


# Proximity of Metastatic Cells Enhances Their Mechanobiological Invasiveness

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(Received 1 December 2016; accepted 11 February 2017; published online 21 February 2017)

Associate Editor Aleksander S. Popel oversaw the review of this article.

**Abstract**—A critical step in metastases formation is cancer-cell invasion through tissue. During invasion, cells change morphology and apply forces to their surroundings. We have previously shown that single, metastatic breast-cancer cells will mechanically indent a synthetic, impenetrable polyacrylamide gel with physiological-stiffness in attempted invasion; benign breast cells do not indent the gels. In solid tumors, e.g., breast cancers, metastases occur predominantly by collective cell-invasion. Thus, here we evaluate the effects of cell proximity on mechanical invasiveness, specifically through changes in gel indentation. Gel indentation is induced by 56, 33 and 2% (in > 1000 cells), respectively, of adjacent high metastatic potential (MP), low MP and benign breast cells, being double the amounts observed in single, well-separated cells. Single cells exhibited a distribution of indentation depths below 10  $\mu\text{m}$ , while adjacent cells also showed a second peak of deeper indentations. The second peak included 65% of indenting high MP cells as compared to 15% in the low MP cells, illustrating the difference in their invasiveness. Thus, proximity of the metastatic cells enhances their mechanical ability to invade, demonstrating why collective cancer-cell migration is likely more efficient. This could potentially provide a rapid, quantitative approach to identify metastatic cells, and to determine their metastatic potential.

**Keywords**—Mechanobiology, *In vitro* invasiveness, Metastatic potential, Cell-substrate mechanical interactions, Breast cancer.

## INTRODUCTION

Cancer is the third cause of death worldwide, where metastases are the main cause of patient mortality. A critical step in formation of metastases is the invasion of cancer cells that have detached from the tumor, through the surrounding tissue. During invasion,

migrating cancer cells can move through the extracellular matrix (ECM), between neighboring cells, and in tissue or blood vessels. During this process the cancer cells change morphology and apply forces to their surroundings.<sup>9,16,26</sup> Those forces depend on the cell and cancer type and the stiffness of surrounding microenvironment.<sup>35,43,44</sup> The ECM surrounding the cells affects invasion, and substrate rigidity has been shown to regulate cancer cell invasiveness at the primary tumor site<sup>40,45</sup>; the ECM mechanics correlates with tumor grade, subtype, and aggression in human breast cancer.<sup>3</sup> As previously shown by our lab, metastatic cells will apply larger forces on stiffer, elastic, and impenetrable substrates<sup>47</sup>, indenting the substrates in attempted invasion.<sup>38,39</sup>

Cell invasion combines both biochemical and biomechanical interactions of the migrating cells with their environment. Invading cells typically require a degradable ECM to modify as they migrate (mesenchymal migration mode<sup>27</sup>) and/or an ECM with pores that are large enough for the cells to squeeze through (amoeboid mode<sup>41</sup>) while they may concurrently remodel the ECM.<sup>22</sup> Highly invasive cancer cells have an enhanced ability to sense (and affect) the rigidity of their surroundings<sup>33</sup> and can readily switch between the mesenchymal and the amoeboid migration modes.<sup>50</sup> These migration modes, and especially amoeboid motion, require cell flexibility and an ability for rapid morphological changes. In amoeboid migration, cell movement includes formation of rounded membrane blebs and elongated protrusions,<sup>54</sup> similar to our previous observations when cells indent a synthetic gel.<sup>39</sup> To rapidly morph, cells must be pliable and dynamic, both externally and internally. Correspondingly, highly migratory and invasive cancer cells have been shown to be externally softer than low metastatic potential and benign cells.<sup>14,30,59</sup> In parallel,

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as we have previously shown, the internal environment of highly metastatic cells is more dynamic and their cytoskeleton is more sparse than both low metastatic potential (MP) and benign cells,<sup>26,28</sup> and the high MP cells are also more receptive to internalizing materials from their surroundings.<sup>25</sup> The enhanced, dynamic pliability of the metastatic cancer cells facilitates their invasion through small pores, as was evaluated by *in vitro* Boyden chamber invasion experiments.<sup>5,6</sup>

