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Mechanobiology of tumor invasion: engineering meets oncology

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Abstract

The physical sciences and engineering have introduced novel perspectives into the study of cancer through model systems, tools, and metrics that enable integration of basic science observations with clinical data. These methods have contributed to the identification of several overarching mechanisms that drive processes during cancer progression including tumor growth, angiogenesis, and metastasis. During tumor cell invasion – the first clinically observable step of metastasis – cells demonstrate diverse and evolving physical phenotypes that cannot typically be defined by any single molecular mechanism, and mechanobiology has been used to study the physical cell behaviors that comprise the “invasive phenotype”. In this review, we discuss the continually evolving pathological characterization and *in vitro* mechanobiological characterization of tumor invasion, with emphasis on emerging physical biology and mechanobiology strategies that have contributed to a more robust mechanistic understanding of tumor cell invasion. These physical approaches may ultimately help to better predict and identify tumor metastasis.

Keywords

Tumor invasion; breast cancer; pathology; mechanobiology; cell forces

1. Introduction to physical cell biology

Physical cell biology is an expanding field of study that involves the measurement of physical properties of molecules, cells, and tissues through the development and use of novel quantitative tools. Increasingly, these approaches are being coupled with strategically-designed tissue models to study mechanisms of disease progression and drug effect in cancer [1–3]. Two general approaches have been described 1) empirical studies that employ complex *in vitro* and *in vivo* model systems to assess and control cell behavior and/or 2) mathematical models that simulate molecular and cellular processes. While this review will focus on the use of empirical strategies, it should be noted that mathematical modeling has played a critical role in the understanding of carcinogenesis, tumor growth and angiogenesis, and in the application of clinical treatment [4–6] summarized in several recent reviews [7,8].

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Conflict of Interest

None declared.

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In their pivotal report, Hanahan and Weinberg suggested that disruption of key physiological processes in epithelial cells results in the acquisition of several abnormal cell functions that are universal to cancer including aberrant growth factor signaling, evasion of apoptosis, sustained angiogenesis, and tissue invasion, which ultimately enable malignant cancer cell growth and development [9]. Many hallmarks of cancer were initially identified through pathological analysis of tumor tissue and were seen as prognostic and predictive markers to guide clinical treatment, but have since been explored in *in vitro* and theoretical models for further study. Tumor cell invasion provides a unique challenge because it is highly diverse and cannot be defined by any single molecular mechanism [10]. There are many coordinated molecular changes that occur prior to and during invasion, and many of the cellular phenotypes observed during invasion are controlled by complex, interrelated signaling networks involving both intracellular and extracellular regulators. Thus, cancer cell invasion, which is a fundamentally physical process, is ideal to study with physical biology tools that can control and manipulate intra- and extracellular variables to gain insight into the factors that initiate and drive tumor invasion. Cellular mechanobiology, a subfield of physical cell biology which uses physical phenotypic metrics to describe the outcome of dynamically-coupled molecular events, has provided a novel perspective to the study of cancer cell progression, and advances in this field have suggested that changes in cellular mechanics, the extracellular matrix (ECM), and cellular mechanotransduction contribute significantly to cancer invasion [11–15]. While such studies are allowing engineers and physical biologists to begin to define an “invasive phenotype” *in vitro*, the characterization of such a phenotype remains somewhat ambiguous in the clinic, where the identification of cellular invasion is of utmost importance.

2. Tumor cell invasion

Metastasis is a highly variable step-wise process that requires local tumor invasion, angiolymphatic transmigration and transport, and finally, establishment of a tumor at a secondary site [10]. Tumor invasion, whereby malignant epithelial cells integrate biochemical and physical cues from their environment with intracellular machinery to dissociate from the primary tumor [10,16], is of interest both clinically and in physical cancer cell biology because it is the first rate-limiting step in metastasis, and thus, is a promising target for clinical cancer management. While tumor invasion and metastasis correlate with high mortality in all types of cancer, this review will focus on breast cancer.

2.1. Mammary gland structure and function

The normal human mammary gland is a highly organized tissue consisting of cellular and noncellular components that exhibit an intimate tie between structure and function. Luminal epithelial cells line the branched ductal epithelial network and are responsible for secretion of milk components into the lumen of ducts and lobules. The basal surface of these cells is surrounded by myoepithelial cells, which are epithelial cells partially differentiated to a myoid phenotype, giving them a contractile function to expel milk from the lobules of the lactating breast [17]. Recently, these cells have become increasingly appreciated for their role in suppressing tumor growth, invasion, and angiogenesis [18,19]. As in all epithelial tissues, a continuous basement membrane primarily consisting of laminin, collagen type IV, and proteoglycans coats the basal surface of ducts and lobules of normal breast tissue, forming a dense meshwork that is maintained by both epithelial cells and stromal fibroblasts [20] and does not permit luminal epithelial and myoepithelial cells from entering the interstitial stroma [16,21,22]. Underlying the basement membrane is the stroma, which is a mixture of cells and connective tissue that can be highly heterogeneous in density and cellular composition [23,24]. Importantly, the mammary epithelium and basement membrane as well as all of the components of the mammary stroma – fibroblasts, ECM,

adipose tissue, inflammatory cells, and blood vessels – are subject to regulation and modification throughout development and tumor progression [25,26].

