Cell-gel mechanical interactions as an approach to rapidly and quantitatively reveal invasive subpopulations of metastatic cancer cells

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Abstract

We present a novel mechanobiology-based invasiveness assay to rapidly and quantitatively evaluate the mechanical invasiveness of metastatic cancer cells and identify invasive subpopulations, without need for chemoattractants and independent of serum content. The commonly accepted assay to determine metastatic potential *in vitro* is the Boyden chamber assay, where the percent of serum-starved cells that can long-term transmigrate/invade through sub-cell size membrane-pores is quantified; those experiments typically take 2-3 days. Those invasive cells are required to be pliable to squeeze through the small pores, yet they are also able to force their way through flexible microenvironments. We have previously shown that metastatic breast-cancer cells will deform and indent soft, impenetrable, elastic gels within 2 hours of seeding, without requiring serum starvation. Specifically, in cell lines with higher metastatic potential, a larger percentage of cells will indent gels and typically also to deeper gel depths. Thus, we are able to rapidly reveal mechanically invasive subpopulations, which are likely those that lead to high metastatic potential. By comparing the Boyden chamber and gel-mechanobiology assays, we show that the indenting cell subpopulations in the gel-assay are part of the group that successfully transmigrate through the Boyden chamber membrane (8μm pores). Thus, we are able to rapidly (within 2 hours of seeding), using the standard cell media, provide a quantitative measure of the mechanical invasiveness of cells, which is correlated to the metastatic potential but is an independent parameter; we evaluate numbers of indenting cells and their indentation depth. Moreover, the mechanical invasiveness assay allows focusing on specific (invasive or non-invasive) cells within the sample to identify specific surface markers, determine invasive mechanisms, and evaluate effects of applied drugs and treatments on the different subpopulations.

Keywords: Mechanobiology, *in vitro* migration and invasion, cancer metastasis, Boyden chamber.

Introduction

Metastasis, the lethal spread of cancer in the body, causes nearly 90% of cancer-associated deaths, despite major advances in molecular biology and genetics of cancer. Stages of metastasis include detachment from the primary tumor, invasion of surrounding tissue, intravasation and dissemination through the blood stream or lymph system, extravasation, and finally the outgrowing of a secondary tumor. This complex process is highly ineffective, yet cells may mutate to increase motility, plasticity and invasive capacities, attaining metastatic competence that results in successful invasion. These stages are marked by significant changes in gene expression, tumors markers and also varying cell-microenvironment interactions. The important roles of the cell microenvironment and the cells' dynamic, mechanical phenotypes have recently been recognized in the context of metastasis. During metastasis the mechanobiology of the cells changes in response to external forces, by dynamically changing the cell mechanics or by modifying their microenvironment (Butcher et al. 2009; Kumar and Weaver 2009).

The mechanobiology, cell mechanics, and cell-microenvironment mechanical interactions have recently been directly correlated with the metastatic potential in different cancer cell types. While tumors have been shown to be stiffer than normal tissue, single metastatic cancer cells are externally and internally softer (Guck et al. 2005; Cross et al. 2007; Gal and Weihs 2012). We have shown that metastatic breast cancer cells are softer, have a more sparse cytoskeleton and exhibit heightened internal dynamics (Gal and Weihs 2010; Gal and Weihs 2012; Goldstein et al. 2013). This also correlates with a reduction in the cell's stiffness for more aggressive and

motile cells (Guck et al. 2005; Cross et al. 2007). Differences in mechanics and physical properties of metastatic, cancer and benign cells have been demonstrated through different physical methods, such as optical stretcher (Lincoln et al. 2004; Guck et al. 2005; Liese et al. 2007; Remmerbach et al. 2009), flow cytometry (Lincoln et al. 2003; Lincoln et al. 2004), magnetic twisting cytometry (Coughlin et al. 2013), micropipette aspiration (Mohammadalipour et al. 2012) and atomic force microscopy (Cross et al. 2007; Li et al. 2008; Fuhrmann et al. 2011; Jonas et al. 2011; Plodinec et al. 2012; Lin et al. 2015; Staunton et al. 2016). For example, high metastatic potential breast cancer cells (MDA-MB-231) have been shown to be more deformable than non-metastatic cells (Guck et al. 2005). When those cells were treated with all-trans retinoic acid, they became less deformable and less aggressive (Wang et al. 2001), and as a result, we showed, that their ability to apply force decreased (Kristal-Muscal et al. 2013). The cells ability to dynamically apply force and rapidly change morphology is what facilitates their enhanced motility and invasiveness - their metastatic potential. The metastatic potential is conventionally evaluated *in vitro* using Boyden chambers. Cells that are able to cross the non-degradable membrane with pores smaller than their diameter are defined as more invasive (Sahai 2005).

