created by agarose overwhelm any gains in traction force associated with higher matrix stiffness.[3,16]

Fiber orientation and morphology

Native ECMs are often composed of networks of fibers, and these constituent structures provide structural integrity to tissues, facilitate cell-ECM interaction *via* integrin-based adhesions, and allow contact guidance cues that facilitate directionally-persistent cell migration along aligned fibrillar structures.[100] Inside 3D matrices, aligned ECM fibers facilitate rapid and persistent cell migration through a 1D-like migratory mechanism that is fundamentally unlike that observed on 2D surfaces.[40] The orientation of the fibers may range from loose and random to interwoven and aligned, which in turn affects the bulk properties of the matrix and the details of cell-ECM interactions at the cellular length scale.[100,101]

Dense packing of these fibers can confine the cell body, in which case invasion requires formation of stable focal adhesions at the cell-fiber interface, deformation of the fiber by contractile forces generated due to attached stress fibers, and degradation of the matrix by MMP-based mechanisms.[11,55,62,66] In 3D collagen matrices, fibrosarcoma and carcinoma cells adopt a mesenchymal mode of motility with integrin-based adhesion and proteolytic degradation of the matrix, and treatment with protease inhibitors completely stops the structural breakdown of matrix fibers. The cell body aligns itself along preformed fiber strands and migrates by conforming to these guidance cues along fibrillar scaffolds; thus, this adaptive migration mechanism facilitates protease-independent amoeboid migration.[59] Fibrillar structures in collagen lattices are also involved in the migratory activity and directional persistence of MV3 melanoma cells.[65] Immediately after encapsulation within 3D collagen matrices, MV3 cells migrate slowly while aggressively remodeling the matrix by forming focal adhesions or stripes of integrins at binding sites that align with collagen fibers at points of attachment. This matrix reorganization creates tube-like migratory tracks surrounded by a dense fiber network, which provides contact guidance cues that enable neighboring MV3 cells to migrate along these pre-formed paths.[65] Similarly, invasive HT-1080 fibrosarcoma and MDA-MB-231 breast cancer cells realign sterically-impeding fibers by proteolyzing them and reorganizing them into parallel microtracks, which greatly enhances the migration of subsequent cells and can even support collective cell migration in which chains of cells translocate forward without breaking cell-cell adhesions.[62]

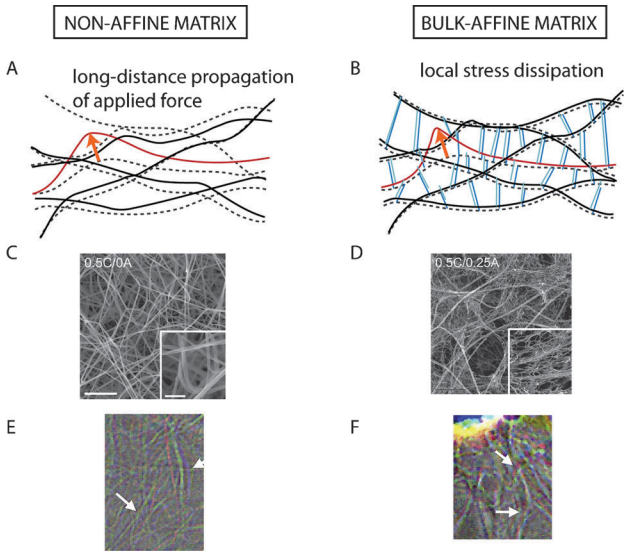

Fig. 3 Non-affine *versus* bulk-affine fibrous ECM. (A) Entangled collagen fibers propagate applied localized force to long distances, which is facilitated by free movement of individual fibers. (B) A network of collagen and agarose forces local dissipation of stresses, and applied local force causes little global impact on the network structure. Scanning electron microscopy (SEM) imaging of collagen-agarose gels with (C) 0% w/v agarose (0A) with (D) 0.5% w/v agarose (0.5A) in a 0.5 mg ml^{-1} collagen gel (0.5 C). (E) Non-affine deformation in agarose-poor matrices (0.5 mg ml^{-1} collagen gel) is shown by slipping and bending (arrows) of individual collagen fibers. (F) Bulk-affine deformation in a agarose-rich gel (0.5 mg ml^{-1} collagen with 0.25% w/v agarose).[16] All images reproduced with permission from Elsevier.[16]