2.2. Malignancy and tumor cell invasion

Malignancy of the breast epithelium is associated with many changes in tissue architecture, cytology (the basic anatomy of a cell), and the stroma that have been summarized elsewhere [25,27]. Here we discuss changes that occur in these components specifically during *tumor cell invasion*. Invasive carcinomas of the breast, which can originate from either the ducts or lobules of the luminal epithelium, generally present as cells exhibiting malignant cytoarchitectural features and infiltrating the stroma either singly or organized into tubules. While it has been shown that invasive tumor cells can be distinguished from their non-invasive malignant counterparts through molecular analysis [28], currently, many of the most dramatic and clinically useful changes that occur during tumor invasion affect other components of the tumor microenvironment. The most significant of these changes are the absence of the myoepithelium and epithelial basement membrane [16,19,29,30]. Importantly, the absence of both of these components serves as a critical diagnostic marker of invasive carcinoma, allowing pathologists to distinguish between invasive carcinoma and carcinoma in-situ [31], which refers to a non-invasive carcinoma that does not disrupt the epithelial basement membrane to infiltrate the surrounding stroma. While the transition between carcinoma in-situ and invasive carcinoma remains a topic of great interest and debate [32,33], recent work by Hu, et al. showed that the loss of myoepithelial cells, which are normally maintained by cell adhesions, growth factors, and transcriptional factors, is sufficient to induce the transition from in-situ to invasive carcinoma [34]. Thus, both the myoepithelium and epithelial basement membrane play important, mechanistically unique, roles in suppressing invasion.

2.3. The invasive tumor stroma

Recently, the tumor stroma, a biochemically and biophysically dynamic microenvironment, has gained increasing attention for its role in cancer progression, and more specifically, tumor invasion [28,35–37]. Invasive carcinoma has a firm texture and an infiltrative appearance due to the development of a fibrocollagenous stroma around the tumor, termed *desmoplasia* [38]. Since carcinoma in-situ lacks a desmoplastic response, there is great interest in determining whether desmoplasia is induced by malignant tumor cell invasion or is itself a precursor to and initiator of tumor invasion [39–41]. Notably, stromal desmoplasia is associated with increased angiogenesis, fibrin and collagen I deposition, and myofibroblast composition, all of which are important for invasive cancer progression [24]. Myofibroblasts, which persistently reside in the tumor stroma and not in benign breast tissue [42], can originate from many differentiated cell types including resting stromal fibroblasts, adipocytes, and myoepithelial cells, as well as mesenchymal stem cells [43]. These cells are thought to contribute to tumor cell invasion through the production and degradation of ECM [43], involvement in critical tumor-epithelium signaling pathways [44], and the generation of contractile forces that result in ECM remodeling [42,45] and release of ECM-bound signaling molecules [46]. Myofibroblast-mediated ECM remodeling contributes to stromal heterogeneity in invasive breast carcinoma, with loose and dense ECM associated with active, ECM-secreting fibroblasts and contractile mature myofibroblasts, respectively [42]. Importantly, the stroma is not merely a passive bystander during tumor invasion; there is strong evidence that it actively participates in a variety of ways [47–51].

3. Pathological characterization of tumor invasion

3.1. Strategies

The identification and characterization of tumor invasion is critical to breast cancer diagnosis and treatment in the clinic since the extent of invasive carcinoma – the biologically relevant component of the tumor – reflects the pathologic tumor size in the TNM staging system and largely determines management strategies. Thus, it is the role of the pathologist to accurately assess the size of this component using a variety of methods, including gross examination and a number of specific histochemical and immunohistochemical stains. Despite the tools pathologists have at their disposal, there are obstacles and pitfalls the pathologist, even one with expertise in breast pathology, faces on a routine basis.

The initial evaluation of invasion in surgical breast specimens is the gross examination, whereby the pathologist can determine a tumor's size, location, and relationship to surgical margins, as well as other tumor characteristics or associated findings such as the presence of necrosis, mucin, cysts and coarse calcifications. Grossly, invasive carcinoma of the breast is described as *scirrhous*, meaning “firm,” and is classically stellate-shaped with a white-gray cut surface, indicative of desmoplasia (Figure 1). Histologically, the desmoplastic response is characterized by a proliferation of spindled fibroblasts and collagen surrounding the invasive tumor, often exhibiting a pale blue color with hematoxylin and eosin staining. The micrograph in Figure 2 shows an invasive tubular carcinoma with well-formed tubule architecture and stromal desmoplasia.