While metastatic cells are soft and flexible, we and others have shown that they are also able to modify their cytoskeleton and apply localized forces to the substrate, either adhesive (traction) or invasive (indenting).<sup>17,37–39,42,60</sup> The adhesive, traction forces applied by cells have typically been measured in 2-dimensions (2D) using synthetic, elastic, polyacrylamide gels. Those gels are produced as a flat substrate with sub-micron fluorescent particles at its surface<sup>2,4,9</sup> or as flexible posts,<sup>24</sup> both coated with ECM-molecules to facilitate cell adhesion. As cells adhere to the gels and anchor themselves they deform the surfaces or posts, with the amount of deformation directly correlated with the force, which is calculated using traction force microscopy (TFM); this has typically been done for monolayers in the context of migration or transmigration, or for single cells interacting with their substrates. Traction forces were shown to increase with gel stiffness and collagen-coating density, with metastatic cells also generating larger tractions than non-metastatic cells.<sup>11,36</sup> In contrast to the more typically observed, lateral traction forces, we have recently identified forces applied by metastatic breast cancer cells normal to the substrate, resulting in indentation of the gel.<sup>17,38,39</sup> Small indentations ( $< 1 \mu\text{m}$ ) have previously been observed in non-cancerous cells,<sup>15,31</sup> likely as a result of lateral traction forces. In contrast, the breast cancer cells evaluated in our previous studies and in the current work have induced indentations up to  $10 \mu\text{m}$ , on the scale of the cell diameter ( $10\text{--}20 \mu\text{m}$ ). While the mechanical interactions of cancer cells with their environment have been evaluated in single cells, most invasive solid tumors predominantly display collective invasion.<sup>12</sup> In collective invasion, cells migrate together and invade their surroundings while remaining physically or functionally connected.<sup>21</sup> Collective invasion of tumor cells is similar to single-cell migration, but also has important differences.<sup>20,23,63</sup> Whereas the molecular mechanisms of collective cancer invasion depend on cancer cells' phenotype and extra-cellular environment,<sup>34</sup> all invading cells need to physically change morphology and push through their microenvironment.<sup>29</sup>

Here, we evaluated the effect of the proximity of cancer cells on their ability to apply force and indent the underlying gels. We seeded cells at high density, generating groups of adjacent cells, and evaluated the changes

in cell morphology and indentation induced in the initially flat gel. We compared the percentage of cells that induce indentations and the indentation depths attained by groups of cells vs. those achieved by single cells. We observe that proximity of the cancer cells enhances their ability to indent the gels. Specifically, the percentage of indenting adjacent cells doubled relative to well-spaced single cells (for both the high and the low MP cells evaluated here), and the indentation depth also increased; benign cells do not significantly indent gels in either configuration. Moreover, we observe measurable differences in amounts of indenting cells and the attained depths between the high and the low metastatic potential cells. Thus, the proximity of the cells affects their invasiveness and the gel indentation assay may potentially be used to classify the malignancy and invasiveness of cancer cells.

