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Charlotte Alibert, Bruno Goud, Jean-Baptiste Manneville. Are cancer cells really softer than normal cells?. *Biology of the Cell*, 2017, 10.1111/boc.201600078 . hal-01489167

HAL Id: hal-01489167

<https://hal.sorbonne-universite.fr/hal-01489167>

Submitted on 14 Mar 2017

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Are cancer cells really softer than normal cells?

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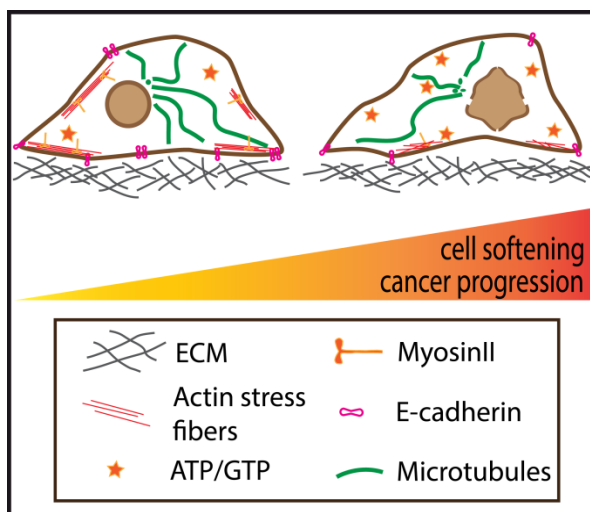
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Running head: Mechanics of cancer cells

Keywords: microrheology, cancer, cytoskeleton, cell mechanics

Summary

Solid tumors are often first diagnosed by palpation, suggesting that the tumor is more rigid than its surrounding environment. Paradoxically, individual cancer cells appear to be softer than their healthy counterparts. In this review, we first list the physiological reasons indicating that cancer cells may be more deformable than normal cells. Next, we describe the biophysical tools that have been developed in recent years to characterize and model cancer cell mechanics. By reviewing the experimental studies that compared the mechanics of individual normal and cancer cells, we argue that cancer cells can indeed be considered as softer than normal cells. We then focus on the intracellular elements that could be responsible for the softening of cancer cells. Finally, we ask whether the mechanical differences between normal and cancer cells can be used as diagnostic or prognostic markers of cancer progression.



Most solid tumors are more rigid than their surrounding environment. In contrast, a growing number of studies show that individual cancer cells are more deformable than their healthy counterparts and that cell softening occurs during cancer progression. The mechanical differences between normal and cancer cells could be primarily due to modifications in the cytoskeleton, but other factors, such as the cell microenvironment, internal membrane trafficking or non-equilibrium active forces, may contribute.

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In the past ten years, the field of mechanobiology has received a growing interest. The number of reports describing techniques to probe cellular mechanics has risen steadily and a variety of theoretical models are now available to interpret experimental data. An emerging field in mechanobiology studies the physics of cancer cells. While molecular biology has produced a plethora of data on tumor biology, it still seems unable to clearly identify the fundamental differences between malignant and benign tumors. Physicists, with more reductionist and universal approaches, have asked whether changes in cell mechanics could be required for malignant transformation (Fritsch et al., 2010). Cells both exert forces on their surrounding environment and sense its mechanical properties. During all stages of tumor progression, from cell proliferation, to tumor development, invasion and metastasis, changes in both cellular and extra-cellular mechanics occur that participate to malignant transformation (Fritsch et al., 2010). As cancer progresses, tumor cells, or at least a subpopulation of cancer cells, appear to become softer (Fritsch et al., 2010). However, cancer cell softening remains a controversial issue. It is still not clear whether cancer cells all have the same material properties with softening being a universal feature or whether mechanical forces and features depend on the tumor type (Jonietz, 2012).

In this review, we focus on the mechanical differences between individual cancer and normal cells and ask whether these differences are significant enough to discriminate cancer cells from normal cells solely based on their mechanical properties (Mierke, 2014; Suresh, 2007; W. Zhang, Kai, Ueno, & Qin, 2013). Individual cancer cell mechanics has been extensively studied in cancer cell lines and, to a lesser extent, in primary cells extracted from tumors, in either 2D or 3D *in vitro* assays. *In situ* mechanical measurements on whole organs or tumor tissues will not be discussed here, although studying cell mechanics in their native tumor environment is obviously relevant and may have a strong clinical impact (Fritsch et al., 2010). We define a ‘normal’ cell as a cell from the same organ as its cancer counterpart but from normal healthy tissue. In the case of cell lines, ‘normal’ cells, although they can proliferate, should not show any invasive or metastatic potential. In the case of primary cells, depending on the isolation and purification procedure, samples may be heterogeneous and contain cancer associated cells. We start by an inventory of the physiological factors that may account for a change in cellular mechanics during cancer development. We then review the experiments and theoretical models developed to measure cancer cell mechanics. Finally we try to identify which cellular elements are altered in cancer that could explain the observed mechanical differences with healthy cells.

1 Why may cancer cells be softer than normal cells?

Several, if not all, biological functions are altered in cancer cells during tumor progression. Hanahan and Weinberg have listed eight hallmarks of cancer which enable tumor growth and metastatic

processes (Hanahan & Weinberg, 2011). During the different steps of the disease, cancer cells acquire biological properties that cannot be found in normal cells. For instance, they have the capacity to replicate indefinitely and to exceed the predefined number of growth-and-division cycles. They can also resist cell death programmed by apoptosis, or evade growth suppressors by inactivating tumor suppressor genes which limit growth and proliferation (Hanahan & Weinberg, 2011). Several signaling pathways, including those involved in membrane trafficking (Orgaz, Herraiz, & Sanz-Moreno, 2014; Tzeng & Wang, 2016), cell adhesion, polarity or migration (Asem, Buechler, Wates, Miller, & Stack, 2016; Leve & Morgado-Díaz, 2012), are deregulated during cancer due to overexpression or downregulation of key signaling molecules. Cell metabolism and energy consumption are also modified (Ghaffari, Mardinoglu, & Nielsen, 2015).

One of the most striking phenomenon in cancer is metastatic spreading, leading to the appearance of secondary tumors in organs located far away from the primary tumor site (Valastyan & Weinberg, 2011). During the metastatic process, the control of cell adhesion, polarity and migration is severely affected. Cancer cells first detach from the primary tumor, then enter the blood circulation by breaking through the basement membrane (intravasation) and survive there. Cancer cells then move out of the blood vessel (extravasation), and finally settle in targeted organs to initiate secondary tumors. Intravasation and extravasation are thought to be facilitated if cells are more deformable. Indeed it should be easier for soft, deformable cells to migrate through small gaps, crowded matrices or entangled matrix fibers, leading to the hypothesis along which the deformability of individual cancer cells should correlate with their metastatic potential.

But this may not be as simple as it seems. First, tumorous tissues are often detected by palpation because they are more rigid than the surrounding tissue. It is not clear whether this is due to cancer cells themselves or to the stroma. As shown by the Weaver group, stiffening of the stroma induces tumor rigidity and tumor progression (Levental et al., 2009), (Lu, Weaver, & Werb, 2012) and promotes all hallmarks of cancer (Pickup, 2014). Cells are mechanosensitive and sense their microenvironment. Cells adapt to their environment in the long term by altering their genetic program (Butcher, Alliston, & Weaver, 2009) but also respond very rapidly to environmental changes by modifying the cytoskeleton and its dynamics (Ananthakrishnan et al., 2006). Second, the epithelial-mesenchymal transition (EMT) and dedifferentiation modify the cell architecture, including the cytoskeleton and thus perturb cell mechanics. Third, since metastatic cells can also invade tissues locally or through the lymphatic circulation (see (Jin, Li, van Dam, Zhou, & Zhang, 2016) for a recent review), a high deformability may not be required for efficient tumor spreading. Fourth, metastatic cells most often do not migrate as single cells but as a cohort during collective migration (Lintz, Muñoz, & Reinhart-King, 2016) and only the so-called leader cells may be more deformable in order to facilitate the passage of more rigid follower cells. The stiffness of a cancer cell may also change according to the various microenvironments it encounters as it leaves the primary tumor, invades surrounding tissues and settles in a new secondary tumor (Kumar & Weaver, 2009). Fifth, to divide in a stiff environment, cells have to overcome the pressure created by the rigid tumor. Confinement stresses were shown to inhibit cell division and proliferation in *in vitro* models (Alessandri et al., 2013; Delarue et al., 2014; Lancaster et al., 2013), suggesting that cancer cells must be stiff enough to divide.

In the next paragraphs, we summarize the physical and mechanical properties of cancer cells that correlate with their metastatic potential before focusing on the experimental techniques and theoretical models developed to study their rheology.

Mechanics of cancer cells

The ability of cancer cells to create metastases is certainly the main reason why cancer cells were thought to be different from healthy cells, not only from a biological point of view but also from a mechanical point of view (Mierke, 2013). During metastasis, cancer cells sense and respond to the mechanical properties of their microenvironment, and generate forces thereon. Since the mechanotransduction events involved in cell movement and migration and their associated signaling pathways are altered during cancer cell transformation (Fernandez-Sanchez, Brunet, Röper, & Farge, 2015), at least some of the mechanical properties of cancer cells could also be altered.

More than 25 years ago, Partin *et al.* showed with Fourier analysis that a high metastatic potential correlates with a high cell motility (Partin, Schoeniger, Mohler, & S., 1989). Since then, many reports have shown a link between cell transformation and their migratory capacities both in 2D and in 3D in several types of cancer. For instance, high grade glioma cell lines are characterized by a faster and less directed 2D migration (Camand, Peglion, Osmani, Sanson, & Etienne-Manneville, 2012). In bladder cancer cell lines, less invasive cells have smaller velocities (Agus *et al.*, 2013; Kraning-Rush, Califano, & Reinhart-King, 2012; Peschetola *et al.*, 2013). In contrast, breast cancer cells exhibit a lower speed and a higher invasiveness in 1D and 3D, but not in 2D (Agus *et al.*, 2013).

