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In Vitro Modeling of Mechanics in Cancer Metastasis

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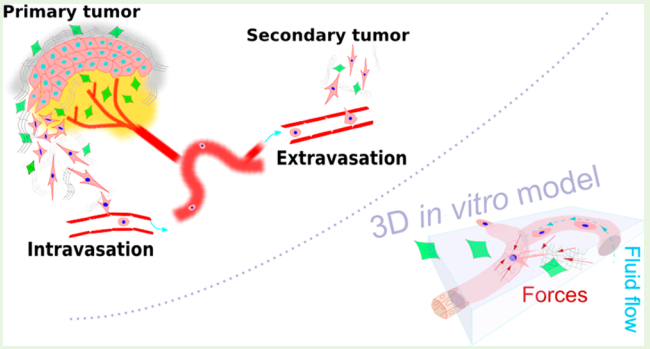
ABSTRACT: In addition to a multitude of genetic and biochemical alterations, abnormal morphological, structural, and mechanical changes in cells and their extracellular environment are key features of tumor invasion and metastasis. Furthermore, it is now evident that mechanical cues alongside biochemical signals contribute to critical steps of cancer initiation, progression, and spread. Despite its importance, it is very challenging to study mechanics of different steps of metastasis in the clinic or even in animal models. While considerable progress has been made in developing advanced in vitro models for studying genetic and biological aspects of cancer, less attention has been paid to models that can capture both biological and mechanical factors realistically. This is mainly due to lack of appropriate models and measurement tools. After introducing the central role of mechanics in cancer metastasis, we provide an outlook on the emergence of novel in vitro assays and their combination with advanced measurement technologies to probe and recapitulate mechanics in conditions more relevant to the metastatic disease.



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INTRODUCTION

Metastatic disease is the major clinical complication in most types of cancer and the cause of more than 90% of cancer-related deaths.[1−3] During this complex, multistep process (Figure 1), tumor cells that acquired an invasive phenotype[4−6] dislodge from the primary tumor,[7] enter the blood or lymphatic microvasculature (intravasate),[8,9] and following survival in blood circulation,[10] possibly exit from microvessels (extravasate) of distal tissues[11] and form secondary tumors (colonize) within vessels and in distant organs.[12] Irregular mechanical alterations in cells and the extracellular environment has made it increasingly apparent that mechanics and mechanical signaling play a central role at all stages of the metastasis cascade.[13−15] However, despite notable progress in the development of in vitro 3D models capable of recapitulating key features of metastasis more realistically,[16−18] the cell mechanics and mechanobiology research have been predominantly concentrated on 2D cells in Petri dish models. Although in in vivo models it is very difficult or almost impossible to probe mechanical features of cancer metastasis particularly at the cellular scale, 2D models, as a first simple reductionist approach, have been pivotal in widening the basic understanding of cancer mechanobiology.[19] Yet, critical steps of cancer metastasis including tumor invasion, intravasation and extravasation are inherently 3D processes occurring in microenvironments rich in complex biomechanical cues. Therefore, furthering our understanding of mechanics in cancer metastasis requires the development of in vitro models and their integration with advanced measurement technologies for quantitative analysis.

MECHANICS IN CANCER METASTASIS

Transformation of tumor cells to an aggressive and migratory phenotype is a key step leading to dissemination of cancer cells in the body.[20,21] Decades of research have been focused on the genetic and epigenetic basis of the oncogenic transformation. Although mechanical signals as an epigenetic factor might have a significant role in tumorigenesis,[22−25] the role of mechanics and mechanical signaling during malignant transformation of tumor cells is critical: (1) Tumor mass is both a mechanically and biologically diverse environment. (2) Cells are mechanosensitive and respond to both biological and mechanical cues. (3) Morphological alteration and inappropriate migratory behavior of tumor cells (such as those observed during tumor cell epithelial−mesenchymal transition and collective migration[26]) are mainly driven by aberrations in cytoskeletal

