Tumor spheroid invasion in epidermal growth factor gradients revealed by a 3D microfluidic device

Young Joon Suh1, Mrinal Pandey1,3, Jeffrey E Segall2 and Mingming Wu1,∗

1 Department of Biological and Environmental Engineering, 306 Riley-Robb Hall, Cornell University, Ithaca, NY 14853, United States of America
2 Anatomy and Structural Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, United States of America
∗ Author to whom any correspondence should be addressed.
3 First author of equal contribution.

E-mail: mw272@cornell.edu

Keywords: tumor spheroid, chemotaxis, microfluidics, EGF, invasion, 3D ECM, tumor microenvironment

Abstract

Epidermal growth factor (EGF), a potent cytokine, is known to promote tumor invasion both in vivo and in vitro. Previously, we observed that single breast tumor cells (MDA-MB-231 cell line) embedded within a 3D collagen matrix displayed enhanced motility but no discernible chemotaxis in the presence of linear EGF gradients using a microfluidic platform. Inspired by a recent theoretical development that clustered mammalian cells respond differently to chemical gradients than single cells, we studied tumor spheroid invasion within a 3D extracellular matrix (ECM) in the presence of EGF gradients. We found that EGF gradients promoted tumor cell detachment from the spheroid core, and the position of the tumor spheroid core showed a mild chemotactic response towards the EGF gradients. For those tumor cells detached from the spheroids, they showed an enhanced motility response in contrast to previous experimental results using single cells embedded within an ECM. No discernible chemotactic response towards the EGF gradients was found for the cells outside the spheroid core. This work demonstrates that a cluster of tumor cells responds differently than single tumor cells towards EGF gradients and highlights the importance of a tumor spheroid platform for tumor invasion studies.

1. Introduction

Tumor cell invasion within a 3D extracellular matrix (ECM) is critical for tumor cells to gain access to the vasculature, an important step for cancer metastasis [1–3]. Epidermal growth factor (EGF) is a potent chemotactic factor that can induce tumor cell invasion and promote metastasis [1, 4]. EGF receptor (EGFR), a transmembrane protein, is activated by binding to its specific ligands including EGF. In normal tissues, EGFR ligands are tightly regulated to control cell proliferation, migration, and as such, maintain tissue homeostasis. In cancer, however, EGFR is overexpressed and/or there can be excessive production of ligands. Often EGF is secreted by cells that are not tumor cells within the tumor microenvironment. For example, macrophages can be obligated partners for tumor invasion and metastasis. Macrophages can secrete EGF to attract tumor cells, while tumor cells produce colony stimulating factor 1 (CSF-1) to activate macrophages to secrete more EGF [5]. Overexpression of EGFR has been implicated in many different types of cancer, including lung, breast, prostate, and colorectal cancer [6–11], and as a result, EGFR has emerged as an important therapeutic target for cancer [12–14].

Much of what we know today about how animal cells respond to cytokine gradients is derived from commercially available assays including the Zigmond chamber [15] and Boyden chamber [16]. These assays are straightforward to implement and have been a work horse for chemotaxis studies. The limitations of these macroscale platforms are that the gradient takes a long time to establish, they cannot maintain a steady gradient for a prolonged period of time, and the platform is difficult to be made compatible with microscopic imaging. Microfluidic platforms have been developed recently to overcome these limitations [17–21].
Tumor cell chemotaxis within EGF gradients has been studied using microfluidic platforms. Using a 2D microfluidic gradient platform, Wang et al found that breast tumor cells (MDA-MB-231 cell line) do not exhibit a chemotactic response to a linear EGF gradient, but migrate along the EGF gradient clearly when the gradient is steep and nonlinear [22]. Recently, our lab studied breast tumor cell chemotaxis in a 3D microfluidic platform, where MDA-MB-231 cells were embedded within a 3D collagen matrix. While chemokinesis was found in the presence of an EGF gradient, no chemotactic response was discovered using linear EGF gradients [23]. In vivo, EGF gradients are typically generated via molecular diffusion within tissues. Thus, a steep and nonlinear gradient is unlikely at the time scale for tumor cells to respond. Recent theoretical work proposed that a cluster of cells responds to chemical gradients differently when compared to their single cell counterparts. They show that a cluster of cells can migrate along the chemical gradient direction by averaging the individual chemotactic responses across the entire cluster body, even when single cells do not exhibit chemotactic behavior in the same gradient [24–27]. Here, a cluster of cells compare the ligand-receptor binding sites at the front and the rear of the cell cluster, whereas a single cell compares its ligand-receptor binding sites at the front and the rear of the cell along the gradient [20]. In an effort to study this phenomenon, studies have been carried out by different groups where they have observed organoids’ branching responses along the SDF-1 and EGF gradients when single cells did not show any significant chemotactic behavior towards the gradient [28, 29].

