

Chemotaxis of Metastatic Breast Cancer Cells in Parallel Gradient Microfluidic Chambers

Wajeeh Saadi^a, Shur-Jen Wang^a, Francis Lin^{ab}, and Noo Li Jeon^{a*}

^aDepartment of Biomedical Engineering, 204 Rockwell Engineering Center, University of California at Irvine, Irvine, CA 92697-2715, USA.

^bDepartment of Physics and Astronomy, 4129 Frederick Reines Hall, University of California at Irvine, Irvine, CA 92697-4575, USA.

*Corresponding author. njeon@uci.edu

ABSTRACT

Growth factor-induced chemotaxis of cancer cells plays a critical role in metastasis. Using a novel microfluidic chemotaxis chamber capable of simultaneously generating multiple growth factor gradients, we quantified and compared the migration of the human metastatic breast cancer cell line MDA-MB-231 in different gradients of epidermal growth factor (EGF). We confirmed the potency of EGF as a chemoattractant, and demonstrated that EGF can direct migration over a large dynamic range of gradients. We also investigated the effect of antibody against the EGF receptor (EGFR) on MDA-MB-231 chemotaxis; both motility and directional orientation were impaired, indicating that cell motility was induced by the activation of EGFR. The microfluidic chamber described in this work will provide a platform for cell-based assays that can be used to compare the effectiveness of different pharmaceutical compounds targeting cell migration and metastasis.

Keywords: microfluidics, metastasis, breast cancer, chemotaxis, epidermal growth factor

1 INTRODUCTION

Cell motility plays an important role in many biological processes, including cancer metastasis [1, 2]. Of particular importance in these processes is chemotaxis: directed motility towards increasing concentrations of soluble factors. Conventional assays of chemotaxis (such as the Boyden chamber, Zigmond chamber, under-agarose assay, and micropipette assay) suffer from various limitations, particularly their reliance on free diffusion between a source and a sink to generate concentration gradients, which are in turn usually unstable and difficult to manipulate [3, 4]. Some of these assays also suffer from being endpoint assays that do not allow visualization of actual migration paths, or from low throughput, single-cell measurements.

Advances in soft lithography and microfluidics enabled the development of chemotaxis chambers that can generate precise, stable concentration gradients, and allow real-time observation of migrating cells [3, 5]. This method was used

to investigate the migration of neutrophils and breast cancer cells in IL-8 [4] and epidermal growth factor (EGF) [6] gradients. In both cases, the chemotaxis chambers were used to produce and maintain precise and stable gradient conditions that allowed quantitative characterization of directed cell migration.

In order for the microfluidic assay to be broadly useful in biology, it should easily lend itself to comparative measurements of chemotaxis in different conditions. To this end, we developed a parallel-gradient microfluidic chemotaxis chamber that can generate soluble gradients of growth factors side by side, allowing comparisons to be carried out simultaneously with a high degree of control. We present two application of this chamber relevant to the metastatic process and therapeutic efforts, hinting at the wealth of information that can be obtained from studies of cancer cell chemotaxis.

2 METHODS

2.1 Microfluidic Cell Migration Assay

The human metastatic breast cancer cell line, MDA-MB-231, was obtained from The American Type Culture Collection (ATCC, Manassas, VA), and cultured in L15 with 10% FBS, as described in Wang et al. [6]. Mouse monoclonal neutralizing anti-EGFR antibody was purchased from Upstate Biotechnology (Lake Placid, NY).

The microfluidic gradient generator was fabricated in poly(dimethylsiloxane) (PDMS) using soft lithography and rapid prototyping; the final microfluidic device consisted of a PDMS top, which contains grooves, covalently sealed against a glass bottom [6, 7]. Detailed procedures of the migration assay have been previously published [6]. Briefly, the microfluidic device was coated with collagen IV and infused with 0.2% BSA/L15 media (with or without growth factor) using syringe pumps. Cells were removed from the culture flask using cell dissociation buffer. For the anti-EGFR experiments, cells were counted after they were taken out of the flask, centrifuged down, and resuspended in anti-EGFR solution in 0.2% BSA/L-15 at a density of 1×10^6 cells/ml prior to filtration. Antibody-treated cells, along with untreated cells, were then incubated on a rotator for 1 hr at room temperature. The cells were loaded into

the microfluidic device from the cell port using a micropipette. For the anti-EGFR experiments, anti-EGFR was added to the solutions flowing over the antibody-treated cells.