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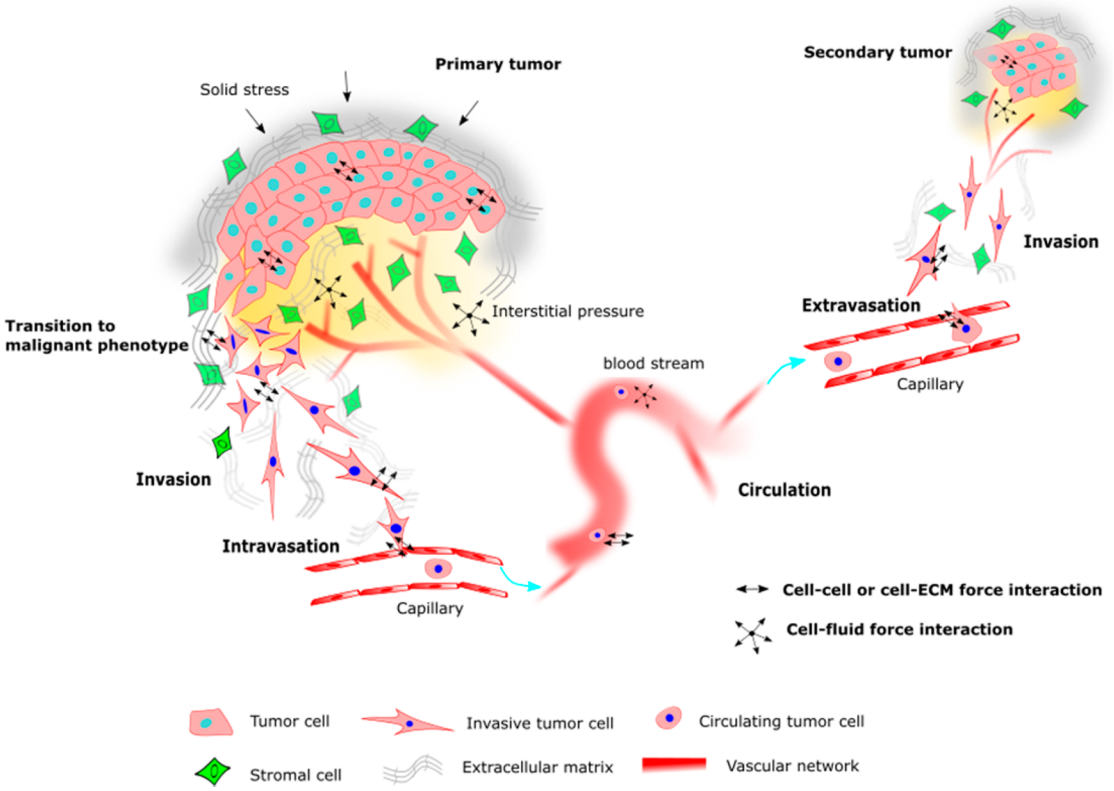

Figure 1. Mechanics in metastatic cascade. A primary tumor, which constitutes a highly abnormal biochemical environment, is formed because of oncogenic mutations and genetic and epigenetic cues. Following tumor formation, some tumor cells acquire a malignant phenotype with inappropriate adhesion, morphology and motility. In addition to biological signals, mechanical cues unique to the tumor microenvironment such as solid stress, interstitial fluid pressure, and ECM structure coordinate acquisition of an invasive phenotype and initiation of the cascade of metastatic events. Invasive cancer cells orchestrate unique force−interaction with cells, ECM, and interstitial fluid, to detach from the primary tumor and migrate through ECM to reach the vascular network and intravasate into the microvessels. Under the forces of blood flow, the intravasated cancer cells disperse into circulation, and those that survive can become lodged in and extravasate from the microvasculature to invade the tissue at the secondary site.

remodelling and cell adhesion,[27] which are clear signatures of malignancy. (4) Cellular morphogenesis, migration, and rheological properties are determinant factors in all post-invasion stages including intravasation, circulation, and extravasation.