The interactions of cancer cells with their microenvironment have also been correlated with the metastatic potential, invasiveness and adaptability of cancer cells. For example, cell subpopulations with stem-like phenotypes are adhered less strongly and express markers of the epithelial-to-mesenchymal transition (EMT) (Morata-Tarifa et al. 2016), indicative of enhanced motility. Cancer cells typically migrate either mesenchymally (by degrading the extracellular matrix) or amoeboid fashion, where the aggressive cells can switch between the modes of motility when necessary (Nguyen-Ngoc et al. 2012; Clark and Vignjevic 2015). A rounded cell morphology is typical for cells that are seeded on a soft gel and also for cells exhibiting amoeboid-type migration (Friedl 2004; Kristal-Muscal et al. 2013); actin rich

protrusions are used by the cell to sense the microenvironment and initiate attachment (Ilina and Friedl 2009). In contrast, on a three-dimensional (3D) matrix invasive cells assumed an elongated spindle-like morphology and present higher contractility when compared to their benign counterpart (Koch et al. 2012).

By evaluating the mechanical interactions of the cancer cells with an elastic, initially flat polyacrylamide (PAM) gel, we have recently shown that cells are rapidly identifiable, and distinguishable from benign cells, (Kristal-Muscal et al. 2013; Dvir et al. 2015; Massalha and Weihs 2016); the gel is physiological-stiffness and impenetrable, as it is non-degradable and has sub-micron pores. We have shown that while benign cells attach and apply small lateral traction forces only, metastatic breast cancer cells may apply either traction forces or a combination of normal and lateral forces, to indent and/or laterally pull the gel. We and others have observed that lateral traction forces exerted by metastatic cells increase linearly with gel stiffness on the range 1-11 kPa, while forces applied by benign cells appear to be stiffness-independent (Kraning-Rush et al. 2012; Massalha and Weihs 2016). Interestingly, we have observed that a subpopulation of the metastatic cells indent the impenetrable gels, when those are soft enough to push, yet stiff enough to grip (Kristal-Muscal et al. 2013; Dvir et al. 2015). The indentation depth attained by single, indenting metastatic breast cancer cells were 2-3-fold larger on 2.3 kPa gels as compared to 1.3 kPa gels, and cells were unable to adhere to 0.5 kPa gels (Kristal-Muscal et al. 2013). Thus, using cell-gel mechanical interactions, we were able to quantitatively distinguished between MDA-MB-231 and MDA-MB-468 lung metastases, breast cancer cell lines, respectively high and low metastatic potential cells, e.g. by indentation depths. Notably, these measurements are independent of the underlying tumor genetics, and rely only on the cell's ability to apply force to their environment as an invasiveness measure. We note that it was a subpopulation of the cells that indented the gels, by applying repetitively larger-and-lower forces with time (Kristal-Muscal et al. 2013; Dvir et al. 2015). That shows

the population heterogeneity and dynamics in the mechanical phenotype of the cells, likely indicating differences in invasiveness.

Here, we show that the rapid and quantitative mechanobiology gel-based assay provides an important quantitative complement to the Boyden chamber transmigration/invasion-assay. Together these assays may be used to evaluate *in vitro* metastatic potential and the ensuing mechanical invasiveness of cancer cells on single-cell level. Both the Boyden chamber and our gel-mechanobiology assay reveal cancer cells that have high capacity to perform amoeboid invasion; the focus of the gel-assay is on the mechanical interactions of cells with their microenvironment, providing a rapid (1-2 hours), quantitative measure of the cell-induced deformation on single-cell level. We show that the subpopulation of cells that migrate through an 8μm Boyden chamber membrane is also subpopulation that will deeply indent the gels; the Boyden chamber effectively concentrates the more invasive cells which then indent the gels. Appropriately, we observe a larger invasive subpopulation in the MDA-MB-231 cells as compared to the MDA-MB-468 cells. Our approach can rapidly reveal subpopulations with mechanically invasive phenotype and may be used to identify and test new cell-specific targets for treatment.

Materials and Methods

Cell culture. We used MDA-MB-231 (HTB-26, ATCC, Manassas, VA) and MDA-MB-468 (HTB-132) both breast cancer lines from lung metastasis. Cells were cultured in DMEM (Gibco, Carlsbad, CA) media supplemented with 10% fetal bovine serum, and 1% vol. each of penicillin-streptomycin, 1-glutamine, sodium pyruvate (all from Biological Industries, Kibbutz Beit Haemek, Israel). Cells were maintained in an incubator at 37°C, 5% CO_2 and high humidity, and were used in passages 10-30 from stock.

Polyacrylamide gel preparation. The gels were prepared according to an established protocol (Raupach et al. 2007; Kristal-Muscal et al. 2013). Glass coverslips, 30 mm diameter, #5 thickness (Menzel, Germany) were hydroxylized using 0.1M NaOH and then activated with 3-aminopropyltrimethoxysilan (both Sigma, St. Louis, MO), and fixed with glutaraldehyde after 24 hours. Gels were prepared on ice, by combining acrylamide (34 µl of 40%vol) and BIS acrylamide monomers (3.8 µl of 2%vol) (both Sigma, St. Louis, MO), in 203 µl of distilled water, we obtain a gel with stiffness (Young's modulus) of 2440±40 Pa, determined with an AR-G2 TA Instruments rheometer (Kristal-Muscal et al. 2013). Prior to initiation of gelation, 2 µl of 200 nm diameter, red fluorescent carboxyl-coated polystyrene particles (Invitrogen, Carlsbad, CA) were added to the solution. Gelation was initiated with 1:200 vol. ammonium persulphate (APS) and catalyzed with 1:500 vol. of tetramethylethylenediamine (TEMED). The gels were prepared by placing 25 µl of the monomer solution inside a plastic frame (Gene Frame, 25 µl, 10 × 10 mm, ABgene Thermo-Scientific, Waltham, MA) adhered on the activated glass coverslip and covered by a plastic coverslip. Slow gelation was performed at 2°C and under 300 g centrifugation (Raupach et al. 2007; Kristal-Muscal et al. 2013) for 30 min. to confine the fluorescent nanoparticles at the gel surface. To facilitate cell adhesion, the surface of the gel is activated with Sulfo-SANPAH (Pierce, Thermo Scientific, Waltham, MA) and coated with rat tail collagen type I (Sigma, St. Louis, MO). Glass coverslips with gels were kept under phosphate-buffered saline (PBS, Sigma, St. Louis, MO) at 4°C until use.