Cell-cell adhesion is regulated during EMT and invasion and the switch from E-cadherin to N-cadherin is characteristic of EMT (Le Bras, Taubenslag, & Andl, 2012). Intuitively, reduced cell-cell adhesion should favor the spreading of cancer cells from the primary tumor (Cavallaro & Christofori, 2001). In agreement, it was shown in an Atomic Force Microscopy (AFM) assay that breast cancer cells show lower cell-cell detachment forces than non-tumorigenic cells (Omidvar, Tafazzoli-Shadpour, Shokrgozar, & Rostami, 2014). However, recent results by Pawlizak *et al.* demonstrate that, in breast cancer cell lines, cell-cell adhesion does not correlate with cadherin density and that a tumorigenic cell line can have higher cell-cell adhesion than a non-tumorigenic cell line (Pawlizak *et al.*, 2015). Theoretical work predicts that cell-cell adhesion induces cell motility in tissues through jamming and glass transitions (Bi, 2016). Another study showed that metastatic cells have a higher adhesion-related particle density than normal cells but also more numerous and smaller cell-matrix adhesion complexes (Rönnlund, Gad, Blom, Aspenström, & Widengren, 2013) suggesting that cancer cells could have higher contractile forces than normal cells (Gad *et al.*, 2012). Indeed invasiveness appears to correlate, at least in some cancer types, with contractile force generation (Mierke, Rösel, Fabry, & Brábek, 2008).

Directly linked to contractile forces, traction forces have also been shown to be increased in cancer cells and to correlate with malignancy (Agus *et al.*, 2013; Kraning-Rush *et al.*, 2012; Peschetola *et al.*, 2013) and invasiveness (Koch, 2012). However, conflicting results were obtained in murine breast cancer cell lines derived from the same primary tumor but possessing different metastatic potentials for which an increasing metastatic capacity correlates with a decrease in traction forces, a decrease in the 2D migration velocity and a decrease in cell adhesion (Indra *et al.*, 2011). These results may be reconciled if the force-velocity curve is not monotonic, i.e. velocity increases with force at low traction forces then decreases at high traction forces. Even if, to our knowledge, such force-velocity relationships are not documented yet in the context of individual cancer cell motility, it was shown that the migration velocity of *Dictyostelium discoideum* decreases

with increasing traction forces due to solid friction (Rieu, Barentin, Maeda, & Sawada, 2005). Similarly, in keratocytes, the force-velocity curves exhibit an optimum as a function of the lamellipodial pushing force (Heinemann, Doschke, & Radmacher, 2011; Prass, Jacobson, Mogilner, & Radmacher, 2006).

It is thus now well documented that motility, adhesion and contractility are altered in cancer cells leading in most cases to a reduced propensity to adhere to other cells and an increased ability to migrate individually or in small groups (Friedl & Gilmour, 2009; Guan, 2015; J. A. Park, Atia, Mitchel, Fredberg, & Butler, 2016). Our main goal here is to review the studies that have focused on the rheology of individual cancer cells and that have compared their rheological properties with those of normal cells. A growing number of experimental techniques are being used to study the mechanics of cancer cell lines or primary cells from biopsies. As we will argue below, the rheological data collected until now clearly point to a softening of cancer cells.

Microrheology of cancer cells: probing cellular rigidity at different scales

A large number of experimental tools have been developed to measure the rheology of complex materials. In the past twenty years, microrheology, i.e. rheology at the scale of the micrometer, has gained a lot of attention. Technologies have appeared to specifically measure the microrheology of cells in suspension or of adherent cells, at the scale of the whole cell or more local scales. Because we focus here on microrheology experimental studies that explicitly compare individual cancer cells with normal cells (for more general reviews on rheology and cell mechanics see for instance (Haase & Pelling, 2015; Wirtz, 2009; Yao, Tassieri, Padgett, & Cooper, 2009)), computational approaches (see (Katira et al., 2013) for a review) or techniques that have been used in the field of cell mechanics but not in the context of cancer are not detailed in the following. This is the case for instance of the uniaxial single cell microplate rheometer (Thoumine & Ott, 1997b); (N. Desprat, 2006), the recent method using magnetic nanowires (Berret, 2016; Chevy, Colin, Abou, & Berret, 2013) or acoustic microscopy (Nijenhuis, Zhao, Carisey, Ballestrem, & Derby, 2014). Techniques that measure the stiffness of a tissue and do not resolve individual cells, such as ultrasound elastography (Chamming's et al., 2013; Tanter et al., 2008) or magnetic resonance elastography (MRE) (Pepin, Ehman, & McGee, 2015) will also not be discussed in detail here. The strength of these techniques is to allow a non-invasive mechanical characterization of whole tumors and their surrounding tissues *in vivo* as opposed to the techniques we describe below. For instance, MRE has been used to probe several organs, such as breast, liver, brain or prostate. It was shown that malignant invasive breast tumors are about twice stiffer than benign breast lesions (Lorenzen et al., 2002). Similar results were obtained with liver or prostate tumors (Garteiser et al., 2012; S. Li et al., 2011; Venkatesh et al., 2008), confirming that tumor stiffening correlates with invasiveness. In contrast, a recent MRE study on intracranial tumors showed that tumorous tissues are softer than normal white matter and that grade IV glioblastomas multiforme are slightly softer than grade III anaplastic astrocytoma (Reiss-Zimmermann et al., 2015).

Rheological techniques can be grouped according to the scale at which they measure cellular mechanics (Fig. 1). At the scale of the whole cell, mechanical parameters integrate the contribution of all cellular elements including potential matrix components, the plasma membrane and its

associated cortex, the cytoskeleton, the nucleus, cytosolic constituents as well as intracellular membrane compartments. At more local scales, depending on the size of the probe used for the measure, only some specific cellular elements may contribute to the mechanical parameters. Micropipette aspiration (Lee & Liu, 2014; Pachenari *et al.*, 2014) or the so-called optical stretcher (Guck *et al.*, 2005) fall in the first category. With these devices, cells in suspension are required. Because a mechanical stretch is applied on the cells, the actin cortex probably contributes predominantly to the extracted physical parameters. Microfluidics devices consisting of microchannels inside which cells have to enter and flow have also been developed to investigate the deformability of normal and cancer cells at the whole cell level (Byun *et al.*, 2013; Hou *et al.*, 2009).

Devices in the second category measure cell rheology of adherent cells by imposing a local deformation (strain) or a force (stress) either extracellularly or intracellularly. Techniques based on AFM have now become widely used and provide measurements of the elastic modulus of a region of interest on the plasma membrane by indenting a probe located at the extremity of the AFM cantilever (see (Mackay & Kumar, 2013; Okajima, 2012); (Lekka *et al.*, 2012) for reviews). Probes of a few 10-70 nanometers in diameter explore very local scales (Cross, Jin, Rao, & Gimzewski, 2007; Lekka *et al.*, 1999; Y. Li, Schnekenburger, & Duits, 2009; Plodinec *et al.*, 2012; Prabhune, Belge, Dotzauer, Bullerdiek, & Radmacher, 2012; Rother, Nöding, Mey, & Janshoff, 2014), but in other studies, micron-sized beads or cells have also been used as tip probes (Bastatas *et al.*, 2012; Darling, Zauscher, Block, & Guilak, 2007; Grady, Composto, & Eckmann, 2016; Ketene, Schmelz, Roberts, & Agah, 2012; Omidvar *et al.*, 2014; S. Park, Koch, Cardenas, Käs, & Shih, 2005; Xu *et al.*, 2012). Similar to AFM-based techniques, indentation of magnetic beads localized on the cell surface using a magnetic field has been used to characterize ovarian cancer cell lines (Swaminathan *et al.*, 2011). In both AFM and magnetic bead indentation, the mechanical measurements are dominated by the rigidity of the plasma membrane and the cortical actin cortex. In contrast, intracellular microrheology quantifies the mechanical properties of the cell interior and the contribution of intracellular elements, including cytoskeletal elements, membrane organelles and soluble components of the cytosol, at typical scales below a few micrometers. Passive intracellular microrheology is performed by tracking the movement of endogenous vesicles or granules or of an external probe internalized inside the cell by endocytosis, microinjection or ballistic injection (Tseng, Kole, & Wirtz, 2002; Wirtz, 2009; Wu *et al.*, 2012; Yamada, Wirtz, & Kuo, 2000). As an example, passive rheology has been used in combination with AFM to characterize breast and pancreas cancer cells (Y. Li *et al.*, 2009). In active intracellular microrheology, a force is applied on the same endogenous or exogenous probes and the resulting movement of the probe is monitored. The force is most often generated by optical or magnetic tweezers and is typically in the pico- to nano-Newton range. Several recent applications have been developed in the field of cancer (Berret, 2016; Guet *et al.*, 2014; Guo *et al.*, 2013).

Rheological parameters and theoretical modelling

Generic terms such as 'soft', 'rigid' or 'deformable' are often used to characterize cell mechanics, even if they do not represent any actual rheological parameter. In the field of rheology, a variety of physical parameters can be measured experimentally, depending on the experimental protocol and the model with which the experiments are interpreted. The two experimental observables are the stress, defined as the force applied on the material over a given cross section area (unit Pa), and the

(dimensionless) strain, defined as the deformation of the stressed material relative to a typical length scale of the system (Fig. 2A). Experimentally, either the strain under a given stress (the so-called creep experiment) or the stress under a given strain (the so-called stress relaxation experiment) can be measured to yield the visco-elastic properties of the material (Fig. 2B).

Visco-elastic materials show behaviors typical of both elastic solids and viscous fluids (Fig. 2C). A Hookean elastic solid is characterized by its spring constant K (in N/m) or its Young modulus E (Pa) and can deform instantly. The strain is proportional to the applied stress. A viscous Newtonian fluid is characterized by its viscosity η (Pa.s) and can flow and undergo unlimited deformation. The flow, i.e. the time derivative of the strain, is proportional to the applied stress. A visco-elastic material responds to deformation both as an elastic solid and a viscous fluid with the ability to deform rapidly and to flow. The ratio between the viscosity and the Young modulus yields a time constant $\tau = \eta/E$ called the relaxation time is used to determine at which time scales the visco-elastic fluid behaves more as a fluid or as a solid. Depending on the mode of deformation, different mechanical moduli can be extracted. If a shear stress is applied (the force is parallel to the cross section), the complex shear modulus G or the creep compliance $J = 1/G$ of the material is measured (Fig. 2A-B). If an extension or a compression stress is applied (the force is normal to the cross section), the Young modulus E of the material is measured. The Young modulus relates to the elasticity of the material, while the complex shear modulus $G = G' + iG''$ contains information on both elasticity and viscosity as its real part G' is related to elastic energy storage and its imaginary part G'' is related to viscous energy loss.