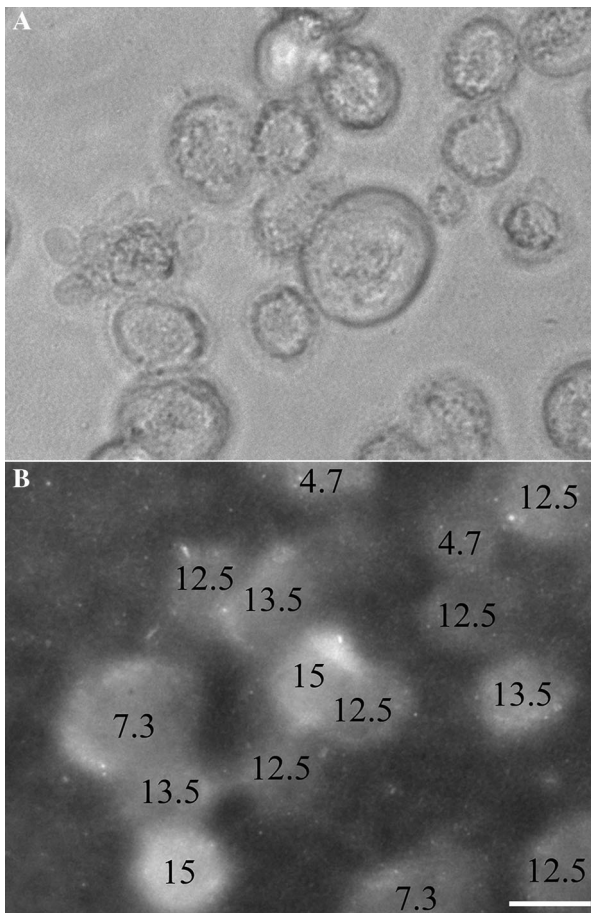
## MATERIALS AND METHODS

### *Cell Culture*

We have used three commercially-available human, epithelial breast cell lines: breast cancer cells from lung metastases with high metastatic potential (MDA-MB-231, all cells from ATCC, Manassas, VA) and with low MP (MDA-MB-468), and benign breast cells (MCF-10A) as control. Cells were cultured in their appropriate media as recommended by the manufacturer. For the MDA-MB-231 and the MDA-MB-468 cell lines we used Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen life technologies, Carlsbad, CA) supplemented with 10 vol.% fetal bovine serum (FBS) (Hyclone, ThermoFisher Scientific, Waltham, MA), 1 vol.% each of L-glutamine, penicillin-streptomycin and sodium pyruvate (all from Biological Industries, Kibbutz Beit Haemek, Israel). For the MCF-10A cell line the media included 1:1 vol:vol DMEM and F12 (Biological Industries, Israel), supplemented with 5 vol.% horse serum (Hyclone, ThermoFisher Scientific, Waltham, MA), 0.05 vol.% Hydrocortisone, 0.01 vol.% Cholera toxin, 0.1 vol.% Insulin (all from Sigma, St Louis, MO), 1 vol.% penicillin streptomycin, 1 vol.% L-Glutamine (both from Biological Industries, Israel), and 0.01 vol.% Human EGF (Peprotech Asia, Israel).

### *Polyacrylamide Hydrogel Preparation*

The polyacrylamide (PAM) gels were prepared at physiological stiffness (Young's modulus  $2400 \text{ Pa}$ )<sup>43</sup> according to an established protocol.<sup>17,38,39,47</sup> In short, gels were prepared on glass coverslips number 5 thickness, 30 mm diameter (Menzel, Germany), with constant ratio of the monomers: Acryl and the cross-linker



**FIGURE 1.** Representative image of adjacent high metastatic potential cells indenting a 2400 Pa polyacrylamide gel. (a) DIC image of cells on the gel; (b) fluorescence image of particles at the gel surface; the numbers on the image represent the indentation depths ( $\mu\text{m}$ ) of each indenting cell, obtained by the difference between the focal heights of the gel and the lowest point where particles are in focus. Scale bar is 20  $\mu\text{m}$ .

BIS-acrylamide (both from Bio-rad, Israel), and water; specifically, we used 34  $\mu\text{l}$  of 40 vol.% acrylamide and 3.8  $\mu\text{l}$  of 2 vol.% BIS acrylamide in 203  $\mu\text{l}$  of distilled water. Polymerization was initiated with ammonium persulfate and catalyzed with tertiary aliphatic amine N,N,N',N'-tetramethylethylenediamine (both from Sigma, St Louis, MO). Red fluorescent (excitation/emission 580/605 nm) carboxylated polystyrene particles (Molecular Probes, Invitrogen Life Technologies, Carlsbad, CA), 200 nm in diameter, were embedded in the gel just below its surface by performing slow gelation at 2 °C during centrifugation.<sup>52,53</sup> Finally, the surface of the gel was shortly activated with Sulfo-SANPAH (Pierce, Thermo Scientific, Waltham, MA), washed with HEPES, and coated with 0.005 mg/ml rat tail collagen type I (Sigma, St Louis, MO) for cell adhesion.

The Young's modulus ( $E$ ) of the gels was determined with a TA Instruments AR-G2 rheometer (New Castle, DE) using a 2-cm parallel plate fixture by the

following relation:  $E = 2G^*(1 + \nu)$  and performing dynamic, frequency and strain sweeps.<sup>39,47</sup> The complex shear modulus,  $G^*$ , was measured by the rheometer and was effectively equal to the elastic modulus  $G'$ , indicating an elastic material.<sup>49</sup> The Poisson's ratio,  $\nu$ , is 0.49 for PAM gels.<sup>8</sup> Rheology measurements of a wide range of polyacrylamide hydrogels have previously been shown to correlate perfectly with localized mechanical properties of the gels, as measured using AFM.<sup>1</sup> In addition, polyacrylamide gels have previously been shown to be homogeneous and to possess remarkably linear mechanical properties, again determined by comparing rheology and AFM measurements.<sup>10</sup> Thus, we consider the gels to have the same Young's modulus at all locations.

