Impact of the Physical Microenvironment on Tumor Progression and Metastasis

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Abstract

The tumor microenvironment is increasingly understood to contribute to cancer development and progression by affecting the complex interplay of genetic and epigenetic changes within the cells themselves. Moreover, recent research has highlighted that, besides biochemical cues from the microenvironment, physical cues can also greatly alter cellular behavior such as proliferation, cancer stem cell properties, and metastatic potential. Whereas initial assays have focused on basic ECM physical properties, such as stiffness, novel *in vitro* systems are becoming increasingly sophisticated in differentiating between distinct physical cues—ECM pore size, fiber alignment, and molecular composition—and elucidating the different roles these properties play in driving tumor progression and metastasis. Combined with advances in our understanding of the mechanisms responsible for how cells sense these properties, a new appreciation for the role of mechanics in cancer is emerging.

Introduction

Cancer has long been understood to arise through a sequence of mutations, leading to the acquisition of key hallmarks such as uncontrolled growth, evasion of apoptosis, and/or induction of angiogenesis [1]. Moreover, the majority of cancer-related deaths are caused not by the primary tumor, but by distant metastasis. Hence, the capability of cancer cells to become migratory and invade into surrounding tissues and distant organs is another crucial hallmark of cancer [1]. But cancer progression is more than just changes in the cancer cells themselves as changes in the tumor microenvironment have also been shown to play a critical role in tumor development and progression [2] as well as drug efficacy [3]. For example, stromal cells, such as fibroblasts [4] or immune cells [5], assist cancer at various stages of tumor development and dissemination. In addition, these stromal cells can work in concert with cancer cells to actively modify physical properties of the extracellular matrix (ECM) [6], which further contributes to tumor progression. Therefore, while cancer research has traditionally focused on biochemical mechanisms, recent studies have now begun to investigate the role of ECM physical properties, such as stiffness, pore size, and viscoelasticity. These advances in understanding also highlight the importance of representing these physical properties accurately in *in vitro* cultures.

Moreover, by appreciating the role of external physical forces on cellular behavior, a key resulting question is how do cells sense, and then integrate, these physical signals into specific behavioral changes? In addition, such behavioral changes can even include alterations in their own mechanical states. Whereas biochemical research has long focused on studying reaction kinetics, it is now understood that many molecules can undergo conformational changes when subjected to forces, exposing additional binding sites and changing the binding kinetics. Hence, molecular pathways and reactions are increasingly studied in the context of mechanical forces using tools and methods developed in the fields of mechanobiology and mechanotransduction.

In this perspective, we will first discuss the tumor microenvironment and how its properties affect tumor progression, migration, and metastasis. We will review recent advances in *in vitro* culture techniques that mimic this complex environment, and emphasize how these and future advances help to understand the different migratory strategies of cancer cells. We will then introduce some of the key intracellular molecules involved in mechanotransduction, illustrate current efforts to uncover the role of physical cues in directing cellular behavior, and comment on the technological advancements needed, in the immediate future, to probe these processes in depth. Finally, we will also highlight how microenvironmental properties may affect the maintenance and migratory capability of cancer stem cells, a highly tumorigenic subpopulation of cancer cells thought to be primarily responsible for tumor formation and propagation.

The Role of the Tumor Microenvironment in Cancer Cell Migration

Physical ECM properties:

Recently, cancer research has focused on the role of physical ECM properties because the stiff tumor microenvironment—caused by tumor-associated ECM remodeling and characterized by increased ECM deposition, fiber alignment and crosslinking—has been shown to actively promote tumor progression and malignancy through increased integrin signaling [7]. This increased malignancy could be counteracted by softening the tumor microenvironment through the inhibition of lysyl oxidase (LOX), an enzyme that covalently crosslinks collagen and elastin in the ECM [8]. Conversely, in a separate study, normal mammary epithelial cells were shown to be driven towards a malignant phenotype by increased matrix

stiffness alone [9*]; further illustrating the active role that ECM mechanical properties play in tumor progression.

In addition, ECM properties also influence cell migration behavior. For instance, key parameters in cell migration, such as cell speed, are crucially affected by matrix stiffness [10]. ECM properties also affect directional motility, as cells have long been known to preferentially migrate along 2D substrates from softer to stiffer environments in a process termed durotaxis [11]. While such behavior was reproduced when the 2D substrate was overlain with a 3D collagen matrix [12], recently developed approaches capable of generating 3D ECM stiffness gradients have revealed that cells, in some cases, are able to migrate from stiff to soft matrices in a process called "reverse durotaxis"[13,14]. Further investigation is required to understand this apparent plasticity in directional motility.