Gel rheology. The gel stiffness was determined using a TA Instruments AR-G2 rheometer (New Castle, Delaware). To determine the shear modulus, gels were prepared directly on the rheometer plate and time sweep experiments were run (oscillatory strain 0.5%, angular frequency 3.14 rad/s) using a 2-cm parallel-plate fixture. The Young's modulus, E, was determined by: $E=2|G^*|\ (1+\nu)$ where G^*, the complex shear modulus is equal to the elastic modulus, $G^* \approx G'$, since the gels are elastic ($G' >> G''$). Using a Poisson's ratio of $\nu=0.49$

(Takigawa et al. 1996), the Young's modulus is $E=3|G'|$. We used a gel with Young's modulus of 2.4 ± 0.2 kPa, as was previously determined at our lab (Massalha and Weihs 2016). The gel's Young's modulus values exhibited small variation and high reproducibility between batches. This low variability is consistent with previous measurements on a wide range of polyacrylamide gels (Abidine et al. 2015; Elosegui-Artola et al. 2016), where rheology and AFM were compared and gels were homogeneous with linear mechanical properties.

Boyden chamber, transwell-migration assay. We determined the number of serum-starved cells that cross through Boyden chamber membranes with 8 μm pores. The transwell inserts were pretreated overnight with collagen type I (5 μg/ml) at 4°C to facilitate cell attachment. Breast cancer cells were serum-starved overnight and 1.0×10^5 cells were seeded in 100 μl of serum-free media in the upper compartment of the transwell inserts (EDM Millipore, Billerica, MA). Serum full DMEM (10% FBS) in the lower chamber was used as a chemoattractant. Cells were allowed to migrate for 72 hours while incubated at 37ºC and 5% CO_2. The cells from the upper and lower compartments were collected, using trypsin or non-enzymatic cell dissociation, for comparison. Cell collection by Trypsin was performed by adding, respectively 200 μl and 300 μl of Trypsin-EDTA Solution A (0.25%, Biological Industries, Cromwell, CT) and incubating for 5 minutes; a larger amount of trypsin (same concentration) was used to fill the Boyden chamber's lower compartment, so as to reach the cells that have traversed the chamber and remain attached to the bottom of the membrane. The trypsin was neutralized by adding 800 μl and 1.2 ml of DMEM growth media to the upper and lower compartments, respectively; these amounts give a ratio of 1:4 trypsin-to-media. As trypsin may alter cell adhesion, we have also used the non-enzymatic Cell Dissociation Solution (Biological Industries, Cromwell, CT). Cells from the top and bottom compartments were incubated, respectively in 200 μl and 300 μl for 10 minutes and then centrifuged and re-suspended in cell media. The cells from both compartments were compared, using two separate experiments:

flow cytometry and the gel-based mechanical evaluation. For the gel-based experiment, to quantify the percentage of indenting cells we perform 4 different experiments with a total of 100-200 for control conditions and for the trespass and no trespass condition we evaluate between 350-500 cells in 6 different experiments. Indentation depth was measured for 50-100 cells for each condition. It is important to note that cells reaching the bottom chamber encounter serum-full media, which after 72 hours may induce significant cell division in cells that are not significantly migrating; the doubling time of both the MDA-MB-231 and MDA-MB-468 is similar, at approximately 24 hours. Thus, we compare the percentage of indenting cells in each chamber, and not the numbers of cells.

Flow cytometry. Cells from the upper and lower compartments of the Boyden chamber membranes were counted using a BD LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ). We counted 10,000 events in 3 experiment for each condition in the two cell lines. Results were plotted on a dot plot of forward-scattered light (FSC) vs. side-scattered light (SSC). We also used the flow cytometry to evaluate cell viability, by quantifying the percent of cells that stain with propidium iodide (PI, Sigma-Aldrich, St. Louis, MO), a marker for dead cells, using 10 μl/ml of a 10 μg/ml stock PI solution. Count were based on 10 μl volume of cell solution.

Microscope imaging. Cells and gels were imaged using an inverted, epifluorescence Olympus 81X microscope using a 60x/0.7NA differential interference contrast (DIC) air immersion objective. Images were captured using a XR Mega-10AWCL camera (Stanford Photonics Inc., Palo Alto, CA) with a final magnification of 107.8 nm/pixel (Kristal-Muscal et al. 2013).