### Cell Staining

Calcein-AM fluorometric assay (BioVision, USA) was used for viability staining,<sup>62</sup> where the compound easily penetrates the cytoplasm of intact and live cells. Hydrolysis of Calcein-AM by intracellular esterases produces fluorescence in the cell cytoplasm which can be observed at excitation/emission of 485/530 nm starting at 30 min after staining. Hoechst 33342 assay (Sigma, St Louis, MO) was used for live-cell nucleus staining,<sup>55</sup> where an enzymatic reaction results in fluorescent labeling (excitation/emission 346/460 nm) of the nucleus after 1–2 h of incubation.

### Microscopy and Imaging

The imaging was done with an inverted, epifluorescence Olympus IX81 microscope, using a 60 $\times$ /0.7NA differential interference contrast (DIC, Nomarsky optics) air-immersion, long working-distance objective lens. The cells were maintained in 37 °C, 5% CO<sub>2</sub>, and high humidity (90%) throughout the entire experiment to sustain their viability. Imaging and indentation depth measurements were started approximately 45 min after seeding to allow cell attachment. Each gel was imaged in at least 9, randomly chosen, fields-of-view. The focal depth of each image was recorded independently during the experiment, using an automated microscope stage connected to a computer and accessed by a custom MATLAB module.

We seeded 300,000 cells on each gel within their respective media, resulting in an average of  $19 \pm 5$  cells per field-of-view (area of 0.016 mm<sup>2</sup>); the cells and gels are immersed in media which reaches 1 cm above gel height to prevent any surface tension or evaporation effects. The cells are adjacent and/or touching, yet typically remain in a monolayer. We define cells as adjacent when they are closely situated to, yet not overlapping, at least one other cell; cells at distances



larger than cell size were not considered adjacent.<sup>2,56</sup> Cell groups were typically <9 cells with 1–6 immediately-adjacent (proximate) indenting cells.

### Indentation Depth Determination

Images were analyzed using a custom-designed module<sup>39</sup> in MATLAB 2012b (The Mathworks, Natick, MA) to determine the number of viable and indenting cells, as well as the indentation depth of each cell (Fig. 1); we determined the number of indenting cells out of the total adhered cells. At each measurement time-point and randomly chosen location on the gel, at least 3 images were acquired: (i) a DIC image of the cells on the gel, (ii) a fluorescence image of the particles embedded at the focal plane of the gel surface, and (iii) a series of fluorescence images at the lowest focal depth where particles are observed, to identify each indenting cells' depth. We typically imaged 5–6 focal depths below the gel-surface height, where 1–8 indenting cells were in focus at each depth (Fig. 1 and Supplementary Materials, Fig. S1). The indentation depth was then calculated by the difference in focal depths between the fluorescence image at the (unindented) gel surface and at the lowest focal plane where particles are in focus, i.e., at the bottom of the specific indenting cell. To demonstrate that the changes in focal depth of the particles embedded in the gel surface correlate with indentations caused by cells, we provide confocal images and side views of the gels with indenting cells (Fig. S2).

The indentation depths of 355 and 506 adjacent, respectively, high and low MP cells, were calculated from at least 6 separate experiments each (see Table S1). Results for indenting single high and low MP cells were adapted from our previous works,<sup>17,39</sup> averaging, respectively, 84 and 41 single cells in 4 separate experiments. We provide only on the indentation depths in the current analysis, without approximating the inducing force as that would require a yet unavailable model. In single cells the force applied to the substrate may be estimated using a Hertz model of a rigid sphere<sup>17,38,39</sup>; this is appropriate due to the cell shape and nucleus placement. However, when cells are adjacent, they synergistically interact and change morphology and the assumptions for the Hertz model do not apply.