Besides stiffness, the ECM architecture itself can direct migratory behavior. For example, collagen fiber alignment is correlated with advanced progression [15], since alignment of fibers away from the tumor enables cancer cells to migrate persistently along those fibers [16**]. Furthermore, the higher mechanical resistance associated with increased ECM density has recently been shown to cause a shift to protease-dependent invasion [17], whereby ECM fibers are degraded by matrix metalloproteinases (MMPs). However, if MMPs are inhibited, cancer cells can switch to an MMP-independent mode of migration [18]. How MMPs and matrix realignment influences migration and relates to other parameters, such as stiffness or pore size, is an ongoing, active area of research [19**].

Increased confinement due to increased collagen concentration has also been shown to be associated with a switch from single to collective cell migration, with leader cells generating proteolytic tracks due to MMP activity [20*]. How collectively migrating cells depend on leader cells, or can self-organize, is itself an ongoing research effort [21]. This research also has potential clinical applications. For instance, if collective migration is largely driven by leader cells, then targeting the leader cell alone might be a promising strategy for preventing metastasis. Therefore, more work needs to be done to investigate which environmental factors drive collective cell migration, and in which situations leader cells emerge and become necessary for migration and metastasis. However, to effectively study how ECM physical properties direct cell migration, further advancements in 3D techniques are needed that can create tunable ECM gradients and vary individual ECM parameters (i.e. mechanical stiffness, ligand density, and fiber alignment) independently. To this end, recent advancements in material design have yielded synthetic fibrous materials, which not only have the desired tunability of synthetic materials, but also mimic the fibrillar structure of the native ECM [22**].

*Figure 1 **The tumor microenvironment.** The behavior of cancer cells is largely influenced by their environment. Hypoxia can induce epithelial-mesenchymal transition (EMT), stromal cells can release chemotactic growth factors, and cell-induced mechanical strains can realign ECM fibers. Moreover, these factors have been implicated with tumor progression, invasion, and metastasis. Furthermore, cells mediate considerable crosstalk between the environmental factors. For instance, in response to hypoxia, cells can stiffen the matrix through increased LOX expression. Additionally, tumor cells interact with fibroblasts causing the deposition of new ECM molecules, and physical forces from strains are associated with fiber alignment, leading to persistent migration and tissue invasion of cancer cells.*

Hypoxia-driven Migration:

Hypoxia has been shown to stimulate the epithelial-mesenchymal transition (EMT), and consequently cancer cell migration, through Notch signaling [23] and Snail-1 upregulation, a critical regulator of EMT. Snail-1 activity is increased by two different, but synergistic, Notch-mediated mechanisms: (1) the intracellular domain of Notch binds to the Snail-1 promoter and consequently increases its expression,

and (2) Notch mediates the recruitment of hypoxia-inducible factor 1 α (HIF-1 α) to the lysyl oxidase (LOX) promoter, thereby elevating LOX expression, which, in addition to its role in crosslinking ECM molecules, stabilizes the Snail-1 protein. Furthermore, hypoxia can also lead to increases in collagen deposition and MMP production [24]. Therefore, hypoxia-driven ECM remodeling may also reinforce cancer cell migration by promoting an invasive phenotype [24,25].

External Mechanical Forces Promoting Migration:

Recent evidence has shown that external mechanical forces also influence cancer cell migration. For example, compressive forces resulting from uncontrolled tumor growth have been found to promote an invasive phenotype. In a study using an *in vitro* 2D model, compressive forces applied to epithelial cell sheets generated a subset of “leader cells” that extended filopodia at the leading edge [26]. The development of these leader cells was acto-myosin independent and characterized by increased fibronectin deposition.

In addition, the compressive forces generated by uncontrolled tumor growth may lead to the collapse of lymphatics and small blood vessels [27], which is thought to elevate interstitial fluid pressure—an indicator of poor prognosis [28]—and drive interstitial flow. Furthermore, this interstitial flow imposes a physical drag on cancer cells that can activate mechanotransductive machinery and lead to directed cellular migration upstream of the flow in a process termed rheotaxis [29**]. These studies have provided valuable insights into how physical determinants can affect cancer cell migration.

