Evolution of Tumor Invasiveness: The Adaptive Tumor Microenvironment Landscape Model

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Abstract

Interactions between cancer cells and their microenvironment are crucial for promoting tumor growth and invasiveness. In the tumor adaptive landscape model, hypoxic and acidic microenvironmental conditions reduce the fitness of cancer cells and significantly restrict their proliferation. This selects for enhanced motility as cancer cells may evolve an invasive phenotype if the consequent cell movement is rewarded by proliferation. Here, we used an integrative approach combining a mathematical tumor adaptive landscape model with experimental studies to examine the evolutionary dynamics that promote an invasive cancer phenotype. Computer simulation results hypothesized an explicit coupling of motility and proliferation in cancer cells. The mathematical modeling results were also experimentally examined by selecting Panc-1 cells with enhanced motility on a fibroblast-derived 3-dimensional matrix for cells that move away from the unfavorable metabolic constraints. After multiple rounds of selection, the cells that adapted through increased motility were characterized for their phenotypic properties compared with stationary cells. Microarray and gene depletion studies showed the role of Rho-GDI2 in regulating both cell movement and proliferation. Together, this work illustrates the partnership between evolutionary mathematical modeling and experimental validation as a potentially useful approach to study the complex dynamics of the tumor microenvironment.

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Introduction

The tumor microenvironment is not an idle bystander but actively participates in tumor progression and metastasis (1, 2). It consists of many components including endothelial, mesenchymal cells, extracellular matrix (EM), and physical parameters such as gradients of oxygen, glucose, pH, and interstitial pressure. An accumulation of evidence has shown that interactions between cancer cells and their surrounding stroma are crucial for promoting the growth and invasiveness of tumor (3–5). Tumor cells promote the development of a tumor-permissive stroma via the aberrant expression of paracrine factors that induce an altered stromal compartment. In turn, alterations in the stromal microenvironment, including enhanced vasculature, modified EM composition, inflammatory responses, and imbalanced protease activity, are essential regulatory factors of tumor growth and invasion (6–8). Thus, it is critical to understand and predict the multistage, nonlinear dynamics of tumor–microenvironment interactions to facilitate the clinical development of novel therapeutic targets that may disrupt the stromal influences on tumor invasion and growth.

It is generally understood that cancers arise through a process characterized as somatic evolution. These Darwinian dynamics consist of competition among evolving tumor phenotypes within environmental selection forces that govern fitness. Although tumor cells are continuously evolving through cumulative genetic and epigenetic changes, it is...
Quick Guide to Main Model Equations and Major Assumptions of the Model

Energy Requirements for Replication

\[ p_{\text{duplication}} = \frac{(ATP - 0.1 \times ATP_0)}{0.9 \times ATP_0} \quad \text{If } 0.1 \times ATP_0 \leq ATP \leq ATP_0; \]

\[ p_{\text{duplication}} = 0 \quad \text{If } ATP \leq 0.1 \times ATP_0; \]

\[ p_{\text{duplication}} = 1 \quad \text{If } ATP \geq ATP_0; \]  

(A)

In the computational model cell duplication occurred only if 3 conditions were met simultaneously: (i) at every generation the cell would be tested for duplication depending on its proliferation phenotype (0 ≤ duplication phenotype ≤ 1.0 results in a probability between 0% and 100%); (ii) there must be at least 1 empty space available next to the mother cell to be occupied by the new cell; and (iii) the cell was tested for its energy production rate and the probability of duplication was represented as a linear function with value 0 for an ATP production rate equal to or below \(8.6 \times 10^{-7}\) M/s (lower values lead to cell death) and 100% for an ATP production rate equal to or higher than \(8.6 \times 10^{-6}\) M/s (ref. 19). \(p_{\text{duplication}}\) is the probability for cell division, ATP is the ATP production rate, and ATP_0 is the minimum energy production rate for 100% probability of cellular division (\(8.6 \times 10^{-6}\) M/s).

Major assumptions for equation A

The model assumes a linear correlation between energy production by glycolytic metabolism and proliferation rate, with a lower threshold and a maximum plateau. This is the simplest representation possible of a monotonic function correlating these 2 properties.