Gel-indentation experiments and depth evaluation. The number of indenting cells and depths were obtained by seeding cells on top of PAM gels (2.4 kPa Young's modulus), within 2ml of cell media. We evaluated gel-indentations for two control conditions with all cells either under serum-starved or serum-full conditions. For the starved control experiments, cells on serum-

starved condition, the cells were cultured for 24 hours on FBS-free DMEM media trypsinized and neutralized with standard DMEM media and placed on the gel for imaging. The serum-full control consisted of cells cultured for 24 hours with standard DMEM media. Controls were compared to gel-indentations induced by the two subpopulations of cancer cells obtained from the upper and lower compartments of the Boyden chamber, respectively, non-trespassing and trespassing (transmigrating) cells.

Cells were imaged one hour after seeding on gels to allow attachment. We imaged several, random fields of view (typically 10-30), collecting three images at each location: (a) a differential interference contrast (DIC, Nomarsky optics) image of the cells on the gel, (b) a fluorescence image of the particles embedded at the gel surface, and (c) when a cell indented the gel, a fluorescence image was obtained at the lowest focal depth where the particles were in focus by eye; these measurements were similar between different users (averaged differ $<0.5\mu m$) and were also verified by confocal microscopy (Dvir et al. 2015), however we are currently also working on developing an automated image processing procedure for this step. The percent of indenting cells in every experiment was quantified. The focal height difference between the two fluorescence images provided the depth of indentation (see e.g., Figure 2).

Statistical analysis. Results are displayed as the mean ± the standard deviation. We average four gel-indentation experiments with a total of hundreds of cells for each cell line in each condition. A two-way ANOVA test with Bonferroni correction was conducted to evaluate the effect of cell type and the condition (i.e., control, starved control, trespass, no-trespass, and cell dissociation method) using a confidence level of 95%. Null hypothesis are identified by p-values less than 0.05.

Results

We show that the mechanical invasiveness of metastatic cells on the synthetic gel system directly correlates and complements with the Boyden chamber assay, providing an enhanced measure of the mechanical, metastatic competence of the cells, on the single cell level. We compare and also combine the two *in vitro* assays: the Boyden chamber transmigration/invasion assay and our gel-based mechanobiological invasiveness assay. Concurrently, we determine the direct correlation between cancer cell migratory and mechanical indentation abilities. We compare the populations of cells that are able to cross an 8 μm Boyden chamber and those that actively indent a soft, elastic and impenetrable polyacrylamide gel, showing the direct correlation (Figure 1). Following 72 hr interaction with the Boyden chamber, we collect the subsets of cells from the upper and lower compartments of the Boyden chamber, respectively, providing the non-trespassing and trespassing (transmigrated) cell fractions. We evaluate indentations induced by the two fractions with those induced by the entire cell population in serum full and serum starved control, to match the Boyden chamber conditions where cells are serum starved 24 hrs prior to seeding.

Flow cytometry counting showed that 69±3% and 48±1%, respectively, of the seeded MDA-MB-231 and MDA-MB-468 breast cancer cells successfully transmigrated through the 8 μm membrane. Viability was determined by PI staining of (initially serum starved) cells collected from the upper and lower chambers of the Boyden membrane, and cells in both compartments were over 95% viable (Figure S1). The two fractions, trespassing and non-trespassing cells, and whole cell-populations controls (serum full and overnight serum-starved) were seeded on the PAM gels to evaluate the mechanical interactions. The cell mechanical interactions with the PAM gel included evaluation of two parameters: the percentage of indenting cells and the biomechanically induced indentation depth.

We observe that a subset of the MDA-MB-468 and MDA-MB-231 cell populations (respectively 35% and 50%) indent the 2.4 kPa PAM gels, likely in attempted invasion; we have previously shown that benign cells do not significantly indent the gels (Kristal-Muscal et al. 2013; Dvir et al. 2015). The gels are impenetrable to the cells, being non-degradable and with sub-micron pores, yet already 1 hour after seeding, single, metastatic breast cancer cells measurably indent the gel. The indentation is apparent as a cell pushes the gel surface downwards to a lower focal plane. Figure 2 shows examples of indenting and non-indenting cells seeded on the PAM gels.

Figure 3 shows the percentage of MDA-MB-468 and MDA-MB-231 cells that indent the PAM gel under control conditions and following Boyden chamber migration. The two whole cell-population controls (serum full and serum starved) were indistinguishable, indicating that the cell's gel-indentation capability is not affected by serum starvation. We observe that the non-trespassing subpopulation exhibited a similar percentage of indenting MDA-MB-468 cells and a reduction in the indenting MDA-MB-231 cells, both with wider variability; this population is expected to be less migratory and less invasive. We have also evaluated the effect of the dissociating solution, as that may affect cell adhesion. Compared the percent of indenting cells following Boyden chamber interaction, collected using trypsin or using a non-enzymatic Cell Dissociation Solution, we observed a larger percentage of indenting cells when using the non-enzymatic cell dissociation. However, the results were qualitatively similar using both collection approaches, and thus we focus on the observed phenomena in general.