Time-lapse differential interference contrast (DIC) images of migrating cells were obtained using an inverted microscope (Nikon, Meville, NY) with a 10X objective. The microscope stage was enclosed in a temperature-controlled box maintained at 37°C. Images were acquired with a CCD camera (Photometrics CoolSNAP cf, Roper Scientific, Tucson, AZ) at 2 min intervals for 3hrs. A computer-controlled motorized stage was used to image multiple positions along the migration channel.

2.2 Data Analysis

Time-lapse images of cells were tracked using MetaMorph (Universal Imaging, Downingtown, PA). A spreadsheet was used to calculate various migration parameters [(1) net cell displacement, (2) total distance, (3) net displacement towards gradient, (4) speed, (5) migration angle, and (6) chemotactic index (CI, net displacement towards gradient divided by total distance)]. Migration angles are defined with respect to the gradient direction (90° = net displacement completely in the gradient direction).

Using Oriana for Windows (Kovach Computing Services, Wales, UK), migration angles were grouped in 10° intervals and summarized in a direction plot. The Rayleigh test of uniformity was applied ($p = 0.01$); when there was significant directionality, the mean angle and the 95% confidence interval were calculated. A Modified Rayleigh test was also applied, in order to test whether deviations from the gradient direction were significant[8]. For each experimental condition, a minimum of 50 cells was analyzed.

3 RESULTS AND DISCUSSION

3.1 Chemotaxis Chamber Design and Layout

Figure 1a shows the design of a microfluidic gradient generator that produces a single gradient of nonlinear polynomial profile [9] (polynomial gradient from here on). The principle behind the generation and control of concentration gradients using microfluidics has been published elsewhere [3, 5]. Briefly, fluid streams are repeatedly split and mixed in the gradient-generating channel network (Figure 1a), the design of which determines the shape of the resulting gradient in the migration channel. In the network shown in Figure 1a, the left inlet splits into four streams, effectively increasing the number of inlets to five. Infusing the device with media and chemoattractant-containing media into the left and right inlet, respectively, with a volumetric flow rate ratio of 4:1, produces a polynomial concentration gradient of the form:

$$C = ax^{4.2} + b$$

where C is the concentration of chemoattractant; x is the position across the migration channel; a and b are constants that depend on the width of the channel as well as the minimum and maximum chemoattractant concentrations.

Each microfluidic network generates a single microfluidic gradient; when two networks are placed in parallel and merged into one channel, two gradients are formed side by side (Figure 1b). The two polynomial gradients in Figure 1b are placed opposite of each other to minimize blurring between the gradients due to diffusion. Alternatively, a physical barrier (Figure 1c) can be used to avoid the blurring interface. A barrier is needed for applications that utilize small molecules (sizes <1 kDa). Relatively large diffusion coefficients of small molecules ($D \approx 5 \times 10^{-5} \text{ cm}^2/\text{s}$) allow them to quickly cross over between adjacent gradients under the flow speeds used in our experiments ($4.2 \times 10^{-2} \text{ cm/s}$). In addition, a barrier is needed for applications that require different pretreatments for separate group of cells. As illustrated in our example, a barrier would allow antibody-treated cells and control cells to be loaded separately while allowing side by side comparison of their migration.

3.2 EGF is a Potent Chemoattractant for Metastatic Breast Cancer Cells

One of the best understood growth factor systems is the EGF/EGFR system, long implicated in cancer development [2, 10]. EGF has been shown to induce chemotaxis of metastatic breast cancer cells, both *in vivo* [11] and *in vitro* [10, 12]. We have previously reported that nonlinear EGF gradients induce chemotaxis of the human metastatic breast cancer cell line MDA-MB-231, while linear gradients fail to induce chemotaxis [6]. Among the polynomial EGF gradients tested (0-25 ng/ml, 0-50 ng/ml, 0-100 ng/ml), 0-50 ng/ml gradient induced optimal directed migration. Compared to the equilibrium dissociation coefficient of EGF ($k_d = 6 \text{ ng/ml}$), these gradients span concentrations that are considerably higher. It is necessary to investigate cell migration in gradients that are shallower in range and closer to the k_d value.

We compared the migration of breast cancer cells in a 0.1-6 ng/ml polynomial EGF gradient alongside a 0-50 ng/ml EGF gradient (Figure 2). Figure 2 shows significant net movement towards the gradient in both ranges, with different efficiencies. The average CI and the percentage of cells that moved towards the gradient were lower in 0.1-6 ng/ml EGF, with a wider distribution of migration trajectories and a larger confidence interval.