Cell Metabolism

Each cell possessed its own energetic metabolism simplified to aerobic conversion of 1 molecule of glucose into 36 molecules of ATP and 6 of CO₂ or anaerobically into 2 molecules of ATP and 2 of lactic acid. The anaerobic or aerobic paths were chosen depending on the availability of oxygen in the extracellular environment according to equations B to G:

Glucose aerobic metabolism in cytoplasm and mitochondria:

\[ \text{Glc} + 6 \times \text{O}_2 \Rightarrow 36 \times \text{ATP} + 6 \times \text{CO}_2 \]  

(B)

Glucose anaerobic metabolism in cytoplasm:

\[ \text{Glc} \Rightarrow 2 \times \text{ATP} + 2 \times \text{Lactic acid} \]  

(C)

In hypoxic conditions (excess of glucose):

\[ \text{ATP}_{\text{Anaer}} = 2 \times \left( \text{Glc} - \frac{\text{O}_2}{6} \right) \]  

(D)

\[ \text{ATP}_{\text{Aer}} = 36 \times \left( \frac{\text{O}_2}{6} \right) \]  

(E)

In hyperoxic or hypoglycemic conditions (excess of O₂):

\[ \text{ATP}_{\text{Aer}} = 36 \times \left| \text{Glc} \right| \]  

(F)

\[ \text{ATP}_{\text{Anaer}} = 0 \]  

(G)

Major assumptions for equations B to G

\(\text{ATP}_{\text{Anaer}}\) and \(\text{ATP}_{\text{Aer}}\) correspond to the production rate of ATP anaerobically and aerobically, respectively, and their sum is the total ATP production rate. The production rate, and not the intracellular ATP concentration, is used because the model assumes that equilibrium is reached long before 1 generation is complete. Thus, the total ATP in a cell, given a fixed time between generations in steady state, is a function of the production rate. The probability of division is thus based on the ATP production rate at equilibrium, whereas the probability of cell death is decided at every metabolic step: If cell ATP production at a given moment is below the minimum necessary for survival, the cell dies.
Diffusion of Species and pH Buffering

The diffusion of species in this model was calculated based on Fick’s first law, which relates the diffusion flux through a surface to the difference of concentration of species in volumes separated by this surface (equation H).

\[ J = -D \frac{\partial \phi}{\partial x} \]  

Where \( J \) is the diffusion flux expressed as \( \frac{\text{mol}}{\mu \text{m}^2 \cdot \text{s}} \), \( D \) is the diffusion coefficient in \( \frac{\text{mol}}{\mu \text{m}^2 \cdot \text{s}} \), and \( \phi \) is the concentration of a species in \( \frac{\text{mol}}{\mu \text{m}^3} \).

Two cubic adjacent volumes in this computer model share a contact surface \( S = 25 \mu \text{m} \times 25 \mu \text{m} \), the distance between centers the two volumes is \( d = 25 \mu \text{m} \) and the time step used in this calculation is \( \Delta t = \frac{1}{10} \text{s} \). Under these conditions, the variation in concentration of the two volumes due to diffusion can be approximated as being the product of the flux through the contact surface, during the time step, divided by the volume \( V \):

\[ C_{t+1} - C_t = \frac{J \times S \times \Delta t}{V} \]  

In the general case, each volume in the model is surrounded by 6 neighboring volumes, thus the general expression used to calculate the diffusion (equation H) can be derived from 10:

\[ C_{t+1} - C_t = \frac{-D \times \sum_{i=1}^{6} (C_t - C_{t_i}) \times S \times \Delta t}{d \times V \times 6} = -\sum_{i=1}^{6} (C_t - C_{t_i}) \times D_N \]  

Where \( D_N \) is a dimensionless constant used to simplify the final expression.

The buffer effect of bicarbonate was calculated as below. In equilibrium the concentrations of the buffer is defined by

\[ \frac{[\text{CO}_3^2^-]}{[\text{HCO}_3^-] \times [\text{H}^+]} = 10^{p\text{Ka}_{\text{bicarb}}} \]  

When \( \text{H}^+ \) (dH) and \( \text{CO}_3^2^- \) (dCO\textsubscript{3}\textsuperscript{2}−) are added to the solution by glycolysis and respiration, there is an unbalance that must be corrected by the transfer (dX\textsubscript{t}) of mass from the exceeding species to the rest of the buffer:

\[ \frac{\text{CO}_3^2^- + d\text{CO}_3^2^- - dX_t}{(\text{HCO}_3^- + d\text{X}_t) \times (\text{H}^+ + d\text{H} + d\text{X}_t)} = 10^{p\text{Ka}_{\text{bicarb}}} \]  

The solution of this equation with variable dX\textsubscript{t} provides the concentrations of species in the new equilibrium.