Inspired by the previous experimental work and theoretical predictions, we developed a 3D tumor spheroid model for tumor chemotaxis studies. Breast tumor spheroids using MDA-MB-231 cells were engineered and embedded within a type I collagen matrix. Under a well-defined linear human EGF gradient, tumor spheroid invasion dynamics were followed and characterized.

2. Materials and methods

2.1. Cell culture

Triple-negative breast tumor cells (MDA-MB-231) expressing EGFP were kind gifts from Dr Joseph Aslan at the Oregon Health and Science University, who obtained them originally from ATCC. They were cultured in high glucose Dulbecco’s modified eagle medium (DMEM) (Cat. #: 11965092, Gibco, LifeTechnologies Corporation, Grand Island, NY) with 10% fetal bovine serum (Cat. #: S11150, Atlanta Biologicals Lawrenceville, GA), 100 units/ml Penicillin, and 100 μg ml\(^{-1}\) streptomycin (Cat. #: 15140122, Gibco). All cells were cultured in T75 flasks (Cat. #: 10062-860, Corning, Lowell, MA, USA), which were placed in a 5% carbon dioxide, 37 °C, and 100% humidity incubator. Cells were passaged every 3–4 days and harvested for experiments when the cell culture reached 70%–90% confluency. MDA-MB-231 cells with 20 or fewer passages were used.

2.2. Tumor spheroids

A specially designed array of microwells was used for making MDA-MB-231 spheroids [30]. Briefly, an array of 36 × 36 microwells was first patterned on a 1 mm-thick agarose gel membrane using a soft lithography method. Each microwell is cylindrical in shape with a diameter of 200 μm and a depth of 250 μm (see figure S1). The agarose gel surface provides low adhesion surfaces to the cells, making it easier for the cells to cluster together and form spheroids. One microwell array was then placed in each well of a 12-well plate (Cat. #: 07-200-82, Corning). Within each well, we placed 1.25 million MDA-MB-231 cells suspended in 2.5 ml of DMEM/F12 media (DMEM/F-12, Cat. #: 11320033, Gibco) supplemented with 5% horse serum (Cat. #: S12150, Gibco), 5% EGF (Cat. #: PHG0311, Gibco), 0.5 mg ml\(^{-1}\) hydrocortisone (Cat. #: H0888-1G, Sigma-Aldrich), 100 ng ml\(^{-1}\) cholera toxin (Cat. #: C-8052, Sigma), 10 μg ml\(^{-1}\) insulin (Cat. #: I1882, Sigma), 100 units/ml Penicillin, and 100 μg ml\(^{-1}\) Streptomycin (Cat. #: 15140122, Invitrogen). The 12-well plate was then kept in an incubator (Forma, Thermo Scientific, Asheville, NC, USA) at 37 °C, 5% carbon dioxide, and 100% humidity for 5 days before harvesting. On day 3, the medium was changed to fresh medium. The spheroids were starved in DMEM supplemented with 1% FBS for 8 h before harvesting to enhance their response to the EGF gradient. The average diameter of the spheroids was about 100 μm at the time of collection. For each experiment, the spheroids were collected from six microwell arrays, and a Falcon\(^\circledR\) Cell Strainer (Cat. #: 352530, Corning) with 70 μm pores was used to collect the spheroids. We note that rich media (DMEM/F12) and long culture time (5 days) are important for the formation of MDA-MB-231 spheroids. More details of the spheroid making process can be found in reference [31].

2.3. 3D spheroid culture

To make 3D spheroid cultures, we suspended tumor spheroids in type I collagen (rat tail tendon Cat. #: 354249, Corning). The collagen stock was first diluted to 5 mg ml\(^{-1}\) with 0.1% acetic acid and stored at 4 °C before the experiments. For each experiment, we typically prepared 200 μl tumor spheroid embedded collagen with a collagen concentration of 1.5 mg ml\(^{-1}\), and a spheroid concentration of about 7800 spheroids/ml. To do this, a 60 μl collagen stock (5 mg ml\(^{-1}\)) was first titrated with 1.32 μl 1 N NaOH and 20 μl 10X M199 (Cat. #: M0650-100Ml, Sigma) to
yield a final pH of ~7.4. Then, 118.7 μl of spheroids culture with DMEM supplemented with 1% FBS was added to reach a final volume of 200 μl. This stock was placed in ice until it was introduced into the microfluidic channel.

2.4. Microfluidic device setup

Surface activation. A standard 1” × 3” glass slide (Fisher Scientific, Pittsburgh, PA) was first treated with 1% PEI for 10 min. After rinsing it with sterile dH2O, it was treated with glutaraldehyde for 30 min. The glass slides were left in a biohood overnight in sterile dH2O. The glass slides were then washed with sterile dH2O and dried before use. Surface activation is a crucial step because it prevents the collagen matrices from detaching from the glass slide due to the cell generated traction forces.