Unlike in other organ systems, documenting invasion microscopically in the breast is not always a straightforward task. Since the microscopic architecture of these lesions can be quite similar to those found in both benign and non-invasive malignant entities, there are invasion-specific changes that are used to identify tumor invasion which can be exploited using adjunctive tools. For example, pathologists frequently take advantage of the absence of myoepithelial cells in determining if a tumor is invasive. Immunohistochemical stains, such as those for p63, smooth muscle myosin, calponin, actin, CD10, and cytokeratin 5/6, are employed to highlight the presence or absence of myoepithelial cells (Figure 3). Additionally, pathologists use cytokeratin immunohistochemical stains to distinguish single infiltrating carcinoma cells from benign stromal or inflammatory cells. Finally, a histochemical stain such as reticulin, or an immunohistochemical stain such as laminin or collagen type IV is used to evaluate the presence and integrity of the epithelial basement membrane.

3.2. Challenges

Pathologists continually face the challenge of assessing tumors for the presence of invasion. Despite the use of myoepithelial-specific cell immunohistochemical stains, there are many obstacles pathologists encounter in the assessment of invasion in the breast. Due to the varying sensitivities and specificities of individual immunohistochemical stains as well as biological diversity of lesions, misinterpretation of staining patterns can occur. For example, there are several benign and non-invasive entities, such as microglandular adenosis, high-grade ductal carcinoma in-situ (DCIS), and variants of papillary DCIS such as solid papillary carcinoma or intracystic papillary carcinoma, which often show absent or attenuated staining with myoepithelial markers, simulating invasive carcinoma [52,53]. Fortunately, these lesions retain their basement membrane, and the pathologist may employ the appropriate stain.

There are also examples of invasive tumors that retain myoepithelial expression or exhibit apparent myoepithelial reactivity, simulating an intact, benign, and invasion-suppressing

myoepithelium. Low-grade adenosquamous carcinoma, for example, is a form of metaplastic carcinoma characterized by an infiltrative glandular pattern that shows variably positive staining with myoepithelial markers. Another interpretive pitfall may arise from the staining of stromal myofibroblasts with certain immunohistochemical stains that are not entirely specific for myoepithelium, such as smooth muscle actin, which may cause them to be mistaken for myoepithelium encasing tumor cells. As these interpretations may result in the misclassification of a tumor as non-invasive, it is prudent to use a panel of myoepithelial markers including p63, a nuclear stain with higher specificity for myoepithelial cells. Further, there are types of invasive breast tumors, such as invasive lobular carcinoma, that can exhibit low-grade cytologic features and lack a desmoplastic response, making them difficult to recognize.

The art in this evaluation is the awareness of the strengths and limitations of the tools available, the clinical context in which they are used, and the implications of the diagnoses rendered. Because of the retrospective, “snapshot” nature of histopathology, the clinical characterization of the “invasive phenotype” is not a “black and white” exercise, and the approach to this characterization is continually evolving through translational and basic science research that aim to assist pathologists and clinicians in accurately staging cancer patients. While morphology and tissue organization remain the standard of diagnosis [31], molecular analysis has helped to advance the field by showing how lesions are related to each other [54–56]. Further advancements in the understanding of invasive carcinoma development will depend on continued clinical and molecular studies as well as analysis of actively-invading tumor cells in suitable *in vitro* and *in vivo* model systems. Together, these efforts will provide a means through which clinical histopathological findings can be integrated with emerging basic science observations, providing critical insight into the molecular and phenotypic characteristics of invasive carcinoma cells.

4. Mechanobiology of tumor cell invasion

4.1. Rationale

Clinically, invasion is often described as an endpoint observation more so than a process, which is appropriate as the primary goals of pathologists and clinicians are to diagnose and effectively manage patients’ tumors. However, since tumor cell invasion is an inherently biophysical process that involves mechanical changes at the cell and tissue scales, extracellular matrix remodeling, and directed locomotion of malignant cells through three-dimensional extracellular matrix [10,16], physical biology and mechanobiology tools can be employed to study cells actively invading. Thus, the role of the cancer mechanobiologist is to develop controlled model systems that incorporate components of the tumor microenvironment and can be used to monitor live cells and quantitatively assess mechanical cell behaviors involved in cell invasion. Results from such models have already contributed to a more robust understanding of tumor cell invasion and continuing work in the field seeks to expand upon the mechanobiological characterization of *in vitro* tumor invasion by elucidating how microenvironmental factors, such as those identified through clinical observations, affect tumor cells’ mechanical state and behaviors such as cytoskeletal dynamics, cell force generation, cell adhesion, and cell migration.