Major assumptions for equations H–I.

The main assumptions of this model are that cells have similar sizes, that diffusion within a cell is fast enough for equilibrium within the simulation time step (0.1s), and that diffusion in all directions in the tumor occur at the same speed.

Microenvironmental selection forces that may determine the optimal phenotypic properties, that is, the cellular characteristics that result in the greatest fitness. These selection pressures are dynamically changed as a result of tumor population growth and evolution (9). The disordered tumor–stroma interactions typically result in reduced and chaotic blood flow that produces hypoxic and acidic microenvironmental conditions. Acidic pH, hypoxia, and fluctuations in nutrition exert strong selection pressures on tumor growth and progression (10).

The evolutionary dynamics of carcinogenesis have been described through a quantitative mathematical model to predict the bidirectional interactions of tumor cells and their surrounding environment during cancer progression (10–12). Our mathematical model shows that evolution of invasiveness occurs by coupling proliferation and motility (9). That is, cancer cell invasiveness can evolve if it is rewarded by proliferation, as enhanced motility allows the cell to escape to a more favorable microenvironment that permits greater proliferation. We hypothesize that enhanced motility is selected as an adaptive mechanism to escape harsh microenvironmental conditions that reduce the cancer cell fitness.

Several studies have shown that a fibroblast-derived 3-dimensional (3D)-matrix system can be utilized to study the influence of the stromal landscape on cell motility (13–15). Fibroblast activation protein (FAP), a serine protease selectively expressed on tumor stromal fibroblasts, but not on carcinomas (16), was used as a platform for studying tumor–stromal interactions. FAP expressing fibroblast-derived matrix was used as an in vivo-like in vitro setting to
evaluate the motility phenotype of cancer cells. In this study, we tested the hypothesis predicted from the mathematical model that cancer cell proliferation is rewarded when enhanced motility is selected by the tumor landscape.

Materials and Methods

**In silico system**

Each cell was represented as a cubic volume with a 25-μm side, or a volume of 15,625 μm³. Cell metabolism was modeled as anaerobic or aerobic glycolysis (17). Outside cover slip, side, or a volume of 15,625 μm³. Oxygen transport was modeled as a 3D structure with 2 layers of cells trapped under a cover slip and move 25 μm (a cell length in this model) every 2 hours. This estimation was obtained from the observed motility in the Panc-1 cell line [American Type Culture Collection (ATCC)] used in this experiment (between 13 and 18 μm/h). Due to the fixed-lattice limitation of our computational model, it is not possible to represent a distance smaller than 25 μm, and thus a time step of 2 hours was used instead. The simulation flow thus consisted of 1 cycle of replication for every 12 events of movement (1 cell long), resulting in a minimum theoretical cell cycle of 24 hours, or a 48-hour cell cycle for the original tumor population (motility value 0.5), which matched the cell cycle in vitro for the Panc-1 cell line used in this experiment (52 hours). No mutations were considered in the cover slip model, and thus the only force acting on population phenotypic values was selection.

A second model representing the growth of a solid tumor in vivo, described in the Supplementary Material, was used to determine whether the evolution of these 2 phenotypes observed in the cover slip model would also be valid in an actual tumor. In this model, we seeded tumors at different densities and allowed mutations to occur with a 1% probability at every replication.

Cell motility was modeled as a dislocation to an empty space randomly chosen immediately next to it. Cell duplication occurred only if 3 conditions were met simultaneously: (i) at every generation the cell would be tested for duplication depending on its proliferation phenotype (0 ≤ duplication phenotype ≤ 1.0 results in a probability between 0 and 1); (ii) there had to be at least 1 empty space available next to the mother cell to be occupied by the new cell; and (iii) the cell was tested for its energy production rate and the probability of duplication was represented as a linear function with value 0 for an ATP production rate equal to or below 8.6 × 10⁻² mol/L/s (lower values lead to cell death) and 1 for an ATP production rate equal to or higher than 8.6 × 10⁻² mol/L/s (19) as described in equation A in the Quick Guide to Main Model Equations and Major Assumptions of the Model.