Device assembly. With a 1 mm thick polycarbonate spacer placed around the device pattern of the master silicon wafer, 2.5% boiled agarose gel was poured over the pattern. The agarose gel was pressed with a standard 1” × 3” glass slide to form a 1 mm-thick agarose gel. After allowing the gel to cool down to room temperature, the gel was then lifted off the pattern gently. The holes for the inlets and the outlets were made with a 2 mm biopsy punch (Cat. #: 21909-132, Miltex Inc. York, PA), and then the gel was submerged in L-15 medium (Cat. #: 11415064, Gibco) with 2.5% FBS, 100 units/ml Penicillin, and 100 μg ml⁻¹ Streptomycin (Cat. #: 15140122, Gibco) for an hour. The agarose gel membrane with the spacer around was placed on a standard 1” × 3” glass slide, which was then sandwiched between a Plexiglas manifold and a stainless-steel frame (see figure 1). All parts except for the Plexiglas manifold and the polycarbonate spacer were autoclaved for sterility, and all channels were primed with L-15 media. In order to prevent evaporation, all the inlets and outlets were then plugged until media or spheroid-mixed collagen gel was introduced.

3D spheroid seeding. 15 μl of well mixed spheroid-embedded collagen was first pipetted slowly into the center channel of the device with an ice pack placed underneath. After plugging the device to prevent evaporation, the device was then incubated at 37 °C and 5% CO₂ for 45 min for collagen polymerization. To prevent the spheroids from settling down at the bottom during the collagen polymerization process, the device was first placed upside-down, where the glass slide was on top. Then, the device was flipped a total of three times at time points 11, 16, and 30 min. Using this protocol, most of the spheroids were located in the middle z plane of the center channel.

Flow control and EGF gradient generation. The flows into the two side channels were provided by two 10 ml syringes (Cat. #: 303134, Becton Dickinson, Franklin Lakes, NJ, USA) pumped with a syringe pump (KDS230, KD Scientific, Holliston, MA) through a medical grade tubing (Cat. #: AY202431-CP, Cole-Parmer, Vernon Hills, IL). All fluids from the syringes passed through a 0.2 μm filter (Cat. #: MS-3301, Pall Corporation, Port Washington, NY) before going into the inlets of the device to prevent air bubbles from entering the microfluidic channels. L-15 medium supplemented with 2.5% FBS was pumped through the sink channels, and the same medium supplemented with 8.33 nM EGF (Cat #: 354052, Corning, Lowell, MA, USA) was pumped through the source channels at a flow rate of 1 μl min⁻¹ to generate the EGF gradient. For control, both the sink channel and the source channel were pumped with L-15 medium supplemented with 2.5% FBS.

2.5. Imaging and data analysis

All images were taken with a 10× magnification objective lens (NA = 0.25, Olympus America, Center Valley, PA, USA) on an inverted epi-fluorescent microscope (IX 81, 40 Olympus America, Center Valley, PA, USA) and a CCD camera (ORCA-R2, Hamamatsu Photonics, Bridgewater, NJ, USA). The light source for fluorescence imaging was provided by the X-Cite series 120PC unit (Excilites Technologies, Waltham, MA, USA). The scope was surrounded by a stage incubator (Precision Plastics Inc., Beltsville, MD, USA) that maintained a temperature of 37 °C, humidity of ~70%, and atmospheric CO₂ level since L-15 medium was used. The device was placed on an automated X-Y microscope stage (MS-2000, Applied Scientific Instrumentation, Eugene, OR), and images were taken every 10 min for 48 h using CellSens software (Olympus America, Center Valley, PA, USA). In each experiment, bright field and fluorescence images were taken at 12 selected positions at each time point. The time point 0 is defined as the time when the imaging started, which is about 1 h after the introduction of the spheroid embedded collagen to the center channel.

The spheroid invasion was characterized by analyzing the area and the center of mass of the spheroid core over time. Here, we define spheroid core as the central group of interconnected cells that can change shape, expand with time, and migrate (see the yellow outlines figure S2). The spheroid core was tracked using the fluorescence images of the MDA-MB-231 spheroid (figure S2) with a particle detection module in ImageJ (National Institute of Health, Bethesda, MD). For each time-series image, all pixels with intensity greater than 400 (the mean intensity of all the images is 333) were selected, of which particles greater than 7000 μm² were segmented. The area and the center of mass of the segment were then calculated using ImageJ. The area of the spheroid expansion was normalized by the initial spheroid area (control: 8513 ± 1139 μm², EGF: 10984 ± 896 μm²). Note that the spheroid area is measured in the x-y plane. The directionality of the spheroid movement was characterized...
Figure 1. Microfluidic setup for tumor chemotaxis experiments and device calibration. (A1) An image of a microfluidic device for 3D tumor spheroid chemotaxis studies. Two functional chips are placed on a microscope stage for multiple position imaging. (A2) and (A3) Designing principles of a microfluidic gradient generator. Four three-channel devices were patterned on a 1 mm thick agarose gel membrane placed on top of a 1” × 3” microscope slide (A2). Each device consists of three parallel channels. Spheroid-embedded collagen was introduced in the middle channel. EGF and media were introduced into source and sink channels, respectively (A3). All channels have a width of 330 μm and a depth of 250 μm, and the distances between adjacent channels are 240 μm each. (B) A cross section view of the three-channel device. Drawing not to scale. (C) A fluorescent image of all three channels at a steady state, t = 60 min. (D) Gradient generation characterization. Time evolution of FITC dextran concentration profiles revealed by the fluorescence images across three channels. It takes about 1 h to reach a steady state. The gradient profile can be kept as long as the side channels are pumped continuously.