4.2. Initiation of invasion

While the molecular mechanisms that initiate invasion are complex [57], it is clear that neoplastic epithelial cells must undergo changes in their adhesion status, morphology, and motility to invade the tumor stroma. Though these changes together resemble a conversion to a classical mesenchymal phenotype, the involvement of epithelial-mesenchymal transition (EMT) in cancer progression is controversial [58–60] because a complete plastic transition

from epithelial to mesenchymal phenotypes is rarely observed clinically. Recently, an emergent perspective suggests that the phenotypic flexibility observed in malignant epithelial cells should not be attributed to EMT as if it were a single conserved process, but rather, that the many biological intermediaries should be considered as grades of “EMT-like” behavior [61–63]. Thus, because malignant cells are able to transiently exhibit select attributes along a spectrum between the epithelial and mesenchymal phenotypes, they can be considered *metastable* [62]. Indeed, this proposition supports the hypothesis that the mode of tumor cell invasion can be “tuned” through intra- and extracellular regulation of cell adhesions, protrusions, and tractions [64,65]. Yang, et al. provided evidence for *neoplastic metastability* by showing that expression of the transcriptional factor Twist is required for metastasis in a mouse model and demonstrating that ectopic Twist expression induces loss of E-cadherin adhesion, a shift from epithelial to mesenchymal molecular marker expression, and increased cell migration *in vitro* [66]. In recent work, Nelson, et al. identified a link between cellular mechanobiology and the epithelial-mesenchymal spectrum, showing that cell spreading positively affects epithelial cells’ sensitivity to the classic EMT-inducing factors MMP3 and Rac1b [67]. A continuation of this work using engineered culture systems, traction force microscopy, and finite element modeling demonstrated that intercellular mechanical tension in multicellular tissue analogs generates gradients of mechanical stress and that regions exposed to the highest endogenous mechanical stresses are most likely to acquire mesenchymal attributes [68]. Further, selective loss of basement membrane was identified at the invasive front of human colorectal cancers and *in vitro* experiments showed that basement membrane loss and the acquisition of a more mesenchymal phenotype are associated at the molecular level [69]. Given this recent work, the relationship among cell morphology and mechanics, basement membrane degradation, and the mesenchymal phenotype remains an intriguing area of research. While a universal initiator of tumor invasion has not been defined, there is mounting evidence that cell mechanics, through cell adhesions, cell and tissue morphology, and both endogenous and exogenous stresses, plays a role in driving this first step in the metastasis process [57].

4.3. Breaching the basement membrane

The epithelial basement membrane is important for maintaining epithelial polarity and differentiation [70], and thus, epithelial tissue homeostasis and structure. The ability of neoplastic cells to proteolytically degrade the basement membrane has long been considered an indicator of metastatic potential [29,71], and there is evidence that synthesis of basement membrane components by epithelial cells is critical for its integrity and function [69,70]. These findings, as well as the clinical importance of basement membrane histopathology as a prognostic indicator of invasion, have motivated a number of studies examining the mechanistic role of basement membrane degradation in tumor invasion. Thus, it is now accepted that invading tumor cells proteolytically degrade the underlying extracellular matrix through specialized actin-rich protrusive structures known as *invadopodia* [72]. *In vitro* studies, in which invadopodia are induced by plating cells on top of extracellular matrix gels, as well as *in vivo* analysis of tumor cell invasion have demonstrated that invadopodia formation and ECM degradation are important in tumor cell invasion [73,74]. The molecular composition, structure, and regulation of invadopodia [75] are of great interest and have been recently reviewed elsewhere [76–78]. Of particular interest is the presence of adhesion, signaling, cytoskeleton, and cell contractility molecules that together impart a mechanosensing capability in invadopodia. Notably, Alexander, et al. demonstrated that increased stiffness of the underlying ECM substrate up-regulates invadopodia number and activity through the signaling molecules FAK and p130Cas [79]. Interestingly, the mechanobiological relationship between extracellular matrix stiffness and the invasive capacity of tumor cells is reminiscent of the clinical observation of fibrocollagenous stromal

ECM surrounding invasive carcinomas. Accordingly, there is considerable support for a mechanistic tie between stromal density and cancer aggressiveness [11,80,81].

4.4. Cell protrusions

While invadopodia are critical for the initial breach of the basement membrane and exposure to stromal ECM, it is the maturation of these structures into larger protrusive extensions that enables invasion into the surrounding stroma [82]. By analyzing the growth rate, orientation, and lifetime of tumor cell protrusions and comparing them to the speed of cell migration, Fraley, et al. showed that tumor cell migration is in part driven by the frequent extension of exploratory membrane protrusions [83]. As in invadopodia, the development of such invasive pseudopodia depends heavily upon proteolytic degradation and the microtubule and actin cytoskeletons [84–86]. Advances in molecular and imaging technologies have contributed to a detailed understanding of how networks of Rho GTPases regulate the actin cytoskeleton [87], and these insights have been applied to the study of tumor invasion both *in vitro* and *in vivo* [12,88]. Of the many known regulatory cascades, the largely antagonistic Rac1/WAVE2/Arp2/3 and RhoA/ROCK pathways, which generally control protrusive and contractile cell processes, respectively, are particularly relevant to mechanobiology [89]. Rac1-mediated signaling, along with effectors of Cdc42 [90] and R-Ras [91], contributes to an elongated, motile phenotype through the induction of membrane protrusions as well as the repression of actomyosin contractility, which, unchecked, tends to revert cells to an amoeboid, or rounded, phenotype [92–94]. The exogenous repression of pseudopod-specific proteins in several metastatic human cancer cell lines reverted these cells to a more epithelial phenotype and inhibited cell invasion [95], demonstrating the dynamic interchange between molecular regulators and effectors of pseudopodia and supporting the finding by Nelson, et al. that changes in tumor cell morphology may be coupled with intermediary phenotypes along the epithelial-mesenchymal spectrum.

