Chapter 4
Cancer Cell Mechanics

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Introduction

Cell mechanics research is rapidly expanding within the cancer community. Its increasing recognition within cancer biology likely stems from the fact that metastasis is an inherently physical process, involving the pushing/pulling of cells away from the primary tumor and through the surrounding stromal environment (Friedl and Alexander 2011; Friedl and Wolf 2003; Wirtz et al. 2011). While cell mechanics has a long history, it has had a significant recent resurgence, largely enabled by new and better technologies for interrogating cells and molecules.

"Cell mechanics" is in some ways a misnomer for a field that encompasses much more than simply cell-scale behaviors and properties. It includes not only the mechanics of individual cells, but also the mechanical forces and mechanical properties at the molecular and tissue scales (Fig. 4.1).

In this chapter, the focus is primarily on mechanics research being performed in Europe, Asia and the United States at the cellular scale; however, it is important to note that the multi-scale contributions of subcellular structures and supracellular tissue properties cannot be overlooked. At the subcellular scale, the cytoskeleton organizes to exert intracellular forces that translate into cell behaviors such as mitosis, intracellular transport, lamellipodial extension, and cell migration. Changes in various molecules within cells are closely tied to mechanical changes in the cell. For example, the mechanical properties and kinetics of cytoskeletal assembly at the molecular level contribute to changes in the mechanical properties of the cell (Kraning-Rush et al. 2011). Similarly, at the supracellular scale, the mechanical properties and architecture of a tissue contribute to cell function and dysfunction. In

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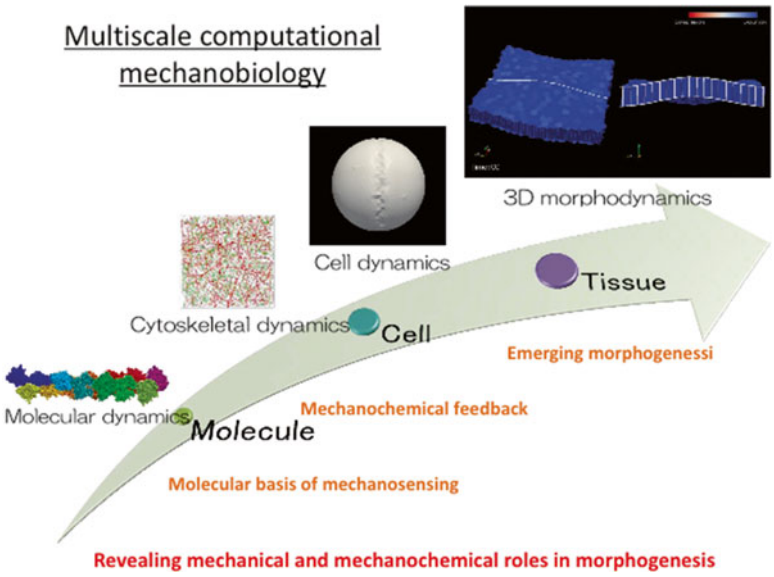

Fig. 4.1 Multiscale computational mechanobiology of epithelial tissue morphogenesis (Courtesy of Y. Inoue, Kyoto University)

the cancer field, this is particularly relevant as it is well-established that most solid tumors are stiffer than normal tissue. This stiffening at the tissue level is not only the basis for many diagnostic methods, but can also contribute to malignancy at the cellular level (Paszek et al. 2005). As such, "cell mechanics" is not limited to the cellular-scale because it spans the molecular, cellular, and tissue level scales. To fully understand and manipulate mechanics at the cellular level, one must consider the mechanics at both the molecular and tissue scales. Biologically, it is this integration that leads to changes in cell function and dysfunction.

Cell mechanics can be divided into three separate subfields: (1) cellular mechanical properties; (2) mechanotransduction (cellular response to forces imposed on cells by the external environment); and (3) cell-generated forces (Fig. 4.2). Studies of cellular mechanical properties have largely focused on either the elastic modulus or deformability of the whole cell or the rheology of the cytoplasm. These measurements are important within the cancer field because they implicate if and how cells will migrate and squeeze through the matrix during metastatic invasion through the stromal matrix and into the vasculature. Mechanotransduction research in cancer has been centered on the response of cells to imposed pressures and fluid shear stresses within the tumor microenvironment which are known to influence tumor growth and metastasis. Lastly, studies of cell-generated forces are critical to our understanding of how cells adhere, traverse, and sense their microenvironment. All of these facets of cell mechanics (mechanical properties, mechanotransduction, and cell-generated forces) influence each other, demonstrating the integration of inside and outside signals. This chapter will address all three of these subfields in oncology

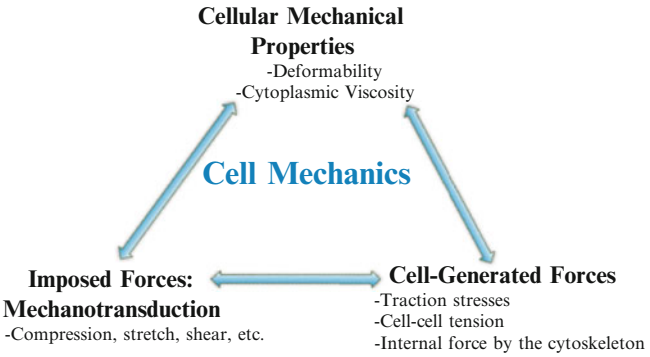

Fig. 4.2 The field of cell mechanics can be divided into three main subsections: cellular mechanical properties, imposed forces, and cell-generated forces (Courtesy of C. Reinhart-King)

and the efforts underway in the United States and those observed during the APHELION study tours in Europe and Asia.

Research

A Brief History of Cell Mechanics

The field of cell mechanics is often thought of as an emerging field, likely because most of the literature on this topic has been published since the 1990s. However, cell mechanics has a long history that dates back almost as far as the invention of the first microscopes. Early reports of cilia by Anton van Leeuwenhoek in the early 1700s noted the movement of particles in the liquid surrounding cells, a phenomenon that became the basis for microrheology studies and the characterization of cytoplasmic viscosity (Pelling and Horton 2008). At the same time, Isaac Newton was conducting separate experiments to define viscosity and descriptors of fluid properties. Even now we consider magnetic tweezers a state-of-the-art technique, when, in fact, the first microscopes equipped with magnetic micromanipulators were reported in the 1920s (Seifriz 1924). Early use of these magnetic systems included measurement of the cytoplasmic viscosity of cells, an active area of oncology research today (Baker et al. 2009; Wu et al. 2012). In 1950, Francis Crick—one of the fathers of molecular biology—published his first two papers on the mechanical properties of chick fibroblasts using a magnetic system with Arthur Hughes (Logothetis 2004). As these papers were published, Crick moved to the Cavendish labs where he switched scientific interests and began his work on the structure of proteins.

Crick was certainly not the only scientist working on questions related to cell mechanics in the pre-genome era. Interest in protoplasm dynamics and viscosity and questions in cell motility appear frequently in the pre-1950 literature. It is

interesting to note that fewer studies performed in the area of cell mechanics were reported in the literature following the discovery of the structure of DNA (determined based on the number of papers published in this time). It is likely that it was this discovery that prompted many scientists to indirectly follow in Crick's footsteps and move away from mechanical studies and into molecular biology. The discovery of the structure of DNA brought about the era of molecular biology, which in many ways may have suppressed what had been the growing field of cancer cell mechanics. However, the importance of cancer cell mechanics has resurged in a very significant way, and it is making great strides in the United States, Europe, and Asia.

Cancer Progression and Metastasis: An Inherently Physical Process

Tumor growth and spread, in addition to being stimulated by genetic, epigenetic, and microenvironmental changes, is a very physical process (Fig. 4.3). From a biophysical perspective, metastasis occurs as cells dissociate from the primary tumor,

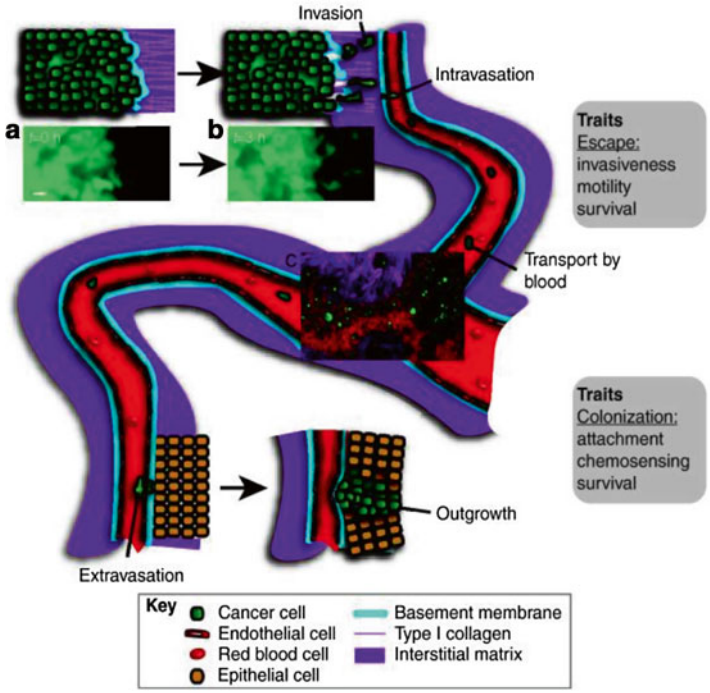

Fig. 4.3 The metastatic cascade (**a**, **b**) Fluorescence micrograph of invasion of a polyoma middle T (PyMT) mammary tumor. (**c**) Fluorescence images of tumor cells within a blood vessel (From Beerling et al. 2011)

breaking cell-cell adhesion bonds. As cells migrate through the matrix-dense stroma, they must push, pull, and degrade matrix fibers to navigate through the fibrous protein mesh. Simultaneously, the cells squeeze and deform to move through the pores within the matrix. As cells intravasate from the matrix into the vasculature, they must squeeze through the vessel wall. Once they are in the circulation, the cells must survive the forces imposed by blood flow. Finally, to colonize a secondary site, metastatic cells must adhere to the lumen of the vessel wall and squeeze through the vasculature during transmigration to seed within a secondary tissue. At each of these major steps in the metastatic cascade, cells both exert force and are exposed to externally imposed forces. The physical nature of these steps naturally leads to numerous questions regarding the mechanical forces involved in cancer metastasis.