Creep or stress relaxation experiments typically yields data in the time domain that have been analyzed using classical viscoelastic models based on the association of a finite number of dashpots and springs. The simplest models associate one spring and one dashpot either in series (Maxwell model) or in parallel (Kelvin-Voigt model). The expected responses of a visco-elastic fluid following these models in a creep experiment are represented in Figure 2C. Models associating more springs and dashpot have also been proposed, such as the Standard Linear Liquid (SLL) –also called the Jeffrey model- or the Standard Linear Solid (SLS) models (Swaminathan et al., 2011); (G. Zhang, Long, Wu, & Yu, 2002); (Darling et al., 2007); (Pachenari et al., 2014). Fitting the data gives the values of the spring constants (in N/m) and of the viscosities (in Pa.s) corresponding respectively to the springs and dashpots included in the model. Depending on the number of dashpots and springs, several relaxation times can be calculated as $\tau_{i,j} = \eta_i/E_j$. Obviously, the quality of the fits increases with the number of viscoelastic elements. However, models with a finite number of relaxation times appear to be too simple to describe cell rheology and models with a continuous distribution of time constants that lead to power-law behaviors have been proposed as described below.

In active microrheology techniques in which an oscillating stress is applied at different frequencies, for instance with the uniaxial rheometer or optical tweezers, the frequency dependence of the complex shear modulus $G(\omega)$ is measured. It is now widely accepted that $G(\omega)$ follows a weak power-law $G(\omega) = A\omega^\alpha$ (Desprat, Richert, Simeon, & Asnacios, 2005; Fabry et al., 2001) both at the whole cell scale and at intracellular local scale, independently of the experimental technique or the cell type (Balland et al., 2006). Still, one study in a hepatocyte carcinoma cell line (Hep G2) shows that a power-law cannot describe well the solid behavior of the cell at low frequencies (B. Wang et al., 2013). Several theoretical models of cell rheology predict that $G(\omega)$ should follow a power-law. In the poroelasticity model, the cell cytoplasm is a biphasic material made of a porous elastic meshwork in a fluid obeying a power law at long time scales

(Moeendarbary *et al.*, 2013). Similarly, power-law stems from soft glassy materials (Kollmannsberger & Fabry, 2009). Even if some reservations have been expressed concerning specific aspects of the soft glassy model (Mandadapu, Govindjee, & Mofrad, 2008; Stamenović *et al.*, 2007), this model is viewed as one of the best to describe cell rheology accurately. A power-law behavior is the signature of a continuous distribution of time constants and cannot arise from discrete models consisting of a finite number of elements. Accordingly, the tensegrity (tensional integrity) model which consists of modelling the cytoskeleton as a stabilized network of prestressed rods ((N. Wang *et al.*, 2001) does not predict the observed power-law dependence of $G(\omega)$ (Ingber, Wang, & Stamenovic, 2014)).

Despite the popularity of viscoelastic models and power-law models, in some experimental techniques, cell rheology can also be measured independently of any model using one or several parameters specific to the experimental technique. For instance, the deformability rate was defined in optical stretcher experiments to quantify cell deformability (Guck *et al.*, 2005). The entry time into microchannels is measured in microfluidics devices (Hou *et al.*, 2009). A so-called rigidity index has been introduced to quantify relaxation experiments using optical tweezers (Guét *et al.*, 2014; Mandal, Asnacios, Goud, & Manneville, 2016). One drawback here is that knowing how the new parameters relate to the Young modulus and/or to the complex shear modulus is not trivial. In any case, cells are complex materials and it seems difficult to describe their mechanics only with a few moduli (Ananthakrishnan *et al.*, 2006). Since several concurrent models are available to analyze the data, one remaining question is how to choose a 'good' model. Of course, the fits performed with the chosen model should adjust the data well, given the experimental errors, on the largest time, frequency and length scales. A 'good' model should also have a small number of free parameters. Because there are only two adjustable parameters in a power-law model (the prefactor A and the exponent α), power-laws have become increasingly popular. To compare the quality of different models to describe the data, some recent studies have used more than one model in their analysis (Chan *et al.*, 2015; Lim, Zhou, & Quek, 2006). In the following, the terms 'soft' or 'deformable' will be used to qualify cellular materials of low elasticity and/or low viscosity hence low values of the Young modulus or of the shear modulus.

2 Cancer cells really appear to be softer than normal cells

Palpation is often the first and simplest way to detect a tumor, for instance in breast tissues, before a more reliable diagnosis by biopsy. It is well accepted that tumor tissues, at the scale of the whole organ, are stiffer than their normal surrounding environment. Working with entire mammary gland, Levental *et al.* showed that the elastic modulus of the tissue indeed increases with tumorigenesis (Levental *et al.*, 2009). However, at the level of the single cell, as discussed above, several lines of evidence suggest that cell stiffness should decrease as a normal cell transforms into a cancer cell. In this section, we review the studies comparing the mechanical properties of cancer cells and normal cells. To our knowledge, very few studies showed that cancer cells may be stiffer than normal cells (see below). The large majority of experiments, whether comparing cells from different organs, from the same organs or even comparing cancer cells of different grades, indicate that cancer cells are softer than normal cells and that cellular rigidity decreases with the progression of the disease.

Only few studies show that cancer cells are stiffer

The first study showing a potential stiffer behavior of cancer cells was made in hepatocellular carcinoma cells (G. Zhang *et al.*, 2002). The authors show that hepatocellular carcinoma cells have higher elastic constants than hepatocytes. However, they appear to have a lower viscosity. Moreover, working with cancer prostate cell lines, Bastatas *et al.* showed that low grade metastatic cells have higher elastic moduli than highly metastatic cells (Bastatas *et al.*, 2012). Less strikingly, Li *et al.* worked with two cell lines of different grades derived from one human pancreas adenocarcinoma (Y. Li *et al.*, 2009). Using AFM, no significant differences in the Young modulus between both cell lines was found whereas a larger mean squared displacement (MSD) was measured for the malignant cell line using passive intracellular microrheology. These are the only papers we know of where cancer cells appear to be more rigid than healthy cells, even if the results are not always statistically very significant. All the other studies focusing on individual cells show that cancer cells are softer than healthy cells.

Mechanics of normal cells vs. cancer cells from the same organ

Most studies in the field have been conducted with breast cancer cells (Agus *et al.*, 2013; Guo *et al.*, 2013; Hou *et al.*, 2009; Y. Li *et al.*, 2009). Fibroblasts (Thoumine & Ott, 1997a); (Wottawah *et al.*, 2005); (S. Park *et al.*, 2005), hepatoma cell lines (G. Zhang *et al.*, 2002), prostate cancer cells (Faria *et al.*, 2008), kidney cell lines (Rebelo, de Sousa, Mendes Filho, & Radmacher, 2013) and bladder cancer cells (Bertseva *et al.*, 2012; Lekka *et al.*, 1999) have also been studied. Cell lines from patients with primary oral or thyroid cancer were used for the first time to our knowledge by Remmerbach *et al.* (Remmerbach *et al.*, 2009) and Prabhune *et al.* (Prabhune *et al.*, 2012) respectively. Together, these results obtained with different techniques, indicate that the Young modulus and the spring constant of tumor cells are lower than those of normal cells and that in a large majority of cases, elastic storage (G') dominates over viscous loss (G'') (see Table 1).

Mechanics of normal cells vs. cancer cells from different organs

Even if a relevant comparison of the mechanical properties of normal cells and cancer cells obviously requires comparing cells from the same organ, some AFM studies compared normal and cancer cells originating from different organs and also found that cancer cells are softer and more deformable. The elastic moduli of three normal cell lines (dermal fibroblasts, chondrocytes, HUVECs) and two cancer cell lines (HT180 fibrosarcoma and HUH-7 hepatocellular carcinoma) were measured by Grady *et al.* (Grady *et al.*, 2016). The data show that both cancer cells have a modulus below 0.5 kPa while moduli of the normal cell lines are above 1.3 kPa. A similar trend was observed when pleural fluids of seven patients with suspected adenocarcinoma from different locations (lung, breast, pancreas, liver and kidney) were studied (Cross *et al.*, 2007). Cytological examinations revealed that four patients were positive for metastatic malignant cells whereas the three others had negative results. The authors pooled the data collected from the seven samples representing 40 cells from tumor samples and 48 cells from normal samples. For cancer cells, a Gaussian fit showed that tumor cells have a mean Young modulus of about 0.5 kPa while a log-normal fit indicated that normal cells have a modulus of about 2 kPa (Cross *et al.*, 2007). Interestingly the distribution of moduli in cancer cells seems to be narrower than in normal cells, suggesting a lower variability in cancer cells (Cross *et al.*, 2007; Grady *et al.*, 2016; Xu *et al.*, 2012).

Mechanics of high grades vs. low grades cancer cells

In order to correlate cell stiffness with malignancy, cell mechanics has been measured in different grades of cancer cells from the same type of cancer. Here again, breast cancer is the most examined cancer type (Gal & Weihs, 2012; Guck *et al.*, 2005; Omidvar *et al.*, 2014; Swaminathan *et al.*, 2011; Xu *et al.*, 2012), but cell lines from other organs such as colon (Pachenari *et al.*, 2014), ovaries (Ketene *et al.*, 2012) or chondrosarcomas (Darling *et al.*, 2007) have also been investigated. The Young modulus and spring constants were shown to decrease with cell malignancy and the deformability rate in optical stretcher experiments increases (see Table 1), showing that cell stiffness inversely correlates with the progression of the disease. A similar behavior was found in primary cells (Plodinec *et al.*, 2012; Swaminathan *et al.*, 2011). Accordingly, cytoskeletal fluidity measured by magnetic twisting cytometry was also shown to increase with the metastatic potential in a variety of cancer cell lines and in cells transformed with oncogenes (Coughlin *et al.*, 2013). However, one AFM study in prostate cancer cells measured a higher Young modulus in highly metastatic cells than in non-invasive cells (Faria *et al.*, 2008).

To conclude, these results (summarized in Table 1) clearly indicate that, even if the absolute values of the mechanical moduli may vary significantly depending on the microrheology technique, cancer cells from a large number of different organs are softer than their normal counterparts. Moreover, with only a few exceptions (Darling *et al.*, 2007; Faria *et al.*, 2008), the decrease in stiffness in cancer cells correlates with their metastatic potential.