Most importantly, we note that for both cell types, regardless of the cell collection method, the cells that have trespassed through the Boyden chamber exhibit a high percentage of indenting cells (>70%), indicating a direct correlation between the Boyden migration and gel indentation. Remarkably, by performing a simple calculation, for example with the trypsinized MDA-MB-468 cells, we observe that since 48±10% of the cells trespassed the Boyden chamber, and

75±3% (N=351) of those then indented the gels, 48%*75% = 36%, which is in fact the percentage of the indenting cells in the overall population; in the trypsinized MDA-MB-231 cells, it is 69±3% trespassing and 72±13% (N= 468) indenting to give 50% of the overall population. This suggest that the Boyden chamber is "concentrating" the more migratory and invasive cells, which are then more likely to indent the gels.

We observed a wide distribution of indentation depths induced in the gels by both MDA-MB-468 and MDA-MB-231 breast cancer cell lines, where the maximal depth following interaction with the Boyden chamber was higher than controls (Figure 4); indentation depths following use of the cell dissociation solution are in Figure S2. The measured indentation depths (up to 12 μm in controls) are significantly larger than previously observed dimples (< 1 μm) exhibited by non-cancerous cells that spread (Hur et al. 2009) or crawl (Delanoe-Ayari et al. 2010); those dimple likely result from mostly traction forces that locally bend the gels. To evaluate the number of "intentionally" indenting metastatic cells, we have set a threshold indentation depth of 2.5 μm, the maximum depth that we had previously observed in benign breast cells (Dvir et al. 2015). Regardless, indentation depths between 0.5-2.5 μm represent less than 6% of the indenting cells in both cell lines. We observe that the serum-full and serum-starved controls are again indistinguishable, where the distributions of the indentation depths were statistically indistinguishable. In contrast, cells that were seeded in the Boyden chambers exhibited wider distributions, reaching larger indentation depths than both controls, regardless of whether the cells trespassed. Notably, in the subpopulation of cells that did trespass the Boyden chamber, the average depth also increased, as compared to the controls and the non-trespassing subpopulation.

Discussion

Our results show that the gel-based system provide an important complementary measure to be combined with the Boyden chamber assay, revealing different aspects of cell invasiveness. The results shown here provide a preliminary indication towards potential use of the gel-indentation assay as an approach to evaluate the *in vitro* metastatic potential of cancer cells; to validate the approach would require further experimentation with cell lines and primary cells. The gel-assay is more rapid, simple, quantifiable, and provides the evaluation of mechanical invasiveness on single cell level, while also providing an average measure of the invasive subpopulations. By combining both methods we shows a subpopulation of cells, within the evaluated breast-cancer cell lines, which is both mechanically invasive (gels) and highly migratory (Boyden chamber).

The Boyden chamber migration assay is typically used to identify the migratory capability and invasiveness of cells, and thus determine their metastatic potential. Boyden chambers may be used in different ways, were changing the pore size or the biochemical functionalization of the membrane allows different features of the migration/invasion to be studies; the equivalent approach in the gel-assay would be to vary gel stiffness (Kristal-Muscal et al. 2013; Massalha and Weihs 2016), its dimensionality or the surface functionalization. Additionally by combining with time lapse imagining, both the gels and the Boyden chamber can be used to characterize cell dynamics. We show by combining the approaches, that passage through a Boyden chamber (8μm pores) concentrates the cells that are able to indent and deform the impenetrable, synthetic, elastic polyacrylamide gel; the fraction of indenting cells significantly increases in cells that have traversed the Boyden chamber. That is, the ability of the mechanically competent cell-subpopulation to successfully trespass through a Boyden chamber is directly correlated with their propensity to apply force and indent the gel, making both methods comparable. Hence, we propose that the mechanobiology gel-assay and Boyden

chamber assay provide complementary information on cell invasiveness, yet the gel- assay has several advantages, such as rapidity and simplicity, as discussed below.

We have observed that serum starvation does not affect the indenting cell subpopulation (i.e. in quantities and attained depths), while it is required to induce cell invasion through the Boyden chamber. The interaction of the serum-starved cells with the Boyden chamber membrane may in fact "activate" some of the cells, making them even more invasive. Specifically, a subset of the subpopulation of indenting cells that had been seeded on the Boyden chamber membrane for 72 hours were able to indent the gels more deeply than the control, in both the trespassing and non-trespassing subpopulations; this shows that cells that do not cross the Boyden chamber may still have the capacity. Serum-starvation has previously been shown to increase the invasiveness of the MDA-MB-231 breast cancer cells used here, resulting in larger percentages cells migrating through a Boyden chamber (Nagelkerke et al. 2013; Ye et al. 2013); serum starvation is typically applied a day prior to the assay. Thus, the serum starvation itself is likely affecting the cell invasiveness.

We note that the experiments run with the gel-indentation assay do not require serum starvation and results are unaffected by it. More importantly, the gel-indentation assay provides rapid results (1-2 hours) and shows the distribution of single-cell behaviors within a cell population.