The Role of Cell Deformability in Cancer Progression

As cells metastasize, they deform to squeeze through the fibers of the matrix-dense stroma. Their ability to deform is related to their mechanical properties (viscoelasticity), and as such there has been increasing interest in how the mechanical properties of metastatic cells differ from non-metastatic or normal cells. One prevailing hypothesis is that metastatic cells are more deformable, which aids in their invasion and motility.

There are multiple methods to measure the mechanical properties of cells: atomic force microscopy, micropipette aspiration (Bao and Suresh 2003), glass cantilevers (Mitrossilis et al. 2010), particle-tracking microrheology (Wirtz 2009), and more recently optical stretching (Fig. 4.4). Measurements of cell deformability have improved significantly over the past several years; however, they are not necessarily new to biology. Early studies of protoplasm viscosity were reported in the 1920s by Heilbrunn (Heilbrunn 1921). He also studied the effects of chemotherapeutics on cellular viscosity in 1957 (Wilson 1957) and showed that ethyl urethane increases the viscosity of cells. This finding is relevant and interesting today in light of newer data regarding the relationship between deformability and metastatic potential. Using an optical stretching device (see Chap. 7 for a description), Guck and colleagues from the University of Leipzig, Germany, showed that deformability increases with metastatic potential in breast cancer cell lines (Fig. 4.5; site report, Appendix B). This effect was later confirmed with cells from human primary tumors. Using atomic force microscopy, Cross and colleagues showed that tumor cells from patients are more compliant than their normal counterparts (Cross et al. 2007). Dr. Sylvie Hénon at the University of Paris, Diderot, France (site report, Appendix B), investigated how cellular mechanical properties change in response to imposed mechanical forces, such as tension, showing that cells can actively stiffen due to imposed forces by recruiting and polymerizing actin (Icard-Arcizet et al. 2008). Together, these data indicate that the mechanical properties of the cell may

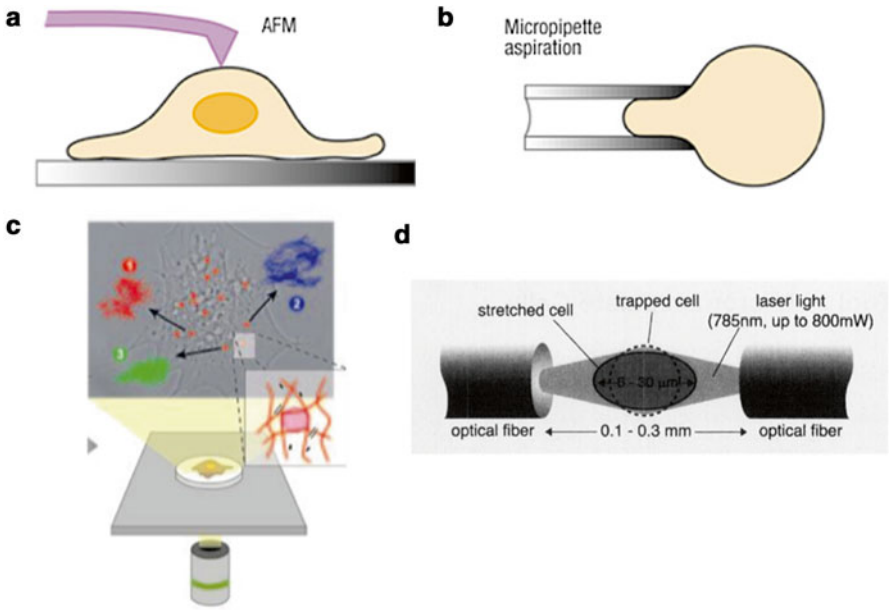

Fig. 4.4 Methods to measure the mechanical properties of cells. (**a**) Atomic force microscopy (Bao and Suresh 2003); (**b**) micropipette aspiration (Bao and Suresh 2003); (**c**) particle-tracking microrheology (Wirtz 2009); and (**d**) optical stretching (Guck et al. 2001)

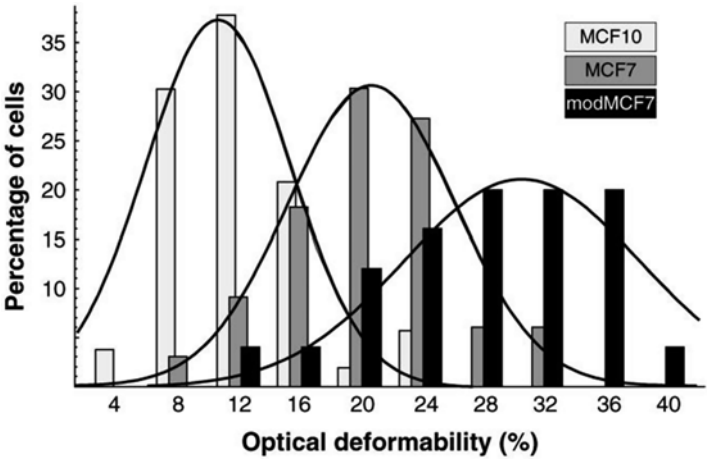

Fig. 4.5 Deformability of cells increases with metastatic potential, as measured using an optical stretcher (From Guck et al. 2005)

be predictors of metastatic potential and have the ability to actively remodel due to externally imposed forces.

The implications of cell stiffening may be both positive and negative with respect to cancer treatments. The data seem to suggest that stiffer cells are less metastatic and that chemotherapeutics stiffen cells, which is a positive effect. However, this stiffening may also have deleterious consequences. Recently, the Fletcher lab (author, Chap. 7) has shown that chemotherapeutic treatments can stiffen leukemic cells that then plug microfluidic channels (Lam et al. 2007). These data suggest that chemotherapy could result in vascular occlusion in capillaries by cancer cells. Therefore, it cannot yet be said that stiffening cells is necessarily a viable, universal method to prevent metastasis due to potentially significant side effects.

For this work to have impact, it must be translated into the clinic. Work being done by Josef Käs' lab at the University of Leipzig, Germany (site report, Appendix B), is continuing research in the area of cell deformability by testing clinical samples and working to move the optical stretching methodology to the clinic. For it to be used in the clinic, the method must be both user-friendly and high throughput. While the method of optical stretching is technically complicated, significant strides are being made to make it tractable to clinical laboratory technicians as a potential mechanism to diagnose the likelihood of metastasis.

In addition to studies of whole cell deformability, data are also emerging on the role of nuclear deformability in invasion. Since the nucleus is the largest organelle within the cell, nuclear mechanics could be a limiting factor in allowing cells to permeate through the pores of a matrix (Friedl et al. 2011). If the nucleus is unable to squeeze through a pore, then the cell cannot invade unless the matrix is degraded. The Lammerding lab at Cornell University has been probing the role of nuclear deformability in 3D cell migration directly using microfabricated platforms (Fig. 4.6). Using a series of microfabricated, constricted channels through which cells can migrate, they have shown that manipulation of the nuclear envelope and lamin A expression alters whether cells can easily pass through the constrictions (Rowat et al. 2013). Given that the nucleus is a limiting factor in the translocation of the cells through tight spaces, a natural extension of this work is to then ask whether the cytoskeleton pushes or pulls the nucleus through constrictions (Isermann and Lammerding 2013).

Recent data from the Wirtz lab at Johns Hopkins University suggests that the nucleus is not only important in migration due to its deformability, but also because its connection to the cytoskeleton. The nucleus connects to the cytoskeleton through the LINC complex and their recent data suggest that this connection plays a critical role in pseudopodial extension during 3D migration (Khatau et al. 2012). Notably, this effect is only observed in 3D migration and not in cells on planar substrates. While there is evidence mounting regarding the importance of the nucleus and nuclear-cytoskeletal coupling in invasion and migration, research on the role of nuclear mechanics in cancer progression is still in its infancy and requires further investigation.