3 Why are cancer cells softer than normal cells?

As detailed above most reports show that individual cancer cells are softer than normal cells. We now try to identify which cellular elements could be involved in these mechanical changes (Fig. 3). The role of the extracellular environment and of the actin cortex in cell mechanics is well-known and has been reviewed previously (Fletcher & Mullins, 2010; Handorf, Zhou, Halanski, & Li, 2015; Salbreux, Charras, & Paluch, 2012) but other cellular constituents could clearly contribute to the observed rheological differences. We focus here more on the intracellular elements that could explain why cancer cells appear to be softer than normal cells.

The cell microenvironment

For the last ten years, mounting evidence shows that the cell microenvironment influences cell behavior. In particular, cells sense the stiffness of the matrix (Lange & Fabry, 2013). Several studies have shown that the matrix stiffness controls lineage specification of mesenchymal stem cells (Engler, Sen, Sweeney, & Discher, 2006), fibroblast polarization (Prager-Khoutorsky *et al.*, 2011), stress fiber orientation (Zemel, Rehfeldt, Brown, Discher, & Safran, 2010) and ordering of the cytoskeleton from a fluid state on soft substrates to a more solid state on stiff substrates (Gupta *et al.*, 2015). In the context of cancer, the tumor microenvironment also influences the behavior of tumor cells (Butcher *et al.*, 2009; Handorf *et al.*, 2015; Tee, Fu, Chen, & Janmey, 2011). For instance, the mechanical properties of the extracellular matrix (ECM) plays an important role in the metastatic process as stiffening of the ECM was shown to promote growth and invasion of mammary tissues both *in vivo* and in culture (Levental *et al.*, 2009). More interestingly in the context of tumor cell

rheology, a 2D or 3D collagen-environment impacts on the intracellular rigidity of malignant cells (Baker, Bonnacaze, & Zaman, 2009). In 3D, a higher matrix stiffness surrounding isolated prostate cancer cells leads to a decrease in the apparent elastic modulus which is an order-of-magnitude higher in 3D than in 2D (Baker et al., 2009). Stiffer 3D environments also promote integrin adhesion, cytoskeletal tension, actin stress fiber formation and cell growth via a signaling pathway involving ERK and ROCK in breast tumor cells (Paszek et al., 2005).

The cytoskeleton

The cytoskeleton is known to play a major role in cell mechanics (Fletcher & Mullins, 2010; Salbreux *et al.*, 2012). Several works comparing the stiffness of normal and cancer cells have thus investigated cytoskeletal organization in cancer cells and tried to correlate changes in the levels of expression of cytoskeletal proteins with the observed decrease in cell stiffness. Most studies have focused on the actin cytoskeleton, especially on the actin cortex at the plasma membrane and on actomyosin complexes, but interest in microtubules and intermediate filaments has also been growing rapidly in the past two decades.

Actin

Modifications of the actin cytoskeleton during the metastatic process are well described (Nürnberg, Kitzing, & Grosse, 2011; Peckham, 2016; Vignjevic & Montagnac, 2008). Notably the relative amount of filamentous actin (F-actin) and monomeric actin (G-actin), which indicates the level of actin polymerization, depends on the cell malignancy. However conflicting results have been reported which seem to be cell-type dependent. For instance, in colon cancer cell lines of different metastatic potential, Nowak *et al.* found that cancer cells derived from the cancerous parental cell line have less G-actin (Nowak, Krawczenko, Duś, & Malicka-Błaszkiwicz, 2002), while the opposite result was observed in keratinocytes and an increase in the G-actin/total actin ratio with invasiveness and metastatic potential was measured (Katsantonis et al., 1994). Metastatic colon cancer cells appear to have increased actin levels (Pachenari *et al.*, 2014). More significantly, the organization of the actin cytoskeleton appears to be different in cancer cells. In breast and ovarian cancer cells and in thyroid cells, the actin network is less dense and contains less stress fibers (Calzado-Martín, Encinar, Tamayo, Calleja, & San Paulo, 2016; Gal & Weihs, 2012; Ketene et al., 2012; Prabhune et al., 2012). Similar observations were made in metastatic MDA-MB-231 breast cancer cells plated on adhesive micropatterns to standardize their intracellular organization when compared to non-tumorigenic MCF-10A cells, with changes in actin organization correlating with a strong decrease in cell stiffness in the metastatic cell line (Mandal *et al.*, 2016). Interestingly, disrupting actin filament polymerization in normal cells induces a decrease in cell stiffness but also reduces the variability of the mechanical measurements (Cai *et al.*, 2013; Grady *et al.*, 2016) as observed in cancer cells (Cross *et al.*, 2007; Grady *et al.*, 2016; Xu *et al.*, 2012).

Microtubules

The role of microtubules in cell rigidity seems less clear than that of actin and, here again, could be cell-type dependent. Microtubule depolymerization by nocodazole only resulted in a slight increase in stiffness in a variety of normal and cancer cells (Grady *et al.*, 2016). A small but opposite effect of nocodazole was observed in retinal pigment epithelial (RPE-1) cells (Mandal *et al.*, 2016) and in HeLa

cells (Wilhelm, Gazeau, & Bacri, 2003), while microtubule stabilization by taxol induced a clear increase in stiffness in RPE-1 cells (Mandal *et al.*, 2016). Even if overexpression of $\beta 3$ tubulin correlates with tumor malignancy and resistance to chemotherapeutic drugs (Ferrandina *et al.*, 2006), whether this is due to a change in cell mechanics has not been investigated. The amount of microtubules relative to actin filaments differs in different grades of colon cancer cells but this difference seems to be due to the total amount of actin (Pachenari *et al.*, 2014). The organization of the microtubule network in three breast cancer cell lines with different metastatic potential did not show any significant variations (Calzado-Martín *et al.*, 2016). However, when non-tumorigenic MCF-10A and metastatic MDA-MB-231 breast cancer cells were plated on adhesive micropatterns, subtle differences in microtubule distribution could be revealed with a more uniform and less intricate network in the metastatic cells (Mandal *et al.*, 2016).

Intermediate filaments

There are six types of intermediate filaments (IFs) differentially expressed depending on the cell type and the IF proteins that compose the filaments. Among these proteins, vimentin, a type III IF protein, is a marker of the EMT and its level of expression increases in cancer cells (Ngan *et al.*, 2007; Ye *et al.*, 2016). IFs have been shown to participate to cell mechanics (Block, Schroeder, Pawelzyk, Willenbacher, & Köster, 2015; Charrier & Janmey, 2016). In particular, knocking-out vimentin in mouse embryonic fibroblasts was shown to decrease cell stiffness (Guo *et al.*, 2013). This seems contradictory to the numerous results we mentioned above showing a decrease in cell stiffness in cancer cells. However, the situation is probably more complex than it seems and correlating the amount of IF proteins with cell rigidity is difficult. For instance, by measuring the vimentin levels in different cancer cells lines, Swaminathan *et al.* reported that stiffer cancer cell lines express less vimentin than compliant cell lines (Swaminathan *et al.*, 2011). In breast cancer cells, vimentin is not expressed in normal M10 and non-metastatic MCF-7 cancer (less deformable) cells, while highly metastatic MDA-MB-231 (more deformable) cells have high endogenous levels of vimentin (C. Y. Liu, Lin, Tang, & Wang, 2015). However, reducing the amount of vimentin in MDA-MB-231 cells decreases cell stiffness while overexpressing vimentin in MCF7 cells increases cell stiffness (C. Y. Liu *et al.*, 2015) as expected if vimentin IFs contribute to cell stiffness.

Similarly to vimentin, keratin IF proteins are involved in cell mechanics and their expression is modified during EMT. The 54 keratins are type I and type II IF proteins. The levels of keratin proteins are downregulated in carcinoma during EMT in epithelial cancers (Savagner, 2010). In breast cancer cell lines, high tumor grade was associated with a loss of cytokeratin and expression of vimentin (Willipinski-Stapelfeldt *et al.*, 2005). Like vimentin, keratins have been used as diagnosis markers of cancer (Karantza, 2011). Most cancer cells are keratin-positive, but different keratins are expressed depending on the tumor type. For instance, colorectal adenocarcinomas appear to be K20+/K7- while malignant salivary gland carcinomas are K7+/K20- (Karantza, 2011). Downregulation of keratin during EMT may play a role in cancer cell softening. In keratinocytes lacking the entire keratin cytoskeleton, optical stretcher experiments demonstrate a clear increase in cell deformability that correlates with increased invasiveness (Seltmann, Fritsch, Käs, & Magin, 2013). The spatial organization of keratin filaments also seems to play a major role. In pancreatic tumor cell lines, the keratin IF network is reorganized in the perinuclear region which induces a decrease in the elastic modulus (Suresh *et al.*, 2005).

Even if vimentin and keratins are involved in cancer cell mechanics, their exact roles and how they are related are not entirely clear yet. Since both keratin depletion (Seltmann et al., 2013) and vimentin depletion (Guo et al., 2013) induce cell softening while vimentin is expressed and keratins are downregulated during EMT, it is not obvious that cells should soften during EMT. Moreover in human melanoma cell lines, in contrast with the classical picture, vimentin and keratin appear to coexpress and the level of coexpression correlates with invasiveness (Hendrix M. J. C. & 1992). Similarly, patients with breast tumors expressing both keratin and vimentin in similar amounts have poorer prognosis and survival rates (Thomas et al., 1999). It is very probable that IF proteins from other classes will be identified as contributing to cell stiffness. The class V nuclear lamins in particular are known to contribute to nuclear mechanics (see below). Since IFs are heteropolymers and bundle within the cell, compensation mechanisms between the different IF proteins may play a role in mechanics when the expression of IF proteins is modified.

The nucleus

At the scale of the whole cell, the nucleus is a major organelle contributing to cell mechanics (Zwerger, Ho, & Lammerding, 2011). As an example, the deformability of the nucleus is the limiting factor when cells migrate through tight constrictions (Lautscham et al., 2015; McGregor, Hsia, & Lammerding, 2016; Raab et al., 2016). In cancer cells, the nucleus morphology is altered compared to healthy cells (Denais & Lammerding, 2014). The volume of the nucleus is larger and its shape is more irregular. The expression of nuclear envelope proteins such as lamins is modified leading to breakage of the nuclear envelope (Bell & Lammerding, 2016; Davidson & Lammerding, 2014). Together these modifications of the nucleus could contribute to the decrease in stiffness observed in cancer cells and could facilitate the migration of cancer cells through narrow pores in the ECM.