All the parameters used in this model and their literature sources are listed in Supplementary Tables S1 and S2. A further description of the computational model implementation appears in the Supplementary Material and in the Quick Guide to Main Model Equations and Major Assumptions of the Model, which accompanies this article.

**Selection of subpopulations in the computational model (in silico system)**

To study the evolutionary dynamics occurring in vitro, we built a computational model that reproduces the geometry of the cover slip. This computational model allowed us to study the fate of thousands of cells individually, and better understand how and which phenotypes are selected as they replicate and move under the cover slip. The computational model was built as a 3D structure with 2 layers of cells trapped under a cover slip. The simulation model was delimited by a volume with dimensions 200 × 200 × 3 cells. The cover slip was a disk with a radius of 150 cells (~4 mm). The original population (O, 10,000 cells) was grown under the center of cover slip in 50% confluence until they reached the distance of 20 cells away from the outer edge of the cover slip and the first 2 subpopulations were collected. In all simulations, the cells selected for the motile population (M) were collected from a distance of 10 to 20 cells from the edge of the cover slip, and the stationary population (S) was collected from those closest to the center of the cover slip (3,000 cells each). These subpopulations were independently reseeded in the center of new cover slips for each new simulation and selected 4 more times. The first simulation lasted 25 generations, whereas the 4 others required 40 generations to reach the same distance limit, due to their smaller initial population size (3,000 vs. 10,000).

**Motility and proliferation in the computational model**

The starting population was initialized with random values for motility and proliferation according to a normal distribution with mean 0.5 and SD 0.1, corresponding to a probability between 0 and 1 of moving or proliferating at every time step. We considered that each cell had a cell cycle of 1 day and could move 25 μm (a cell length in this model) every 2 hours. This estimation was obtained from the observed motility in the Panc-1 cell line [American Type Culture Collection (ATCC)] used in this experiment (between 13 and 18 μm/h). Due to the fixed-lattice limitation of our computational model, it is not possible to represent a distance smaller than 25 μm, and thus a time step of 2 hours was used instead. The simulation flow thus consisted of 1 cycle of replication for every 12 events of movement (1 cell long), resulting in a minimum theoretical cell cycle of 24 hours, or a 48-hour cell cycle for the original tumor population (motility value 0.5), which matched the cell cycle in vitro for the Panc-1 cell line used in this experiment (52 hours). No mutations were considered in the cover slip model, and thus the only force acting on population phenotypic values was selection.

Production of a fibroblast-derived 3D matrix

Matrices were produced by FAP-expressing fibroblasts that were cotransfected with murine fap gene under the Tet-responsive promoter and rtTA regulatory element into NIH-3T3 cells. The NIH-3T3 cell line was obtained from the ATCC. ATCC has verified the identity of this cell line by methods including short tandem repeat profiling. As described by Amatangelo and colleagues (25), fibroblasts (7 × 10⁵) were seeded onto gelatin-coated glass cover slips (18 mm), and confluent fibroblastic cultures were treated with media supplemented with ascorbic acid (50 μg/mL) and doxycycline (2 μg/mL) every other day for 8 days. Alkaline detergent

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treatment (0.5% Triton X-100, 20 mmol/L NH₄OH in PBS) gave rise to cell-free in vivo-like 3D matrices.

Speciation of motile Panc-1 cells by enhanced cell migration

The Panc-1 cell line was obtained from the ATCC. ATCC has verified the identity of this cell line by methods including short tandem repeat profiling. Panc-1 cells (1 × 10⁵) were incubated onto matrix-coated glass cover slip for 3 hours to allow for attachment to the matrix. Then glass cover slips were placed upside down onto a plate for allowing cells to grow between cover slip and plate, resulting in the metabolic constraints by limited diffusion of nutrients (26). After 2 weeks of incubation, motile (M) cells, migrated out and away from underneath the cover slip, whereas stationary (S) cells, which resided near the cover slip, were isolated using Cloning Disks (Labcor Products). These cells were independently replated and reselected more than 6 times. Speciated cells from the fifth (M₅ and S₅) or sixth (M₆ and S₆) selections were further analyzed by the following studies.