Molecules in Mechanical Sensing

Sensing ECM properties:

As we have discussed, physical properties and external forces from the cellular environment can drastically affect cell behavior. A fundamental question is thus how do cells sense these physical signals? To this end, integrins are important receptors involved in sensing the extracellular environment. Not only do they bind chemically to ECM ligands, but they also feel forces on the order of 1-40 pN for individual integrins, as measured with FRET sensors [30*,31**]. These forces are thought to originate from actin-myosin mediated tension [31], but cell membrane tension also plays a role during initial stages of adhesion [30*]. Moreover, it has become clear that differences in the binding strength of different types of integrins to ECM molecules are crucial for rigidity sensing [32]. Recent work has highlighted that the glycocalyx can also regulate integrin clustering and signaling, and is associated with increased cancer malignancy [33].

Intracellular molecules associated with integrins and adhesion complexes have also been implicated in force sensing. For instance, increasing ECM stiffness has been found to stabilize the vinculin-talin-actin complex, which increases PI3K signaling and leads to increased malignancy [34*]. Vinculin also plays a prominent role in F-actin regulation, adhesion formation and maturation, and ECM traction force organization in cell protrusions and migration [35]. These, and related intracellular mechanotransductive molecules, have also been implicated in migration in response to extracellular fluid flow [29].

Sensing neighboring cells:

However, mechanotransductive signaling is not just limited to cell-ECM interactions, but also extends to cell-cell interactions as well. Here, E-cadherin is a major molecule involved in cell-cell coupling, and its downregulation in cancer has long been associated with the epithelial-mesenchymal transition, and

subsequently, migratory and metastatic behavior [36,37]. More recent focus has been on the role of E-cadherin in force sensing and transmission. Using a FRET sensor, it was shown that the actin-myosin cytoskeleton constantly exerts forces on E-cadherin [38]. Surprisingly, E-cadherin feels these forces even outside of cell-cell contacts [38]. The link of E-cadherin to actin is facilitated by catenin molecules, and the strength of the resulting bond increases under applied force through what is termed a catch-bond [39*]. We anticipate that these intercellular forces play an important role in regulating processes such as EMT and/or collective cell migration, and future work will include quantitative measurements of cell-generated forces [38].

*Figure 2. **Intracellular signaling pathways integrate cell responses:** Cancer cells integrate a multitude of external signals to direct their responses. Besides well-established growth factor stimuli, for instance, LPA, which binds to G-protein coupled receptors (GPCR), physical forces also affect cell behavior. Cell-cell or cell-matrix adhesion molecules such as E-cadherin or integrins have a dual role: On the one hand, they provide structural support by linking the intracellular cytoskeleton with other cells or ECM, respectively. On the other hand, these molecules are also involved in signaling cascades, with a substantial overlap with the cascades mediated by growth factors. They, as well as some molecules binding to them, such as focal adhesion (FA) molecules, are constantly exposed to forces which, for instance, originate from myosin-mediated intracellular tension. Downstream of the cascades, molecules such as YAP integrate those different stimuli and direct transcriptional programs involved in proliferation, stem cell properties or metastasis.*

Cancer-associated alterations in cell mechanical properties:

Interestingly, despite tumors being generally stiffer than comparable healthy tissue [7], individual cancer cells are typically softer than their healthy counterparts [40,41]. Whereas this increased stiffness is often attributed to increased ECM deposition in cancer, recent work has shown that cancer tissues have a bimodal distribution of stiffness, compared to unimodal distributions in healthy tissue, with the soft peak of the bimodal distribution corresponding to cells which, under hypoxia, exhibit high metastatic potential [42]. It is commonly believed that softness enables cells to migrate more easily through their environment. However, a simple classification into stiff or soft cells might not be sufficient to characterize a cell's ability to migrate. Hence, the focus is now shifting towards uncovering subcellular contributors to overall cell mechanics. For instance, nuclear stiffness, mediated by Lamins, can be rate limiting when cells migrate through confined environments [43]. Intermediate filaments stiffen the cytosol, but not the cortex [44], and their role in spreading and migration is an ongoing research topic [45]. Future work is needed to delineate more clearly the roles of the different subcellular compartments in cell mechanics.