The role of nuclear deformation in cell physiology extends beyond its role in being a physical impairment in the ability of cells to invade and migrate through small pores. Recent work from G.V. Shivashankar at the Mechanobiology Institute in Singapore has shown that nuclear deformations due to actomyosin contractility

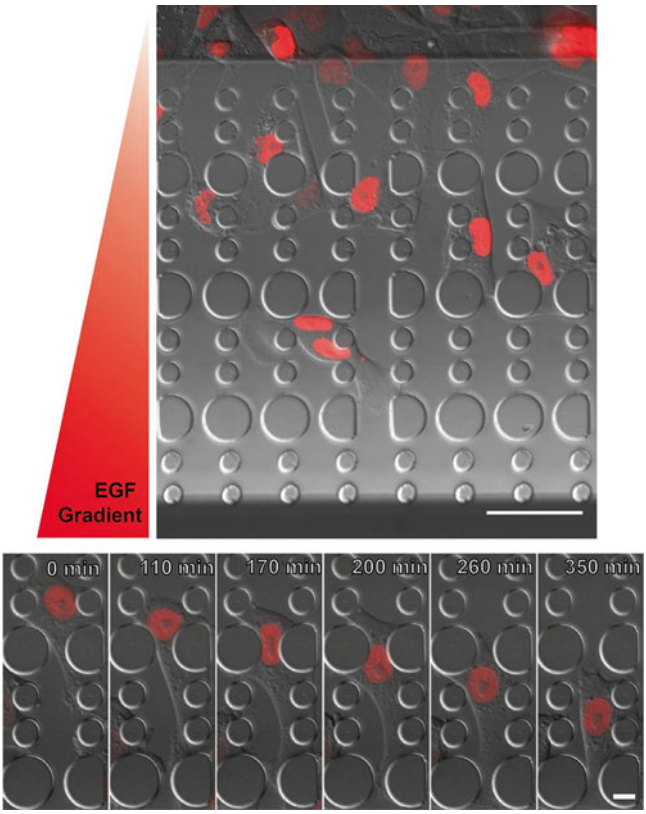

Fig. 4.6 Breast cancer cells (MDA-MB-231) migrating along chemotactic gradient (EGF) through microfluidic constriction channels. *Top panel*: overview of the device with constrictions of decreasing width. Scale bar: 50 um. *Bottom*: time-lapse sequence of cells with fluorescently labeled nucleus (mCherry-Histone-4) passing through 5 um wide constriction, displaying substantial nuclear deformations in the process. Scale bar: 10 um (Figure courtesy of Celine Denais, Lammerding Lab)

and geometric constraints can alter gene expression (Jain et al. 2013; Gupta et al. 2012; site report, Appendix C). Changing cell shape and nuclear size correlates with changes in global acetylation levels and transcriptional profiles (Fig. 4.7). These data suggest that the shape changes cells undergo during metastatic invasion may alter transcription profiles.

Cell-Generated Forces in Adhesion and Migration

Cells generate traction stresses against their matrix to adhere and migrate. These forces aid in remodeling the matrix and propelling cells forward during migration. Cell migration is necessary for metastatic invasion, and as such there has been recent interest in characterizing and understanding cell-generated traction stresses.

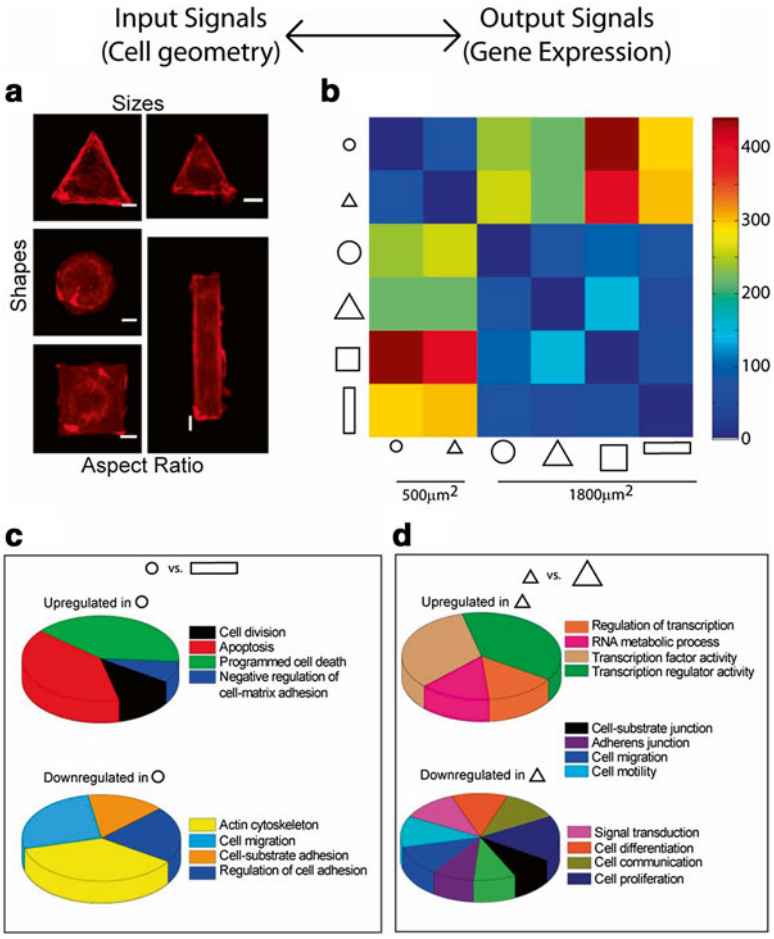

Fig. 4.7 Gene expression is regulated in part by cell geometry (From Jain et al. 2013) (**a**) Cells can be pattered in various, sizes, shapes, and aspect ratios. (**b**) Color-coded matrix showing differential gene regulation as a function of cell shape and size. (**c, d**) Gene ontology analysis from (**c**) cells in small circle compared with larger cells and (**d**) small triangle compared with large triangle

There are a number of methods that have been developed to measure cellular traction stresses, including wrinkling substrates (Harris et al. 1980), traction force microscopy (Dembo and Wang 1999), micropatterned elastomeric substrates (Balaban et al. 2001), and micropillar arrays (mPADS) (Fig. 4.8, Tan et al. 2003). Original versions of traction force microscopy allow for only the measurement of individual cells or cell pairs. More recent modification by Dr. Xavier Trepat, University of Barcelona, Spain (site report, Appendix B), allow for the measurement of forces in cell sheets (Tambe et al. 2011). These methodologies have enabled several interesting insights in the area of cell migration and adhesion.

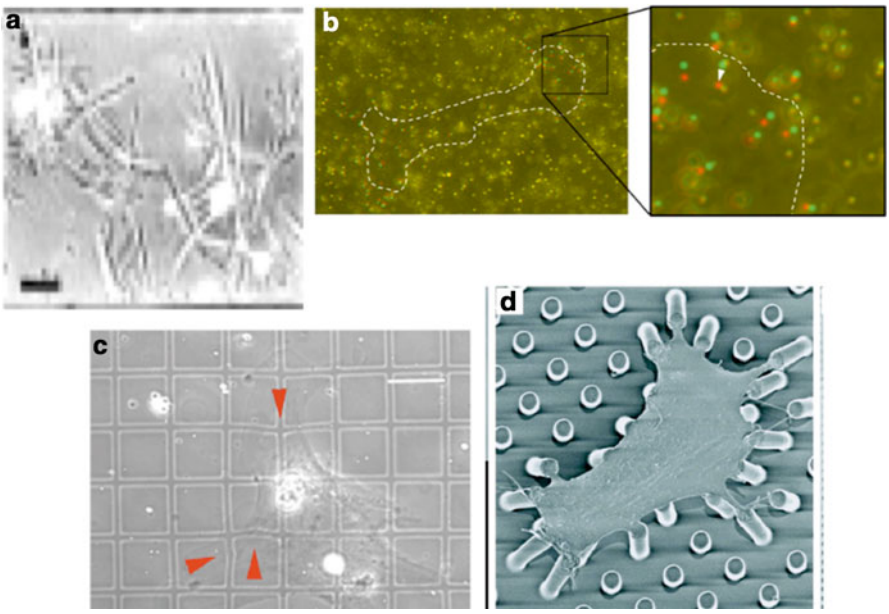

Fig. 4.8 Methods to measure cell-generated traction stresses
(**a**) Wrinkling substrates (Adapted from Harris et al. 1980); (**b**) Traction force microscopy, originally described by Dembo and Wang (1999); image adapted from Kraning-Rush et al. (2012b); (**c**) micropatterned elastomeric substrates (Adapted from Balaban et al. 2001); and (**d**) micropillar arrays (Adapted from Tan et al. 2003)

Traction stresses play a key role in migration, and there has been significant work investigating the relationship between cell contractility and malignancy. When traction stresses of lung, breast, and prostate metastatic cell lines and their non-metastatic counterparts were measured, it was reported that metastatic cells exert increased forces as compared to the non-metastatic cells (Fig. 4.9). These forces increase with matrix stiffness (Kraning-Rush et al. 2012a). Similar to the results discussed earlier on cell deformability, these data suggest that traction stresses may also be a mechanical biomarker of metastasis. A study published recently by Ben Fabry's lab at the University of Erlangen-Nuremberg, Germany (site report, Appendix B), indicates that in addition to the force magnitude, the anisotropy and polarization of the force may also be an important indicator of metastatic potential using a similar panel of metastatic and non-metastatic cell lines (Koch et al. 2012).