While these data show that the chemotactic efficiency is dose-dependent, they also demonstrate that EGF induces chemotaxis in metastatic cells over a broad dynamic range of concentrations, and confirms its potency as a chemoattractant [10]. The ability to adapt to a broad range

of EGF concentrations may have important implications for therapeutic efforts to treat metastasis.

3.3 Effect of EGFR-Blocking Antibody on Cancer Cell Migration in EGF Gradient

One of the strategies that have targeted the EGF/EGFR signaling pathway in cancer therapy utilizes EGFR-blocking antibodies [13]. Using the parallel gradient chamber (Figure 1c), we examined the effect of anti-EGFR treatment on the migration of metastatic breast cancer cells alongside untreated control cells. Antibody treatment abolished the chemotactic response of the cancer cells and resulted in random migration. Morphologically, antibody-treated cells were mostly unpolarized, while untreated cells had well defined lamellipodia that were oriented towards increasing EGF concentration (data not shown). This polarization is characteristic of cells undergoing chemotaxis [1]. Moreover, antibody-treated cells had a very low average CI, and a uniform distribution of trajectories (Figure 3), while control cells had significant clustering of trajectories in the gradient direction.

The microfluidic chemotaxis chamber allows the migration process to be examined in real time, providing details of individual cell trajectories as well as distributions of cell populations. We can thus evaluate both motility and directionality independent of each other, which cannot be directly observed using conventional endpoint assays. We found that the anti-EGFR antibody targets both motility and directional sensing: in the presence of anti-EGFR, cells moved randomly with speeds similar to those at basal levels (non-EGF stimulated, data not shown). This verifies that the observed migratory response occurs specifically via EGFR, and that blockade of this receptor does not affect basal migration.

This approach can be used to categorize different types of inhibitors based on their mechanism of action. Using the microfluidic chemotaxis chamber, the effects of different inhibitors can be investigated in a quantitative manner, allowing comparisons of effectiveness based on quantitative data, in addition to qualitative observations.

4 CONCLUSION

We have developed a parallel-gradient microfluidic chemotaxis chamber capable of generating different experimental conditions in parallel, allowing individual cells to be investigated in detail while providing quantitative, statistically meaningful data for the whole cell population. This chemotaxis chamber represents a platform to investigate the effects of pharmaceutical compounds on cell migration (in terms of speed, chemotactic index, and directional orientation) and provides a means to evaluate their effectiveness in relation to metastasis. Coupled with conventional cell based assays, this approach may provide information about the different pathways involved in the

migration of cancer cells and how they are modulated. This knowledge may result in novel approaches for treatment of metastasis that target cell motility.

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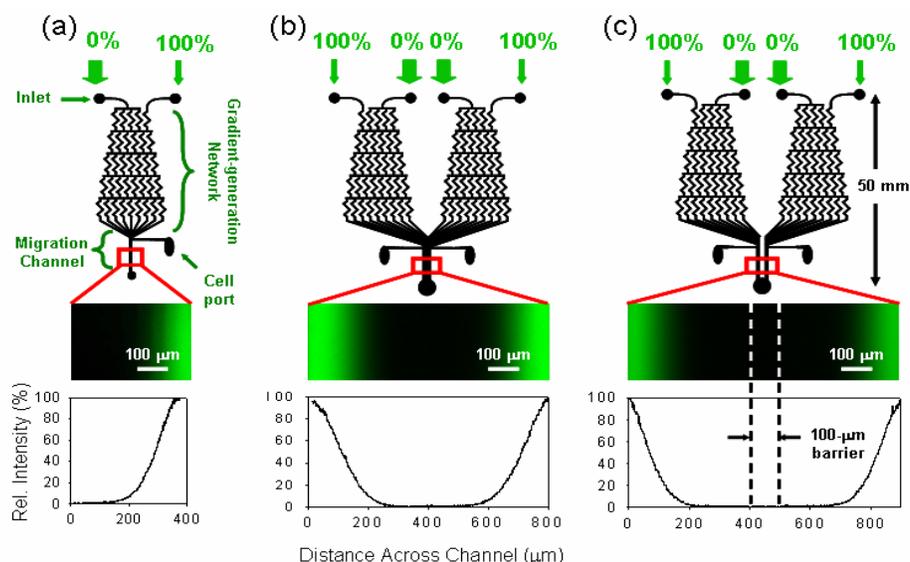


Fig. 1 Schematic diagrams of the microfluidic chemotaxis chambers. (a) A single-gradient chemotaxis chamber that produces a polynomial gradient. The gradient was visualized by injecting media with 0% and 100% FITC-dextran from the inlets, with a volumetric flow rate ratio of 4:1 respectively. A fluorescence (pseudo colored) image of the migration channel and a corresponding intensity profile are shown at the bottom. (b) A parallel-gradient chemotaxis chamber producing two opposite gradients side by side. (c) A parallel-gradient chemotaxis chamber with a 100- μm barrier in the middle of the migration channel.

