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COMBINED EXPERIMENTAL AND MATHEMATICAL APPROACH FOR DEVELOPMENT OF MICROFABRICATION-BASED CANCER MIGRATION ASSAY

Saheli Sarkar¹, Bethany L. Bustard², Jean F. Welter³, and Harihara Baskaran^{2,*}

¹Department of Biomedical Engineering, Case Western Reserve University, Cleveland, Ohio 44106

²Department of Chemical Engineering, Case Western Reserve University, Cleveland, Ohio 44106

³Department of Biology, Case Western Reserve University, Cleveland, Ohio 44106

Abstract

Migration of cancer cells is a key determinant of metastasis, which is correlated with poor prognosis in patients. Evidence shows that cancer cell motility is regulated by stromal cell interactions. To quantify the role of homotypic and heterotypic cell-cell interaction on migration, a two-dimensional migration assay has been developed by microfabrication techniques. Two breast cancer cell lines, MDA-MB-231 and MDA-MB-453, were used to develop micropatterns of cancer cells (cell islands) that revealed distinct migration profiles in this assay. Although the individual migration rates of these cells showed only a seven-fold difference, MDA-MB-453 islands migrated significantly lower than MDA-MB-231 islands, indicating differential regulation of migration in isolated cells vs. islands. Island size had the greatest impact on migration, primarily for MDA-MB-231 cells. Migration of MDA-MB-231 islands was decreased by interaction with homotypic cells, and significantly more by heterotypic non-cancer associated fibroblasts. In addition, a mathematical model of island migration in multi-cellular population has been developed using Stefan-Maxwell's equation. The model showed qualitative agreement with experimental results and predicted a biphasic relation between cell densities and island sizes. The combined experimental and mathematical model can be used to quantitatively study the impact of cell-cell interactions on migration.

Keywords

Microfabrication; breast cancer; stromal cells; mathematical model; multi-component transport; soft lithography; homotypic interactions; heterotypic interactions

INTRODUCTION

Cell migration plays critical roles in development, immunologic responses, wound healing and tumor progression. Cells can migrate either individually (*e.g.*, immune cells and germ cells) or collectively (*e.g.*, epithelial cells). In either event, cell-cell interactions play a critical role in migration, which can be observed during wound healing, sprouting angiogenesis and tumor invasion. Down-regulation of cell adhesion molecules leads to the loss of such interactions resulting in altered cell migration. A typical example is the

*Corresponding author Harihara Baskaran Associate Professor of Engineering Case Western Reserve University 111C A.W. Smith Building 2102 Adelbert Road Cleveland, OH, 44106-7217 Tel: 216-368-1029 Fax: 216-368-3016 hari@case.edu.

epithelial-to-mesenchymal transition (EMT), where collective migration is replaced by disseminated single-cell migration. Cell migration strategies are highly diverse and have been well documented, particularly in case of tumor migration¹⁴. It is interesting to note that the analysis of human cancer genes has not identified one (or more) specific gene(s) that can exclusively regulate cell migration, although many genes that impact metastasis have potential roles in altering motility³³. This indicates that cell motility is rarely autonomous and is strongly dependent on the balance of pro- and anti-migratory signals received by the cells from their microenvironment. Given the significance of cell-cell interactions on migration, it is imperative to quantify this effect during migration.

A number of two- (2D) and three-dimensional (3D) *in vitro* assays have been developed to study cell migration^{11, 12}. Although 3D assays can potentially mimic the *in vivo* environment more closely than the 2D assays, they are also more difficult to image and quantify. Two-dimensional (2D) *in vitro* assays include migration on a surface, scratch wound model and fence assay. These assays have been widely used to study migration of various cell lines, but they suffer from a number of drawbacks. First, they do not allow precise control over spatial patterning of cells, particularly when multiple cell types are involved. This is frequently observed in cancer cell migration, which is highly regulated by stromal cells such as fibroblasts and macrophages via paracrine signaling^{9, 15}. However, the current quantitative assays measuring migration only allow a random distribution of cancer cells co-cultured with stromal cells. This does not represent the site-specific localization of stromal cells, particularly at the edges of a tumor as revealed by multi-photon microscopy of live animals⁴⁰. This implies that homotypic interaction (between cancer cells) is predominant within the core of the tumor and heterotypic interaction (between cancer and stromal cells) primarily occurs at the tumor periphery. These dynamics of cell-cell interaction and their effect on migration cannot be quantifiably measured in a standard co-culture assay. Second, the impact of tumor size or shape on cell migration cannot be investigated in such assays since most 2D migration assays begin as a monolayer of cells. Tumor size may potentially play a role in metastasis. The linear progression model (LPM) of metastasis states that the primary tumor develops, becomes large in size (2–5 cm) and then metastasizes¹⁹. The parallel progression model (PPM) states that cancer cells may detach from the primary tumor at any stage of tumor progression, thus metastases can develop in parallel with the primary tumor⁸. The growth course of cancers has frequently shown that metastases are found more rapidly after detection of primary tumor, which theoretically fits with PPM. In breast, prostate and esophageal cancer, disseminated tumor cells show less genetic mutation compared to primary tumors; this also fits with early dispersal of cells from the primary tumor. On the other hand, certain mutations seem to validate the LPM. For *e.g.*, p53 gene is rarely mutated in tumors <2cm but significantly altered in tumors >5cm¹⁸. Genomic analyses of matched tumor-metastasis pair from patients have revealed extremely high discrepancy at all levels, thus ruling out a direct conclusion from these studies. Nevertheless, it seems apparent that tumor size does have a key role during growth and metastasis, and that smaller tumors may just as well initiate metastasis as larger tumors. Third, scratch wound assays can result in injury of the cells and underlying matrix at the boundary of the wound. This can trigger wound-healing responses, which are difficult to decouple from migration. Fourth, not only is it difficult to create multiple identical scratches in the same monolayer, cell proliferation is also a concern. Filter-based assays such as Boyden and Dunn chambers are suitable for studying gradient-based migration such as haptotaxis and chemotaxis. However, these are end-point assays and are constrained by pore dimensions, which only accommodate active migration of individual cells and not of cell clusters. Thus, they do not provide a quantifiable way to determine the impact of cell-cell interaction on migration.