4.5. Cell adhesions

As cells generate invasive protrusions into the surrounding ECM, they develop integrin-based adhesions along the cell body, stimulating RhoA/ROCK activity, which causes actomyosin contractility and locally suppresses Rac-based actin polymerization and cell protrusion [96]. Integrin activation via attachment to extracellular matrix ligands is part of a bidirectional signaling network between the ECM and the cytoskeleton that drives mechanotransduction, molecular switching, and signal transduction, and plays a critical role in cancer progression [97,98]. The contribution of integrin signaling to tumor invasion regulation is mediated through receptor tyrosine kinases such as focal adhesion kinase (FAK) and its many effectors, which control, among other things, the orchestration of motility machinery, cell-cell and cell-matrix adhesion modulation, gene transcription, and ECM degradation enzymes [98–101]. Although the loss of the cell-cell adhesion molecule E-cadherin is classically linked to the acquisition of a more mesenchymal phenotype in breast cancer [102,103], a diversity of cell-cell and cell-matrix expression phenotypes are observed in invasive human breast carcinomas, precluding the use of such markers in the clinical identification of invasive cancer [104–106]. Nonetheless, cell adhesion dynamics play preventative and stimulatory roles in tumor cell invasion both *in vitro* and *in vivo* [107–109] and from a mechanobiology perspective, cell-matrix and cell-cell adhesion molecules serve key functions in the transmission of intra-, inter-, and extracellular forces between the actin cytoskeleton and the microenvironment [110].

Recently, an *in vitro* tumor migration model was used to show that the “tactile” ability of tumor cells is achieved through orchestration of cell protrusions, adhesions, and cytoskeletal contractility [83]. The authors suggested that the ability of cells to negotiate through their fibrillar three-dimensional microenvironment is dependent on 1) the extension of protrusive

processes into the matrix, 2) the generation of short-lived adhesions and localized contractile cell forces to “feel” the physical and biochemical properties of the ECM, and finally, as determined through this assessment of the microenvironment, 3) the selection of a suitable direction in which to further engage the ECM and spread [83]. This study demonstrated the critical ability of integrins and their downstream effectors to act as intermediary regulators between the Rac and Rho pathways and contribute to both the protrusive/contractile, and thus, migratory behavior of tumor cells [96].

Several recent mechanobiological studies have revealed a strong relationship among the biophysical properties of the extracellular matrix, dysregulated integrin signaling, and cancer progression. Increased expression and activation of $\beta 1$ -integrin-dependent signaling regulators contribute to a number of cancers [100], and the $\beta 1$ -integrin subunit itself is required for tumorigenesis in a mouse breast cancer model [109]. One proposed mechanism for this observation is that, in addition to regulating Rac and Rho activity [96], abnormal integrin/receptor-tyrosine-kinase signaling induces loss of cell-cell adhesions, which normally have a role in maintaining tissue organization and structure [111]. Indeed, ECM stiffness-dependent over-activation of FAK and extracellular-signal-regulated kinase (ERK) has been shown to lead to dysregulation of *in vitro* mammary tissue structure analogs through destabilization of cell-cell adhesions and enhanced cell contractility [11]. Further, high-density (stiff) collagen gels can induce an invasive phenotype through a FAK/ERK mechanotransduction network, with ERK acting to regulate transcriptional responses as well as cell contractility [112]. Additionally, stiffening of the tumor stroma via crosslinking enhances tumor invasion through amplification of integrin and phosphoinositide 3-kinase (PI3-K) signaling both *in vivo* and *in vitro* [113]. Together, these results present compelling evidence that maintenance of breast tissue structure is highly dependent on cellular adhesion homeostasis and that the mechanosensing role of cell-matrix adhesions and their downstream effectors can contribute to the physical disruption of mammary epithelium in an ECM-dependent manner.