Importantly, ECM fibers can sometimes restrict cell migration rather than facilitating it. For example, when glioma cells are dispersed into a 3D collagen I matrix, increasing collagen concentration within a specific range results in faster invasion, more extensive remodeling of the matrix into aligned collagen bundles, and more directionally persistent migration.[18] Along with the changes in fiber morphology, increased collagen concentration concurrently alters pore size, which also affects cell migration phenotypes in 3D, as discussed earlier. However, increasing collagen concentration beyond this range introduces steric barriers that begin to limit invasion speed.[18] Similar principles are observed in studies in which collagen fibrillogenesis is manipulated by inclusion or exclusion of telopeptides, which are peptide sequences at the end of the collagen molecule that facilitate fibril extension and bundling. Telopeptide-free collagen type I gels consist of a much more diffuse meshwork of thin fibers than their telopeptide-intact counterparts and strongly promote invasion of breast carcinoma cells (MDA-MB-435S cell line) through a Rho/ROCK-dependent mechanism. These results clearly demonstrate that the mechanical and structural properties of fibrillar networks regulate the degree of invasion of breast carcinoma cells.[102] A key lesson from all of these studies is that the cell's ability to interact with, deform, and align the fiber network plays a critical role in cell invasion.

5. Non-linear elasticity of matrix components

While "continuum" elastic materials deform in a linear, continuous and isotropic manner at all scales,[103] fibrous materials respond non-uniformly to stress and strain at macro- and microscales.[104,105] Many biological materials fall into this category, which means that they can display unexpected properties such as strain-stiffening; *i.e.*, these materials can be extremely resistant to large deformations even if their "bulk" stiffness is relatively low. These nonlinear properties derive from the fact that these materials can dissipate stresses through the local slipping, sliding and bundling of specific fibers.[13,104–107] As expected, the degree of strain-stiffening depends on the specific chemical and mechanical properties of the fibers, as well as the degree to which these fibers are crosslinked.[106]

The strain-stiffening nature of 3D biological ECMs in turn has profound consequences for cell motility through these matrices. Because cells deform the matrix locally, the effective stiffness that they experience is expected to be different than the stiffness one would measure in bulk. To complicate matters further, the mesenchymal mode of motility in collagen matrices is associated with successive deformation and relaxation of individual collagen fibers, suggesting that these cells actually experience a time-dependent range of stiffness throughout the motility cycle.[59] In addition, cells exert contractile forces *via* cell-ECM binding sites to locally stiffen the matrix, align the fibers[108] and modify the microenvironment of nearby cells,[107] which leads to alignment of cells along bundled fibers.[18,107] This matrix strain-stiffening mechanism may lead to directionally persistent glioma invasion in collagen matrices by creating a positive feedback loop guiding the cell in one direction,[18] similar to the stiffness-preferential migration behavior observed on 2D surfaces of gradient stiffness.[27] In addition, strains on the order of 10% may physically disrupt and weaken the collagen matrix at the microscale, which could prime the matrix around the cell for remodeling and further enhance cell invasion—mechanically analogous to proteolytic matrix degradation.[18] The importance of nonlinear elasticity is also apparent in the collagen-agarose system described earlier. When agarose is added to collagen, it leads to restriction of deformation and movement of collagen fibers, thereby converting matrix rheology from a non-affine to a bulk-affine-like regime. When the available degrees of freedom for individual fiber deformation and coupled movement of neighboring fibers are restricted, cells are unable to deform and remodel individual ECM fibrils reducing topological communication between cells (Fig. 3).[16]

6. Conclusion

Although extensive studies with 2D culture paradigms have made it clear that ECM stiffness strongly regulates cell motility, this relationship has proven to be much more complex to investigate in 3D. This stems from the fact that manipulations that alter the stiffness of 3D ECMs often also alter other matrix parameters that can independently (and synergistically) modulate cell migration. A deeper

understanding of the effects of ECM properties on cell invasion phenotypes requires a more detailed understanding of the relative contributions of these ECM properties, such as fiber morphology, pore size, and non-linear deformability of the fibrous scaffold.

In order to decouple effects due to microstructural ECM properties and ECM stiffness, new matrix systems need to be developed that permit orthogonal control of these parameters. In addition, new mathematical models and tools to quantify mechanical properties of matrix and mechanical interactions between cells and matrix at the microscale will facilitate further dissection of the relationship between ECM properties and cell invasion mechanisms.[15,23,101,109] Some recent efforts in this direction include controlled variation of pore size in collagen matrices by varying gelation temperature,[99] calibration of pore and fiber dimensions based on collagen content, incorporation of microfluidic control for spatial and temporal delivery of specific soluble factors to cells in a 3-D environment,[110] and organized confinement and alignment of cells encapsulated in microengineered 3D hydrogels.[111] In order to achieve more precise control over individual mechanical properties of 3D scaffolds, tissue patterning strategies originally pioneered in 2D settings[112] are now being translated into 3D. Along these lines, 3D PEG hydrogel scaffolds of defined pore sizes have been developed through crystalline templating of microspheres,[113–115] photo-patterning,[116] and paraffin sphere-dissolution[117] techniques. Separately, the effects of fiber architecture on cell adhesion, morphology, alignment, and persistent motility have been characterized by studying cell interactions with surfaces of oriented, biocompatible nanofibers fabricated by electrospinning technology.[118–121]