Microfabrication has been used extensively in the past decade to study various types of cell motility. It has also been used to obtain micropatterns of cell clusters and single cells¹³ as well as to control the localization of cells in a multi-cellular population, such as hepatocytes and fibroblasts⁴. However, very few studies have exclusively focused on studying migration from cell patterns in 2D cultures. An assay motivated by the wound-healing model was recently developed to investigate migration of uninjured epithelium from rectangular patterns³¹. Subsequently, microfluidic wound-healing assays have been developed^{27, 36}, but none of these assays investigated migration of cancer cells in a heterogeneous microenvironment.

In this study, a combined approach has been taken to model breast cancer migration. A microfabrication-based 2D migration assay was developed by spatially patterning breast cancer cells within cell islands and surrounding them with fibroblasts. Furthermore, the impact of cancer island size on cell migration in 2D was evaluated by including a microarray containing various sizes and shapes of cell islands. A mathematical model of cell-cell interactions, based on Stefan-Maxwell's equations was also developed to study migration profiles of cell islands.

MATERIALS AND METHODS

Reagents and Cell Culture

The human breast adenocarcinoma cell line MDA-MB-231 (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco's Modified Eagle Medium (DMEM, 1.5g/L glucose). NIH-3T3 mouse fibroblasts (ATCC) were cultured in DMEM (4.5g/L glucose). The human breast cancer line MDA-MB-453 was cultured in RPMI 1640. All media (Invitrogen, Carlsbad, CA) was supplemented with 10% Fetal Bovine Serum (FBS) and the cell lines were maintained at 37°C and 5% CO₂. For heterotypic cultures of MDA-MB-231 and NIH-3T3 cells, a 1:1 combination media was used. For cells cultured on fibronectin (Fn), petri-dishes (P-60, BD Falcon, Franklin Lakes, NJ) were coated with a 20µg/ml solution of Fn (Invitrogen) for 1 hr at 37°C and 5% CO₂ prior to use. TGF-β1 (PeproTech, Rocky Hill, NJ) was used at a final concentration of 4 ng/ml.

Microfabrication and Development of cell islands

The procedure used to develop MDA-MB-231 and MDA-MB-453 cell islands has been described previously³⁴. Photolithography was performed with photomasks (Advanced Reproductions, North Andover, MA) containing arrays of square patterns (area: 1.25–0.025 mm²) to obtain photoresist (Su-8 2075, Microchem Corp, Newton, MA) features on silicon wafers. Subsequently, soft lithography techniques were used to obtain poly(dimethyl siloxane) (PDMS) membranes (150µm) consisting of the same designs. About 3×10⁶ cells were seeded on membranes treated with 1% BSA solution and allowed to attach overnight to form confluent cell islands, after which the membranes were removed and discarded. Wherever applicable, complete MDA-MB-231 media was supplemented with TGF-β1 (4ng/ml) immediately after removal of the membrane.

Cell Migration Assay

For individual cell migration assays, 5×10⁵ cells were seeded onto Fn-coated (10µg/ml) P-100 petri-dishes. Migration was measured in a temperature and humidity-controlled custom-built incubator on the microscope stage. Images were obtained every 5 min for 15 hrs for MDA-MB-231 cells and every 10 min for 66 hrs for MDA-MB-453 cells. Cell motility was analyzed with the Cell Tracker feature of ImagePro software (Media Cybernetics, Inc., Bethesda, MD).

Cell migration from confluent cell islands was studied for 10 hrs. Islands were imaged at $t=0$ (initial time point) and at $t=10$ hrs (end time point). This duration was chosen to minimize cell proliferation effect on island expansion (since the doubling time for MDA-MB-231 cells in culture was found to be ~24 hrs). For homotypic and heterotypic experiments, MDA-MB-231 cell islands labeled with CFDA-SE (10 μ M) cell tracer (Vybrant, Invitrogen) were developed as described and surrounded with additional cells outside the islands after the PDMS membrane was removed. The additional cells were either MDA-MB-231 cells in case of homotypic, or NIH-3T3 cells in case of heterotypic interactions. Migration was quantified at $t=10$ hrs as before. Phase-contrast and fluorescent images of the cell islands were taken with an Olympus IX71 inverted microscope (Olympus, Japan) using UPlanFl 10 $\times$ and LCPlanFl 20 $\times$ objectives.