### Statistical Analysis

Statistical analysis was performed to compare between the cells with high or low metastatic potential and to compare adjacent and single cells of the same type. We used the general, linear mixed model is a multivariate regression method that helps to generalize

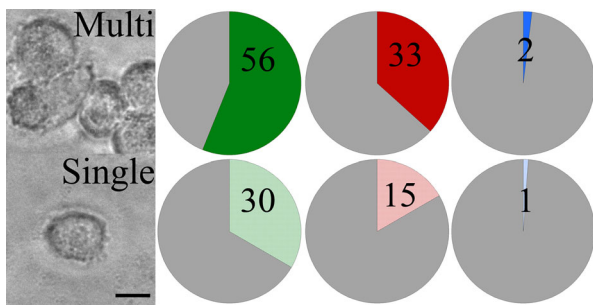
the analysis of variance (ANOVA).<sup>18</sup> In all cases, statistical significance was determined when the  $p$  value was less than 0.05.

## RESULTS

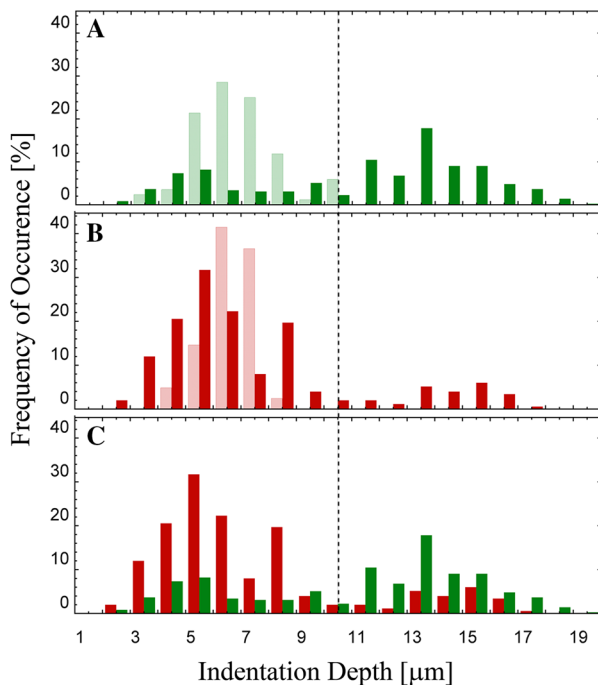
We have evaluated the indentations induced on 2400 Pa PAM gels by groups of closely situated, non-aggregated cells (Figs. 1 and S2). We evaluated the number of indenting cells and the indentation depths of each cell within the group, comparing high metastatic potential, low MP, and benign cells. We observed that 56 and 33%, of high MP and low MP cells, respectively, indented the gels; negligible amounts of benign cells indented, similar to our previous observations using single cells.<sup>39</sup> Interestingly, for both metastatic cell types, the percentage of indenting cells that are densely seeded and organized in groups is approximately double the percentage of single indenting cells (Fig. 2). We note that the cells in groups are viable (Fig. S3) throughout the experiment (up to 6 h). In addition to the increase in percentage of indenting cells, adjacent cells attain larger indentation depths than single cells, likely by synergistic interactions; the number of neighboring cells did not appear to correlate with obtained depths (not shown).

We observe that the indentation depths of the adjacent cells did not change during the observation time. This is in contrast to our previous observations with single cells,<sup>39</sup> and could indicate different energetic capabilities of single cells vs. clusters. We have measured the indentation depths of each indenting cell within adjacent cell groups starting at 45 min after seeding and up to 6 h (Fig. S4). The indentation depths at early and late times were statistically indistinguishable ( $p > 0.19$ ) for both high and low MP cells; we evaluated at least 160 indenting cells in groups at each time point for each cell line. Hence, we merge the results of adjacent cells from all experiment times to improve statistics; cells may move on the gel and thus the imaged cells may differ between time points, making the results effectively independent.