Tumor Microenvironment: A Biomechanically Aberrant Tissue. Disruption in key physiological cellular processes such as cell cycle leads to loss of tissue homeostasis and tumor formation, a precursor to invasion and metastasis. A solid tumor with recognizably increased stiffness is made of primary cancer cells and a collection of stromal cells such as immune cells, stromal fibroblasts, and vascular endothelial cells that are embedded in the extracellular matrix (ECM) and nourished through the vascular system resulting from angiogenesis (Figure 1). The special composition of tumors and the complex interactions among cancer cells, immune cells, stromal cells, and the ECM lead to a structurally and mechanically irregular microenvironment unique to solid tumors.[28,29] For example, cell morphology and organization exhibit different patterns compared to normal tissue, the composition and 3D structure of the ECM that consists of a fibrous mesh of proteins are remodeled and continuously evolve during tumor progression, microvessels are often excessively branched and exhibit abnormal dilated, tortuous, elongated, and sacculated shapes. In addition to the irregular heterogeneous architecture of tumor microenvironment, the rapid proliferation of cancer cells pushes against the surrounding normal tissue and causes a buildup of pressure within the tumor that is known as growth induced solid stress.[30,31] Furthermore, stress accumulates within the interstitial fluid phase of the tumor mass as a result of morphological and architectural abnormalities of blood (leading to increased permeability) and lymphatic microvessels and unnatural interaction of the fluid with solid phase of the tumor.[32]

Mechanical Signaling, Mechanosensitivity, and Mechanotransduction. During both physiological and pathological conditions, cells sense different mechanical cues through various complex sensory machinery located on different cellular sites such as stretch-gated ion channels and focal adhesions.[33,34] Mechanical cues can originate from direct application of different types of forces on the cell, such as tensile, compressive, pressure, and shear forces, or structural and mechanical properties of the cellular environment such as stiffness and the microstructural architecture of the ECM.[35−37] Signals detected via mechanosensory systems activate intracellular signaling cascades which result in transduction of mechanical cues into intracellular biochemical events that in turn regulate cellular behavior and function such as the cell cycle, morphogenesis, and migration.[38] In the context of cancer invasion, unique biomechanical signals generated during tumor initiation and progression perturb the normal behavior of cells within and adjacent to the tumor, and the initiation of complex biomechanical signaling and mechanotransduction processes can expedite the transformation of primary cancer cells to a

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malignant phenotype.[22,39−42] Furthermore, after acquisition of invasive behavior, to move through surrounding tissue, respond to signals, and overcome a variety of biomechanical barriers, cancer cells must employ robust cellular migration and shape change strategies through regulation of their cytoskeletal dynamics.[43]

Cytoskeleton, Cell Migration, and Rheology. Cytoskeletal processes drive cancer metastasis by enabling the invasion and spread of cancer cells.[44] Because cytoskeleton drives cell division and regulates cell cycle, it has a prominent role in tumor initiation and progression. Furthermore, cytosketal remodelling via actin polymerization and acto-myosin contraction together with cell adhesion are the fundamental determinants of cellular morphogenesis, migration, and mechanical properties.[45] Hence almost all steps of cancer dissemination that require cell motility are influenced by cytoskeleton dynamics. Although cytoskeletal reorganization provides the forces for morphogenesis and migration, the maximal rate at which shape change and migration can occur is dictated by the rate at which the cell can be deformed.[46,47] Hence, in addition to physical influences of the extracellular environment,[48] the dynamic mechanical properties or rheology of the cell is a rate limiting factor for cancer cell migration.[47] Fascinatingly, it is becoming increasingly recognized that cells with higher metastatic potential are softer[49,50] but generate stronger forces[51,52] compared to nonmetastatic cells, allowing them to squeeze through 3D ECM and metastasize more readily.[53] On the other hand, it has been suggested that cancer cells may also become stiffer because of increased actomyosin contractility.[54] Dissecting effects of different mechanical factors such as cellular adhesions, force generation, and stiffness is, however, not conclusive and requires wider examination.

PROBING THE MECHANICS: CHALLENGES AND EMERGING OPPORTUNITIES

Investigating mechanics of cellular systems requires probing (1) morphological changes, (2) mechanical properties, and (3) force interactions of live cells and extracellular environment. Light microscopy including confocal and epifluorescence techniques have been extensively used to investigate cell morphological changes and migration.[55] Measurement of mechanical properties is based on the application of forces or deformations and probing the concomitant deformations, or forces, respectively.[56] A variety of mechanical measurement techniques such as magnetic twisting cytometry, magnetic tweezers, optical tweezers, substrate cell stretchers, shear flow rheometry, and atomic force microscopy have been utilized to investigate cell mechanics and mechanical models.[57,58] However, inherent limitations in these technologies such as imaging depth and necessity of physical contact restrict their application to investigate cell mechanics mostly in a well-plate, 2D assay.