The mechanically invasive subpopulation that is highlighted here is likely comprised of cells that are capable of performing amoeboid migration. Boyden chamber membranes are non-degradable with sub-cell diameter pores (here 8 µm pores and cells ~20 µm diameter), which requires amoeboid migration, including significant cell pliability and dynamic changes in cell morphology. Single, MDA-MB-231 breast cancer cells are softer both externally and internally, as compared to non-metastatic cancer and benign breast cells (Guck et al. 2005; Cross et al. 2007; Gal and Weihs 2012), due to heightened cytoskeleton dynamics and more

sparse intracellular structure (Mierke et al. 2010; Goldstein et al. 2013). Concurrently, the same metastatic cells are also adaptable, and can modify their cytoskeleton and morphology (Dvir et al. 2015), and apply strong mechanically invasive forces to their microenvironment (Mierke et al. 2008; Kristal-Muscal et al. 2013; Kristal-Muscal et al. 2015). Most of the indenting cells (~85%) in the current work exhibited transient bleb-like membrane protrusions a characteristic of amoeboid migration (Krakhmal et al. 2015), as also observed previously at our lab (Kristal-Muscal et al. 2013); the gels are also non-degradable, making them a non-proteolytic assay similar to Boyden chambers. Cells use such membrane-blebs to actively sense suitable adhesion sites or tracks to successfully invade (Krakhmal et al. 2015).

In summary, our mechanobiology gel-assay approach may be in combination with the Boyden chamber assay to evaluate different aspects of *in vitro* mechanical invasiveness and metastatic potential of cancer cells. The combined testing approach used here, merging mechanobiology with Boyden chamber migration assays, has revealed a mechanically aggressive subpopulation, being highly migratory and indenting deeply it is likely the metastatic competent subpopulation within these cell lines. The ability to understand and predict cell responses and behavior in different microenvironments is of fundamental importance in tissue engineering, for the design of biologically relevant tissues and scaffolds. Cell motility plays an important role in tissue engineering and in the current work, we highlight a specific aspect of interest that can affect tissue engineered scaffolds, i.e. the mechanical invasiveness exhibited by highly migratory/invasive metastatic cells.

The correlation between migration and mechanical invasiveness in cancer cells is highlighted in this work. The gel-indentation mechanobiology approach reveals complementary aspects of cell invasiveness *in vitro* to those available using the Boyden chamber assay. An advantage of the gel-indentation assay is that it is more rapid (1-2 hours vs. 3-4 days) and provides quantitative information on single cells in the population, a strength of the mechanobiology

approach. The gel-assay may be used independently to determine mechanical aspects of the cell invasiveness, e.g. their “strength”. Furthermore, the approach can be used to evaluate the effect and efficacy of anti-metastatic drugs and also to identify the underlying biochemical and epigenetics associated with the mechanically invasive phenotype, focusing on single cells within the population. There are likely to be several different phenotypes within the indenting cell population; genetically different subpopulations of breast-cancer cells that promote collective invasion has been observed in 3D spheroids (Westcott et al. 2015). The isolation and identification of metastatically competent cell subpopulations is essential to understand the underlying causes for metastasis, and to provide accurate prognosis. Thus, using a rapid, quantitative mechanobiology approach could shed light on the nature and abilities of aggressive subpopulations within a tumor sample.

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Author Disclosure Statement

No competing financial interests exist

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Figures

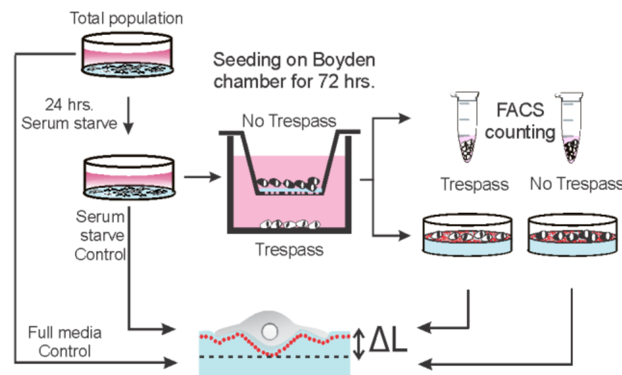

Figure 1. The experimental flow used to evaluate the mechanical, metastatic competence or metastatic potential of the breast cancer cell lines. Cells are serum starved for 24 hrs, seeded on Boyden chambers, and collected after 72 hrs from the top (no-trespass, black fill) and bottom (trespassing, light fill). Cells from top and bottom were counted using flow cytometry and their mechanobiology was evaluated on our gel-based system. Results from the Boyden chamber-separated populations were compared to the entire cell-population, both serum full and serum starved.

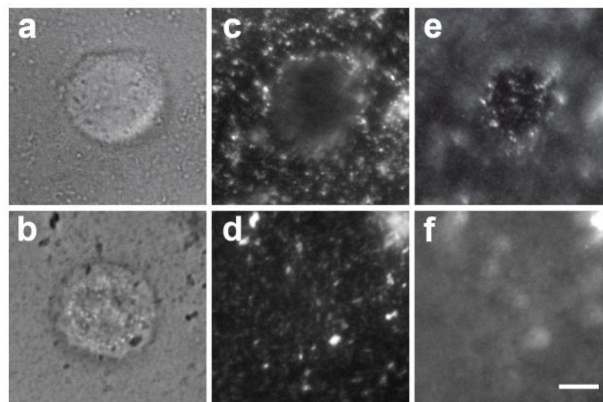

Figure 2. Single, metastatic, breast cancer cells (MDA-MB-231) seeded on 2.4 kPa stiffness polyacrylamide gel either (top row) indent the gels (bottom row) adhere but do not indent. (a-b) DIC image of indenting and non-indenting cells, showing that both maintain a rounded morphology; (c-d) fluorescence image of 200nm diameter fluorescent beads embedded at the gel surface. In (c) beads directly under the cell are displaced to a lower plane due to cell-induced indentation; (e) beads are in focus at 6.28 μm below gel surface, indicating the indentation depth generated in the gel; (f) for the non-indenting cell the gel remains flat, and beads are out of focus below the gel surface. Scale bar is 10 μm.