Cell polarity during cell migration dictates the direction and persistence of movement. Samuel Safran's group at the Weizmann Institute of Science, Israel (site report, Appendix B), has explored the effects of the microenvironment on polarity, showing that it increases in single cells on stiff substrates (De et al. 2008, 2010; Safran and De 2009). Asymmetries that develop during cell spreading can affect actin-myosin polarity and the extent of alignment of forces in response to matrix rigidity (Zemel et al. 2010). This polarity is often accompanied by polarized

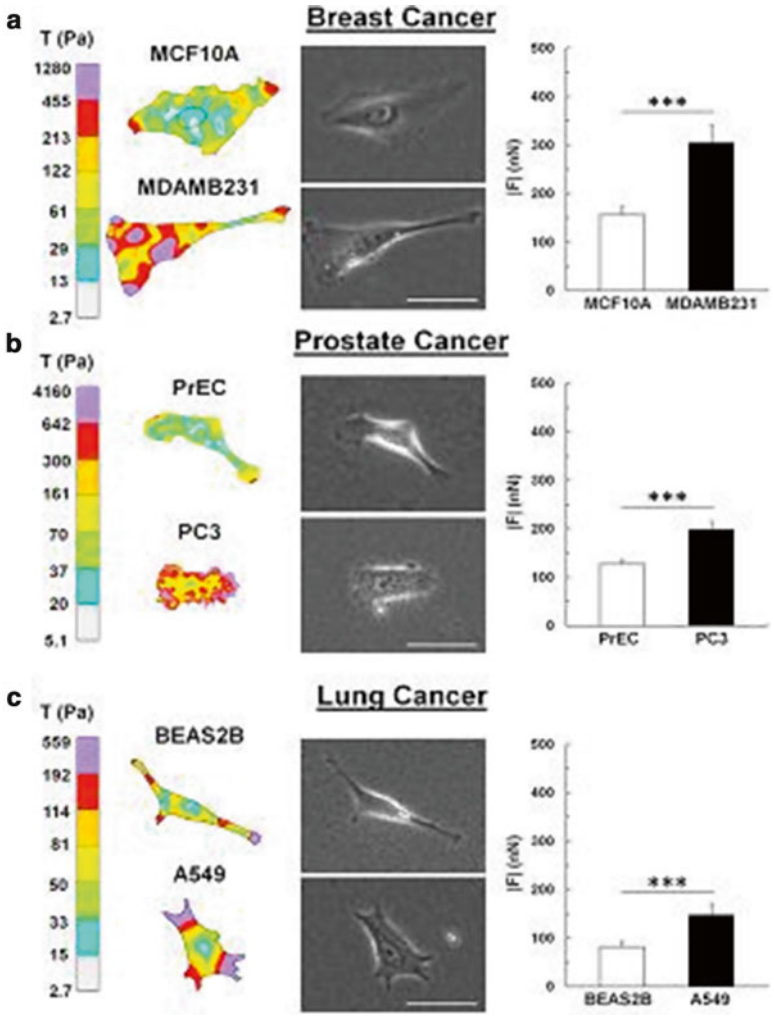

Fig. 4.9 Traction stresses increase with metastatic potential (Kraning-Rush et al. 2012a) Traction maps and corresponding phase images of metastatic and non-metastatic cells from (**a**) breast, (**b**) prostate, and (**c**) lung

remodeling of the matrix and may be important to our understanding of the role of elongation and polarity in mesenchymal modes of metastatic cell migration.

Studies of early polarization events in single cells have also been extended into studies of collective cell movements. For example, du Roure and colleagues have shown that cells along the edge of an adherent population exert the highest forces during collective migration (du Roure et al. 2005). These forces are significantly higher than forces at the edge of single cells. In light of the leader-follower migration dynamics described by Peter Friedl and colleagues at the Radboud University

Nijmegen Medical Centre, Netherlands (site report, Appendix B), where cell invasion from a tumor can occur in a sheet-like movement with "leader" cells at the front, these data may also explain a more cooperative mechanism of cell migration, where force is concentrated within leader cells (Khalil and Friedl 2010; Lee et al. 2012). In fact, Carey recently used a spheroid co-culture of non-metastatic, low force-producing epithelial cells mixed with highly metastatic, high force-producing cells to directly probe the leader-follower behavior. When the co-culture spheroids are embedded into collagen, the highly metastatic cells move to the outside of the spheroid, reorganize the surrounding collagen into tracks that run perpendicular to the spheroid, and invade into the surrounding collagen followed closely by the typically non-invasive, non-metastatic cells. The high-force producing cells emerge as early leaders in the leader-follower dynamic, remodeling the matrix to enable collective movements (Fig. 4.10). Notably, inhibition of force or MMP activity using Y27632 or GM6001 respectively ablates these effects. With pharmacological inhi-

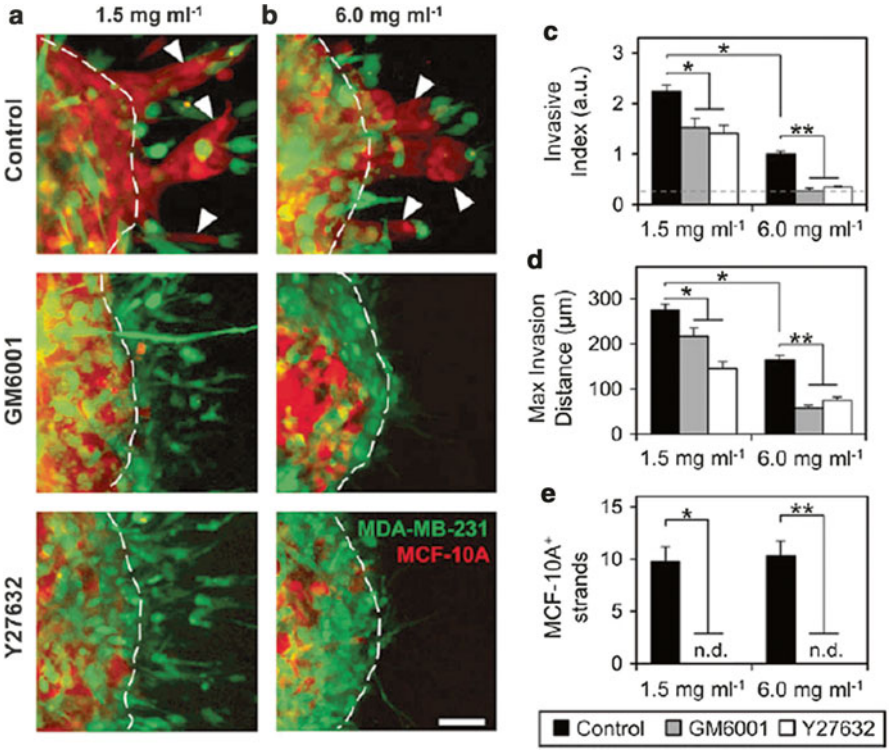

Fig. 4.10 Co-culture spheroid model of invasion (From Carey et al. 2013)
Highly invasive epithelial cells (MDA-MB-231, *green*) were mixed with non-invasive epithelial cells (MCF-10A, *red*) and embedded in (**a**) 1.5 or (**b**) 6.0 mg/ml collagen and treated with GM6001 or Y27632. *Dashed lines* indicate original spheroid boundary and arrowheads indicate invasive strands containing non-metastatic cells. Scale bar equals 50 μm. Invasion was quantified in terms of (**c**) invasive index, (**d**) maximum invasion distance, and (**e**) number of invasive strands containing non-metastatic MCF10A cells

bition, invasive cells move into the surrounding matrix but there is no matrix remodeling, and non-invasive cells do not move into the surrounding matrix. Together, these studies implicate force and polarization of force as key events to collective cell movements both in planar cultures and 3D spheroid cultures.

In addition to a clear focus on the role of forces in migration, there has also been significant interest in the basic biology of force generation and the genesis and transmission of these forces at focal adhesions. Work to quantify the force-transducing ability of individual focal contacts and focal adhesions has been largely pioneered by Drs. Benjamin Geiger and Alexander Bershadsky at the Weizmann Institute, Israel (site report, Appendix B). Their early work in this area made the first attempts at correlating focal adhesion and size with force magnitude (Balaban et al. 2001). A number of studies have followed, including work by Dr. Ulrich Schwarz at the University of Heidelberg, Germany (site report, Appendix B), investigating adhesion size and geometry with respect to force generation, and it continues to be an area of intense interest (Schwarz et al. 2002, 2006; Stricker et al. 2010). Additional work by Dr. Michael Sheetz has provided unique insights into the role of individual focal adhesion proteins in adhesion and contraction of adherent cells (site report, Appendix C). Using nanopatterned substrates, they investigated the time course of integrin clustering, intracellular focal adhesion protein clustering, and cells contraction (Yu et al. 2011; Roca-Cusachs et al. 2013). This approach, focused on identifying the critical molecular players for adhesion and force generation, may lead to the identification of therapeutic targets to prevent cell migration during metastasis.