Furthermore, cancer metastasis occurs in a complex physicochemical environment. In all metastatic subevents, mechanosensing and mechanotransduction take place in a landscape composed of diverse cell types dynamically interacting with spatiotemporally evolving ECM of heterogeneous composition and mechanical properties. Therefore, forces generated by endothelial, stromal and cancer cell types, and forces acting among these cell types and mediated by the surrounding ECM are also dynamic, and thus challenging to measure. Compositionally, intracellular and extracellular elements are multiphasic; stresses and forces can be exerted by both the fluid (e.g., fluid flow shear forces, hydrostatic interstitial pressures) and the solid domains (e.g solid stress, cell−cell, and cell−matrix force interactions), encompassing mechanical feedback in many cell motility events under chemical (chemotaxis), compositional (e.g., haptotaxis, durotaxis), or even electrical (galvanotaxis) stimuli, to name a few. These complexities push researchers toward multifaceted experimental and computational techniques. One central issue is thus in the choice of proper in vitro methodologies for the observation and characterization of mechanics in metastasis. Here, we identify five main challenges that are also emerging opportunities for future research.

Imaging. A key challenge for imaging technologies is to precisely capture relevant metastatic events while additionally obtaining mechanical readouts from these images or other independent mechanical tools. Major advances in imaging have often had a profound impact on our ability to quantify mechanics in metastatic processes. At the subcellular scale, satisfactory resolution within intact tissues is currently hard to achieve. Optical super-resolution imaging has given insights at the molecular scale, e.g., imaging clustering and localization of single molecules.[59] Although relevant, these high-resolution assessments are currently limited to specific applications, certainly far from being capable of measuring mechanics over a large dynamic range of metastatic events. Advanced in vivo imaging techniques are enabling assessment of tumor pathophysiology at a higher resolution, and in an intact host. For instance, intravital imaging, requiring an appropriate animal model, can measure anatomical and functional parameters linked to the mechanics of metastatic events.[60] Intravital imaging can assess how invasive tumor cells move, and more generally the mechanisms of cell migration during invasion and intravasation.[61] Because of the capability for imaging deeper within a sample than other light microscopy, intravital measurements might prove effective to study mechanics in cancer metastasis, despite the inability to control key parameters during in vivo imaging. Other interesting approaches are the (micro)elastography methods that are mostly focused at establishing the correlations between intra- and extra-cellular stiffness with cancer malignancy. These methods are classically applied along with magnetic resonance imaging,[62] or more recently using optical coherence tomography.[63] New discoveries can be achieved from the above-mentioned advances in the imaging field and from powerful combinations of different techniques, especially in the creation of new 3D models of metastatic processes in deep tissues with a multiscale possibility of precisely quantifying relevant spatio-temporal events,[64] and properties (such as stiffness in elastography). Noninvasive imaging with affordable in vivo models of 3D tissue microenvironments relevant for cancer metastasis[65] can boost the therapeutic and drug screening relevance, provided limitations related to immunocompromised microenvironment and histological appearance are targeted.[66] Moreover, reproducing and imaging 3D models of metastatic processes at the micrometer and submicrometer scale to understand force exchanges among the cellular entities involved requires an elevated level of control in real-time 3D imaging, currently achievable with proper re-engineered metastatic microenvironments.