by displacement of the spheroid center of mass along the gradient direction with respect to the initial position at t = 0. Here, the center of mass is the brightness-weighted average of the x and y coordinates of the spheroid core.

Cell trajectories were obtained using the manual tracker in ImageJ (National Institutes of Health) using the time-lapse images. Single-cell migration parameters, speed, velocity, persistence length, and mean squared displacement (MSD) were calculated using these trajectories [23]. The cell speed was defined as the total length of each track divided by the time duration. The cell velocity was defined as the distance between the cell starting and ending locations divided by the time duration. The cell persistence length was defined as the distance between the cell starting and ending locations divided by the length of the cell trajectory. MSD was defined as the average of the distance traveled between neighboring time points squared. To minimize experiment-to-experiment variability, data acquired were normalized for each experiment. The cell speed in the EGF condition is normalized by the average cell speed of the control condition from each experiment (0.182, 0.067 μm min⁻¹), and the cell velocity along the direction of gradient (x-velocity) is normalized by first subtracting the x-velocity from the average cell velocity along the x-direction of the control group (0.011, −0.003 μm min⁻¹) then dividing by the average speed of the cells in the control condition (0.182, 0.067 μm min⁻¹). The persistence length of the cells in the EGF condition is normalized by the average persistence of the cells in the control condition (0.46, 0.51), and the persistence length of the cells in the EGF condition along the gradient direction (x-plength) is normalized by subtracting the average persistence length along the gradient direction of control (0.03, −0.05). For calculation of aspect ratio, the ellipse function from ImageJ was used to fit a single cell. Then, the ratio of major over minor axis was used to obtain the aspect ratio values. Cells with aspect ratio less than 2 were considered amoeboid and greater than 2 mesenchymal [32, 33]. Non-parametric t-test (Mann–Whitney test) was carried out using Prism (GraphPad Software, Inc., La Jolla, CA).
3. Results and discussion

3.1. Microfluidic experimental setup, EGF gradient generation and calibration

A hydrogel based microfluidic chemical gradient generator previously developed in our lab was adapted here for tumor spheroid chemotaxis experiments (see figure 1) [19, 34]. Briefly, a pattern of four three-channel devices was imprinted in a 1 mm thick agarose gel and sandwiched between a Plexiglas manifold and a stainless-steel frame (see figures 1(A) and (B)). MDA-MB-231 tumor spheroids embedded in type I collagen at a final concentration of 1.5 mg ml⁻¹ were introduced into the middle channel. After collagen polymerization, L-15 medium with 2.5% FBS with or without 8.33 nM EGF was introduced to the source and sink channels, respectively. This is comparable to the reported apparent $K_d$ (2–15 nM) of EGFR towards its ligands [35, 36]. As a result, a linear EGF gradient of 10.3 nM mm⁻¹ was established in the middle channel via molecular diffusion through the agarose gel, resulting in about 1.03 nM EGF concentration difference from the front and the back end of the spheroid in the EGF gradient.

To verify that the microfluidic device can establish and maintain a steady EGF (MW: 6400 Da) gradient across the center channel for a prolonged period of time (~24 h), we characterized the gradient generation using FITC dextran (MW: 4000 Da). Here, a 0.1 mM FITC dextran solution was pumped through the source channel, and blank PBS was pumped through the sink channel at a flow rate of 1 μl min⁻¹. Time-lapse fluorescence images of all three channels were taken every minute for over 24 h. We define $t = 0$ to be the time when the syringe pump was started right after an initial flush of the fluids in their respective channels to remove all air bubbles within the channels. The fluorescence concentration profiles in figure 1(D) show that it takes about 1 h to reach a steady state. This is consistent with the theoretical calculation using a first order approximation for the establishment time, $\sim L^2/2D$, where $L$ is the distance between the two side channels (0.81 mm), and $D$ is the diffusion coefficient of FITC dextran (94.77 μm² s⁻¹). The gradient profile was kept steady and linear for more than 24 h in our experiments as long as the flows in the side channels were maintained. The gradient of 10.3 nM mm⁻¹ is estimated assuming the EGF concentration in source channel is 8.33 nM, sink channel 0 nM, and the EGF gradient is linear.