4.6. Cell contractility and mechanics

4.6.1. Cell contractility—Cell contractility occurs when the motor protein myosin II forms oligomeric complexes that engage two overlapping actin filaments, translating them in anti-parallel directions and generating cytoskeletal tension [114]. The phosphorylation of the myosin light chain (MLC) signifies a key controlling step in this process and involves a number of regulators, most notably, the Rho-associated kinase (ROCK) and myosin light chain kinase (MLCK) [115]. The kinase activity of ROCK is enabled by binding the active form of either of the small GTPases RhoA or RhoC and is responsible for directly phosphorylating MLC [116] and deactivating the negative regulator of MLC, myosin light chain phosphatase [116,117]. Notably, RhoA and RhoC are generally over-expressed in tumors and play a number of critical, but distinct [118], roles in tumor progression [119,120]. MLC-dependent contractility can also be mediated through MLCK activation by the mitogen-activated protein kinase (MAPK)/ERK linkage, which is downstream of adhesion- and growth-factor-dependent signaling through FAK/Src [121]. This FAK/ERK/MLCK pathway was shown to promote cell motility *in vitro* [122] through enhanced adhesion turnover [123]. Further, Provenzano, et al. recently showed that the FAK/ERK/contractility network enables an ECM density-dependent protrusive and invasive phenotype in cultured breast cells [112]. Importantly, both the FAK/ERK/MLCK-contraction [124] and Rho/ROCK-contraction [125] pathways have been shown to be part of bidirectional mechanotransduction circuits, and it has been suggested that dysregulation of these positive feedback circuits can contribute to tumorigenesis and tumor invasion [11,13]. These findings, coupled with malignancy-induced changes in the mechanics of the breast stroma

[27,81], have helped cellular mechanobiology emerge as a major theme in the process of tumor invasion.

Mechanosensing, which has been introduced above in the context of other invasive cell behaviors, is a cell's ability to sense and respond to the mechanical properties of its microenvironment. Through its biochemical linkage with, and dependence upon, cell adhesions, the cytoskeleton, and Rho GTPases, cell contractility plays a central role in coupling both the "sensing" and "responding" mechanisms [126]. Stiffness, which is the resistance of a material to deform under application of an external load, is of intense interest in tumor mechanobiology, and a great deal of work has been done to determine how cells use mechanotransduction to modify their phenotype in response to properties of the ECM such as stiffness [127–132].

In addition to its role in mechanotransduction, MLC-based cell contractility is involved in a number of molecular tumor cell functions including regulation of proliferation [133] and differentiation [127] as well as many more classically mechanical cell functions such as cell morphology determination and traction generation [134]. The mechanical outcome of cell contractility depends on where and when it is generated as well as the combined states of the Rho GTPase family of cytoskeletal regulators [87]. Generally, cytoskeleton dynamics are responsible for establishing and supporting cell morphology, which largely determines physical cell function [135,136]. Of particular interest to the mechanobiological study of tumor cell invasion is contractility in the cell cortex, which is a complex cytoskeleton-enriched structure on the inner surface of the cell membrane that plays a critical role in cell morphology determination [137]. ECM stiffness-dependent Rho-based contractility in the cortical actin network has been shown to inhibit cell protrusions, and local depletion of this myosin II activity sufficiently destabilizes the stressed cortical cytoskeleton network to launch pseudopodia [138]. These findings demonstrate that cell contractility machinery can play a permissive role in enabling Rac- and Cdc42-mediated protrusions, and indeed, it has been suggested that actin-myosin contractility exhibits a dominant effect on cell morphology and migration in three-dimensional environments [92,93,139,140].

When cells transfer cytoskeletal contraction to the extracellular matrix through integrins, forces known as cell *traction forces* are produced. Recently, there has been increasing interest in characterizing the molecular and extracellular regulators of these traction forces and factors such as substrate mechanics, cell adhesion, and traction-dependent ECM deposition are increasingly well understood for cells on planar substrates [129,141–144]. However, the regulation and outcome of cell traction forces in the three-dimensional microenvironment of *in vitro* model systems and *in vivo* tissues are more obscure because of their spatial complexity and dimensionality [83,84,134,145,146]. Nonetheless, it is clear that tumor cells generate such myosin-dependent traction forces both *in vitro* and *in vivo* to modify cell-matrix adhesions and probe their mechanical environment as previously discussed, as well as to deform and remodel the extracellular matrix and translate the cell body during migration [94]. Cell-induced extracellular matrix reorganization is functionally important for tumor cells and occurs when cells repeatedly "gather" extracellular matrix towards the cell body via Rho/ROCK-based myosin contractility [147]. Notably, such cellular contractility has been shown to cause radial realignment of ECM adjacent to an *in vitro* tumor model, enabling a phenomenon known as *contact guidance*, whereby cells preferentially migrate through aligned regions of the ECM [148,149]. This cell-induced alignment closely resembles the stromal ECM remodeling seen in invasive tumors *in vivo* [36], and microscopically pre-aligned collagen gels support Rho/ROCK-independent tumor cell invasion [148]. In addition to inducing extracellular matrix reorganization, cell tractions can enable translation of the cell body through the 3D ECM [139,150]. Importantly, these *propulsive traction forces* are spatially, temporally, and functionally distinct from the

traction forces that regulate matrix probing and remodeling [151]. A cell chooses among the distinct outcomes of cell traction forces in a context-dependent manner and, while it remains poorly understood, mechanobiology models and the ability to quantitatively assess physical cell behavior have improved understanding of this selection process.