Other cellular responses to mechanical signals:

The transcription factors YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) have recently been implicated in transducing physical cues, such as matrix stiffness or cell size [46,47], into specific cellular responses, which include stiffness-dependent differentiation patterns [46], proliferation, cancer stem cell properties [48], resistance to anoikis [49] and metastasis [50]. Interestingly, YAP/TAZ signaling also acts as a memory in stem cell differentiation when extracellular stiffness is changed over time [51*]. Furthermore, YAP activity also plays a crucial role in the matrix stiffening and increased cancer invasion known to be facilitated through cancer associated fibroblasts (CAFs)[52]. Since, in turn, increased ECM stiffness is associated with increased YAP activity, [46], CAFs mediate a positive feedback loop of cell stiffness and YAP activity [52]. Likewise, there is crosstalk of YAP/TAZ with other important pathways implicated in cancer. For instance, Lamin-A and B, proteins associated with nuclear mechanics, are regulated by tissue elasticity, and subsequently regulate activity of YAP and SRF [53]. Moreover, β -catenin, a molecule known to have a dual role as both an intracellular messenger and a structural linker coupling Cadherins to the cytoskeleton, has been

implicated in mediating crosstalk between YAP/TAZ and Wnt signaling [54,55]—a pathway known to be involved in regulating stem cell pluripotency during development and cell fate in cancer. Furthermore, it has recently been shown that a growing tumor can also affect β -catenin signaling in neighboring healthy cells due to the pressure exerted by the growing tumor [56**].

Migrating Cancer Stem Cells

Despite clear evidence showing the lethality resulting from cancer cell migration and metastasis, the exact mechanisms are still not completely understood. For example, there appears to be incongruent evidence showing that, while metastatic tumor formation is a relatively rare event, cancer cell invasion away from the primary tumor can occur early and often during tumor progression [57,58]. To explain this apparent paradox, some have proposed that only the migration of a rare and highly malignant subpopulation of tumor cells, the cancer stem cells (CSCs), can lead to the formation of tumors at distant metastatic sites [59]. Here, it is thought that the stem cell-defining capacity of self-renewal, which enables CSCs to proliferate indefinitely [60], is required to colonize the distant metastatic site.

Importantly, there is further evidence suggesting that CSCs may also have enhanced motility [61]. For example, in two recent studies that utilized fluorescent-based reporter systems to study CSC behavior in real-time, both studies found that cells identified as CSCs were more invasive within 3D matrices compared to their more differentiated counterparts [62,63]. Furthermore, the epithelial-mesenchymal transition (EMT), a key developmental program commonly activated during cancer invasion and metastasis, has been shown to generate CSCs [64,65]. Thus, the EMT cellular program may generate cancer cells that essentially pose a dual threat by exhibiting increased migration and the capacity to form new tumors.

In addition, investigations into the biophysical properties of both normal stem cells and CSCs have shown these cells to be significantly softer than their differentiated counterparts [66–68*]. For example, in a study that was able to isolate breast cancer cells based on their deformability, the most deformable cells were found to be correlated with a stem-like gene expression signature [69]. These findings raise the intriguing possibility that CSCs may be better able to migrate through their surrounding ECM due to their unique biophysical properties; namely their increased deformability. However, this hypothesis remains to be vigorously tested, and resolving this issue will rely on improved 3D culture models used in coordination with techniques rooted in biophysics and basic cancer biology. These findings motivate further investigations into the biochemical and biophysical mechanisms governing CSC migration, and could lead to an improved understanding of metastasis and the development of better therapies focused on targeting CSCs.

Future Directions

It has now been firmly established that physical cues from the tumor microenvironment play a fundamental role in tumor progression and metastasis. However, we are only beginning to understand the intricate details of the role of these forces. What exactly is causing these physical changes, and when do they arise in tumor progression? Moreover, is it possible, through therapeutic intervention, to revert these physical changes, and could this help to achieve a better prognosis?

The investigation of these questions will be greatly aided by the ongoing improvements of *in vitro* cell cultures. 3D cell cultures are increasingly becoming the norm, and recent breakthroughs have led to better independent control of physical properties, such as pore size and fibrous structure. Techniques able to control the physical ECM microenvironment will be combined with techniques capable of accurately controlling the ECM chemical composition, soluble growth factor concentrations, nutrient availability, and/or oxygen tension. Furthermore, these techniques should also be incorporated into co-culture assays that probe the interaction of cancer cells with stromal cells, immune cells or endothelial cells. In addition, similar to how mathematics has greatly accelerated progress in physics and traditional engineering, mathematical and computational approaches are increasingly capable of capturing biological complexity, and are becoming a third arm of biomedical research; aiding and complementing *in vivo* and *in vitro* experiments. We envisage that such new innovations—driven by collaborations between biologists, clinicians, engineers, physicists, mathematicians, and material scientists—will lead to novel high-throughput assays that can mimic various tumor microenvironments representing different tissues, different stages of tumor development, and different patients; and thus revolutionize both the basic understanding of tumor progression as well as drug screening and testing.

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Figure 2