3.2. An EGF gradient promoted spheroid core spreading and mild chemotaxis into the 3D ECM

Tumor spheroids were shown to spread out significantly more in the presence of EGF gradients than in the absence of EGF within the 48 h observation time (see figure 2(A) and figure S2, and movies S1 and S2). When examining the movies of tumor spheroid invasion carefully, tumor spheroids were seen to initially spread out, and then individual cells started to detach from the spheroid core and invaded into the ECM. We define the spheroid core as the main tumor body where all the tumor cells are connected to each other. In the following, we quantify the spheroid invasion processes in two steps: (i) the spheroid core dynamics; (ii) motility of the cells that invaded out of the spheroid core.

For spheroid core dynamics, we examined the area and the center of mass of the spheroid core as a function of time. Figure 2(B) shows that the area of the spheroid core spread out much more in the presence of EGF than those in the absence of EGF. Interestingly, spheroid spreading leveled off at around $t = 30$ h in the presence of the EGF gradient, whereas in control, the spheroids were spreading throughout the entire 48 h. The leveling off maybe a consequence of the dissolving of the tumor spheroid with time, which is consistent with the area spreading of each individual spheroid as shown in figure S4.

When examining the displacement of the center of mass of the spheroid core as a function of time, we found a mild chemotaxis along the EGF gradient. In figure 2(C), a significant deviation in $x$-displacement of the center of mass of the spheroid core from its initial position appeared after 16 h, and then leveled off when $t \sim 30$ h. We conjecture that it takes time for the spheroid to respond to EGF, and this chemotactic response became less sensitive when the spheroid core is fully dissolved around $t = 30$ hrs. In the control, on the other hand, the center of mass of the spheroid core did not move significantly, indicating that there was no significant chemotactic response.

One interesting phenomenon that we observed during the experiment was that often tumor spheroids generated enough cell traction force to detach collagen from its boundaries. Despite the application of the surface treatment process on the glass slide to prevent collagen gel detachment, the force that the spheroids exerted on the collagen gel was sufficient to detach the collagen from the agarose gel wall for most spheroids (figure S3). Another observation that we made in figure S3 is that the detachment area is larger in the EGF case than its counterpart without EGF, indicating that tumor spheroids generate more traction force in the presence of EGF than in control. The greater cell traction force can lead to high levels of local collagen alignment [37], which in turn, can lead to enhanced cell motility as seen in the section below.

In our experiments, only the spheroids that did not detach the collagen gel were included in our data analysis because the center of mass of the spheroids were seen to have a corresponding shift with the gel peeling. This yielded a total of four spheroids for the control condition and a total of five spheroids for the EGF gradient condition for the data analysis shown.
Figure 2. EGF gradient promoted tumor spheroid invasion within a collagen matrix. (A) Bright-field time lapse images of MDA-MB-231 spheroids embedded in collagen in the presence or absence of an EGF gradient. Using the particle detection module in ImageJ, we segmented the tumor cells that are still connected with each other (marked by the yellow outline) and named it the spheroid core. The segmentation is carried out using the fluorescence images, and the outlines are superimposed onto the bright field images as shown here. The center of mass of the spheroid core was tracked for the entire duration of the experiment (marked by the red dot). The EGF gradient (5.14 nM mm\(^{-1}\)) was imposed along the \(x\)-direction. The tumor spheroids were embedded within a 1.5 mg ml\(^{-1}\) collagen matrix. (B) Normalized area of the spheroid core in the presence and absence of EGF gradient. (C) The displacement of the center of mass of the tumor spheroid core with respect to its initial position along the gradient direction. In (B) and (C), the time between two consecutive data points was 10 min. The blue dots are experimental results from the control case, and the red dots are those from the EGF gradient case. The lighter shades represent the SEM for each case. A total of four spheroids and five spheroids were analyzed for control and EGF gradient case, respectively.

3.3. The EGF gradient promoted motility of the cells that invaded away from the spheroid core

The motility of the cells that invaded away from the spheroid core was examined using the bright field time-lapse image series as those shown in figure 2(A). Cell trajectories of each migrating cell in the presence/absence of the EGF gradients were obtained using manual tracker in ImageJ (see figures 3(A1) and (A2)). Note that the starting point of each cell track was when the cell first detached from the spheroid core. Clearly, cell motility was enhanced in the presence of the EGF gradient when compared to the control condition (figures 3(A1) and (A2)). It is interesting to note that the cell trajectories in figure 3(A) displayed an alignment along the \(y\) or channel direction. This was more evident in presence of the EGF gradient than without. This phenomenon was caused by the collagen alignment along the channel due to flow shear stress when unpolymerized collagen was introduced into the narrow channel as reported in early work [38]. Collagen fiber alignment was confirmed with images taken with brightfield microscopy (see figure S5).