In addition to examining the forces generated by cells against their substrate, several groups have researched forces at cell-cell contacts and their roles in collective cell movements and the initiation of signaling at junctions. Significant work in this area has been done by Chen and colleagues at the University of Pennsylvania using mPADs (Liu et al. 2010), and Trepat using a modified version of traction force microscopy (Trepat and Fredberg 2011). Trepat has shown that cell-cell contacts contribute to epithelial collective migration and that cells in a cluster can each contribute to collective movements (site report, Appendix B). More recently, an international collaboration between Ladoux (France), Trepat (Spain), and Lim (Singapore) have shown that cell-cell contacts can dominate in areas of low cell-matrix adhesion and can function as bridges to maintain cell sheet integrity (Vedula et al. 2014).

These experimental approaches to investigate cellular force generation and its effects on cell-cell dynamics have been complemented by computational approaches to understand how individual mechanical interactions at the cellular level translate into 3D morphogenesis events. Mechanobiologists at Kyoto University, Japan, have developed models that span from cell to tissue to understand morphogenesis (Okuda et al. 2013; site report, Appendix C). Treating cells as interacting polyhedrons with formal boundaries and taking into consideration the mechanical interactions of the cell with their surroundings and each other, Okuda and colleagues have recapitulated the large-scale tissue deformations that occur during morphogenesis *in silico* (Fig. 4.11). In an analogous *in silico* approach, Dr. Yoshikiro Morishita's group at the RIKEN Center for Developmental Biology, Japan, has taken information regarding large-scale tissue-level deformations and used Bayesian statistical models to predict the individual cell movements and deformations and morphogen gradients

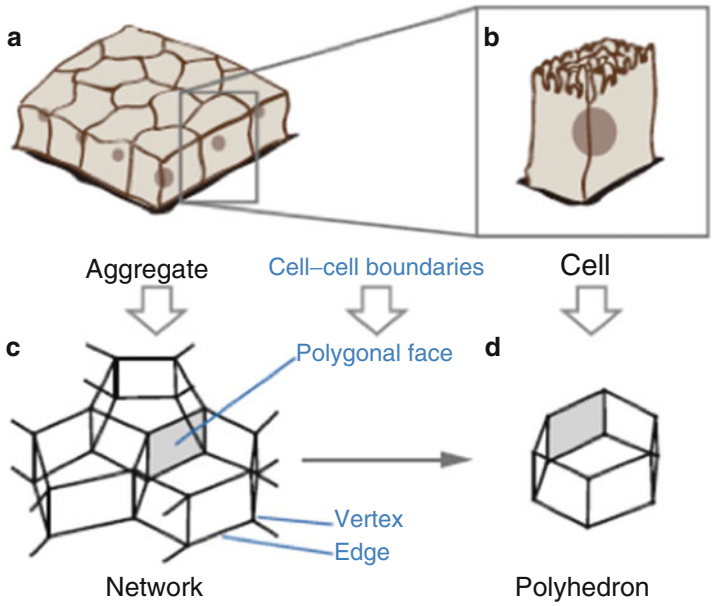

Fig. 4.11 Model shapes of a multicellular aggregate (From Okuda et al. 2013)
(**a**) Aggregate composed of cells. (**b**) Single cell. (**c**) Network that represents a cell aggregate composed of vertices and edges (*solid lines*). Polygonal faces (*gray area*) compartmentalize the network. (**d**) A polyhedron represents a single cell

that cause these tissue-level changes (Hironaka and Morishita 2012; Morishita and Iwasa 2011). These computational models have a specific benefit in their ability to capture the multi-scale (molecular, cellular, tissue) contributions of mechanobiology to tissue structure. Adapting these models to tumor formation and spread may prove to be a powerful approach to integrating the multi-scale contributions of the microenvironment to tumor biology.

More recently, there has been increasing attention on experimentally measuring cellular forces of cells within fully 3D matrices. Chen and colleagues at the University of Pennsylvania have published the first work in this area, developing both PEG-based hydrogel materials and a computational approach for the measurement of forces exerted by cells in 3D matrices (Fig. 4.12). Numerous groups are developing alternate methods for 3D traction measurements. Keng-hui Lin's lab at Academia Sinica, Taiwan, for instance, described 3D foam-like materials containing spherical cavities to study 3D cell contractility (Lee et al. 2013; site report, Appendix C). The cavities are on the cell-size scale, and as such, cells can adhere to and pull on the walls of the cavity (Fig. 4.13). The use of well-defined, flexible materials lays the foundation for the calculation of 3D forces based on the deformations of the cavity walls due to cell contraction.

Measurements of cell-generated forces have resulted in both the identification of cellular force as a mechanical biomarker of metastasis and key insights into how cells move. Further investigation into the mechanisms of force generation in metastasis may lead to therapeutics that target force generation during metastasis.

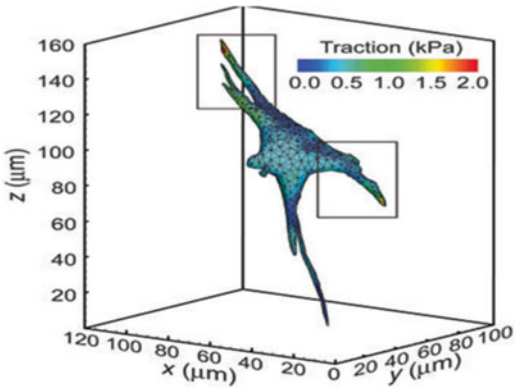

Fig. 4.12 3D traction force microscopy. Contour plot of the tractions exerted by the cell (From Legant et al. 2010)

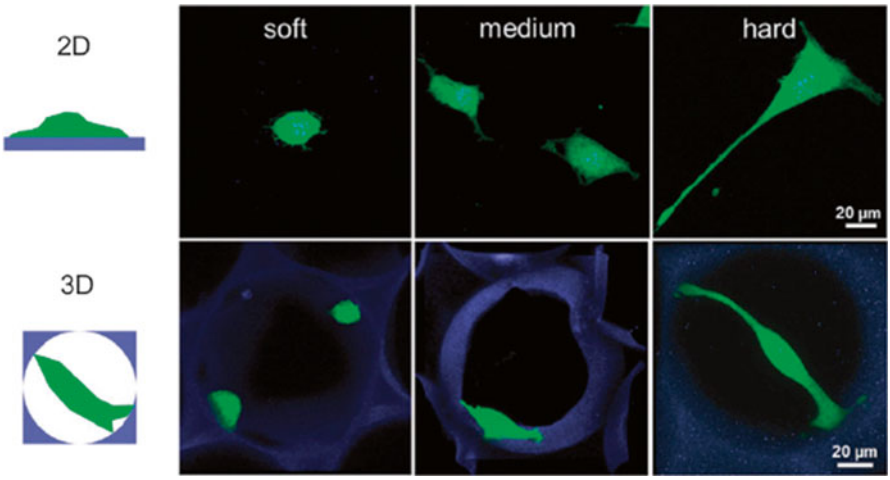

Fig. 4.13 Elongation of fibroblasts on stiff substrates (Adapted from Lee et al. 2013) Micrographs of fibroblasts on 2D flat substrates (*top*) and on 3D spherical pores of 100 mm (*bottom*). Micrographs in the same column are made of the same AC/BIS (5/0.2 %, 7/0.2 %, and 12/0.3 % for soft, medium, and hard gels, respectively). The cell body is shown in *green* based on expression of whole-body GFP. Fibronectin on the pore surface is labeled with Cy5 (*purple*) to visualize the pore

Mechanotransduction in Cancer: Tumor Response to Imposed Forces

Mechanotransduction is a broad term that describes the transduction of external mechanical cues into chemical signals within the cell. These cues can include (but are not limited to) changes in the mechanical properties of the extracellular matrix, compressive pressures, and tensional forces.

Most types of solid tumors are stiffer than normal tissue. There has been extensive work investigating the effects of matrix stiffness on cell behavior, some of which date back to 1990 when it was first noted that cell spreading increases on

stiffer substrates (Keese and Giaever 1991). With the advent of tractable systems to control matrix stiffness, including the development of polyacrylamide substrates (Pelham and Wang 1998; Wang and Pelham 1998), several studies have pointed to the role of matrix stiffness in promoting malignancy (Kraning-Rush et al. 2012a; Levental et al. 2009; Paszek et al. 2005). Integrins and focal adhesion have been implicated as the mechanosensors of matrix stiffness. Additionally, the laboratories of both Dr. Daniel Fletcher (author, Chap. 7) and Dr. Atef Asnacios from the University of Paris, Diderot, France (site report, Appendix B), have used modified cantilever systems to investigate cellular force response to active changes in stiffness (Crow et al. 2012; Mitrossilis et al. 2010). Interestingly, Dr. Ming-Jer Tang's lab at Tunghai University, Taiwan, has shown that transformed cells display altered mechanosensitivity, resisting soft substrate-induced apoptosis (Wang et al. 2007; site report, Appendix C).