The plasma membrane

The bending rigidity of the plasma membrane could contribute to the overall cell stiffness. The rigidity of the plasma membrane is controlled by its lipid composition and lipid synthesis and metabolism are known to be implicated in tumor development (Baenke, Peck, Miess, & Schulze, 2013). More than twenty years ago, Mittelman et al. showed a correlation between the metastatic potential of lymphoma derived cell lines and their bending rigidity through measurements of membrane shape fluctuations (Mittelman, Levin, Verschueren, Debaetselier, & Korenstein, 1994). Fluctuations were larger in cell lines with the highest metastatic potential. Supposing that fluctuations are of thermal origin, larger fluctuations indicate a lower rigidity in the highly metastatic cells. Softening of the plasma membrane was also reported recently in breast and cervical cancer cells using fluctuation analysis of membrane blebs or of giant plasma membrane vesicles (Händel et al., 2015). Conversely a pharmacological inhibitor that increases membrane rigidity also decreases invasiveness of a mammary carcinoma cell line (Braig et al., 2015). Interestingly, thermal noise is not the only source of membrane shape fluctuations and active non-equilibrium energy-dependent forces are thought to enhance such fluctuations (Betz, Lenz, Joanny, & Sykes, 2009; Boss et al., 2012; Manneville, 1999; Tuvia, Levin, Bitler, & Korenstein, 1998). A softening of the plasma membrane in metastatic cancer cells could thus also be due to a higher non-equilibrium activity.

Intracellular compartments and internal membranes

Besides the nucleus, other intracellular compartments could also participate in intracellular rigidity. It was shown for instance that the local intracellular stiffness increases in the proximity of the Golgi apparatus (Guet *et al.*, 2014). Moreover, dispersion of Golgi membranes by brefeldin-A has recently been reported to slightly increase intracellular rigidity (Mandal *et al.*, 2016). Interestingly, similarly to experiments performed at the scale of the whole cell (Bonakdar *et al.*, 2016), when a stress is applied on the Golgi apparatus, this organelle does not relax back to its original shape and actin contributes to its rheology (Guet *et al.*, 2014). To date no other data directly support a role for intracellular compartments in cell mechanics, but given the cytosolic abundance of internal membranes from the endoplasmic reticulum, the endosomal or lysosomal compartment or the mitochondrial networks, it would be surprising that internal membranes do not significantly contribute to intracellular stiffness (Ohashi, Hagiwara, Bader, & Knight, 2006). Since several signaling pathways involving membrane trafficking or mitochondria are affected during cancer cell transformation (Fehrenbacher & Jäättelä, 2005; Goldenring, 2013; Kamp, Shacter, & Weitzman, 2011; X. Wang, Peralta, & Moraes, 2013), internal membranes may also be implicated in the decrease in cancer cell stiffness.

Signaling proteins

The interplay between cellular rigidity and the signaling pathways associated with the cytoskeleton and membrane trafficking is complex. From a fundamental point of view, understanding the modifications in cell rigidity in cancer cells could help identifying which signaling pathways or proteins are involved during tumorigenesis. For example, it has been shown that ROCK-dependent modifications of the keratin network play a role in cell stiffness (Bordeleau, Myrand Lapierre, Sheng, & Marceau, 2012). Another example is given by the adhesion protein E-cadherin, an epithelial marker which levels differ in healthy and cancer cells. Breast highly invasive MDA-MB-231 (softer) cancer cells do not express E-cadherin but express N-cadherin instead, while non-invasive MCF-7 and slightly invasive T470 (stiffer) cell lines exhibit E-cadherin levels that inversely correlate with their invasiveness (Omidvar *et al.*, 2014). A similar correlation was observed in ovarian cancer cell lines with less invasive, stiffer cells expressing more E-cadherin (Swaminathan *et al.*, 2011).

Non-equilibrium active forces

In recent years, the role of active forces, such as the forces exerted by ATP-consuming molecular motors, in cell mechanics has attracted a lot of attention. From a statistical physics point of view, because they require an energy source, these out-of-equilibrium forces are of a different nature from thermal passive forces. Recently, a new technique based on optical tweezers called Force Spectrum Microscopy has been introduced to measure the effect of fluctuating forces caused by active processes on the cytoplasm rheology (Guo *et al.*, 2014). Using this technique, the authors confirmed that malignant breast cancer cells (MCF-7 and M6C cells) have a softer cytoplasm than benign cells (MCF-10A and M28 cells) and that this decrease in stiffness correlates with higher intracellular forces, suggesting that non-equilibrium active forces may be enhanced in cancer cells. In cancer cells, ATP is predominantly produced by glycolysis rather than by mitochondria as it is the case for normal cells (Hanahan & Weinberg, 2011). However the link between ATP-dependent forces and cell rigidity is not clear and conflicting results have been reported. Optical tweezers active microrheology using 0.5 μm diameter beads oscillating in the 0.1-100 Hz frequency range has shown

that ATP depletion softens the cell interior about two-fold (Guo *et al.*, 2014), while an opposite effect was measured with a protocol based on the relaxation of 2 μm diameter beads within an optical trap (Mandal *et al.*, 2016). The last result is consistent with the lower tracer particle displacement and lower intracellular forces measured in the absence of ATP (Guo *et al.*, 2014). The influence of myosin II activity on cancer cell mechanics was investigated more specifically by inhibiting myosin II with blebbistatin or downregulating myosin II. In adherent ovarian cancer cells and melanoma cells, blebbistatin decreases intracellular stiffness and increases invasiveness (Guo *et al.*, 2014; Swaminathan *et al.*, 2011). In contrast, a recent study in a variety of cells in suspension shows that myosin II inhibition increases cell stiffness measured by optical stretcher experiments (Chan *et al.*, 2015), pointing to a central role of cell adhesion components, such as focal adhesions, or actin stress fibers, in controlling cell stiffness.

Concluding remarks and open questions

Most studies which have compared the mechanics of individual normal and cancer cells have found that cancer cells are softer than healthy cells. More importantly, the decrease in stiffness seems to correlate with the progression of the disease. Cell rigidity is not the only physical parameter that is modified in cancer cells. Cell adhesion, traction forces or internal tensile stresses are also affected. The mechanical properties of cancer cells could thus be used as novel diagnostic and/or prognostic markers to complement histological exams and genetic phenotyping of the tumor. To achieve this aim, several technical limitations and conceptual issues will first have to be resolved.

First, studies in the field should now concentrate on primary tumor cells from patients to validate the use of cell mechanics as a relevant diagnosis or prognosis tool. Until now, most of the work has been performed in cancer cell lines, some of which were established several decades ago. The grade of a given cell line is evaluated based on the World Health Organization Classification ("World Health Organization Classification of Tumours," 2004). Even if this classification is regularly updated, the grades of some of the earlier cell lines, which have most often been deduced from histological observations, may no longer be accurate. Moreover cell culture conditions and the number of cell passages can induce cross-contaminations and genetic drifts, or even alter the mechanical properties of the cells (Prabhune *et al.*, 2012; Torsvik *et al.*, 2014). Primary cancer cells from biopsies should provide more relevant systems despite the possible technical difficulties in maintaining them in culture. In recent years, patient derived xenografts (PDX) and patient derived tumor cells (PDTc) have been increasingly used as validated models for different types of cancer (Cassidy, Batra, Greenwood, & Bruna, 2016; Xie *et al.*, 2015). In the field of cancer cell rheology, the next step clearly involves the use of such models. The dimensionality may also play a crucial role (Mak, Kamm, & Zaman, 2014). Until now, most studies have been performed on suspended cells or on cells in 2D cultures. Efforts should now be directed towards rheological measurements on cells in a 3D environment.

However, since cells can adapt very rapidly to their environment, cell softening could be due to the fact that cells are studied in an artificial *in vitro* environment and not in their native tumor environment. As discussed above, cancer cell lines may have different properties from their original tumor cells due to their culture conditions. Patient-derived cells or cells obtained by fine needle

aspiration from solid tumors are extracted from the primary tumor and transferred to a new environment. The mechanical parameters measured *in vitro* may thus not be representative of the mechanical state of the cells inside the tumor. However because significant differences are observed between normal and cancer cells, *in vitro* measurements could still be useful for diagnosis and/or prognosis. Cell softening seems also at odds with findings showing that tumor cells have a higher contractility and exert higher traction forces. But again, this may be induced by the stiffer tumor environment or depend on the type of cancer.

Even if the different microrheology techniques qualitatively give similar results by showing that cancer cells are softer than normal cells, the absolute values of the measured physical parameters appear to vary significantly, even between studies performed with the same instrument (Table 1). Measurements on suspended cells obtained with the optical stretcher or external measurements obtained with AFM may be dominated by the actin cortex and may not fully probe the internal cytoskeleton which is predicted to be more contractile in cancer cells. Associating several techniques, especially techniques operating at different scales, should help resolve this issue. For instance, AFM has been applied together with particle tracking (Agus et al., 2013; Y. Li et al., 2009) or with acoustic microscopy (Nijenhuis *et al.*, 2014). The variability of the measurements is mostly due to variability in cell morphology, for instance cell size or volume, spread area of adherent cells, positioning of intracellular organelles or nucleus size. This is particularly true for techniques that operate at local scales. For example, the results of AFM experiments not only strongly depends on the zone which is probed in the cell, but also on the cell shape and on the geometry of the probe tip used for indentation (Darling *et al.*, 2007). To standardize cell geometry and organization, adhesive micropatterns have been combined with AFM (Mandal et al., 2016; Rigato, Rico, Eghiaian, Piel, & Scheuring, 2015) or with optical tweezers microrheology (Mandal et al., 2016; Rigato et al., 2015). Micropatterning allows a better reproducibility of the experiments but requires a fine tuning of the cell aspect ratio (volume/surface) with the micropattern area to ensure meaningful intracellular mechanical measurements. The temperature is also a critical parameter in rheological experiments. Cell heating in optical tweezers has been known for a long time (Y. Liu et al., 1995) but has recently been used in thermorheology experiments based on an optical stretcher to assess the mechanical parameters of breast cancer cells (Kießling, Stange, Kas, & Fritsch, 2013; Schmidt et al., 2015).