We computed cell speed, velocity and persistence using the cell trajectories shown in figure 3(A). Distributions of cell speed as well as their average speed (figures 3(B1) and (B2)) show that cells are much more motile in presence of the EGF gradient than in absence of the EGF gradient. The average normalized speed of the cells is 1.54 in the presence of EGF, in contrast to 1.00 in the absence of EGF, yielding a 54% increase. To evaluate whether the cells that invaded away from the spheroid core are chemotactic to the EGF gradient, the distributions of cell \(x\)-velocity (figure 3(C1)) and average \(x\)-velocity with and without EGF gradient were plotted (figure 3(C2)). Both plots (figures 3(C1) and (C2)) showed that the detached cells did not show a distinguishable chemotactic response. The normalized persistence length is 1.17 in presence of the EGF gradient, in contrast to 1.00 in absence of EGF, which
Figure 3. Single tumor cells display distinct chemokinesis but no obvious chemotactic behavior in EGF gradient (A) trajectories of cells in control (A1) and in EGF gradient (A2) condition. Each colored line is a cell trajectory, and cells were tracked after they detached from the spheroid. 55 and 90 cells were tracked in (A1) and (A2), respectively, and the track duration was between 3–45 h with an average of 20.5 ± 10.0 h for the EGF gradient case, and 2–45 h with an average of 21.8 ± 11.1 h for control. The dotted lines represent the walls of the channels. The EGF gradient was generated by flowing 8.33 nM EGF and medium in source and sink channel, respectively. (B1) Distribution of normalized cell migration speed in control versus in EGF gradient. (B2) Normalized cell migration speed. ∗∗∗∗: p = 0.0002. (C1) Distribution of normalized velocity $V_x$ along the EGF gradient direction. (C2) Normalized cell velocity along the direction of gradient. (D1) Normalized cell migration persistence. ∗: p = 0.0104. (D2) Normalized cell migration persistence along the direction of gradient. Results from (B)–(D) were computed from trajectories shown in (A1) and (A2). The stars were obtained using a nonparametric t-test compared to the control group (Mann–Whitney test with ∗∗∗∗: $P < 0.0001$, ∗∗∗: $P < 0.001$, ∗∗: $P < 0.01$ and ∗: $P < 0.05$).

is an increase of 17% (figure 3(D1)). Again, there was no significant difference in the persistence of the detached cells along the direction of the EGF gradient in presence vs absence of the EGF gradient (figure 3(D2)).

Previously, we reported a study on the role of EGF gradients in single cell migration within a 3D collagen gel at a concentration of 1.5 mg ml$^{-1}$ [23]. The results presented in this study were consistent with our previous report in that chemokinesis was observed in the presence of the EGF gradient, but no chemotaxis for single cells invading in a collagen matrix. Interestingly, we did find that tumor cells were more sensitive to EGF in the spheroid assay in contrast to the single cell assay. We note that an overall 54% increase in speed was observed in the spheroid assay from this study, whereas only ~12% increase in speed was observed in the 3D single cell assay. It is also interesting to note that an overall ~17% increase in persistence length was observed in the spheroid assay, whereas only ~10% increase in persistence length was observed in the single-cell assay.

It is interesting to ask the question of where the enhanced sensitivity of tumor cell motility to the EGF gradients comes from in the spheroid assay in contrast to the single cell assay. We note that the impact of EGF to tumor cell motility is twofold. The first is a direct impact, where the tumor cells respond to the EGF gradients through the EGF-EGFR binding kinetics; and the second is an indirect impact, where the ECM architecture is remodeled by the tumor cell generated traction force, and the aligned collagen matrices promote tumor cell invasion. While the chemokinetic response to EGF gradients is similar for tumor spheroid assay and the single tumor cell assay, the collagen matrices are much more aligned in the case of tumor spheroid assay due to the large traction force. It is conceivable that the aligned matrices promote tumor cell invasion. Further work is required to decouple the contribution of
biochemical and biophysical impact of EGF gradients on tumor cell motility.

3.4. EGF promoted an elongated cell shape over rounded cell shape and single cell spreading in space

We found that cells were more elongated and exhibited more mesenchymal over amoeboid cell motility in the presence of the EGF gradient, consistent with our previous work [39]. This is shown in the distribution of cell aspect ratios in presence/absence of the EGF gradient (figure 4(A)). In this analysis, we labeled a cell as amoeboid if the aspect ratio was less than 2 to be consistent with previous publications [32, 33].

To analyze the individual cells spreading in space, MSDs of the MDA-MB-231 tumor cells were computed using the cell trajectories (figure 4(B)). Though the experiments were done in 3D, the cells were tracked in 2D. Thus, a first-order approximate mean squared displacement equation, \( \text{MSD} = 4Dt \), was used, where MSD is the average of the displacement squared of the cells at various time points, \( D \) is the diffusion coefficient of the cells, and \( t \) is time in minutes. The diffusion coefficients for the cells in presence and absence of the EGF gradient were 7.04 \( \mu \)m\(^2\) min\(^{-1}\) and 1.45 \( \mu \)m\(^2\) min\(^{-1}\), respectively. The drastic difference in the diffusion coefficients showed that EGF promoted cell spreading in space significantly.