Data Analysis

The initial and final island areas were quantified by ImagePro software. The area occupied by each cell island was measured at the start- and end- points of the experiment. Migration was quantified as the area of island at any given time normalized to the initial area, with margins of cell islands tracked manually. This parameter was termed Migration Index (MI) and expressed as mean $\pm$ SEM of at least 9 cell islands ($n=9$) from three different experiments. Island data for each size was obtained in triplicate from each experiment. The data was statistically analyzed by using Student's t-distribution. A p-value of <0.05 was considered statistically significant.

Mathematical model

A mathematical model of island migration was developed based on Generalized Stefan-Maxwell's (S-M) equation²⁰. We considered the macroscopic spatiotemporal distribution of cancer cell density ($n(x, y, t)$), stromal cell density ($p(x, y, t)$), ECM concentration ($f(x, y, t)$) and matrix metalloproteinase (MMP) concentration ($p(x, y, t)$) within a square domain. Cell migration is assumed to occur due to random motility and haptotaxis on ECM. Although the cell islands were seeded on a uniform layer of Fn, cancer cells are known to secrete proteases that degrade matrix locally, thus creating bound Fn gradients which give rise to haptotactic flux² ($J_n = \chi(n\nabla f)$). We did not consider chemotaxis since soluble factor gradients were unlikely to be present in our assay as any factor produced by the cells can freely transport at a rate much faster than the cells themselves. Likewise, given that cells are patterned in monolayers, mass transport of nutrients (glucose/oxygen supply) was not included. A basic assumption of this model is that both cell islands and stromal cells have sufficient available space (ϵ) in the Petri dish to migrate; hence we consider a multi-component ((n) , (p) and (ϵ)) diffusive system allowing us to utilize the S-M equation. The conservation equations for cancer (1) and stromal cell (2) densities are depicted below (derivations shown in Appendix):

$$\frac{\partial n}{\partial t} = \nabla \cdot \left[\frac{c}{c - n - p} \left\{ \frac{n}{\left(\frac{n}{D_n}\right) + \left(\frac{p}{D_p}\right)} \nabla n + \frac{n}{\left(\frac{n}{D_n}\right) + \left(\frac{p}{D_p}\right)} \nabla p \right\} \right] - \chi \nabla \cdot (n \nabla f) \quad (1)$$

$$\frac{\partial p}{\partial t} = \nabla \cdot \left[\frac{c}{c - n - p} \left\{ \frac{p}{\left(\frac{n}{D_n}\right) + \left(\frac{p}{D_p}\right)} \nabla n + \frac{p}{\left(\frac{n}{D_n}\right) + \left(\frac{p}{D_p}\right)} \nabla p \right\} \right] - \chi \nabla \cdot (p \nabla f) \quad (2)$$

Here, D_n and D_p are the constant diffusion coefficients of cancer and stromal cells respectively; χ is the haptotactic coefficient; c is defined as the sum of initial values of the three variables ($c = n_0 + p_0 + \varepsilon_0$).

The governing equations for ECM degradation (3) and MMP concentration (4) are obtained from previously developed models².

$$\frac{\partial f}{\partial t} = -\delta m f \quad (3)$$

$$\frac{\partial m}{\partial t} = D_m \nabla^2 m + \mu n - \lambda m \quad (4)$$

Here, δ is the ECM degradation rate; D_m is the constant diffusion coefficient of MMPs; μ and λ are the MMP production and decay rates respectively.

In the model, both length and time scales are normalized and the equations non-dimensionalized before implementation. It is assumed that the outer boundaries of the domain are impenetrable, so no-flux conditions are imposed on all boundaries for all the variables. The equations are solved in COMSOL Multiphysics software (COMSOL, Inc., Burlington, MA). The values of various parameters and reference variables used for modeling are listed in Table 1.

Sensitivity Analysis

We performed sensitivity analysis for the following parameters: D_n , D_p , D_m , χ , δ , μ and λ . An island size of 0.0625 mm^2 was utilized. Each parameter was increased individually by 10 % of the values listed in Table 1 while maintaining the other parameters at the baseline values. Migration Indices (MI) were obtained from the simulations and the percentage change in MI with respect to the baseline MI is shown. The analysis was carried out for two cell density ratios: 1:0.1 and 1:1.

RESULTS

Quantification of island migration in breast cancer cell lines

MDA-MB-231 cells were patterned in confluent square-shaped cell islands in a number of sizes ($0.025 - 1.25 \text{ mm}^2$) based on the microarray design (Fig. 1A). Consistent cell density was obtained in all the island sizes (Fig. 1C). Fig. 1B shows the typical migration of MDA-MB-231 cell islands. Cell migration was observed in all cell islands, but the smaller islands showed significantly larger ($p < 0.01$) Migration Index (MI) compared to the larger islands (Fig. 1D). The smallest square island (initial area: 0.025 mm^2) showed a maximum MI of 2.72 ± 0.27 (mean $\pm$ SEM) while the largest square island (initial area: 1.25 mm^2) showed an MI of 1.20 ± 0.07 over 10 hrs. The two largest islands (0.625 and 1.25 mm^2) had the same MI despite a two-fold initial size difference, implying that the size effect had reached a threshold.