There are several requisites for an efficient diagnostic method for cancer. First, techniques requiring a low amount of tissue or ideally allowing *in situ* diagnosis are highly advantageous. Time is also an important factor and measurements and subsequent data analyses should be fast. Finally, the technique should yield statistically significant data. When working with individual cells, large ensemble statistics can be obtained with high throughput methods. High-throughput analysis of cell rheological properties has been comprehensively reviewed recently (Darling & Di Carlo, 2015; Weaver et al., 2014). Microfluidic approaches and optical-tweezers based approaches are most amenable to high-throughput. Measurement of the transit time of cells through constrictions or channels combined with microfluidic flow (Byun et al., 2013; Hou et al., 2009; Lange et al., 2015) allows the analysis of 1-100 cells/s (Darling & Di Carlo, 2015). Stretching cells using hydrodynamic stresses (Dudani, Gossett, Tse, & Di Carlo, 2013) gives the highest analysis rates, up to 20000 cell/s. The optical stretcher has also recently been developed into a high-throughput technique with typically 1 cell/min to 1 cell/sec rates of measurement of cell deformability (Darling et al., 2007; Otto et al., 2015). Other approaches based on acoustic waves, osmotic shocks or automation of AFM have

been devised (Darling & Di Carlo, 2015) but their application to cancer cell mechanics remain limited. Of note, coupling standing acoustic wave with microfluidics showed that cancer cells of various origins are more compressible than normal cells (Hartono et al., 2011).

A limiting factor here may be tumor heterogeneity (Meacham & Morrison, 2013). Differences not only exist between tumors of the same type in different patients, but also between cells within a tumor. For a given stage in cancer development, cells of different grades can coexist, depending on their localization within the tumor. Correlating with known genetic or epigenetic differences, mechanical differences may appear. Cells of the same grade could thus have different mechanical properties. Moreover, soft and stiff regions coexist in a tumor, as shown for instance by MRE (Pepin, Ehman, & McGee, 2015), which may contain both soft and stiff cells and/or extracellular matrix. Even if malignancy seems to correlate with the decrease in cell stiffness, the tumor grade may not be the relevant parameter for a precise mechanics-based diagnosis. In this context, can cell rheology really lead to new diagnostic or prognostic methods for cancers? Clearly several technical challenges still remain to be overcome. However given the evident mechanical differences between normal and cancer cells, chances of success in a near future are high. From a fundamental point of view, understanding why cell mechanics is altered during cancer progression should also give extremely valuable information to better understand this disease.

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Figure and table legends

Figure 1

Experimental techniques used to compare the rheological properties of healthy and cancer cells at the scale of the whole cell (left) or at the local scale (right). Micropipette aspiration (a) or optical stretcher (b) probe mechanics by imposing a stretch deformation of the whole cell, while microfluidic devices (c) force cells to flow or migrate through channels of imposed geometries. Local indentation of the cells can be performed using an atomic force microscope (AFM) cantilever to which a bead or another cell is attached (d). Intracellular passive microrheology measures the local mechanics of the cell interior by tracking the movement of an internalized probe (e). In active microrheology, a force is applied on the probe using magnetic or optical tweezers (f).

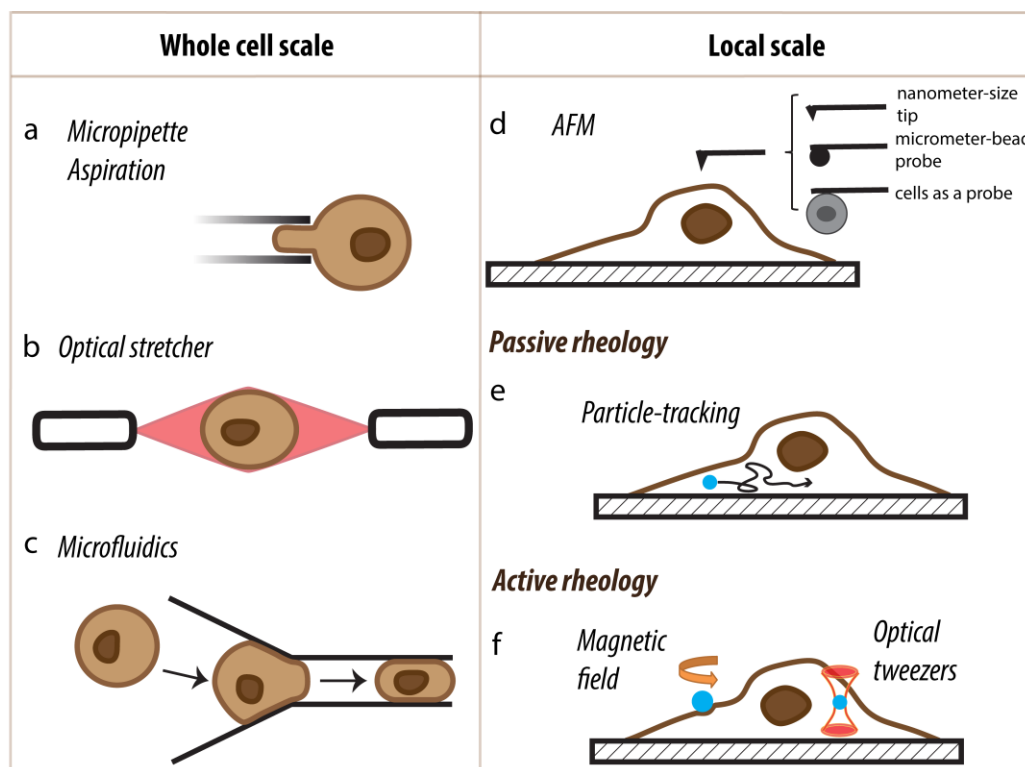


Figure 2

Basic rheology: concepts, models, mechanical parameters and their time-dependent or frequency-dependent behavior in a creep experiment. (A) Definition of the stress σ (Pa) and the strain ϵ (dimensionless) in a material submitted to a shear stress. The bottom plane of the material is fixed while a force F is applied to the top plane in a direction parallel to the surface S . (B) Creep and stress-relaxation experiments. In a creep (resp. stress relaxation) experiment a constant stress (resp. strain) is applied. From the evolution of the strain (resp. stress) with time, the creep compliance $J(t)$ of the material can be deduced. $J(t)$ is the inverse of the shear modulus $G(t)$. (C) Simple models in rheology. a- An elastic Hookean solid is symbolized by a spring of spring constant K (N/m) and Young modulus E (Pa). The stress is proportional to the strain and the proportionality constant is the Young modulus E (Pa). b- A Newtonian fluid of viscosity η (Pa.s) is symbolized by a single dashpot. The stress is proportional to the time derivative of the strain and the proportionality constant is the viscosity η (Pa.s). c- Maxwell model of a visco-elastic material. The material behaves as an elastic solid at short time scales ($t \ll \tau$, where τ is the time constant $\tau = \eta/E$) and as a viscous fluid at long time scales ($t \gg \tau$). d- Kelvin-Voigt model of a visco-elastic material. The material behaves as a viscous fluid at short time scales ($t \ll \tau$) and as an elastic solid at long time scales ($t \gg \tau$). (D) More complex models used to describe cellular rheology. a- The Standard Linear Solid (SLS) model is composed of a Kelvin-Voigt element in series with a spring. b- The Standard Linear Liquid (SLL) model is composed of a Kelvin-Voigt element in series with a dashpot. c- Models with a continuous distribution of time scales are represented by an infinite number of Kelvin-Voigt or Maxwell elementary elements and lead to a power-law behavior.