Using the 3D spheroid invasion assay, we observed a strong motility enhancement and a mild chemotactic response of tumor spheroids to EGF gradients. In contrast to previous chemotaxis studies where single cells were embedded within an ECM, tumor cells associated with tumor spheroids exhibited an enhanced sensitivity to the EGF gradients compared to single cells embedded within an ECM.

4. Future perspective

The enhanced tumor cell motility sensitivity to EGF gradients in the spheroid versus single cell assay highlights the importance of a physiologically realistic spheroid assay. Looking ahead, a number of important questions need to be addressed. First are the roles of cell-ECM interaction in tumor sensitivity to EGF gradients. It will be important to decouple the biophysical contribution and biochemical contribution of EGF to tumor invasion. A critical step here is to follow the ECM architecture during the tumor invasion processes. Second is the relative contribution of cell-ECM and cell–cell adhesion molecules in the chemotactic response. For cell-ECM interactions, we can monitor integrin expression as a function of EGF concentration. For cell–cell adhesion, we can use spheroids made of cell lines that have higher cell–cell adhesion molecules (E-cadherin) such as MCF-7. It is likely that these cell lines will be able to better mimic the behaviors presented in the theoretical studies on cell cluster migration [24–26]. Third is the inclusion of a natural control where the spheroids are exposed to a uniform EGF concentration, which could help isolating the effect of gradient sensing from the chemokinetic effect of EGF.

Acknowledgments

This work was mainly supported by a grant from the National Cancer Institute (Grant No. R01CA221346); partially supported by the Cornell Center on the Microenvironment & Metastasis (Award No. U54CA143876 from the National Cancer Institute), and the Cornell NanoScale Science and Technology. JES is the Betty and Sheldon Feinberg Senior Faculty Scholar in Cancer. We thank Dr Beum Jun Kim, Yu Ling Huang, and Brian Cheung for assistance.
in experiments, and Professor Minglin Ma’s lab for sharing their microwell assay for spheroid formation.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

ORCID iDs

Young Joon Suh https://orcid.org/0000-0002-8281-3042
Mrinal Pandey https://orcid.org/0000-0001-8941-4290
Jeffrey E Segall https://orcid.org/0000-0001-5425-9315
Mingming Wu https://orcid.org/0000-0001-7846-3580