Several methods have been developed to quantify cell traction forces in complex, *in vivo*-like 3D model environments, and the majority of these depend on the microscopic visualization and tracking of either beads embedded within a hydrogel matrix or fibrillar ECM components [146]. The displacement or compaction of these markers – over time or compared between the stressed and relaxed states – can be interpreted as a traction force metric or derived into strain energy and/or stress fields. Bead displacement maps have been used to describe three-dimensional cell contractility and cell-mediated matrix remodeling in terms of adhesions [83,152] and their role in cell migration [150,153,154]. Recently, Legant, et al. developed a highly-quantitative method to assess cell tractions using a mechanically-defined, PEG hydrogel engineered to enable degradation and remodeling by embedded cells. They found that spread cells exert contractile stresses ranging from 100 - 5000 Pa, with the strongest tractions generated at the tips of pseudopodial protrusions [155]. While this method represents the most rigorous numerical description of 3D traction forces to date, it does not account for the fibrillar structure and bioactivity of native ECM, which is known to exhibit complex mechanics and control cell behavior [156]. Thus, our lab and a number of others have measured extracellular matrix deformation and reorganization in natural biopolymer matrices to describe cell contractility in three-dimensional environments [134,157–159]. Such matrices allow *in vivo*-like interactions between cultured cells and the environment and enable ECM deposition, remodeling, path-making, and path-finding, all of which occur frequently *in vivo* [156,160]. Early work with confocal reflectance [145,161,162] and differential interference contrast (DIC) [163] microscopy established the utility of ECM fiber visualization for probing how cells physically interact with their environment, and recently, mechanobiology models have provided better understanding of the regulation and outcome of single-cell traction forces. For example, several groups have studied how cytoskeletal dynamics in cell pseudopodia bring about traction forces and induce ECM fiber alignment [158,159]. Local matrix deformations have been used to describe the temporal and spatial evolution of ECM remodeling by cells embedded within 3D hydrogel scaffolds [139,164]. Further, three-dimensional biomechanics have been incorporated into a 3D Incremental Digital Volume Correlation (IDVC) model that enables correlation between tissue-scale stresses and cell-scale changes in ECM structure [165] as well as quantification of cell traction-mediated volumetric strains [157]. Finally, since the matrix alignment and remodeling events described above result in ECM compaction around contractile cells, our lab and others use pericellular collagen compaction to measure 3D cell tractions [134,158,159,166]. The ability to quantitatively assess cell contractility is of utmost importance in cancer mechanobiology, which strives to identify and understand the molecules, cell structures, and phenotypes that are involved in the generation of 3D forces [160,167], and, thus, in tumor cell invasion.

4.6.2. Cell mechanics and deformability—There is considerable interest in understanding how the biomechanical state of tumor cells influences, and is influenced by, invasion [168]. While subcellular components and biomolecules possess mechanical properties that dictate some cell-scale biomechanics [169], whole-cell biomechanical properties such as deformability and viscosity are of particular relevance to tumor cell invasion and are ultimately controlled by cell contractility and cytoskeleton dynamics. The molecular basis, assessment, and implications of such tumor cell biomechanics have been discussed comprehensively in a several recent reviews [168,170].

The cytoskeleton is organized into diverse and intricate networks of molecules that exhibit elegant form-function relationships and allow them to participate in sensing, resisting, and responding to internal and external mechanical forces [170–172]. The adaptability and dynamics of these networks allows cells to mechanically respond to the mechanical, structural, and biochemical properties of the ECM [133,135,173,174] and many methods have been developed to probe the subcellular and whole-cell mechanical reaction to both intracellular and extracellular factors [57,168]. Tools such as optical tweezers, microplate stretchers, and micropipette aspiration can be used to assess whole-cell deformability [168] and techniques such as atomic force microscopy, magnetic bead rheology, and cytoindentation are regularly used to probe the stiffness of particular regions of the cell and determine how much force is required to mechanically stimulate cells to induce a desired experimental phenotype [168,175]. Optical tweezers were used to characterize the deformability of normal and metastatic human breast cell lines, and it was found that cell stiffness decreases with metastatic potential [176]. In support of these results, atomic force microscopy experiments with live human cells demonstrated that metastatic cancer cells are considerably less stiff than their normal epithelial counterparts [177]. Interestingly, the finding that tumor cell stiffness and metastatic potential are inversely related has been reported in a number of epithelial cancer models [178–181], with the opposite relationship observed in a non-adherent leukemia cell line [182]. Toward elucidating a molecular mechanism for these observations, it has been shown that inhibition of myosin II decreases cell stiffness and increases invasiveness while expression of the metastasis suppressor T β RIII/betaglycan increases cell stiffness and decreases invasiveness [181]. Still, the connection between cell stiffness and metastatic potential is diverse and cell-type specific and, thus, remains debated [168].