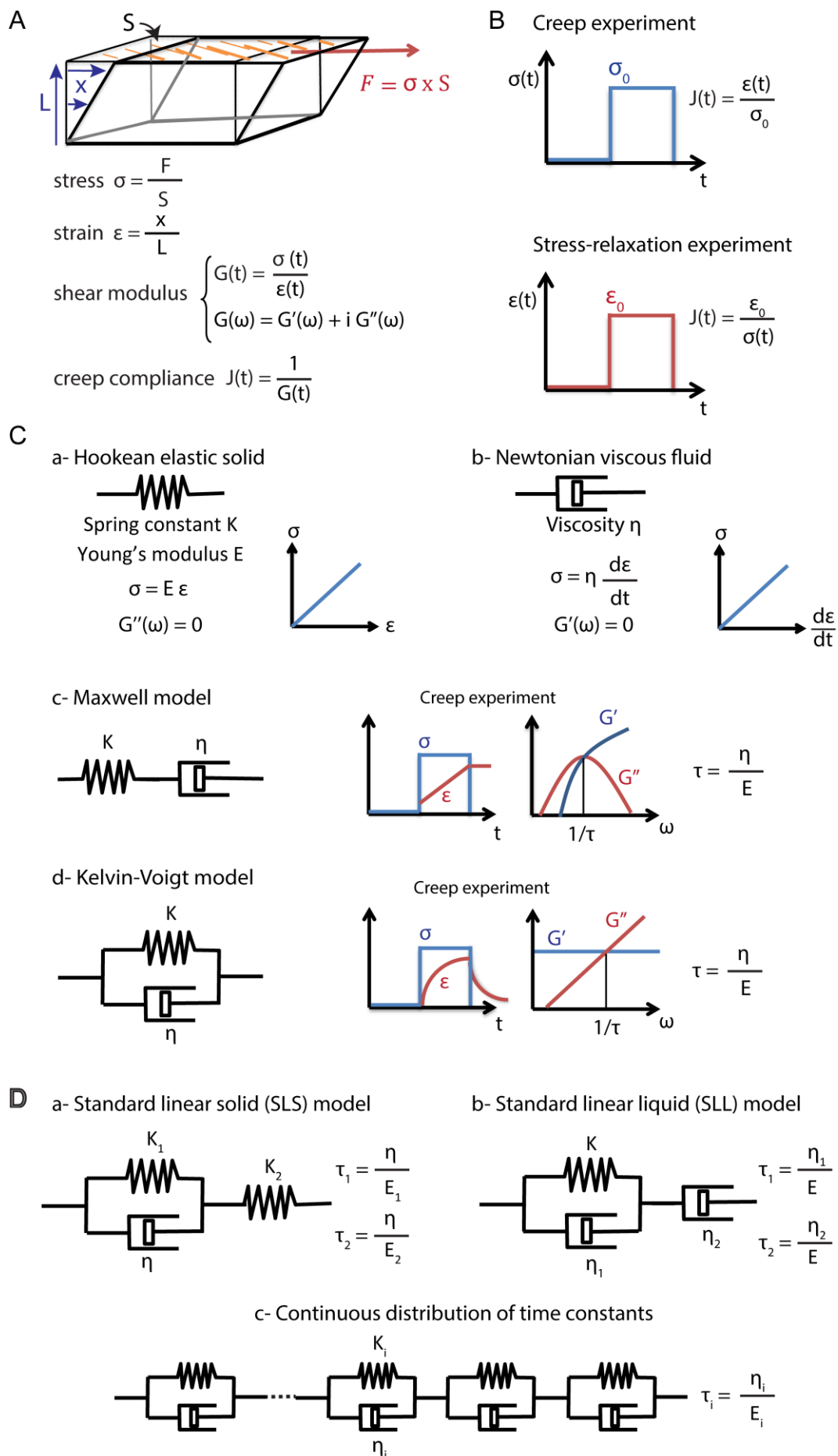


Figure 3

Cellular constituents that contribute or could contribute to the differences in rheological behaviors observed between healthy cells and cancer cells. Elements labelled with a star (*) change their characteristics during cell transformation. Elements listed on the left (ECM, actin, intermediate filaments) have been shown to directly impact on cell stiffness. In contrast, elements listed on the right (myosin II, E-cadherin, microtubules, energy sources such as ATP or GTP, internal membranes and trafficking) are known to contribute to cell stiffness but the mechanism and whether the contribution is direct or indirect is not clear yet. The cartoon compares the organization of a normal cell (left) with that of a cancer cell (right). In cancer cells, the nucleus volume is larger and the nuclear envelope is abnormally deformed and perforated; stress fibers are less dense and less organized; extra centrosomes appear with an elongated shape and abnormal positioning; and E-cadherin expression is reduced. Several studies have established that modifications of the actin cytoskeleton contribute to the mechanical differences between normal and cancer cells. Similarly, changes in the extracellular matrix (ECM) stiffness and of the expression levels of intermediate filament proteins, could explain changes in cell mechanics observed in cancer. Cancer cells are also known to sustain higher levels of ATP produced by glycolysis rather than by mitochondria as in normal cells. Intracellular out-of-equilibrium (active) forces which depend on the energy produced by ATP or GTP hydrolysis, such as the forces exerted by molecular motors, could thus be increased in cancer cells. Other cellular constituents altered in cancer, such as internal membrane organelles and their associated signaling molecules, myosin II or the microtubule network, have been proposed to impact on cell rigidity but their exact contribution is still debated.

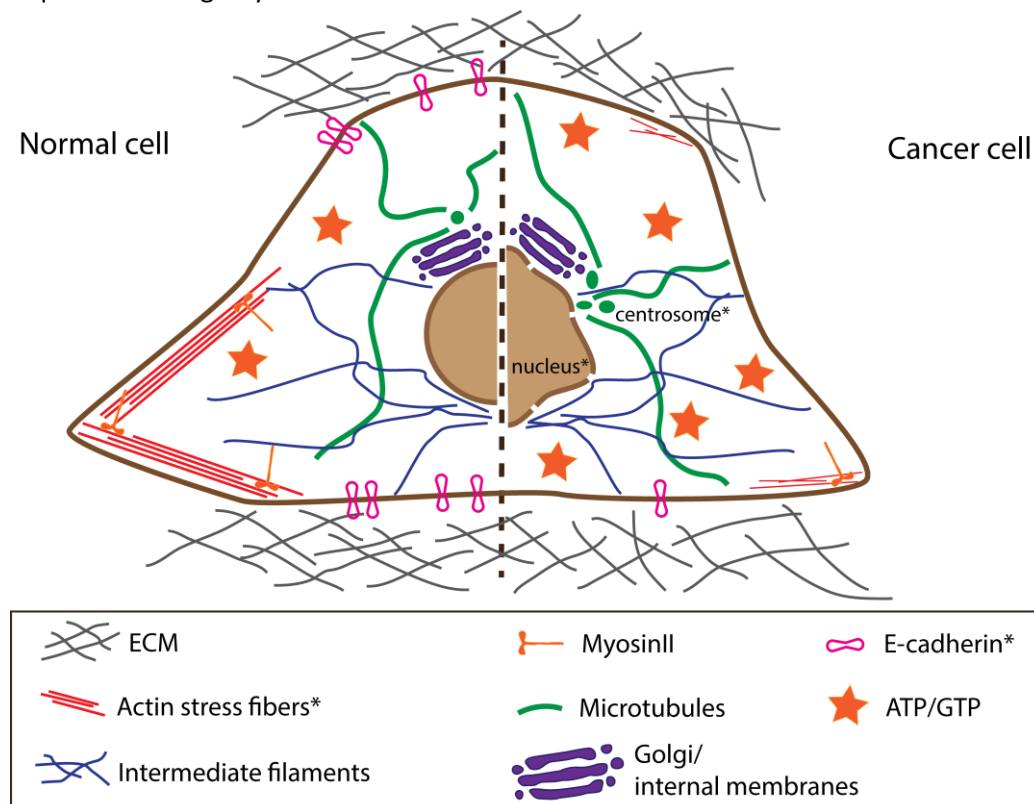


Table 1

Summary of the studies showing that individual cancer cells are softer than normal cells and/or that softness correlates with malignancy. References are sorted according to the main experimental technique used in the paper. For each reference, we report the cancer type and cell lines, the measured rheological parameters, the theoretical model and, when applicable, the cytoskeletal element specifically targeted in the study. E (Pa) represents the Young modulus or the elastic constant depending on the analysis, K (N/m) represents the elastic spring constant when a model using springs is used to describe cells or cellular environment, and μ (Pa.s) corresponds to the viscosity. J , G , G' , G'' and G_0 are respectively the compliance (in Pa^{-1}), the complex shear modulus (in Pa), its real and imaginary parts and its modulus. A star on the reference indicates Atomic Force Microscopy (AFM) experiments done with micron-sized beads as the probe. Colors represent the progression of the disease in the same organ or at the same localization inside the body (green = normal cells or low metastatic potential, orange = intermediate metastatic potential, red = highest metastatic potential). Abbreviation used: SLS, standard linear solid; SLL, standard linear liquid.

Acknowledgements

We thank Atef Asnacios and Kalpana Mandal for stimulating discussions. C.A is funded by a grant from UPMC University Paris 6 (Programme Doctoral "Interfaces Pour le Vivant"). We acknowledge funding from INCa/Cancéropôle (#2013-1-PL BIO-07-ICR-1). We thank the reviewers for their constructive comments. We apologize to colleagues whose work we could not cite due to space limitations. The authors declare no conflict of interest.

Rheological technique	reference	Cell lines	Parameters studied			model	Element of the cytoskeleton studied		
			E(kPa)	K (Pa)	μ (Pa.s)			Other	
Aspiration pipette	Pachenari <i>et al.</i> , 2014	Human colon cancer cells: HT29 (grade I) SW48 (grade IV)		$K_{1,HT29} = 80$ $K_{1,SW48} = 60$ $K_{1,HT29} = 150$ $K_{2,SW48} = 350$	$\mu_{HT29} = 7000$ $\mu_{SW48} = 6000$	- time constant τ - deformability rate	SLS	Organization of actin cytoskeleton Levels of actin and microtubule	
	Thoumine <i>et al.</i> , 1997	human fibroblast : normal AS3 SV-40 transformed fibroblast: AS3WT2			$\mu_{AS3} = 1.15 \cdot 10^4$ $\mu_{AS3WT2} = 0.8 \cdot 10^4$				
Particle tracking and AFM	Li <i>et al.</i> , 2009	Human breast cells : MCF10A (non-malignant) MCF 7 (malignant) Human pancreas cells : PA-T (non-malignant) PA-S (malignant)	Young modulus at 1Hz: $E^*_{MCF10A} = 6$ $E^*_{MCF7} = 0,3$				$MSD_{tumor\ cells} > MSD_{begin\ cells}$	Modified Hertz model	
	Agus <i>et al.</i> , 2013	Human breast cells: MCF10A MDA-MB-231 (tumorigenic)	Young modulus (depth of 0.8 μ m at lamella) $E_{MCF-10A} = 1.75$ $E_{MDA-MB-231} = 0.3$				$MSD_{MDA-MB-231} > MSD_{MCF10A}$	Hertz model	
AFM	Park <i>et al.</i> , 2005*	normal fibroblast : BALB 3T3 malignantly transformed fibroblast: SV-T2, H-ras	Elastic constant at the leading edge: $E_{BALB3T3} = 1.01 \pm 0.40$ $E_{SV-T2} = 0.48 \pm 0.51$ $E_{Hras} = 0.42 \pm 0.35$					According to the region, Hertz model or Chen model	Actin density Motility of lamellipode
	Ketene <i>et al.</i> , 2012*	Mouse ovarian surface epithelial: Early(E) : 15-25 passages Intermediate(I) : 75-80 passages Late(L) : 155-171 passages	Young modulus: $E_E = 0.554 \pm 0.349$ $E_I = 0.472 \pm 0.306$ $E_L = 0.395 \pm 0.136$	$K_{1,E} = 371 \pm 229$ $K_{1,I} = 310 \pm 220$ $K_{1,L} = 261 \pm 90$ $K_{2,E} = 117.5 \pm 85$ $K_{2,I} = 73.36 \pm 65$ $K_{2,L} = 49.14 \pm 31$	$\mu_E = 145 \pm 102$ $\mu_I = 103 \pm 54$ $\mu_L = 51 \pm 30$	- time constant τ - $E_{elastic}$, $E_{relaxed}$, $E_{instantaneous}$	Hertz model SLS	Actin organization	
	Rother <i>et al.</i> , 2014	Cells lines from different localizations : Kidney (K), adrenal gland (AG), lung epithelial layer (L), mammary gland (MG) Beginn : MDCKII (K), NMuMG(MG), MCF-10A(MG), NIH3T3 Malign: CaKi-1 (K); MDA-MB-231(MG); MCF-7; A549(L); SW13 (AG).			$\mu_{begin} = 4.65 \pm 0.28$ $\mu_{malign} = 5.60 \pm 0.18$	-Shear modulus $G_{begin} = 1.28 \pm 0.1$ $G_{malign} = 0.62 \pm 0.07$ -loss tangent $\eta = G''/G'$ Higher for malign cells	power-law structural damping model		
	Darling <i>et al.</i> , 2007*	Chondrosarcomas of grade II: 105KC (no metastasize) FS090 (less aggressive than JJ012) JJ012 (highest malignancy)	Young modulus: $E_{105KC} = 0.800$ $E_{FS090} = 1.27$ $E_{JJ012} = 0.480$	$K_{1,105KC} = 530 \pm 220$ $K_{1,FS090} = 850 \pm 570$ $K_{1,JJ012} = 230 \pm 150$ $K_{2,105KC} = 530 \pm 360$ $K_{2,FS090} = 720 \pm 710$ $K_{2,JJ012} = 210 \pm 130$		time constant τ	Hertz model SLS	Actin fibers	
	Cross <i>et al.</i> , 2007	Pleural fluid of seven patients with suspected adenocarcinomes : After analysis : 3 normal cells from liver, hepatic and oedema 4 cancer cells from lung, breast and pancreas	Young modulus: $E_{normal} = 1.970$ $E_{cancer} = 0.530$					Hertz model	
	Plodinec <i>et al.</i> , 2012	Mice lung primary cells: Cells from primary tumor	Stiffness 1.26+0.43						