Fibronectin (Fn) is known to promote adhesion and migration of MDA-MB-231 cells via $\beta 1$ integrin³. The effect of adhesion to Fn on MDA-MB-231 cell islands was quantified in this migration assay. All sizes of cell islands showed an increased MI, which was significant in most sizes ($p < 0.01$ for $0.0625 - 1.25 \text{ mm}^2$) compared to control (Fig. 2). MDA-MB-231 cells are also known to express TGF- β type I and type II receptors, so the effect of TGF- $\beta 1$ on island migration was assessed. TGF- $\beta 1$ significantly increased MI ($p < 0.01$) in all island

sizes (Fig. 2). In both these situations, the effect of initial island area on migration was still apparent.

To determine whether other cell types depicted the same island dynamics in this assay, we developed MDA-MB-453 cell islands. This breast cancer cell line is non-metastatic and depicted significantly lower MI than the MDA-MB-231 cells over a period of 24 hrs (Fig. 3A, 3C). Within this duration the effect of initial island area on MI was minimal (Fig. 3B). The doubling time of the two cell lines was determined to be very similar: 1.05 days for MDA-MB-231 cells and 1.50 days for MDA-MB-453 cells. Thus the effect of proliferation on island area increase after 24 hrs became a limiting factor. A comparison of the migration rates of individual cells of the two lines revealed that MDA-MB-231 cells migrated ~7 times more than the other cell line ($0.0639\mu\text{m}/\text{min}$ vs. $0.0093\mu\text{m}/\text{min}$). However, a comparison of the typical migration rates of cell islands showed that MDA-MB-231 islands migrated at least 40–150 times (Table 2; Fig. 3C) more than MDA-MB-453 islands of equal size. This implies that in addition to the differences in individual cell motility, cell-cell interactions also play a role in regulating island migration.

Homotypic and heterotypic interaction

Based on experiments with MDA-MB-231 cells alone, experimental conditions that yielded maximal MI were chosen for subsequent interactive studies. Also, MDA-MB-453 cells were not included in these experiments since their MI was extremely low under control conditions at $t=10$ hrs. Homotypic interaction was investigated by seeding an additional population of breast cancer cells outside MDA-MB-231 islands at $t=0$ hrs (Fig. 4A). Homotypic MDA-MB-231 cancer cells attached within 4–5 hrs after seeding. Quantitative analysis revealed that the addition of homotypic cells resulted in a significant decrease ($p < 0.01$) in MI compared to control under all experimental conditions (Fig. 4C). The data shown here are for one square island size (0.025 mm^2). Similar decreases in MI were observed in all other island sizes. The addition of Fn or TGF- β 1 did not significantly increase MI in most island sizes.

Heterotypic interactions were obtained by developing MDA-MB-231 cell islands and seeding NIH-3T3 fibroblasts outside the islands (Fig. 4B). NIH-3T3 cells attached within 2–3 hrs after seeding. There was a significant decrease in MI compared to control in all island sizes (Fig. 4C). This decrease was also significant compared to homotypic interaction in islands grown on Fn and in the presence of TGF- β 1 ($p < 0.001$), thus indicating that untransformed fibroblasts were able to inhibit migration of cancer cells in 2D. Since fibroblasts have been shown to have a density dependent effect on cancer cell lines, we used two different densities of NIH-3T3 cells outside islands (cancer: fibroblast density ratio=1:0.1 and 1:0.2). The small increase in cell density inhibited migration significantly in most island sizes ($p < 0.01$) (Fig. 4D).

Mathematical simulation

A 2D model of the migration assay was developed for control conditions and homotypic/heterotypic interactions. The control condition, where only one cell population (cancer) is modeled, is simulated using equations previously described for solid tumors² as mentioned in Appendix. Our model results show that cancer cell islands temporally expanded into the surrounding space (Fig. 5A). Here, a value of dimensionless time, $t=1$ represents 10 hours, the end-point in the migration experiments. Fig. 5C shows a cross-sectional plot of one island expansion along the x-axis. ECM decay began within the island as the cancer cells secreted MMPs. Due to the rapid diffusion of MMPs over time the model showed widespread ECM degradation throughout the domain (Fig. 5D). Fig. 5E shows the increase

in concentration of MMPs over time. The effect of initial island area on migration was also shown by simulation (Fig. 5B) and matched the trend seen in experimental results.

Cell-cell interaction was modeled using coupled PDEs derived from S-M equation. Fig. 6A shows island migration as a function of initial island area at various ratios of cancer to stromal cell density. At low density of stromal cells (normalized ratio of cancer to stromal cell density=1:0.1), the trend in island migration with respect to the area-dependent effects was similar to that of control conditions in Fig. 5B. The typical migration pattern of one such cancer cell island (initial area: 0.025 mm^2) and the surrounding stromal cells is shown in Fig. 6B. The results were remarkably different when the cancer: stromal ratios were 1:1 and 1:10. In both cases, larger cancer cell islands still showed similar migration but smaller islands (initial area $\leq 0.025 \text{ mm}^2$) showed substantially decreased MI, thus depicting a biphasic relationship between island size and migration (Fig. 6A). The altered migration pattern for an island of initial area 0.00625 mm^2 (Fig. 6C–H) is shown here for the various density ratios. When the normalized cell densities are equal (ratio 1:1), the smaller islands initially migrated outward ($\zeta_t = 0.5$), but then migrated inward ($\zeta_t = 1$) (Fig. 6D). When the stromal cell density is larger (ratio 1:10), the smaller islands migrated inward immediately ($\zeta_t = 0.5$) and migrated outward with time ($\zeta_t = 1$) (Fig. 6E). The corresponding stromal cell migration is also shown (Fig. 6G and 6H respectively).