While the single cells<sup>39</sup> exhibited a single-peak Gaussian-like distribution of indentation depths, adjacent cells exhibited two peaks, below and above 10  $\mu\text{m}$ , with the lower range overlapping with that of the single cells (Fig. 3). The indentation depths induced by the single, well-spaced, high and low MP cells were <10  $\mu\text{m}$  and their averages were statistically indistinguishable ( $p > 0.3$ ); averages of the indentation-depth distributions are provided in Table S1. In contrast, the adjacent cells exhibited a distribution with (at least) two peaks separated by a minima at 10  $\mu\text{m}$ , which is the highest indentation depth of single



**FIGURE 2.** Percentage of indenting adjacent (top row) and single cells (bottom row), indicated by representative images; single cell results are adapted from Kristal-Muscal *et al.*<sup>39</sup> From left, high metastatic potential (green), low MP (red) and benign (blue) cells that indent the 2400 Pa PAM gels in varying percentages. Scale bar is 10  $\mu\text{m}$ .



**FIGURE 3.** Distribution of indentation depths of all indenting cells. (a) Adjacent (dark bars) and single (light bars) high metastatic potential cells; (b) adjacent (dark bars) and single (light bars) low MP cells; (c) comparison of adjacent high (green bars) and low (red bars) MP cells. All single cell results adapted from Kristal-Muscal *et al.*<sup>39</sup> Dashed line marks the local minima of the distribution of adjacent cells at 10  $\mu\text{m}$ .

cells. Interestingly, the first peak of the adjacent cells was slightly lower than that of the single cells for both high and low MP cells (see Table S2 for statistical analysis), yet the minima overlap at 10  $\mu\text{m}$ .

We observe that the high and low MP cell lines differ when seeded densely and group indentation ensue (Fig. 3c). The higher metastatic potential cells induce deeper indentations in groups, as compared to the low MP cells. Specifically, about 65% of the indenting high

MP cells exhibit indentation depths at the higher range of indentations ( $>10 \mu\text{m}$ ), while only 15% of the indenting low MP cells exhibit depths in the second peak; in the single cells the cell amounts and indentation depths were indistinguishable. This may indicate different capabilities and synergistic interactions between adjacent high and low MP cells.

## DISCUSSION

We observe that close proximity of metastatic cancer cells increases their ability and propensity to forcefully indent soft, elastic, impenetrable gels, likely in attempted invasion; in a recent work, we show that mechanical indentations correlate with cell motility and invasiveness through a Boyden chamber.<sup>7</sup> When seeded on collagen-coated gels with 2400 Pa stiffness, a subset of the metastatic cells will indent, but not cross the gel, as it is non-degradable. It is important to note that the forces applied by cells during gel indentation are sensitive to both substrate stiffness and ECM-ligand coating type and density.<sup>61</sup> We and others have shown that increased gel stiffness using collagen coating induces stronger, cell-applied traction forces, as does increased collagen density<sup>36,47</sup>; the increased force generation is directly mediated by enhanced cell spreading. Hydrogel coating with other ECM ligands, e.g., fibronectin, has been shown to induce comparable traction forces to collagen coating<sup>46</sup> and even to promote cell invasion,<sup>48</sup> hence we expect similar and even enhanced indentations with different gel coatings.

We have shown that the percentage of indenting cells doubles when seeding density is high and cells are adjacent and in close contact, as compared to single, well-spaced cells; benign cells do not indent the gels regardless of seeding density. In addition, the indentation depths attained by adjacent cells are higher, suggesting synergistic cell interactions with neighboring cells and with the substrate. We note that this synergistic, mechanobiological effect of cell proximity appears to be amplified in the high metastatic potential cells (as compared to the low MP cells), likely correlating with and facilitating their increased invasiveness.

We observe that more of the adjacent cells indent gels and are able to reach larger indentation depths than the single cells. In general, the measured indentation depths of both the single metastatic cells and clusters are an order of magnitude greater than dimples previously observed to be induced by non-cancerous cells while spreading<sup>31</sup> or crawling.<sup>15</sup> It is important to note that the indentation depths observed here do not result from cell weight effects (gravitation) on the 2400 Pa gels, as the benign cells do not indent the gels to any measurable depth. We have also previously