Cell contractility and deformability are of significant functional importance to tumor cell invasion in that they enable cells to use various modes of migration as they encounter steric obstructions in the extracellular matrix. For example, if the fibrillar three-dimensional ECM contains architectural features that restrict cell migration, the tumor cell is forced to either degrade or squeeze through these features. Thus, cells may use their cytoskeletal contractility machinery to deform the cell body sufficiently to negotiate the restrictive ECM [160,183,184]. Notably, the cell nucleus, which is larger and stiffer than other organelles, may prevent a cell from successfully migrating through regions with particularly small pores or deformation-resistant extracellular matrix [185]. Therefore, there is also interest in measuring the mechanical properties of the cell nucleus [186] and determining its role in tumor cell migration [187].

5. Tumor cell invasion: integration of mechanobiological phenotypes

While epithelial cells migrate during morphogenesis, benign tissue remodeling, proliferative disorders, and carcinoma *in situ*, they do so without crossing the basement membrane into the stromal compartment [16]. As previously discussed, tumor invasion is characterized by the presence of malignant epithelial cells within the stromal compartment, and there is tremendous interest in the field of mechanobiology to learn why and how these cells migrate to form the invasive tumor lesions that are observed clinically. Notably, invasive tumor migration is dependent upon the integration of the previously-described functions of the “invasive phenotype” including initiation of invasion, breaching the basement membrane, generation of cell protrusions, development of cell-matrix adhesions, and generation of cell contractility. By sequentially employing the *pushing*, *sticking*, *pulling*, and *squeezing* actions described above, tumor cells, acting either alone or in collaboration with one another, are able to invade and negotiate through the stromal extracellular matrix. Importantly, tumor cells apply these behaviors to varying degrees in a cell- and context-dependent manner, which results in diverse and dynamic migration phenotypes [64,65]. The

tumor stroma, which can be both dense and loose [42], with varying amounts of fibrillar alignment and stiffness [36,113], likely provides extracellular control over tumor invasion *in vivo* [65]. The extracellular matrix influences proteolysis, cell-cell, and cell-matrix adhesions, as well as Rho GTPase-mediated cell mechanobiology, and thus, serves as a major determinant of migration strategy [92,139,188–190]. Accordingly, the adaptability of three-dimensional tumor cell migration is of intense interest and has been studied extensively in both *in vivo* and *in vitro* models.

6. Summary

Clinically, the identification of tumor invasion occurs as an endpoint observation and depends primarily upon qualitative characterization of malignant epithelial cells and components of the tumor microenvironment such as the basement membrane, myofibroblasts, and stromal extracellular matrix. While this perspective is important for diagnosis and management of patients' tumors in the clinic, understanding the *process* of tumor invasion will ultimately enable more effective diagnostic and therapeutic strategies for improved patient care. Toward this, mechanobiologists have begun to define the “invasive phenotype”, which represents the integration of functional states of interconnected molecular and mechanical networks that participate in the regulation of mechanotransduction, cytoskeletal dynamics, cell adhesions, and thus, cell shape, mechanics, and migration. The mechanobiological study of tumor cell invasion has enabled physical cell biologists to identify the mechanical cellular functions that are involved in each step of tumor cell invasion and demonstrate how histopathological phenomena, including desmoplasia and ECM remodeling, affect each step of the process through a complex mechanical-biochemical feedback system broadly termed *mechanotransduction*. Continued work with mechanobiology models of cancer invasion and metastasis should seek to more fully integrate the biochemical and biophysical properties of the tumor microenvironment to better characterize the effects of therapeutic strategies, identify mechanobiological biomarkers of cancer progression, and further define the “invasive phenotype”.

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Highlights

- Tumor invasion, the process by which cells physically dissociate from primary tumors, is a rate-limiting step in metastasis.
- Invasion depends on coordination of intracellular molecular machinery and response to biophysical extracellular cues.
- Mechanobiology and engineering have been used to study physical tumor cell behaviors that comprise the “invasive phenotype”.
- Understanding the invasive phenotype should enable more effective diagnostic and therapeutic strategies in the clinic.

Figure 1. Gross assessment of invasive carcinoma of the breast

Cut section of an invasive ductal carcinoma of the breast from an excisional biopsy specimen shows an infiltrative, stellate-shaped mass with a white cut surface.

Figure 2. Microscopic assessment of invasive carcinoma of the breast
Invasive tubular carcinoma of the breast, characterized by well-formed tubules and desmoplastic stroma (hematoxylin-eosin, 200X).

Figure 3. Immunohistochemical staining to distinguish between invasive carcinoma and benign breast tissue

Immunohistochemical staining for p63 shows absence of myoepithelium in invasive carcinoma (right), and highlights the nuclei of myoepithelium in benign breast ducts (left) (200x).