Tumors are also subjected to pressure and compressive stresses when confined during growth. Recent work from Dr. Lance Munn's group (author, Chap. 5) suggests that these forces enhance invasion by increasing cell-matrix adhesion (Tse et al. 2012). However, data from Dr. Jean-François Joanny's group at the Institute Curie, France (site report, Appendix B), suggests that imposed mechanical stress can inhibit tumor growth by inhibiting cell proliferation (Fig. 4.14). Clearly, there is a significant need to continue work in this field to better understand how mechanical stresses affect tumor growth.

As is the case in most fields investigating mechanotransduction in various physiological systems, there is growing interest in identifying and characterizing molecular mechanotransducers within the cell. Given the important role of cadherins in maintaining tissue structure and their location at cell-cell junctions, there is significant research ongoing to understand the role of cadherins in mechanosensing. Using a magnetic twisting cytometry system, Johan de Rooij's lab at the Hubrecht Institute,

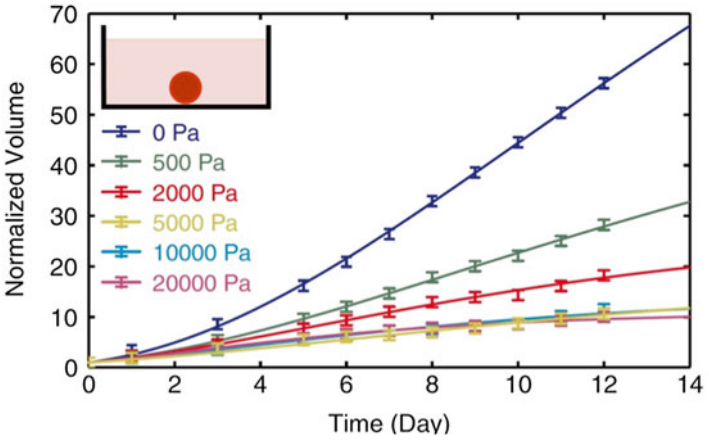

Fig. 4.14 Mechanical stress inhibits tumor growth. Spheroids were subjected to imposed pressures and their growth monitored over 2 weeks (From Montel et al. 2011)

Netherlands (site report, Appendix B), has found that E-cadherin is a mechanotransducer that causes cell stiffening when under tension (le Duc et al. 2010). This response is mediated by vinculin, a protein commonly associated with focal adhesions that is also known to localize to cell-cell junctions in structures termed focal adherens junctions. François Gallet's laboratory at the University of Paris, Diderot, France (site report, Appendix B), has also investigated cadherin mechanics, specifically the cross-talk between cadherins and integrins (Al-Kilani et al. 2011). Interestingly, their data suggest a negative feedback loop between cadherin binding and integrin binding. A similar relationship has been shown for endothelial cells, where integrin-matrix adhesion is altered by matrix stiffness and VE-cadherin engagement is disrupted (Huynh et al. 2011). There is work being done in the United States that is also investigating cadherin-binding mechanics, including a collaboration between Deborah Leckband's group at the University of Illinois at Urbana-Champaign and Johan de Rooij at the Hubrecht (site report, Appendix B; Leckband et al. 2011; le Duc et al. 2010). Together, these data suggest that changes in matrix stiffness could facilitate cancer cell invasion by enhancing integrin adhesion and disrupting cell-cell adhesion. These results are important in our understanding of the microenvironmental cues that stimulate metastasis because they indicate that increased matrix binding may actively lead to decreased cell-cell adhesion.

Mechanotransductive cues in cancer are not limited to pressure and increased stiffness. In lung cancer, for example, cells are exposed to periodic cyclic, tensile stresses due to lung expansion during breathing. Recent work from Chau-Hwang Lee's laboratory at the Academia Sinica has shown that cyclic stress reduces myofibroblast activation of cancer cell migration (Huang et al. 2013; site report, Appendix C). This work was enabled by the development of a novel device that allows for co-culture of fibroblasts and cancer cells, optical observation of cell behavior, and the application of well-defined tensile forces (discussed in Chap. 7).

Mechanoactivation of cells has also been a focus of G.V. Shivashankar's lab in the Mechanobiology Institute in Singapore. Specifically, they have an interest in understanding how physical forces alter transcriptional activity. Using magnetic particles attached to the surface of the cell membrane, they imposed well-calibrated forces and investigated the subsequent effects on actin polymerization, chromatin remodeling and nuclear transport (Fig. 4.15). Forces imposed on the membrane can result in changes in actin polymerization and chromatin reorganization. The altered F/G actin ratio can alter nuclear transport of transcription factors to the nucleus. This work was enabled by a device that could both impose forces and image fluorescence anisotropy. In fact, most mechanotransduction experiments require the integration of a device with cells, and approaches to create tractable devices will help transition mechanotransduction experiments into cell biology labs.

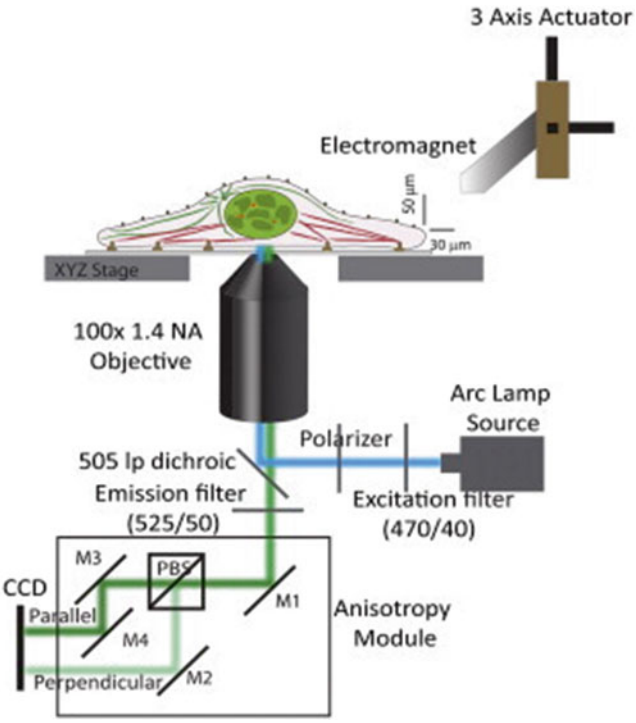

Fig. 4.15 Schematic of experimental setup application of force on single living cells (From Iyers et al. 2012)

Discussion

Efforts in the United States, Europe, and Asia have demonstrated that cell mechanics is a critical component to our understanding of cancer progression. Mechanics cannot be viewed as a complement to molecular biology, but rather as a partner. The biophysical properties of cells and the response of cells to mechanical forces is intimately involved in tumor growth and the metastatic process. Efforts to train scientists at the interface of biology and mechanics, such as those being pioneered at the Mechanobiology Institute in Singapore, will produce an entirely new breed of investigator that is able to work at the interface of the physical sciences and oncology. Close connections with clinicians, like those found at the University of Leipzig, are essential to translating cell mechanics studies to patient diagnosis and treatment. There are many questions that have not yet been answered, but we are now well-positioned to tackle them with the advent of new, tractable methodologies to probe cell mechanics. Connecting the biophysical properties of cells to the invasive behaviors of cells is a critical step in bringing cell mechanics work closer to therapeutic treatment.

Challenges to the Field of Cell Mechanics

Assessing the state-of-the-art research throughout Europe, Asia, and the United States has revealed several universal challenges in the field of cell mechanics.

Adapting Cell Mechanics Concepts to Biology Labs and the Clinic

While the importance of mechanics is increasingly appreciated, most of the current oncology studies continue to focus exclusively on molecular biomarkers, signaling pathways, and small molecule inhibitors. Significant technology development has occurred in the molecular biomarker field, producing more user-friendly, high-throughput methods for analyzing the molecular signature of cells. These methodologies are readily adaptable by scientific labs and have found their way into clinical assays. In contrast, there has been less done to make measurements of cell mechanics tractable to scientists outside of the mechanics field. As a result, the impact of cell mechanics has been more limited as fewer research labs have adopted methods or become familiar with the conceptual framework of cell mechanics.

Likewise, unlike molecular properties, mechanical properties are difficult to manipulate. There is no analogous technique to siRNA or knockdown in mechanics. Intervention in pathways related to mechanics often alters multiple signaling pathways. Therefore, it is difficult to test whether a specific cell behavior (e.g., invasion, migration, or proliferation) is specifically due to mechanical changes.

High-Throughput Screening of Single Cells

Cell mechanical testing is traditionally done by probing cells on an individual basis and has not been widely translated into high throughput methods. Most mechanics assays—both the actual experimental testing and the analysis required to convert the measurements into meaningful values—are typically very time consuming because each cell is tested and analyzed individually. Analysis of populations becomes difficult simply due to the time required to collect the amount of data necessary to analyze statistical differences. In contrast, many molecular techniques such as Western blotting and PCR are designed to test cell populations and are relatively high-throughput compared to mechanical testing techniques. For mechanics to be widely adopted, higher throughput methods will be beneficial.