Cell migration assay

Speciated Panc-1 cells (5 × 10⁴) in Dulbecco's modified Eagle's medium (DMEM) with 1% FBS were plated onto a Transwell system insert (8-µm pore size; Falcon) in a 24-well plate. The lower chamber was filled with DMEM supplemented with 10% FBS in the presence or absence of recombinant human hepatocyte growth factor (HGF; 100 ng/mL; ref. 27). After 24 hours of incubation, unmigrated cells were removed from the upper surface of the insert membrane with a cotton swab, and migrated cells on the lower membrane were stained using DiffQuick Stain Kit (IMEM Inc.). Cells from 5 regions per filter were randomly selected and counted under the microscope. Each experiment was conducted in triplicate and repeated 3 times.

Invasion assay within 3D matrices

As described by Castello-Cros and colleagues (15) and by Cukierman (28), speciated Panc-1 cells (1 × 10⁶/24-well plate) were replated onto the matrices and incubated overnight. Approximately 10 to 15 cells were selected under inverted microscope (Nikon TE-2000U), and images were acquired every 10 minutes for 12 hours. Individual cell dynamics were analyzed using the MetaMorph program following factors: (i) the net path distance (D, µm) by calculating the number of µm/pixel; (ii) the path trajectory (T, µm) of the individual cell during the recording period; (iii) average velocity (AV, µm/h) as the path distance divided by the elapsed time; and (iv) directionality (D/T ratio) as the ratio of the net distance divided by trajectory path and the means to determine random (<0.5) versus directional invasion (close to 1) of individual cells.

MTT assay for cell proliferation

Speciated Panc-1 cells (2,000, 4,000, and 8,000 cells per well) were grown on a 96-well plate in triplicates for 4 days. Cells were incubated with MTT solution (40 µL of 5 mg/mL Sigma) for 2 hours at 37°C in the dark and lysed by SDS solution (100 µL of 20%) overnight. A color change from yellow to purple caused by metabolically active cells was detected in the Multiskan plate reader at 570 nm.

Differential gene expression profile by microarray analysis

The Agilent Expression array was done at the Microarray facility in Fox Chase Cancer Center (Philadelphia, PA). Briefly, 500 ng of total RNA from speciated Panc-1 cells (the fifth and sixth selections) was labeled using the Agilent Low RNA input linear amplification kit following the manufacturer’s instructions. Eight hundred and twenty-five nanograms of labeled targets were hybridized onto Agilent 4 × 44K whole genome arrays for 17 hours at 65°C. Microarray scan images were processed using Feature Extraction software (Agilent) and subjected to further statistical analysis. To avoid dye bias, RNA samples were done as dye-flip replicates.

Gene profile analysis

Agilent Human 4 × 44K Whole Genome arrays were preprocessed with Agilent Feature Extraction (FE), version 9.5. Expression measurements used for analysis were the FE Processed Signal values, which are background corrected and dye normalized. Probes were removed if they lacked a valid Entrez gene identifier or Gene Ontology (GO) annotations, or if they had intensities near background across more than 75% of all channels. Differential expression was assessed using empirical Bayes moderated 1-sample t statistics implemented in the Bioconductor (29) package limma (linear modeling of microarray data; ref. 30). P values were adjusted for multiple testing using the method of Benjamini and Hochberg (31) to estimate false discovery rates (FDR). Lists of differentially expressed probes were generated separately for the 2 sets of samples, M₅ versus S₅ and M₆ versus S₆.

Gene depletion by siRNA transfection

Speciated Panc-1 cells (2 × 10⁵ cells per 6-well plate) grown in antibiotic-free DMEM overnight were transfected with 80 pmol of siRNA duplexes of RhoGDI2 (Rho GDP dissociation inhibitor 2, Rho-GDIβ). Amphregulin, Sgf1 (secretogranins), SPARC (Secreted Protein Acidic and Rich in Cysteine), and scrambled control siRNA following the manufacturer’s instruction (Santa Cruz Biotechnology). Levels of gene expression knocked down by each siRNA were confirmed by OneStep reverse transcriptase PCR (RT-PCR) Kit (Qiagen; ref. 32).

Results

Selection of subpopulations for the in silico system

In a 3D computer structure with cells trapped under a cover slip, oxygen and nutrients flowed from the media surrounding the cover slip and diffused to the cells (Fig. 1A). As oxygen and nutrients were metabolized by cells under the cover slip, a gradient was formed through the outer regions by decreasing oxygen and glucose concentrations and increasing acidity generated by anaerobic glucose metabolism in the hypoxic regions of the model. In these simulations, the M population...
Simulation was run 5 times. The computer simulations showed values for motility and proliferation according to a normal coupling of motility and proliferation in cancer cells. Computer simulation results hypothesized an explicit simulation and selected 5 additional times. Proliferation and motility values are graphically represented by white (higher) and blue (lower).