Realistic in Vitro Models: Engineered Microenvironment. In vitro models dramatically increase the potential for precise control of parameters and accurate mechanical quantification in cancer metastasis and open its unique cellular

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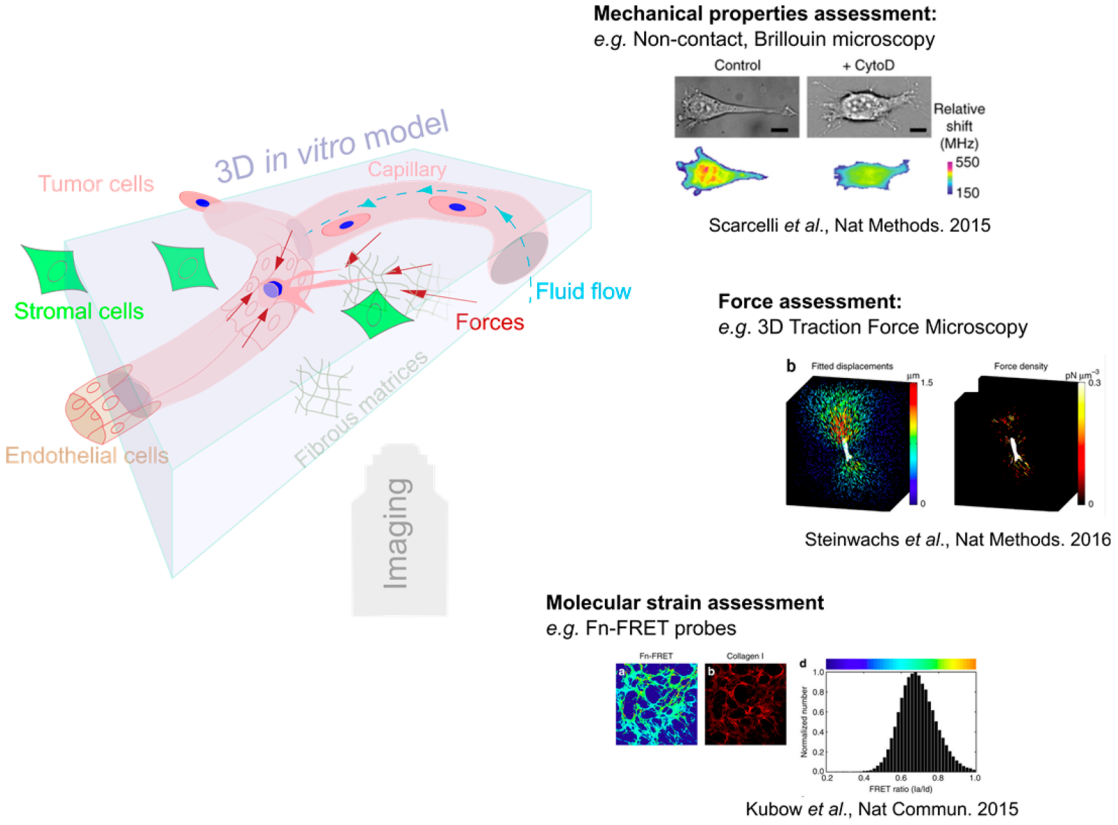

Figure 2. Engineering of a metastasis-mimicking microenvironment and examples of compatible tools for probing the mechanics. (Left) Schematic of a proposed platform integrating imaging and force measurement methodologies with an in vitro model of cancer metastasis. The model has microenvironmental features such as the coexistence of a microvascular network with stromal cells and biomechanical stimuli such as fluid flow and fibrous biopolymers. (Right) Three recent examples of stiffness, ECM deformation, and molecular force assessments. Reproduced with permission from refs 85−87. Copyright 2015−2016 Nature Publishing Group.

environment for experimental attack. A plethora of in vitro models is available for malignant cells in 2D.[67] While these 2D cancer models are consolidated high-throughput tools for many applications (e.g., drug screening), more realistic cellular environments have provided very different clinical outcomes when compared to the 2D situation.[68] In 2D, cancer cells organize as a monolayer, structurally distinct from the 3D physiological situation. This dimensional preconditioning produces very different mechanobiological signaling.[69,70] Ubiquitous characteristics of 3D tumors, such as the hypoxic environment inside tumor masses, are not possible to recapitulate in 2D cell cultures. Moreover, a metastasis-mimicking microenvironment must include the vasculature or the endothelial monolayer, and the stromal extracellular environment (Figure 2). Ad hoc 3D culture systems can be designed depending on the research question; for instance, the use of 3D spheroidal cultures, mimicking early events of metastasis and intravasation, can unravel interesting mechanobiology-dependent phenomena that are depending on cell−cell or cell−matrix adhesions and possible to target therapeutically.[71,72] Realistic models of microvasculature on a chip have been developed using microfluidics, and have been combined with other complex environments and interactions, including immune cells.[73−76] Such organ and disease models have provided novel characteristics and outputs such as physiologically relevant capillary morphologies and values of vascular permeability, excellent imaging and real-time monitoring capabilities, all extremely valuable for the study of metastasis. The possibility of controlling applied fluid flows and pressures—ubiquitous stimuli in physiology and disease—is another advantage of microfluidics-based engineered microenvironments.[77,78]