estimated gravitational indentation due to a single cell to be on the scale of  $1.2 \times 10^{-16} \mu\text{m}$ , which is 16–17 orders of magnitude smaller than the indentation depths observed here.<sup>2</sup> While single cell indentations were limited to  $<10 \mu\text{m}$ , adjacent cells induced a second indentation-depth peak centered on  $\sim 13 \mu\text{m}$ . Interestingly, we are able to distinguish between the high and low MP cells based on the amounts of cells attain the larger indentation depths. Specifically, we have observed 65% of the high MP clusters with indentations  $>10 \mu\text{m}$ , as compared to only 15% of the low MP cells. A similar phenomenon was recently observed during *in vivo* cancer-cell migration,<sup>51</sup> where the velocities of single highly invasive (MDA-MB-231) and more moderately invasive (TN1) breast cancer cells did not significantly differ, yet the adjacent cells of the highly invasive cells exhibited significantly larger velocity. A recent work from our lab directly correlates the two phenomena of cell motility and mechanical, gel indentations.<sup>7</sup> Thus, the more invasive cells exhibit increased aggressiveness when clustered both *in vivo* and *in vitro*, as demonstrated by motility and invasiveness, i.e., indentation capacity.

The doubling percentage of indenting adjacent cells and the increased indentation depth are a strong indication of an increase in the invasive capacity of adjacent cells. Such groups have previously been shown to be more invasive, with cells collectively migrating as loosely adhered groups, sheets, and clusters.<sup>13</sup> Amplified invasiveness of clusters of cancer cells was demonstrated when, for example, more metastases formed in mice injected with clumps of tumor cells as compared to mice injected with an equal total number of single cells.<sup>19</sup>

We<sup>39,47</sup> and others<sup>11</sup> have shown that (single) metastatic cancer cells apply stronger forces to stiffer microenvironments. The forces applied by single, indenting high and low metastatic breast cancer cells on 1300 Pa gels were 2–3-fold smaller than those applied to a 2300 Pa gel<sup>39</sup>; cells were unable to adhere properly to softer, 500 Pa gels. Similarly, the lateral traction forces exerted by the same metastatic cells increase with gel stiffness, as verified for gels 1000–11,000 Pa.<sup>36,47</sup> Moreover, dynamic stiffening of a surrounding hydrogel induced invasive phenotypes even in benign cells.<sup>58</sup> More generally, non-cancerous fibroblasts preferentially migrate toward stiffer regions<sup>45</sup> and may adapt their stiffness to match that of their environment.<sup>57</sup> In cancerous fibrosarcoma cells, mechanical (magnetic) stimulation increased the percentage of invading cells.<sup>48</sup> Thus, we propose that the enhanced indentation capacity of adjacent metastatic cells observed in the current study, is related to mechanical (tension-induced) interactions of the cells likely through the gel; the gels are expected to remain elastic, likely with unchanging Young's modulus, un-

der the strains applied by the cells (not shown). Force application by cells, and especially such synergistic interactions likely require changes in cell morphology and intracellular structure. We have previously shown a coordinated restructuring of the metastatic cells, likely intended to facilitate force application, i.e., remodeling the actin and the microtubules and moving the nucleus and changing its shape.<sup>17</sup> Coordination of actin dynamics facilitates not only cell migration and force application, but also development of cell–cell junctions in formation of clusters,<sup>32</sup> which could allow the cells to act together. While of great interest in many works, the exact synergistic mechanism of collectively migrating cells has yet to be elucidated.

In conclusion, we have shown that adjacent metastatic cancer cells are able to more forcefully interact with their microenvironment, highlighting the increased invasiveness of clusters as compared to single cells. We have shown that the gel-indentation platform used here may potentially be used to identify the metastatic potential of cancer cells, to provide a sample-specific, or patient personalized, predictive prognosis. We note that the gel-platform may facilitate rapid (few hours) and quantitative evaluation of the metastatic potential, through the mechanobiological interaction of the cells with their substrate. Mechanobiology and specifically the mechanical interactions of cancer cells with their microenvironment can thus provide a means to elucidate important aspects in cancer cell migration and invasion, such as the collective nature of invasion.

## ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (doi: [10.1007/s10439-017-1814-8](https://doi.org/10.1007/s10439-017-1814-8)) contains supplementary material, which is available to authorized users.

## ACKNOWLEDGMENTS

The authors thank Mrs. Rakefet Rozen for her assistance in analyzing the results. The work was partially supported by The Technion EVPR Funds—The Elias Fund for Medical Research and The Karbeling Fund for Bio-Medical Engineering Research, and also by a grant from the Ministry of Science, Technology and Space, Israel, and the National Science Council (NSC) of Taiwan.

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