References

[1] Roussos E T, Condeelis J S and Patsialou A 2011 Chemotaxis in cancer Nat. Rev. Cancer 11 573–87
[2] Wu M and Swartz M A 2014 Modeling tumor microenvironments in vitro J. Biomech. Eng. 136 021011–7
[3] Chambers A F, Groom A C and MacDonald I C 2002 Dissemination and growth of cancer cells in metastatic sites Nat. Rev. Cancer 2 563–72
[4] Zhou Z N et al 2014 Autocrine HBEFG expression promotes breast cancer intravasation, metastasis and macrophage-independent invasion in vivo Oncogene 33 5784–93
[5] Goswami S, Sahai E, Wyckoff J B, Cammer M, Cox D, Pixley F J, Stanley E R, Segall J E and Condeelis J S 2005 Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor factor paracrine loop Cancer Res. 65 2738–83
[6] Berger M, Greenfield C, Gullick W, Haley J, Downward J, and F J, Stanley E R, Segall J E and Condeelis J S 2005 Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor factor paracrine loop Cancer Res. 65 2738–83
[7] Berger M, Greenfield C, Gullick W, Haley J, Downward J, and F J, Stanley E R, Segall J E and Condeelis J S 2005 Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor factor paracrine loop Cancer Res. 65 2738–83
[8] Lemoinne N R, Hughes C M, Gullick W J, Brown C L and Wynford-Thomas D 1991 Abnormalities of the EGF receptor system in human thyroid neoplasia Int. J. Cancer 49 558–61
[9] Libermann T A et al 1985 Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin Nature 313 144–7
[10] Salomon D S, Brandt R, Ciardiello F and Normanno N 1995 Epidermal growth factor-related peptides and their receptors in human malignancies Crit. Rev. Oncol. Hematol. 19 183–232
[11] Tillotson J K and Rose D P 1991 Endogenous secretion of epidermal growth factor peptide stimulates growth of DU145 prostate cancer cells Cancer Lett. 60 109–12
[12] Rauch J, Volinsky N, Romano D and Kolch W 2011 The secret life of kinases: functions beyond catalysis Cell Commun. Signaling 9 23
[13] Wang Y, Deng W, Zhang Y, Sun S, Zhao S, Chen Y, Zhao X, Liu L and Du J 2018 MICAL2 promotes breast cancer cell migration by maintaining epidermal growth factor receptor (EGFR) stability and EGFR/P38 signalling activation Acta Physiol. 222 e12920
[14] Seshacharyulu P, Ponnusamy M P, Haridas D, Jain M, Ganti A K and Batra S K 2012 Targeting the EGFR signaling pathway in cancer therapy Expert Opin. Ther. Targets 16 13–31
[15] Zigmond S H 1977 Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors J. Cell Biol. 75 606
[16] Boyden S 1962 The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes J. Exp. Med. 115 483–66
[17] Jeon N L, Dertinger S K W, Chiu D T, Choi I S, Stroock A D and Whitesides G M 2000 Generation of solution and surface gradients using microfluidic systems Langmuir 16 8311–6
[18] Kim B J and Wu M 2012 Microfluidics for mammalian cell chemotaxis Ann. Biomed. Eng. 40 1316–27
[19] Haessler U, Kalinin Y, Swartz M A and Wu M 2009 An agarose-based microfluidic platform with a gradient buffer for 3D chemotaxis studies Biomed. Microdevices 11 827–35
[20] Haessler U, Pisano M, Wu M and Swartz M A 2011 Dendritic cell chemotaxis in 3D under defined chemokine gradients reveals differential response to ligands CCL21 and CCL19 Proc. Natl Acad. Sci. USA 108 5614–9
[21] Abhyankar V V, Toepke M W, Cortesio C L, Lukota M A, Huttenlocher A and Beebe D J 2008 A platform for assessing chemotactic migration within a spatiotemporally defined 3D microenvironment Lab Chip 8 1357–65
[22] Wang S-I, Saadi W, Lin F, Minh-Canh Nguyen C and Li J 2004 Differential effects of EGF gradient profiles on MDAMB-231 breast cancer cell chemotaxis Exp. Cell Res. 300 180–9
[23] Kim B J, Hannanta-anan P, Chau M, Kim Y S, Swartz M A and Wu M 2013 Cooperative roles of SDF-1α and EGFR gradients on tumor cell migration revealed by a robust 3D microfluidic model PLoS One 8 e64822
[24] Camley B A 2018 Collective gradient sensing and chemotaxis: modeling and recent developments J. Phys.: Condens. Matter 30 223001
[25] Varenes J, Han B and Mugler A 2016 Collective chemotaxis through noisy multicellular gradient sensing Biophys. J. 111 640–9
[26] Gopinathan A and Gov N S 2019 Cell cluster migration: connecting experiments with physical models Semin. Cell Dev. Biol. 93 77–86
[27] Debets E V, Janssen L M C and Storm C 2021 Enhanced persistence and collective migration in cooperatively aligning cell clusters Biophys. J. 120 1483–97
[28] Ellison D et al 2016 Cell–cell communication enhances the capacity of cell ensembles to sense shallow gradients during morphogenesis Proc. Natl Acad. Sci. USA 113 E679–88
[29] Hwang P Y, Brenot A, King A C, Longmore G D and George J 2016 Collective migration and dynamic collective behaviour in chemotaxis Biophys. J. 120 1130–1138
[30] Song W, Tung C-K, Lu Y-C, Pardo Y, Wu M, Das M, Kao D-I, Chen S and Ma M 2016 Dynamic self-organization of microwell-aggregated cellular mixtures Soft Matter 12 5739–46
[31] Lu Y-C, Song W, An D, Kim B J, Schwartz R, Wu M and Ma M 2015 Designing compartmentalized hydrogel microcarriers for cell encapsulation and scalable 3D cell culture J. Mater. Chem. B 3 353–60
[32] Huang Y L, Tung C-K, Zheng A, Kim B J and Wu M 2015 Intestinal flows promote amoeboid over mesenchymal motility of breast cancer cells revealed by a three dimensional microfluidic model Integr. Biol. 7 1402–11
[33] Petrie R J, Gavara N, Chadwick R S and Yamada K M 2012 Nonpolarized signaling reveals two distinct modes of 3D cell migration J. Cell Biol. 197 439–55
[34] Cheng S-Y, Heilman S, Wasserman M, Archer S, Shuler M L and Wu M 2007 A hydrogel-based microfluidic device for the studies of directed cell migration *Lab Chip* 7 763–9

[35] Macdonald-Obermann J L and Pike I J 2018 Allosteric regulation of epidermal growth factor (EGF) receptor ligand binding by tyrosine kinase inhibitors *J. Biol. Chem.* 293 13401–14

[36] Schlessinger J 1988 The epidermal growth factor receptor as a multifunctional allosteric protein *Biochemistry* 27 3119–23

[37] Hall M S, Alisafaei F, Ban E, Feng X, Hui C-Y, Shenoy V B and Wu M 2016 Fibrous nonlinear elasticity enables positive mechanical feedback between cells and ECMs *Proc. Natl Acad. Sci. USA* 113 14043–8

[38] Lee P, Lin R, Moon J and Lee I P 2006 Microfluidic alignment of collagen fibers for *in vitro* cell culture *Biomed. Microdevices* 8 35–41

[39] Geum D T, Kim B J, Chang A E, Hall M S and Wu M 2016 Epidermal growth factor promotes a mesenchymal over an amoeboid motility of MDA-MB-231 cells embedded within a 3D collagen matrix *Eur. Phys. J. Plus* 131 8