The Need for Interdisciplinary Training

Cell mechanics and mechanobiology differ from fields like molecular biology in pedagogy as well. Mechanobiology, while an old field, is not offered as a unique degree program unlike many other biology sub-disciplines. While there are a

plethora of biology textbooks available, there are few that could be considered the "authority" on mechanobiology. The Mechanobiology Institute in Singapore has made significant strides in this area in the creation of MBInfo (mechanobio.info), a multi-media resource, containing chapters describing mechanobiology across scales. The information is written and reviewed by expert scientists in the field. Its wiki-based format allows for information to be continually updated, a necessary element in a constantly changing field. Training students and scientists in both biology and mechanics is essential to making significant strides in tying oncology to the physical sciences.

Perceptions of the Integration of Mechanical Measurements with Molecular Biology

A potential limitation to the efforts being made in the field of cell mechanics is related to the perceptions of the larger biological community about cell mechanical measurements relative to what is already known. Molecular biology approaches and molecular biomarkers are often the dominant concepts in biomedical research. As such, there is a tendency for researchers to try to link mechanical changes to specific genetic and molecular changes. While it is logical that physical changes have their roots in genetic changes, it is possible that this may not always be the case. For instance, any number of different genetic or signaling changes can result in the same mechanical phenotype. Therefore, screening a population could result in the identification of a certain mechanical phenotype without finding a unifying underlying molecular biomarker. Additionally, the mechanical traits of a cell are transient—changing in time as a function of migration and protrusive activity, cell cycle state, matrix properties, and other factors. Therefore, it may not be possible to capture individual molecular or signaling changes that are responsible for mechanical properties. Given the lack of success in identifying universal molecular biomarkers of cancer progression and the recent surge of data showing that metastatic cells are more deformable and exert stronger forces, it is possible that mechanical biomarkers may be a promising avenue for diagnosing and treating cancer. A greater understanding of cell behavior may come from viewing mechanics as complementary to, but not necessarily rooted in, molecular biology.

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References

Al-Kilani, A., de Freitas, O., Dufour, S., Gallet, F.: Negative feedback from integrins to cadherins: a micromechanical study. Biophys. J. **101**, 336–344 (2011)

Baker, E.L., Bonnecaze, R.T., Zaman, M.H.: Extracellular matrix stiffness and architecture govern intracellular rheology in cancer. Biophys. J. **97**(4), 1013–1021 (2009)

Balaban, N.Q., Schwarz, U.S., Riveline, D., Goichberg, P., Tzur, G., Sabanay, I., Mahalu, D., Safran, S., Bershadsky, A., Addadi, L., Geiger, B.: Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates. Nat. Cell Biol. **3**, 466–472 (2001)

Bao, G., Suresh, S.: Cell and molecular mechanics of biological materials. Nat. Mater. **2**, 715–725 (2003)

Beerling, E., Ritsma, L., Vrisekoop, N., Derksen, P.W., van Rheenen, J.: Intravital microscopy: new insights into metastasis of tumors. J. Cell Sci. **124**, 299–310 (2011)

Carey, S.P., Starchenko, A., McGregor, A.L., Reinhart-King, C.A.: Leading malignant cells initiate collective epithelial cell invasion in a three-dimensional heterotypic tumor spheroid model. Clin. Exp. Metastasis **30**(5), 615–630 (2013)

Cross, S.E., Jin, Y.S., Rao, J., Gimzewski, J.K.: Nanomechanical analysis of cells from cancer patients. Nat. Nanotechnol. **2**, 780–783 (2007)

Crow, A., Webster, K.D., Hohlfeld, E., Ng, W.P., Geissler, P., Fletcher, D.A.: Contractile equilibration of single cells to step changes in extracellular stiffness. Biophys. J. **102**, 443–451 (2012)

De, R., Zemel, A., Safran, S.A.: Do cells sense stress or strain? Measurement of cellular orientation can provide a clue. Biophys. J. **94**, L29–L31 (2008)

De, R., Zemel, A., Safran, S.A.: Theoretical concepts and models of cellular mechanosensing. Methods Cell Biol. **98**, 143–175 (2010)

Dembo, M., Wang, Y.L.: Stresses at the cell-to-substrate interface during locomotion of fibroblasts. Biophys. J. **76**, 2307–2316 (1999)

du Roure, O., Saez, A., Buguin, A., Austin, R.H., Chavrier, P., Silberzan, P., Ladoux, B.: Force mapping in epithelial cell migration. Proc. Natl. Acad. Sci. U. S. A. **102**, 2390–2395 (2005)

Friedl, P., Alexander, S.: Cancer invasion and the microenvironment: plasticity and reciprocity. Cell **147**, 992–1009 (2011)

Friedl, P., Wolf, K.: Tumour-cell invasion and migration: diversity and escape mechanisms. Nat. Rev. Cancer **3**, 362–374 (2003)

Friedl, P., Wolf, K., Lammerding, J.: Nuclear mechanics during cell migration. Curr. Opin. Cell Biol. **23**, 55–64 (2011)

Guck, J., Ananthakrishnan, R., Mahmood, H., Moon, T.J., Cunningham, C.C., Käs, J.: The optical stretcher: a novel laser tool to micromanipulate cells. Biophys. J. **81**, 767–784 (2001)

Guck, J., Schinkinger, S., Lincoln, B., Wottawah, F., Ebert, S., Romeyke, M., Lenz, D., Erickson, H.M., Ananthakrishnan, R., Mitchell, D., Käs, J., Ulvick, S., Bilby, C.: Optical deformability as an inherent cell marker for testing malignant transformation and metastatic competence. Biophys. J. **88**, 3689–3698 (2005)

Gupta, S., Marcel, N., Sarin, A., Shivashankar, G.V.: Role of actin-dependent nuclear deformation in regulating early gene expression. PLoS One **7**, e53031 (2012)

Harris, A.K., Wild, P., Stopak, D.: Silicone rubber substrata: a new wrinkle in the study of cell locomotion. Science **208**, 177–179 (1980)

Heilbrunn, L.V.: Protoplasmic viscosity changes during mitosis. J. Exp. Zool. **34**, 417–447 (1921)

Hironaka, K., Morishita, Y.: Encoding and decoding of positional information in morphogen-dependent patterning. Curr. Opin. Genet. Dev. **22**, 553–561 (2012)

Huang, J.W., Pan, H.J., Yao, W.Y., Tsao, Y.W., Liao, W.Y., Wu, C.W., Tung, Y.C., Lee, C.H.: Interaction between lung cancer cell and myofibroblast influenced by cyclic tensile strain. Lab Chip **13**, 1114 (2013)

Huynh, J., Nishimura, N., Rana, K., Peloquin, J.M., Califano, J.P., Montague, C.R., King, M.R., Schaffer, C.B., Reinhart-King, C.A.: Age-related intimal stiffening enhances endothelial permeability and leukocyte transmigration. Sci. Trans. Med. **3**, 112–122 (2011)

Icard-Arcizet, D., Cardoso, O., Richert, A., Henon, S.: Cell stiffening in response to external stress is correlated to actin recruitment. Biophys. J. **94**, 2906–2913 (2008)

Isermann, P., Lammerding, J.: Nuclear mechanics and mechanotransduction in health and disease. Curr. Biol. **23**(24), R1113–R1121 (2013)

Iyer, K.V., Pulford, S., Mogilner, A., Shivashankar, G.V.: Mechanical activation of cells induces chromatin remodeling preceding MKL nuclear transport. Biophys. J. **103**(7), 1416–28 (2012)

Jain, N., Iyer, K.V., Kumar, A., Shivashankar, G.V.: Cell geometric constraints induce modular gene-expression patterns via redistribution of HDAC3 regulated by actomyosin contractility. Proc. Natl. Acad. Sci. U. S. A. **110**, 11349–11354 (2013)

Keese, C.R., Giaever, I.: Substrate mechanics and cell spreading. Exp. Cell Res. **195**, 528–532 (1991)

Khalil, A.A., Friedl, P.: Determinants of leader cells in collective cell migration. Integr. Biol. (Camb.) **2**, 568–574 (2010)

Khatau, S.B., Bloom, R.J., Bajpai, S., Razafsky, D., Zang, S., Giri, A., Wu, P.H., Marchand, J., Celedon, A., Hale, C.M., Sun, S.X., Hodzic, D., Wirtz, D.: The distinct roles of the nucleus and nucleus-cytoskeleton connections in three-dimensional cell migration. Sci. Rep. **2**, 488 (2012)

Koch, T.M., Munster, S., Bonakdar, N., Butler, J.P., Fabry, B.: 3D Traction forces in cancer cell invasion. PLoS One **7**, e33476 (2012)

Kraning-Rush, C.M., Carey, S.P., Califano, J.P., Smith, B.N., Reinhart-King, C.A.: The role of the cytoskeleton in cellular force generation in 2D and 3D environments. Phys. Biol. **8**, 015009 (2011)

Kraning-Rush, C.M., Califano, J.P., Reinhart-King, C.A.: Cellular traction stresses increase with increasing metastatic potential. PLoS One **7**, e32572 (2012a)