To complement these more sophisticated matrix platforms, methods are also needed that permit measurement of both matrix properties and cell-matrix force interactions in 3D and on the microscale. Important advances along these lines have been made with particle tracking microrheology (PTM), a technology in which the viscoelastic properties of a material are inferred from the Brownian motion of embedded tracer microparticles.[122,123] Because this method requires only the ability to visualize the cell and does not require direct physical access, it has translated well to the study of cellular mechanics in 3D matrices.[124,125] PTM has also been extended to probe the local real-time deformation of the collagen matrix during mesenchymal tumor cell migration in 3D, and to correlate these changes with specific components of the motility cycle, including actomyosin contractility, protrusion of the leading edge, and retraction of the trailing edge.[126] Another promising direction lies in the 3D application of traction force microscopy (TFM),[6] which has exhaustively been used to study traction forces generated by cells against the matrix during 2D motility.[5,127] Recently, tools have been developed to extend TFM into 3D settings by utilizing confocal microscopy to obtain 3D images of strain markers embedded in the gel and digital volume correlation algorithm to track deformations in 3D volume elements.[128] This combination of techniques takes the thickness of the compliant substrate into account and enables spatial and temporal force measurements in the normal direction,[129,130] in contrast to 2D TFM where only in-plane forces can be measured after cell detachment. Such measurements reveal how cells exert forces in all three dimensions, even during migration on compliant 2D surfaces, and that normal forces, in addition to the in-plane forces at the leading and trailing edges of a migrating cell, also contribute to the net propulsive force required for cell migration.[131] While these 3D TFM tools have provided new insights into the role of 3D traction forces in 2D cell migration that could not be achieved by traditional 2D TFM, traction force measurements during cell invasion in a true 3D environment remain a challenge.[132] More sophisticated 3D TFM tools need to be developed that will enable a better understanding of how cells engage and communicate with their ECM by exerting forces and deforming the matrix.

In addition to the development of new culture paradigms and material scaffolds, a much deeper quantitative understanding is needed of the biophysical mechanisms through which cells invade 3D ECMs and how these mechanisms relate to specific biophysical properties of the ECM that include, but are not limited to, bulk stiffness. Progress in this direction is crucially limited by the lack of mathematical models that integrate spatio-temporal gradients of mechanosensitive signaling pathways, cellular motility and ECM properties. Although several recent efforts have started to address this gap in modeling and mechanics, most of the existing models have either treated the entire cell as a continuum structure or focused on microscale molecular components without complete integration.[22–24,133–135] Notably, few of these models incorporate the biophysical properties of the ECM in the study of cell-ECM interactions in 3D settings, where cells must simultaneously generate traction against the ECM and squeeze themselves through steric barriers in order to productively migrate. Among the relatively few recent efforts in this area is a finite element bio-chemo-mechanical model that integrates ECM geometry into a mechanochemical description of cell contractility and cell-ECM adhesion dynamics to simulate cell behavior on micropatterned ECMs[136] and predict the gradients of contractile forces exerted by the cell-collagen micro-tissues tethered on microfabricated tissue gauges.[137] While these early efforts have shown great promise, there is a dire need for new models capable of relating tissue geometry and mechanics with subcellular adhesive and contractile mechanisms, particularly given the increasingly appreciated importance of these cues in limiting tumor cell invasion and metastasis.[15,23,101,109]

It is important to note that much of our biophysical understanding of cell migration in 3D ECMs is based almost entirely on fibrous matrices, such as the highly collagenous matrix found in breast and other connective tissues. However, many tissues in the body are composed of noncollagenous materials that are significantly less structured. For example, brain ECM is primarily composed of hyaluronic acid and proteoglycans which form a more amorphous matrix.[138] Similarly, liver tissue consists of only ~3% ECM by cross-sectional area and consists of a complex mixture of collagens I–IV, fibronectin, glycoproteins, and proteoglycans.[139,140] Given that both of these organs are critical sites for both metastatic disease and highly aggressive primary tumors, it is important to understand how their unique architecture contributes to

tumor spread. We expect that novel cell biological and pathophysiological insights into invasive and metastatic disease will emerge from careful consideration of matrix regulation of cell motility in these environments. Systematic consideration of three-dimensional matrix properties that are likely to regulate cell invasion—including those discussed here—should serve as an informative set of design criteria in the development of material scaffolds that recapitulate key features of the native tissue.

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