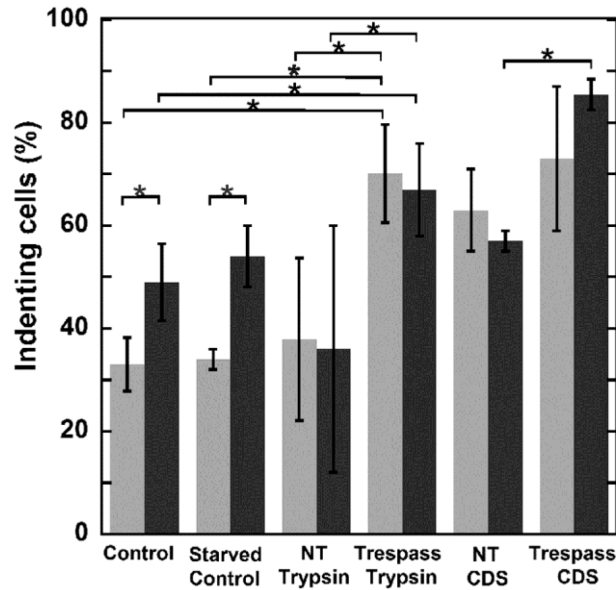

Figure 3. Percentage of MDA-MB-468 (light gray) and MDA-MB-231 (dark gray) metastatic potential cells that indent the 2.4 kPa polyacrylamide gels. Controls include all cells plated on the gels under either serum-full or serum-starved conditions; control cells were dissociated with trypsin. We compare the whole-population controls to two cell populations that are plated on the gels following Boyden chamber migration for 72 hours being, respectively, the trespassing subpopulation (collected from the lower chamber) and the non-trespassing (NT) subpopulation (upper chamber); the experiment is performed once with trypsin and once with a non-enzymatic cell dissociation solution (CDS). Results are presented as the mean ± standard deviation. Asterisks indicate statistically significant differences ($p<0.05$).

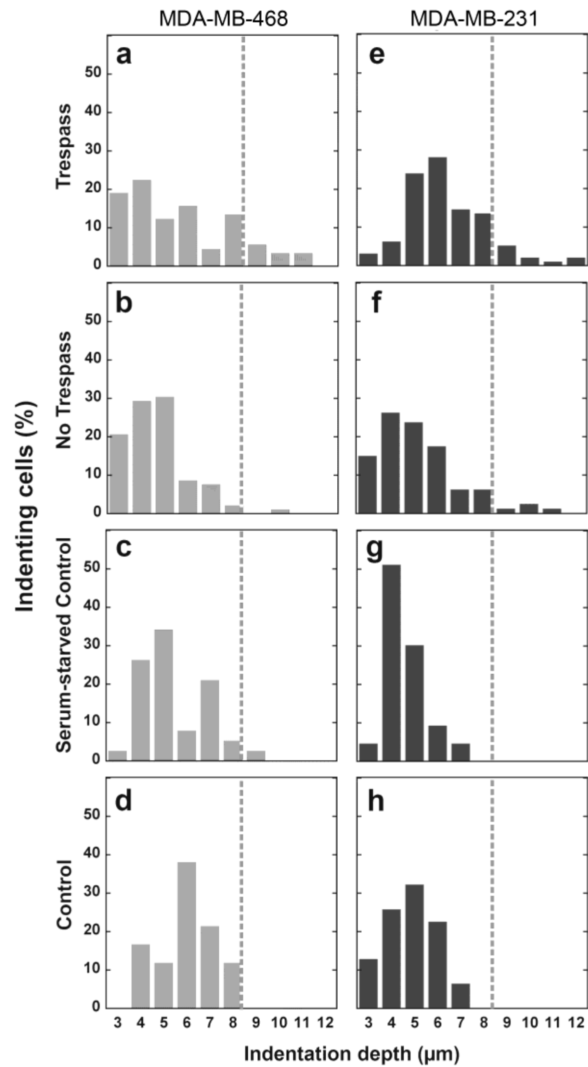

Figure 4. Indentation depth distributions of MDA-MB-468 (a-d, light gray, left) and MDA-MB-231 (e-h, dark gray, right) breast cancer cells. We compare cells that (a, e) trespassed through an 8 μm Boyden chamber; (b, f) non-trespassing cells collected from the top of the Boyden chamber membrane; (c, g) serum-starved controls including all cells; and (d, h) serum-full control with all cells. Dashed lines are guides to the eye noting the maximal observed indentation depths of the controls in both cell types, at least 50-100 single cells were evaluated in each condition.