Biomaterials Technologies. Designing, fabricating, and integrating tumor-recapitulating ECM is the essence of transforming 2D assays to 3D realistic in vitro models of metastasis. However, having a custom-designed biomaterial for in vitro setting that is fully compatible with the complex in vivo ECM while appropriate for testing different mechanical effects is a critical challenge of the field because every single feature of the biomaterial that is dissimilar, such as composition, structural organization, architecture, and mechanical stiffness, can potentially influence both biological and physicals behaviors of the cell.[79,80] For example, although the use of ECM-mimicking natural biopolymers, such as collagen, fibrin, or matrigel, could increase the clinical relevance of the in vitro environment via reproducing realistic biochemical and mechanical signaling,[81] they lack tight control of mechanobiology-related cues such as mechanical stiffness, degradation rate, porosity, and cell adhesion site number.[79] An increasing number of studies that used synthetic biomaterials, have instead been able to precisely tune environmental cues by controlling MMP-degradable sequences, the density of adhesions sites or signaling ligands, and gradients of stiffness.[82−84] Systematically targeting the effect of these microenvironmental parameters on adhesion, migration, and cellular forces in cancer metastasis, can generate relevant experimental throughput; provided these microenvironments are compatible with the tools for imaging and measuring mechanical quantities and that care is taken to

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clearly distinguish the effects of mechanical stiffness from those of ligand density. Notably, because it is impossible to recapitulate the complex 3D biomechanical characteristics of wide variety of cancer types, a reductionist approach, for example, by designing hybrid biomaterials taking certain advantages of natural and synthetic materials, can pave the way to study and dissect effects of specific features of the tumor microenvironment.

Mechanical Measurement Techniques. *Force Measures.* The accurate and relevant measurement of mechanical quantities in vitro needs a proper combination of high-resolution imaging capabilities, mechano-sensitive readouts, and a metastasis-mimicking microenvironment. In one 2D study of this kind, researchers quantified traction forces of several malignant cells on different substrates, and concluded that contractile forces exerted by these cells are higher on stiff substrates and at the later stage of the disease.[88] The authors employed 2D traction force microscopy (TFM), a tool that includes the imaging of the deformation of fiducial markers tethered to the cellular and extracellular matrix, and the computational procedure to back-calculate the cellular forces that generated the deformation. 2D TFM has been extensively used in cell mechanobiology; however, to tackle the mechanics of metastasis, TFM in 3D is essential,[89] with application to collective multicellular entities,[90] and the measurement of traction force in physiologically relevant 3D ECMs, i.e. elastically nonlinear and spatiotemporally remodelled.[86,91] For that, an accurate mapping of mechanical properties in remodelled ECMs at the cell level needs advanced imaging to probe the local composition of cellular and extracellular domains.

Stiffness Measures. More locally and deeper in tissue than AFM, magnetic and optical tweezers in microscope setups are powerful tools for local assessment of mechanical properties[29,92] and could be directly used in 3D metastasis-mimicking in vitro studies. Another example is confocal Brillouin microscopy which is a promising noncontact method for characterization of both extra- and intracellular compositionally and mechanically driven remodelling during metastatic events. The Brillouin scattering technique measures interaction between light and spontaneous acoustic phonons (i.e., thermally generated longitudinal vibrational waves) and determines the optical frequency shift of the scattered light indicating the longitudinal compressive modulus of the material.[93] This technique has been recently integrated with confocal microscopy and provided a robust noncontact tool for determining local hydro-mechanical stiffness properties in cell and tissue constructs.[85]