Kraning-Rush, C.M., Carey, S.P., Califano, J.P., Reinhart-King, C.A.: Quantifying traction stresses in adherent cells. Methods Cell Biol. **110**, 139–178 (2012b)

Lam, W.A., Rosenbluth, M.J., Fletcher, D.A.: Chemotherapy exposure increases leukemia cell stiffness. Blood **109**, 3505–3508 (2007)

le Duc, Q., Shi, Q., Blonk, I., Sonnenberg, A., Wang, N., Leckband, D., de Rooij, J.: Vinculin potentiates E-cadherin mechanosensing and is recruited to actin-anchored sites within adherens junctions in a myosin II-dependent manner. J. Cell Biol. **189**, 1107–1115 (2010)

Leckband, D.E., le Duc, Q., Wang, N., de Rooij, J.: Mechanotransduction at cadherin-mediated adhesions. Curr. Opin. Cell Biol. **23**, 523–530 (2011)

Lee, M.H., Wu, P.H., Staunton, J.R., Ros, R., Longmore, G.D., Wirtz, D.: Mismatch in mechanical and adhesive properties induces pulsating cancer cell migration in epithelial monolayer. Biophys. J. **102**, 2731–2741 (2012)

Lee, Y.H., Huang, J.R., Wang, Y.K., Lin, K.H.: Three-dimensional fibroblast morphology on compliant substrates of controlled negative curvature. Integr. Biol. **5**, 1447–1455 (2013)

Legant, W.R., Miller, J.S., Blakely, B.L., Cohen, D.M., Genin, G.M., Chen, C.S.: Measurement of mechanical tractions exerted by cells in three-dimensional matrices. Nat. Methods **7**, 969–971 (2010)

Levental, K.R., Yu, H., Kass, L., Lakins, J.N., Egeblad, M., Erler, J.T., Fong, S.F., Csiszar, K., Giaccia, A., Weninger, W., Yamauchi, M., Gasser, D.L., Weaver, V.M.: Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell **139**, 891–906 (2009)

Liu, Z., Tan, J.L., Cohen, D.M., Yang, M.T., Sniadecki, N.J., Ruiz, S.A., Nelson, C.M., Chen, C.S.: Mechanical tugging force regulates the size of cell-cell junctions. Proc. Natl. Acad. Sci. U. S. A. **107**, 9944–9949 (2010)

Logothetis, N.K.: Francis Crick 1916–2004. Nat. Neurosci. **7**, 1027–1028 (2004)

Mitrossilis, D., Fouchard, J., Pereira, D., Postic, F., Richert, A., Saint-Jean, M., Asnacios, A.: Real-time single-cell response to stiffness. Proc. Natl. Acad. Sci. U. S. A. **107**, 16518–16523 (2010)

Montel, F., Delarue, M., Elgeti, J., Malaquin, L., Basan, M., Risler, T., Cabane, B., Vignjevic, D., Prost, J., Cappello, G., Joanny, J.F.: Stress clamp experiments on multicellular tumor spheroids. Phys. Rev. Lett. **107**, 188102 (2011)

Morishita, Y., Iwasa, Y.: Coding design of positional information for robust morphogenesis. Biophys. J. **101**, 2324–2335 (2011)

Okuda, S., Inoue, Y., Eiraku, M., Sasai, Y., Adachi, T.: Reversible network reconnection model for simulating large deformation in dynamic tissue morphogenesis. Biomech. Model. Mechanobiol. **12**, 627–644 (2013)

Paszek, M.J., Zahir, N., Johnson, K.R., Lakins, J.N., Rozenberg, G.I., Gefen, A., Reinhart-King, C.A., Margulies, S.S., Dembo, M., Boettiger, D., Hammer, D., Weaver, V.: Tensional homeostasis and the malignant phenotype. Cancer Cell **8**, 241–254 (2005)

Pelham Jr., R.J., Wang, Y.L.: Cell locomotion and focal adhesions are regulated by the mechanical properties of the substrate. Biol. Bull. **194**, 348–349 (1998); discussion 349–350

Pelling, A.E., Horton, M.A.: An historical perspective on cell mechanics. Pflugers Arch. **456**, 3–12 (2008)

Roca-Cusachs, P., del Rio, A., Puklin-Faucher, E., Gauthier, N.C., Biais, N., Sheetz, M.P.: Integrin-dependent force transmission to the extracellular matrix by α-actinin triggers adhesion maturation. Proc. Natl. Acad. Sci. U. S. A. **110**(15), 1361–1370 (2013)

Rowat, A.C., Jaalouk, D.E., Zwerger, M., Ung, W.L., Eydelnant, I.A., Olins, D.E., Olins, A.L., Herrmann, H., Weitz, D.A., Lammerding, J.: Nuclear envelope composition determines the ability of neutrophil-type cells to passage through micron-scale constrictions. J. Biol. Chem. **288**(12), 8610–8618 (2013)

Safran, S.A., De, R.: Nonlinear dynamics of cell orientation. Phys. Rev. E Stat. Nonlin. Soft Matter Phys. **80**, 060901 (2009)

Schwarz, U.S., Balaban, N.Q., Riveline, D., Bershadsky, A., Geiger, B., Safran, S.A.: Calculation of forces at focal adhesions from elastic substrate data: the effect of localized force and the need for regularization. Biophys. J. **83**, 1380–1394 (2002)

Schwarz, U.S., Erdmann, T., Bischofs, I.B.: Focal adhesions as mechanosensors: the two-spring model. Biosystems **83**, 225–232 (2006)

Seifriz, W.: An elastic value of protoplasm, with further observations on the viscosity of protoplasm. J. Exp. Biol. **2**, 1–11 (1924)

Stricker, J., Sabass, B., Schwarz, U.S., Gardel, M.L.: Optimization of traction force microscopy for micron-sized focal adhesions. J. Phys. Condens. Matter **22**, 194104 (2010)

Tambe, D.T., Hardin, C.C., Angelini, T.E., Rajendran, K., Park, C.Y., Serra-Picamal, X., Zhou, E.H., Zaman, M.H., Butler, J.P., Weitz, D.A., Fredberg, J.J., Trepat, X.: Collective cell guidance by cooperative intercellular forces. Nat. Mater. **10**, 469–475 (2011)

Tan, J.L., Tien, J., Pirone, D.M., Gray, D.S., Bhadriraju, K., Chen, C.S.: Cells lying on a bed of microneedles: an approach to isolate mechanical force. Proc. Natl. Acad. Sci. U. S. A. **100**, 1484–1489 (2003)

Trepat, X., Fredberg, J.J.: Plithotaxis and emergent dynamics in collective cellular migration. Trends Cell Biol. **21**, 638–646 (2011)

Tse, J.M., Cheng, G., Tyrrell, J.A., Wilcox-Adelman, S.A., Boucher, Y., Jain, R.K., Munn, L.L.: Mechanical compression drives cancer cells toward invasive phenotype. Proc. Natl. Acad. Sci. U. S. A. **109**, 911–916 (2012)

Vedula, S.K., Hirata, H., Nai, M.H., Brugués, A., Toyama, Y., Trepat, X., Liu, C.T., Ladoux, B.: Epithelial bridges maintain tissue integrity during collective cell migration. Nat. Mater. **13**(1), 87–96 (2014)

Wang, Y.L., Pelham Jr., R.J.: Preparation of a flexible, porous polyacrylamide substrate for mechanical studies of cultured cells. Methods Enzymol. **298**, 489–496 (1998)

Wang, Y.H., Chiu, W.T., Wang, Y.K., Wu, C.C., Chen, T.L., Teng, C.F., Chang, W.T., Chang, H.C., Tang, M.J.: Deregulation of AP-1 proteins in collagen gel-induced epithelial cell apoptosis mediated by low substratum rigidity. J. Biol. Chem. **282**, 752–763 (2007)

Wilson, L.V.H.A.W.L.: A rational approach to the problem of cancer chemotherapy. Biol. Bull. **113**, 388–396 (1957)

Wirtz, D.: Particle-tracking microrheology of living cells: principles and applications. Annu. Rev. Biophys. **38**, 301–326 (2009)

Wirtz, D., Konstantopoulos, K., Searson, P.C.: The physics of cancer: the role of physical interactions and mechanical forces in metastasis. Nat. Rev. Cancer **11**, 512–515 (2011)

Wu, P.H., Hale, C.M., Chen, W.C., Lee, J.S., Tseng, Y., Wirtz, D.: High-throughput ballistic injection nanorheology to measure cell mechanics. Nat. Protoc. **7**(1), 155–170 (2012)

Yu, C.H., Law, J.B., Suryana, M., Low, H.Y., Sheetz, M.P.: Early integrin binding to Arg-Gly-Asp peptide activates actin polymerization and contractile movement that stimulates outward translocation. Proc. Natl. Acad. Sci. U. S. A. **108**(51), 20585–20590 (2011)

Zemel, A., Rehfeldt, F., Brown, A.E., Discher, D.E., Safran, S.A.: Cell shape, spreading symmetry and the polarization of stress-fibers in cells. J. Phys. Condens. Matter **22**(19), 194110 (2010)