Supplementary Materials

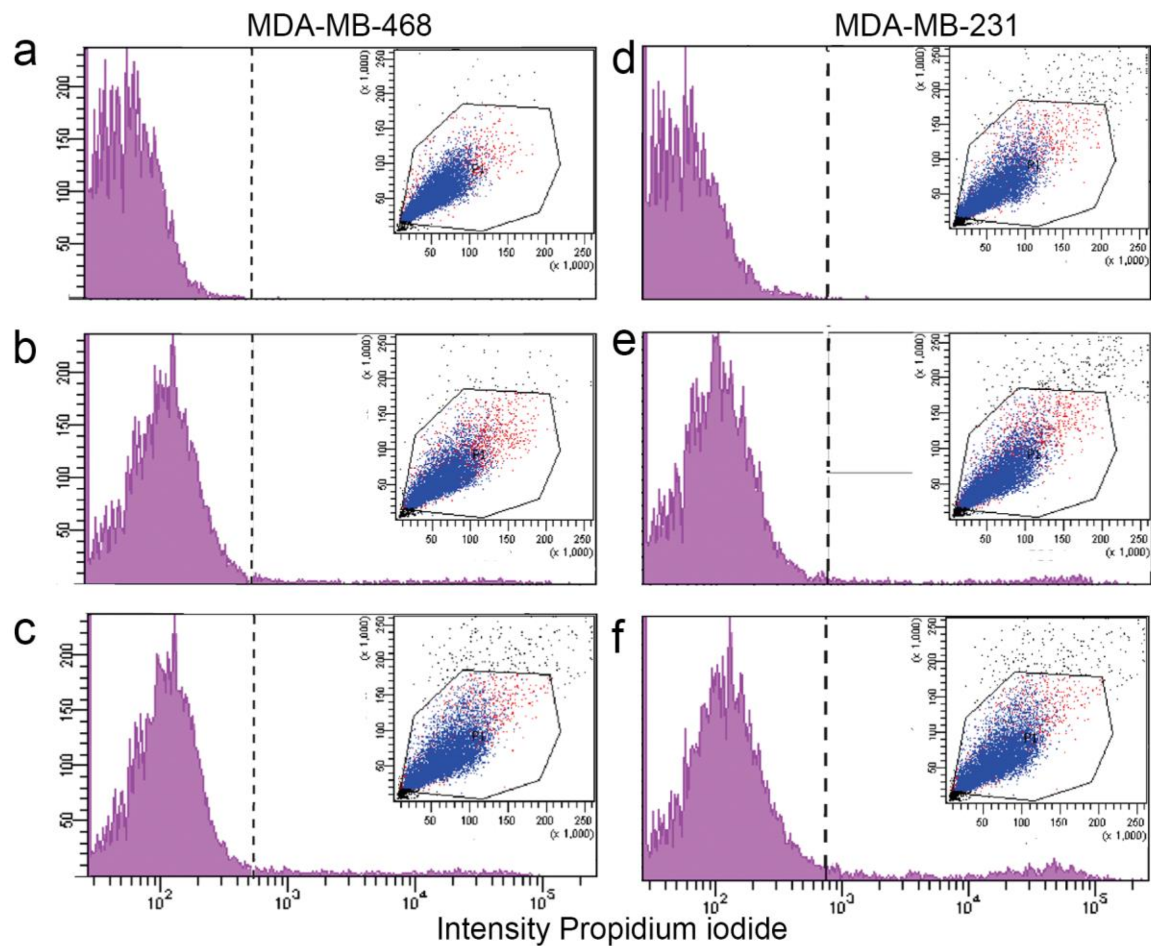

Figure S1. Cell viability following Boyden chamber assay. Whole population of unstained (a) MDA-MB-231 and (d) MDA-MB-468 cells are used as control histograms for gating. Insets are forward scatter (FSC) vs. side scatter (SSC) dot plots with gating on live cells. Cells were collected following Boyden chamber assay and were stained with propidium iodide as a marker for dead cells. MDA-MB-231 and MDA-MB-468 cells were collected from the upper chamber are (b) 98% and (e) 95% viable, respectively, and from the lower chamber are (c) 96% and (f) 96% viable.

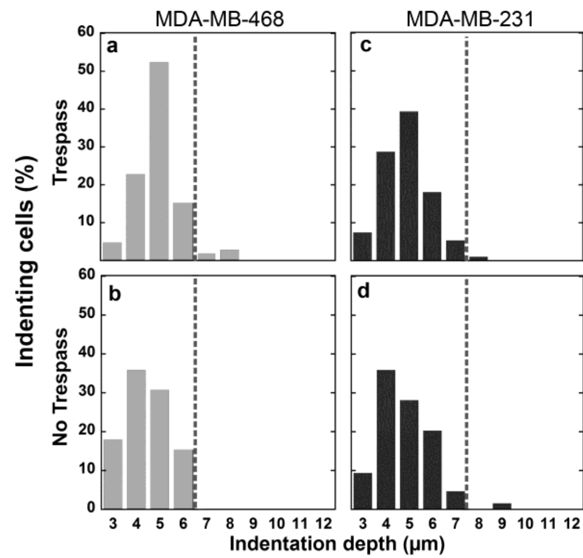

Figure S2. Indentation depths attained by using cell dissociation solution following interaction with Boyden chamber. MDA-MB-468 (a-b, light gray, left) and MDA-MB-231 (c-d, dark gray, right). Cells that (a, c) trespassed through an 8 μm Boyden chamber and (b, d) non-trespassing cells collected from the top of the Boyden chamber membrane. Dashed vertical lines match those in Figure 4.