The mathematical model relied on reference and parameter values available in literature. This led to a difference in model predictions and experimental data. In Fig. 7, we compared islands of initial area $0.025\text{--}0.25 \text{ mm}^2$ for both cases. Heterotypic interactions led to low experimental MI at relatively small stromal densities (cancer: stromal ratio = 1:0.1 and 1:0.2) whereas the mathematical model predicted that higher stromal densities (1:1 or 1:10) would be required for inhibition of migration. Further, to study the relevance of various parameters used in the model, we carried out a sensitivity analysis of the model parameters. The results show that the motility coefficients of the cancer and stromal cells significantly affected the MI (Fig. 8). In case of the cancer cell motility coefficients D_n and D_p , the model response was greatly dependent on the cell density used although the changes in MI were not large (Fig. 8, Box). The model is sensitive to the haptotactic parameter δ and the MMP diffusion coefficient D_m but not to δ , μ and λ . For all parameters except the cell motility coefficients, the model responded similarly irrespective of the cell densities used. The MI decreased for an increase in the cancer or stromal cell motility when the cancer cell to stromal cell density ratio was 1:1.

DISCUSSION

The microfabrication-based migration assay was used to experimentally model cancer cell migration *in vitro* in a 2D system. It was found to be highly reproducible, efficient and easy to use. It allowed the study of migration in an “injury-free” environment. Cell migration occurred from all sides of the islands, thus allowing rapid quantification of migration. This limits the potential effect of proliferation even in rapidly dividing cells, such as the breast cancer lines studied. A primary advantage of this microfabrication-based model compared to that of other barrier-type assays^{10, 22, 37} was the inclusion of multiple sizes of islands within the same PDMS membrane, which substantially increased the efficiency of the assay. This assay is also highly suitable for cancer migration studies, since it depicted distinct localizations of cancer cells within the islands and an interfacial layer of stromal cells at the periphery, thus providing a more accurate 2D simulation of *in vivo* mammary tumors.

This assay was used to investigate the migration of two breast cancer cell lines: the highly invasive MDA-MB-231 and non-tumorigenic, weakly invasive MDA-MB-453⁵. The rate of cell island migration was strongly dependent on initial island areas as well as the cell line

studied. The smaller MDA-MB-231 islands had significantly greater MI compared to the larger islands (Fig. 1), which was not observed in MDA-MB-453 cell islands (Fig. 3). This effect can be partially explained by the fact that smaller islands have a greater perimeter to area ratio compared to larger islands, implying that smaller islands have a larger migratory population available. However, this does not explain the differences between the two cell lines. We surmise that the lack of E-cadherin and other tight junction proteins in the fibroblast-like MDA-MB-231 cells contributed to the rapid dispersion of cell islands. MDA-MB-453 cells are classified as “weakly luminal epithelial-like phenotype” since they express low levels of E-cadherin and desmoplakin I/II²³. This could have contributed to their significantly decreased migration from cell islands and the tendency to move more collectively than MDA-MB-231 cells while resulting in a relatively small difference between individual cell migration speeds. This observation clearly shows that cell-cell interaction, or lack thereof, can have a significant impact on migration, which will be difficult to quantify in other monolayer or filter-based assays.

Interestingly, the migration profiles of epithelial breast cancer cells were significantly different than that of keratinocytes in cell islands, as shown previously³⁴. Larger keratinocyte islands showed greater MI than smaller islands. This effect was seen to some extent in MDCK cells, where migration was slower in 100 μm rectangular patterns compared to the migration in 150 μm (or wider) patterns³¹. The authors concluded this to be the effect of crowding of neighboring patterns impacting migration, although this was not the case in our migration assay. We hypothesize that a combination of cell adhesion and migration gives rise to two distinct cell populations in the islands: a highly motile outer layer and a relatively quiescent inner layer. The inner layer can be recruited by the rapidly advancing outer layer in a migratory population retaining strong cell-cell adhesion (such as keratinocytes). This is optimal in an intermediatesized island, as the smaller islands lack the inner layer and very large islands simply have too small perimeter to area ratio. If cell adhesion has minimal role in migration, smaller islands migrate faster (as seen in MDA-MB-231 islands).

Heterotypic cell interactions between MDA-MB-231 and NIH-3T3 cells significantly lowered MI compared to homotypic cancer cell even in the presence of ECM and growth factors (Fig. 4). The effect of stromal fibroblasts on cancer growth and progression has been studied extensively in recent years. It has been shown that normal stromal fibroblasts differ in many respects from the cancer-associated fibroblasts (CAF), which have been shown to promote invasion, angiogenesis and tumor growth^{6, 28}. Normal fibroblasts were shown to suppress proliferation of MCF10AT cells while CAFs failed to do so³². It is noteworthy that in addition to fibroblasts, other stromal cell types such as macrophages and endothelial cells are also known to regulate tumor progression via crosstalk between the malignant cells and their local microenvironment^{15, 24, 15, 40}. Endothelial cells are essential not only in the development of tumor vasculature, which promotes tumor growth, but also in mediating chemokine signaling that enables tumor migration and survival^{24, 26}. Our microfabrication-based assay can be utilized to study the effect of such stromal cells in regulating 2D migration of cancer cells.