Rheological technique	reference	Cell lines	Parameters studied				model	Element of the cytoskeleton studied
			E (kPa)	K (Pa)	η (Pa.s)	Other		
		Metastatic cells Mouse mammary gland primary cells: Normal and early cancer cells	0.56+0.26 1.02+0.42 0.45+0.12					
AFM	Lekka <i>et al.</i> , 1999	Human epithelial cells Non-malignant : Hu609 (ureter) HCV29 (bladder urothelium) Cancer : Hu456 (bladder carcinoma) T24 (bladder carcinoma) BC3726 (v-ras transformed HCV29)	Young modulus: $E_{Hu609} = 12.9 \pm 4.8$ $E_{HCV29} = 10.0 \pm 4.6$ $E_{Hu456} = 0.4 \pm 0.3$ $E_{T24} = 1.0 \pm 0.5$ $E_{BC3726} = 1.4 \pm 0.7$				Sneddon's model	
	Grady <i>et al.</i> , 2016*	normal cells: Dermal fibroblasts Boval chondrocytes HUVEC Cancer cells: Fibrosarcoma hepatocellular carcinoma	Elastic modulus Normal $E_{HUVEC} = 1.37$ $E_{Chondrocyte} = 2.07$ $E_{Fibroblast} = 2.19$ Cancer cells: $E_{Fibrosarcoma} = 0.48$ $E_{Carcinoma} = 0.30$				Hertz model	Actin and microtubules polymerization (nocodazole and cytochalasin D)
	Omidvar <i>et al.</i> , 2014*	Human breast cancer cells: MCF-7 T47D MDA-MB-231	Young modulus $E_{MCF-7} = 1.04 \pm 0.27$ $E_{T47D} = 0.94 \pm 0.25$ $E_{MDA-MB-231} = 0.62 \pm 0.21$			Cell-cell detachment force (nN) $F_{MCF-7} = 13.5 \pm 2.3$ $F_{T47D} = 5.8 \pm 0.6$ $F_{MDA-MB-231} = 4.6 \pm 0.6$	Hertz model	Expression of N-cadherin and E-cadherin Actin distribution
	Xu <i>et al.</i> , 2012*	Ovarian normal cells : IOSE Ovarian cancer cells: HEY HEY A8 (highly) OVCAR3 OVCAR4	Young modulus $E_{IOSE} = 2.472 \pm 2.048$ $E_{HEY} = 0.884 \pm 0.529$ $E_{HEYA8} = 0.494 \pm 0.222$ $E_{OVCAR3} = 0.576 \pm 0.236$ $E_{OVCAR4} = 1.120 \pm 0.865$				Hertz model	Actin distribution (density and alignment of fibers)
	Prabhune <i>et al.</i> , 2012	Primary thyroid cells normal cells : S748 (1 donor) cancer cells : S277 (1 donor)	Elastic modulus $E_{S748} = [2,2-6,9]$ $E_{S277} = [1,2-1,4]$			Looked at E on three consecutive days of culture	Hertz-Sneddon model	Actin distribution
	Bastatas <i>et al.</i> , 2012	Prostate cancer cells: LNCaP (lowly metastatic, non-metastatic) CL-1, CL-2 (highly metastatic)	Elastic modulus $E_{S748} \sim 200$ $E_{CL-1} \sim 400$ $E_{CL-2} \sim 260$			Cell migration [Ca ²⁺] _i dynamics	According to the region, Hertz model or Chen model	
	Faria <i>et al.</i> , 2008	Benign prostate cells: BPH Malignant prostate cells: LNCaP clone FGC (non invasive) PC-3 (highly invasive)	Young modulus $E_{BPH} = 2.8 \pm 0.5$ $E_{LNCaP} = 0.3 \pm 0.05$ $E_{PC-3} = 1.4 \pm 0.2$				Hertz model	
	Ramos <i>et al.</i> , 2014	Bladder cells : Non-cancerous : HCV29 Cancer : T24 transitional cell carcinoma HTB-9 (grade II, carcinoma) HT1376 (grade III, carcinoma)	Young modulus (biggest peak) $E_{HCV29} = 16.0 \pm 0.9$ $E_{HTB-9} = 3.0 \pm 0.1$ $E_{T24} = 2.9 \pm 0.5$ $E_{HT1376} = 5.2 \pm 0.1$				Sneddon's model	Different studies on actin inside cell lines: (presence of stress fiber, F-actin expression levels, effect of actin polymerization with cytochalasin D)
	Abidine <i>et al.</i> , 2015	Bladder cells : RT112 moderately differentiated T24 higher malignancy potential J82 higher malignancy potential				transition frequency f in perinucleus region (crossing of G' et G'') in Hz $f_{RT112} = 160 \pm 15$	Hertz model	Actin polymerization (latrunculin A and Y27632)

						$f_{T24} = 150 \pm 15$ $f_{82} = 205 \pm 20$ Plateau modulus at low frequency at perinucleus region in Pa $G_{RT112}^0 = 2756 \pm 102$ $G_{T24}^0 = 1856 \pm 120$ $G_{82}^0 = 1350 \pm 45$		
Rheological technique	reference	Cell lines	Parameters studied				model	Element of the cytoskeleton studied
			E(kPa)	K (Pa)	Mu (Pa.s)	Other		
Optical stretcher	Guck <i>et al.</i> , 2005	Human breast cells: MCF10 MCF7 modMCF7 MDA-MB-231, modMDA-MB-231 Fibroblast : BALB 3T3 SV-T2				Optical deformability (%): OD _{MCF-10} = 10.5±0.8 OD _{MCF-7} = 21.4±1.1 OD _{modMCF-7} = 30.4±1.8 OD _{MDA} = 33.7±1.4 OD _{modMDA} = 24.4±2.5 OD _{BALB3T3} = 8.4±1.0 OD _{SV-T2} = 11.7±1.1		
	Remmerbach <i>et al.</i> , 2009	Primary oral cancer cells : Normal (PHK) : 4 donors cancer (OSCCs): 5 patients Oral cell lines : Normal :OKF-4TERT1, OKF-6TERT1 Cancer :HN, BHY, CAL-27, CAL-33				Compliance J (Pa ⁻¹) at t = 0.5sec J _{all normal lines} = 7.18±0.43 J _{all cancer lines} = 18.2±1.1		
	Wottawah <i>et al.</i> , 2005	normal fibroblast : NIH/3T3 malignantly transformed fibroblast: SV-T2				G' _{NIH=3T3} = 100 ± 10 Pa G' _{SV-T2} = 63 ± 7 Pa		Reduced actin cytoskeleton for SV-T2
Optical tweezers	Guo <i>et al.</i> , 2014	Human breast cells: MCF10-A MCF-7				MSD _{MCF-7} above MSD _{MCF-10A}	No model Found power law	Inhibition of Myosin II
	Bertseva <i>et al.</i> , 2012	Bladder cells Non-cancerous : HCV29 Cancer : T24				MSD _{T24} above MSD _{HCV29}	Found power law	
Optical tweezers + micropatterns	Mandal <i>et al.</i> , 2016	Human breast cells: MCF10-A MDA-MB-231				Shear modulus (Pa) G _{MCF-10} = 3.6 G _{MDA} = 1.6	SLL	Microtubules Actin
Microfluidics	Hou <i>et al.</i> , 2009	Human breast cells : MCF10A MCF 7				Entry time Elongation Transit velocity		
Magnetic	Swaminathan <i>et al.</i> , 2011	Primary ovarian cancer cells from ascites: OV445 (least invasive III), OV207 (most invasive IV), Ovarian cancer cell lines: IGROV (less inv --) Ovca420 (+/-) HEY (most inv ++),SKOV3 (++)				Max compliance J (Pa ⁻¹) J _{IGROV} = 0.25 J _{Ovca420} = 0.45 J _{HEY} = 1 J _{SKOV3} = 1 J ₂₀₇ = 3.2 J ₄₄₅ = 0.2	Jeffrey's model	Actin distribution Presence of phosphorylated myosin light chain (pMLC) Expression of E-Cadherin and Vimentin Inhibition of Myosin II
Acoustic wave	Hartono <i>et al.</i> , 2011	Normal cells: breast = 3.77 ± 0.09, fibroblast = 3.78 ± 0.17				Compressibility (x10 ⁻¹⁰ Pa ⁻¹) : C _{breast} = 3.77 ± 0.09 C _{fibroblast} = 3.78 ± 0.17		

		Cancer cells from different organs: MCF-7 = 4.22 ± 0.19 , HEPG2 = 4.28 ± 0.12 , HT-29 = 4.04 ± 0.16 (grade I)				$C_{\text{MCF-7}} = 4.22 \pm 0.19$ $C_{\text{HEPG2}} = 4.28 \pm 0.12$ $C_{\text{HT-29}} = 4.04 \pm 0.16$		
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