Another interesting opportunity arises in the use of molecular strain sensors, for instance the ones functionalizing structural biological molecules (such as cadherins) involved in disease with fluorescent resonance energy transfer (FRET) technologies.[94−96] For instance, extracellular fibronectin can be successfully engineered as a mechano-sensitive FRET probe.[87] The physiological unfolding of structural molecules reflects local microenvironmental mechanics, despite the difficulties in the correlation between force and molecular strain and unfolding. Finally, other force measurement tools could be used to unravel the force interactions in metastatic events, such as 3D laser ablation,[97,98] cellular force inference,[99] or extracellular liquid droplets, where deformation is inferred from the counter-action of cellular forces and surface tension of the droplet.[100]

Computational Modeling Advances. Computational (in silico) methods should be used in combination with in vitro approaches in any comprehensive biophysical study and modeling mechanics during metastasis is no exception. Computational models are essential for the analysis and interpretation of experimental and imaging data (e.g., in 3D TFM), provide insight and generate new hypotheses, facilitate the design of new experiments, enable systematic parameter variation, and test ideas hardly testable with experiments. The reliability of a computational model must be extensively evaluated with experiments through prediction, experimental verification, and model revision, often requiring an iterative approach. Several computational models of cancer metastasis exist, spanning from atomistic to continuum, and have been extensively reviewed elsewhere.[101−103] These have predicted specific aspects of cancer metastasis such as extracellular remodelling-induced proliferation, cell protrusive growth along stiffness gradients, or the positive correlation of cell stiffness and contractility with migration and growth rates.

Future efforts should point to novel model formulations for (1) the understanding of cell−ECM force interactions, particularly paving the way for numerical frameworks for ECM degradation and remodelling, (2) linking spatiotemporal modeling scales (and generating multiscale models), (3) integrative hybrid discrete-continuum modeling strategies[104] toward a systems biology perspective that focuses on emergent properties of collective entity (applicable to cancer spheroids, tumorigenesis, or the endothelial barrier) rather than on the reductionist study of the parts. Computational advances along these lines will continue to provide new insights into the mechanobiology of the complex metastatic milieu.

INTEGRATIONS AND FUTURE DIRECTIONS

Finding a cure for a complex disease such as cancer necessitates understanding of the disease from every aspect, from complex genetics and biological signaling to physical interactions and mechanical properties of individual cells. So far, we have summarized the state-of-the-art characterization of mechanical interactions in metastasis—an increasingly critical issue to understand cancer dissemination. The force interactions between tumor cells and ECM and the concomitant effects of stromal and immune cell types can be accessed by finely tuning the biomaterials encapsulating these cell types in 3D. Several stimuli can be applied, and a first integration with computational models is necessary to design the experiments and quantify how ECM properties, fluid flows, and external forces affect the mechanobiological interactions. Deviations from physiological values of such stimuli and correlation with several pathologies or drugs can be studied. Computational models should continuously challenge these experiments and help in redefining the mechanobiology significance of results; for instance, new mechanobiological readouts can be predicted from pathways implemented in computational network models validated by the experimental output, therefore suggesting new exciting experiments and accelerating discoveries.

For all these applications, tissue engineering and biomaterials advances provide continuous inspiration for in vitro modeling. Assembly techniques such as bioprinting and controlled spheroid formation will dictate how to produce sophisticated 3D environments that realistically recapitulate the metastatic niche;[66] moreover, we have shown how force interactions are measurable with a plethora of methods. Thus, a further integration among techniques should focus on those assemblies

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and functionalized natural or synthetic polymers that can accommodate molecular force and stiffness sensors, and increase their throughput by controlling the biological complexity, e.g., the use of 3D stiffness gradients in ECM-mimicking polymers.

Beyond characterization, using and integrating techniques from the described palette with other advanced genetically and biologically focused approaches can answer clinically relevant questions and effectively pave the way for personalized cancer medicine. A promising avenue for future research is to combine genetic and functional profile information in determining cancer progression and drug efficacy. For example further to directly investigating mechanobiological hypotheses, because high-throughput in vitro models of mechanics in cancer metastasis provide more advanced biomechanical readouts, they can be readily used in combination with transcriptional profiling as platforms to screen drugs targeting other pathways.

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Notes

The authors declare no competing financial interest.

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