Mathematical models of tumor growth and migration have long employed Fickian diffusion-based approach to describe cell motility^{1, 25, 30}. Although a number of models addressing different aspects of tumor progression such as avascular growth^{38, 39}, angiogenesis⁷ and chemotaxis¹⁶ have been developed, relatively little work has been done on interacting cell populations. In the past, mathematical models of interacting cell populations have been developed by using terms representing extension of linear³⁵ and nonlinear diffusion²⁹ in reaction-diffusion equations. The fundamental limitation of this type of diffusion-based modeling is that Fick's Law is applicable specifically (i) in binary systems and (ii) in dilute

multi-component mixtures²¹. Physiologically, cells exist in a dense (i.e., non-dilute) mixture of various cell types, ECM matrices and soluble factors. Thus, models of interacting cell populations implementing Fick's Law suffer from an inherent limitation. S-M equation constitutes a more general description of diffusion in multi-component mixtures by virtue of interrelated diffusion coefficients for all components in the mixture. It has been used to describe transport in a binary system interacting with a support when both species-species and species-support interactions are significant²⁰. To our knowledge, this is the first time that S-M equations have been used as a basis to study multiple cell populations. An advantage of using the above equations is that both single cell (cell-void space) and island migratory processes (cell-cell interactions) can be quantified. The model results showed a strong dependency of migration not only on island size but also on cell density. At low stromal densities (cancer:stromal=1:0.1) the cancer islands migrated irrespective of stromal cells, simulating diffusion in a dilute multi-component mixture, *viz*, Fickian diffusion. In contrast, the biphasic relation between island size and migration at equal or higher stromal cell density observed was unique to S-M equations. In S-M equations, unlike Fickian-type diffusion, the migration rate of one cell depends on the migration rate as well as the densities and density gradients of both cell types, resulting in these interesting behaviors. Indeed, results from the sensitivity analysis (Fig. 8) confirmed that cancer cells in smaller islands can migrate up the cell density gradient in the presence of stromal cells. All three cell-density ratio conditions showed similar values of migration for the two largest islands tested. This implies that at larger initial sizes of islands, cell-cell interaction is not a contributing factor in migration in our experiments. But for smaller islands, heterotypic interactions are very critical. Extrapolating to *in vivo* conditions, one may speculate that a relatively smaller tumor depends more heavily on local conditions than a larger tumor for migration.

While the simulation and experimental results show qualitative agreement, there were some quantitative variations between the two. Experimental results (Fig. 7) indicated that the stromal inhibitory effect on migration was achieved at lower densities (cancer:stromal=1:0.1) than that indicated by the model (1:1 ratio). An increase of stromal population (1:0.2 ratio) resulted in further decrease in migration despite the addition of fibronectin. We cannot rule out the possibility of inhibitory paracrine signaling pathways. Contact inhibition of migration can lead to cell motility values that are strongly dependent on cell densities.

CONCLUSIONS

In this work, we showed that the effect of cell-cell interactions can alter the migration profile of cells, which is crucial to the evaluation of cancer migration as it is highly regulated by stromal cell interactions. Since non-CAFs suppressed migration of highly metastatic cancer cells, we may conclude that cancer cells must promote transformation of stromal cells before invasion or metastasis. The microfabrication-based assay provided an improved alternative to random co-culture migration. The S-M model equations addressed some key limitations of Fickian-diffusion models of cancer migration. This model can be used and extended for studies of different types of cell motility in multi-cell environments such as in wound healing. This work also has potential implications in tissue engineering applications, most notably in cartilage where lateral integration of engineered tissue into the host remains problematic. We suggest that similar studies of the effects of local geometry on cell migration at the junction of engineered and host tissues can promote the design of implants to optimize integration.

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APPENDIX

The Generalized Stefan-Maxwell's equation for uncharged substances behaving in thermodynamically ideal manner is expressed as:

$$-\nabla x_i = \sum_{j=1}^I (x_j N_i - x_i N_j) / c D_{ij} \quad i=1, 2, \dots, I \quad (5)$$

where x_i = mole fraction of component i , N_i = flux of component i , c = total concentration and D_{ij} = interdependent S-M diffusion coefficients. Here, we designate $i = 1$ as cancer, $i = 2$ as stromal and $i = 3$ as empty space. We obtain $x_1 + x_2 + x_3 = 1$. We also designate $c \nabla x_1 = \nabla n$, $c \nabla x_2 = \nabla p$. We assume that motility of cancer cells in empty space ($D_{13} = D_n$) and motility of stromal cells in empty space ($D_{23} = D_p$) are significantly larger than motility of cancer cells through stromal compartment (D_{12}), therefore $D_{12} \rightarrow 0$.