Figure 1. In silico system. A, the 3D computational model was built with cells trapped under a cover slip. Cyan, gray, and red layers represent cells, cover slip, and the surface of media, respectively. B, the original (O) population is seeded at 50% confluence and grown under a cover slip (top). In all simulations, subpopulations were selected from the regions delimited by the red arcs in the figure (bottom) and independently reseeded in the center of cover slips for each simulation and selected 4 more times. Proliferation and motility values are graphically represented by white (higher) and blue (lower).

Figure 1. In silico system. A, the 3D computational model was built with cells trapped under a cover slip. Cyan, gray, and red layers represent cells, cover slip, and the surface of media, respectively. B, the original (O) population is seeded at 50% confluence and grown under a cover slip (top). In all simulations, subpopulations were selected from the regions delimited by the red arcs in the figure (bottom) and independently reseeded in the center of cover slips for each simulation and selected 5 additional times.

Computer simulation results hypothesized an explicit coupling of motility and proliferation in cancer cells

The original (O) population was initialized with random values for motility and proliferation according to a normal distribution with mean 0.5 and SD 0.1, corresponding to a probability from 0 to 1 of moving or proliferating. Each simulation was run 5 times. The computer simulations showed that the M5 final subpopulation had higher proliferative values than the O cells (M5 = 0.7 ± 0.07, S5 = 0.52 ± 0.1, and O = 0.5; Fig. 2A, top). Whereas M5 cells also had increased motility (0.76 ± 0.01) compared with the O (0.5), S5 cells did not show enhanced motility (0.48 ± 0.1; Fig. 2A, bottom). These results suggested that proliferation and motility are selected by environmental pressures by M5 cells leaving the acidic and hypoxic environment of the cover slip, resulting in greater proliferation. The selection for proliferation of M cells as compared with S cells was likely due to the lower metabolic and spatial restrictions (gaps of up to 20 cells) which allow the M5 cells to grow unrestrained. In the interval evaluated, cells that have higher proliferative potential outgrow their competitors as 3D simulations after 5 selection rounds make explicit the difference in progression and phenotype distribution between the 2 populations (Fig. 2B). The results suggest that the invasive phenotype and proliferative phenotype may not be able to be separated in populations growing in a spatially confined area such as a solid tumor or in our experimental model.

The simulations with the solid tumor model, in which no reseeding was allowed, led to cells with increased values for proliferation and motility in the tumor–host interface: 0.54 ± 0.01 for proliferation and 0.58 ± 0.003 for motility when no mutations were allowed, and 0.68 ± 0.02 for proliferation and 0.75 ± 0.01 for motility for a mutation rate of 1%. No significant differences were observed when the original tumor was seeded at a density of 20% when compared with a fully confluent original tumor.

The mathematical modeling results were experimentally examined by selecting motile Panc-1 cells

We tested our mathematical modeling experimentally by selecting Panc-1 cancer cells for enhanced motility/invasion on a 3D matrix, away from the metabolic constraints of limited diffusion imposed by a glass cover slip over multiple iterations (Fig. 3A). Speciation was accomplished by serially selecting M cells that migrated away from the glass cover slip, as opposed to S cells that were viable but did not migrate. After 6 consecutive rounds of selections, M6 cells traveled greater distances away from the cover slip compared with S6 cells (Fig. 3B).

Speciated M Panc-1 cells retain enhanced motility/invasion in vitro and in 3D matrix

To characterize the invasive phenotype, chemoattractant induced migration of M6 and S6 cells was measured using a Transwell assay. As shown in Fig. 4, there was a 2-fold increase in the number of M6 cells migrated into the lower chamber because of a FBS gradient (1% FBS in top chamber and 10% FBS in lower chamber, \( P = 0.014 \)). Consistent results were also seen with HGF supplementation in the lower chamber as an additional chemoattractant (\( P = 0.001 \)). These observations suggest that speciated Panc-1 cells could be selected that retain enhanced motility in vitro.