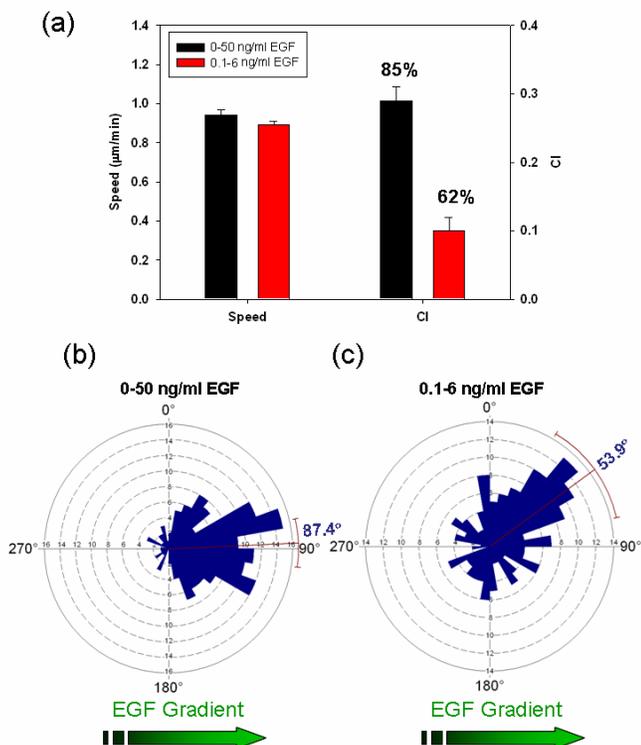


Fig. 2 Chemotactic response of MDA-MB-231 in 0-50 ng/ml and 0.1-6 ng/ml polynomial EGF gradients in parallel. (a) Average speed and chemotactic index (CI) values. Error bars represent standard error. Percentages indicate the number of cells that migrated towards the gradient. (b-c) Direction plots showing the distribution of migration angles. A Rayleigh test shows that migration was directional in both ranges, with the mean angles of 87.4° and 53.9° for 0-50 ng/ml and 0.1-6 ng/ml, respectively. Arcs indicate 95% confidence intervals. Cells that migrated a net distance of less than 25 μm were excluded.

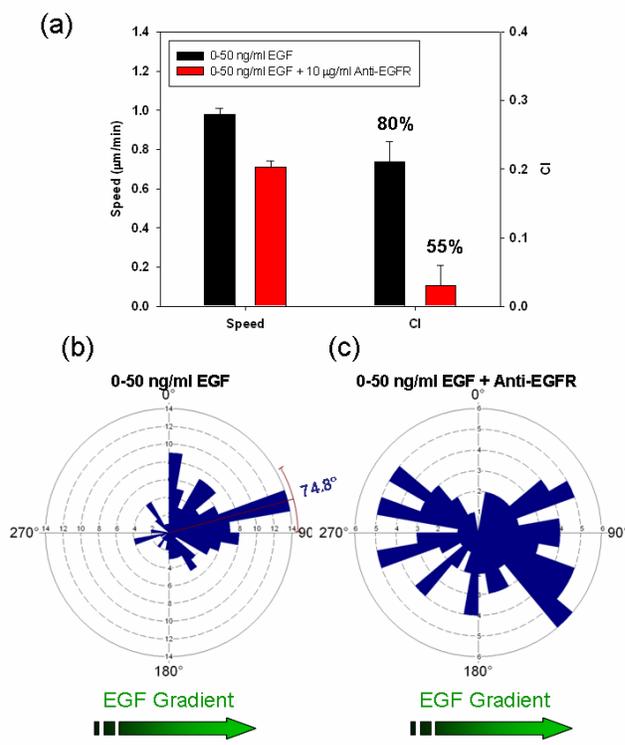


Fig. 3 Anti-EGFR antibody inhibits chemotaxis of MDA-MB-231 in a 0-50 ng/ml EGF gradient. (a) Average speed and CI for antibody-treated cells, compared with control cells side by side. Error bars represent standard error. Percentages indicate the number of cells that migrated towards the gradient. (b-c) Direction plots showing the distribution of migration angles. A Rayleigh test showed that untreated cells migrated directionally, with a mean angle of 74.8° (arc indicates 95% confidence), while antibody-treated cells had no directional preference.