Expanding (5) and incorporating the above-described terms, we obtain (1) and (2). We use the following non-dimensionalization scheme:

$$\bar{n} = \frac{n}{n_0}, \bar{p} = \frac{p}{p_0}, \bar{m} = \frac{m}{m_0}, \bar{f} = \frac{f}{f_0}, \bar{x} = \frac{x}{L}, \bar{y} = \frac{y}{L}, \bar{t} = \frac{t}{\tau}$$

Here, L is the length of cell island and τ is the experimental duration (=10 hrs). The variables are rescaled with their respective initial densities (n_0 , p_0 , f_0 and m_0). The values used for each of these reference variables are listed in Table 1. The non-dimensionalized equations (after removing the overbars) for cancer cells, stromal cells, ECM and MMPs respectively are:

$$\frac{\partial n}{\partial t} = d_n \nabla \cdot \left[\frac{1}{1 - n \left(\frac{p}{c_0} \right) - \frac{p}{c_0}} \left\{ \frac{n}{n + \left(p \frac{D}{\varphi} \right)} \nabla n + \frac{1}{\varphi} \left(\frac{n}{n + \left(p \frac{D}{\varphi} \right)} \right) \nabla p \right\} \right] - \rho \nabla \cdot (n \nabla f) \quad (6)$$

$$\frac{\partial p}{\partial t} = d_p \nabla \cdot \left[\frac{1}{1 - n \left(\frac{p}{c_0} \right) - \frac{p}{c_0}} \left\{ \frac{p}{n + \left(p \frac{D}{\varphi} \right)} \nabla n + \frac{1}{\varphi} \left(\frac{p}{n + \left(p \frac{D}{\varphi} \right)} \right) \nabla p \right\} \right] - \rho \nabla \cdot (p \nabla f) \quad (7)$$

$$\frac{\partial f}{\partial t} = -\eta f m \quad (8)$$

$$\frac{\partial m}{\partial t} = d_m \nabla^2 m + \kappa n - \sigma m \quad (9)$$

Here, the parameter groups are as follows:

$$d_n = \frac{D_n \tau}{L^z}, d_p = \frac{D_p \tau}{L^z}, d_m = \frac{D_m \tau}{L^z}, \rho = \frac{\chi f_o \tau}{L^z}, \eta = \delta m_o \tau, \kappa = \frac{\mu n_o \tau}{m_o}, \sigma = \lambda \tau, \varphi = \frac{n_o}{p_o} \text{ and } D = \frac{D_n}{D_p}$$

The initial conditions become:

$$n = \begin{cases} 1, & \text{Inside islands} \\ 0, & \text{Outside islands} \end{cases}, p = \begin{cases} 0, & \text{Inside islands} \\ 1, & \text{Outside islands} \end{cases}, f = 1 \text{ and } m = 0.$$

To model control cell islands (in the absence of secondary cell populations outside islands) the following equation was used to simulate cancer migration instead of the S-M equation:

$$\frac{\partial n}{\partial t} = D_n \nabla^2 n - \chi \nabla \cdot (n \nabla f) \quad (10)$$

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1.

Development of cell islands. (A) MDA-MB-231 cell islands of varying initial areas at $t=0$ hrs: (a) 0.025mm^2 , (b) 0.125 mm^2 , (c) 0.25 mm^2 , (d) 0.625 mm^2 and (e) 1.25 mm^2 . (B) Migration of cell island (initial area: 0.0625 mm^2) at $t=0$ hr and $t=10$ hrs. Scale bars: $100\mu\text{m}$. (C) Quantification of average cell count in islands at $t=0$ hrs. Data presented for $n \geq 21$ islands $\pm$ SEM from 8 different experiments. (D) Quantification of island migration on control (untreated) surfaces. Migration Index (MI) is defined as island area at $t=10$ hrs normalized to the initial island area at $t=0$ hrs. Cell migration from all island sizes is significantly lower than migration from 0.025 mm^2 island ($p < 0.05$ for 0.125 mm^2 island and $p < 0.01$ for larger islands ($0.25\text{--}1.25\text{ mm}^2$)). Data presented for $n = 9$ islands from 3 different experiments.

2. Quantification of migration from MDA-MB-231 cell islands in the presence of fibronectin (Fn) and TGF- β 1. (A–C) Cell Islands of initial size 0.125 mm^2 migrating on (A) control surface, (B) Fn-treated surface and (C) in the presence of TGF- β 1 supplemented media imaged at $t=10$ hrs. (D) MI of square islands at $t=10$ hrs under each condition compared to control. Data is presented as Migration Index (MI) $\pm$ SEM for $n=9$ islands from 3 different experiments. Scale bars: $100 \mu\text{m}$.

3. Migration of MDA-MB-453 cell islands. (A) MDA-MB-453 cell island of initial area 0.125 mm² at t=0 hrs, t=24 hrs and t=48 hrs. Scale bar: 100μm. (B) Quantification of cell island migration at days 1, 2, 3 and 4. (C) Comparison of MI of MDA-MB-231 and MDA-MB-453 cell islands at various time-points.

4. Effect of homotypic and heterotypic cell-cell interactions on MDA-MB-231 island migration. (A) MDA-MB-231 cells were seeded both inside (green; labeled with CMFDA-SE dye) and outside islands (red; pseudo-color overlay) in homotypic interaction. (B) NIH-3T3 fibroblasts (red) were seeded outside MDA-MB-231 cell islands (green) in heterotypic interaction. Cell islands of initial area 0.025 mm^2 are shown here at $t=10 \text{ hrs}$. Scale bars: $100 \mu\text{m}$. (C) Quantification of homotypic and heterotypic interaction compared to control. Both types of interaction significantly lowered MI under all experimental conditions. The decrease in MI induced by heterotypic condition is significant in Fn and TGF- β 1 categories (* indicates $p < 0.05$ compared to homotypic interaction). Data is shown here for square islands of initial size 0.025 mm^2 . (D) Increase in fibroblast density from 1:0.1 (Ht LD: Heterotypic Low Density) to 1:0.2 (Ht HD: Heterotypic High Density) with respect to MDA-MB-231 cell density significantly lowered MI ($p < 0.01$ for islands 0.0625 – 0.25 mm^2) on untreated surfaces.

5.

Dynamics of island migration obtained by mathematical modeling. (A) Cancer cell islands migrating in control condition (no cells outside islands) at $t=0$ hrs ($\bar{t}=0$), $t=5$ hrs ($\bar{t}=0.5$) and $t=10$ hrs ($\bar{t}=1$) respectively. The time scale has been normalized to experimental end-point ($t=10$ hrs). (B) Numerical Migration Index of cell islands obtained by normalizing island area at $\bar{t}=1$ to initial area at $\bar{t}=0$. (C–E) Cross-sectional view of a typical cell island along x-axis, showing the dynamics of (C) cancer cell density, (D) ECM density, and (E) MMP concentration at different time-points. Parameter values mentioned in Table 1 were used to simulate these graphs. Initial and Boundary conditions were as specified in the Appendix.

6.

Modeling of cell-cell interaction. (A) Varying cell densities of cancer and stromal population show different migration profiles. The normalized ratio of cancer and stromal cells are 1:0.1, 1:1.0 and 1:10 respectively. (B) Cross-sectional view of a cell island (initial area 0.025 mm²), showing changes in cancer density within island and complementary changes in stromal density outside island at $t=0$ hrs ($t=0$), $t=5$ hrs ($t=0.5$) and $t=10$ hrs ($t=1$). (C–E) Changes in cancer cell density in island of initial area 0.00625 mm² surrounded by varying density of stromal cells (cancer: stromal ratios = 1:0.1 (C); 1:1.0 (D); 1:10 (E)). (F–H) Corresponding changes in stromal cell density outside island (F = 1:0.1 ratio; G = 1:1.0; H = 1:10).

7.

Comparison of mathematical and experimental data of cell-cell interactions. (A) The mathematical model data is obtained as described in Fig. 6, but limited to the following sizes of cell islands: 0.025–0.25 mm². (B). The experimental data is obtained from heterotypic cellular interactions at the following cancer:stromal densities: 1:0.1 and 1:0.2. All cells were seeded on fibronectin and the initial sizes of cell islands are 0.025–0.25 mm². Here, control represents no stromal cells outside cell islands.

8. Sensitivity of Stefan Maxwell equations to model parameters. For an island size of 0.0625 mm^2 , the change in the numerical Migration Index was calculated for a 10% positive change in the parameters: D_n , D_p , D_m , δ , χ , μ , and λ . Two cancer to stromal cell ratios: 1:0.1 and 1:1 were used. The box highlights the switch in trends for the Migration Index when the cell ratio was changed from 1:0.1 to 1:1.

Table 1

Parameters and variables used in migration model

	Description	Value
<i>Motility Coefficients</i>		
D_n	Cancer cells	$1.38 \times 10^{-10} \text{ cm}^2 \text{ sec}^{-1}$ ■
D_p	Stromal cells	$1.38 \times (10^{-10} - 10^{-8}) \text{ cm}^2 \text{ sec}^{-1}$ ■
D_m	MMP	$5 \times 10^{-8} \text{ cm}^2 \text{ sec}^{-1}$ ■
<i>Haptotactic coefficient</i>		
χ	Haptotaxis parameter	$2600 \text{ cm}^2 \text{ sec}^{-1} \text{ M}^{-1}$ ▶
<i>Production/decay rate</i>		
δ	ECM decay by MMPs	$3 \times 10^3 \text{ cm}^2 \text{ gm}^{-1} \text{ sec}^{-1}$ ■
μ	MMP production by cancer cells	$6.94 \times 10^{-8} \text{ sec}^{-1}$ ▼
λ	MMP natural decay rate	$5 \times 10^{-7} \text{ sec}^{-1}$ ■
<i>Reference variable values</i>		
τ	Time scale	36000 sec ●
L^2	Island area	$0.0125 - 0.0025 \text{ cm}^2$ ●○
n_0	Cancer cell density	$35 \times (10^3 - 10^4) \text{ cells/cm}^2$ ●○
P_0	Stromal cell density	$35 \times (10^3 - 10^4) \text{ cells/cm}^2$ ●○
f_0	ECM density	$1 \times 10^{-11} \text{ M}$ ▶
m_0	MMP concentration	$1 \times 10^{-7} \text{ gm/cm}^2$ ■
ε_0	“Empty cell” concentration	$0.9 \times 10^6 \text{ cells/cm}^2$ ■

▶ values obtained from Anderson².▼ values obtained from Kim *et al.*¹⁷.

● Obtained from experimental data.

■ This work.

○ When appropriate, additional values were used solely for simulation.

Table 2

Typical increases in MI of breast cancer cell islands in 24 hours.

Island Area (mm ²)	Increase in Migration Index/day	
	MDA-MB-231	MDA-MB-453
0.125	4.893	0.04
0.3125	3.234	0.032
0.625	2.91	0.031