To further assess the motility phenotype of speciated Panc-1 cells in an in vivo-like setting, 3D matrices were generated by culturing FAP expressing fibroblasts for 8 days before denuding the confluent fibroblasts leaving an
acellular EM. These matrices contain a meshwork of long fibronectin fibrils that can be utilized as roadways by tumor cells (14). Random versus directional motility of individual cells was quantified by determining the ratio of the net distance over trajectory path (D/T). In this analytical method, the range is 0 to 1 with a ratio of 1 representing the maximum value for ideal directional motility, whereas ratios significantly less than 0.5 are cells migrating in a random pattern. Figure 5A and B are representative examples of migration/invasion tracks of Panc-1 cells over 12 hours and quantified for average velocity and directionality, respectively. M6 cells had greater velocity and directional motility compared with S6 cells (velocity ¼ 17.6 ± 2.1 vs. 13.4 ± 1.0 μm/h, P = 0.04; directionality ¼ 0.70 ± 0.04 vs. 0.46 ± 0.05, P < 0.001 for M6 and S6 cells, respectively). In addition, speciated M6 cells showed greater average velocity and directional invasion compared with parental Panc-1 cells (velocity ¼ 11 ± 1.8, directionality ¼ 0.66 ± 0.02).

**Speciating M Panc-1 cells have greater intrinsic proliferative phenotype**

Given the mathematical model prediction of an intrinsic relationship between motility and proliferation, a cell proliferation assay was done of the speciated Panc-1 cells in an MTT assay (Fig. 5C). After 4 days of incubation, M6 cells had a nearly 2-fold greater intrinsic proliferation rate compared with S6 cells (P < 0.001). Speciated M6 cells also had a 2-fold greater proliferation rate compared with parental Panc-1 cells. Thus, speciating M6 cells resulted in greater intrinsic proliferative phenotype that was coupled with its enhanced motility, providing experimental evidence confirming the mathematical model prediction.

**Identification of altered gene expression in speciated subpopulations**

To characterize the potential molecular mechanism regulating the observed coupling of cell motility and proliferation in speciated M cells, gene expression profiles of 2 independent samples (fifth and sixth selections) were analyzed using an Agilent Expression Microarray. From the 34,127 genes in the microarray, genes that showed a statistically significant difference were identified as described in Materials and Methods. The resulting set (~2,400 genes) having more than 2-fold differential expression between M and S cells was analyzed by GO enrichment process for clustering genes within the cell motility and proliferation categories. After adjusting P values of differential expression to an estimated FDR < 1%, a list of 23 differentially expressed genes was generated (Supplementary Table S3). Four candidate genes upregulated in M Panc-1 cells were selected for further study. These candidate genes are Rho-GDI2, sparc, amphiregulin, and sgl2.
Rho-GDI2 plays a role in regulating both cell movement and proliferation

To test the role of candidate genes in regulating cell migration and proliferation, transcripts of candidate genes were knocked down in speciated cells using RNA interference with siRNA, and the transfected cells analyzed for in vitro migration and proliferation assays in 3 independent experiments. Endogenous RNA levels of each gene in M6 and S6 cells were analyzed by quantitative RT-PCR (Fig. 6A). Compared with control transfection with scrambled RNA duplexes, each siRNA was able to downregulate gene expression by approximately 70% for amphiregulin, approximately 60% for Rho-GDI2, and approximately 50% for sgII. However, only minor depletion was seen in sparv siRNA transfected cells (<10%, data not shown).

In a Transwell experiment to analyze the effects of specific gene depletion on cell migration (Fig. 6B), consistent results across multiple experiments showed amphiregulin, Rho-GDI2, and sgII depletion caused a significant reduction in the cell migration of both M6 and S6 cells (all \(P<0.001\)) compared with control transfection.

siRNA-transfected cells were further analyzed by the functional role that they may play in cell proliferation (Fig. 6C). Depletion of amphiregulin and sgII had no significant inhibitory effect on cell proliferation. In contrast, Rho-GDI2 depletion caused a significant decrease in cell proliferation in M6 cells (\(P=0.02\)) but had minimal effect on S6 cells (\(P=0.15\)). These results suggest that because Rho-GDI2 positively regulates both the cell migration and proliferation of motile pancreatic cancer cells, it may be the mechanism by which tumor cells invade and proliferate to escape the harsh microenvironment.

Discussion

Mathematical modeling of the adaptive tumor microenvironment landscape is helpful because nonlinear dynamics of tumor–microenvironment interactions are difficult to predict intuitively. Mathematical models can assist in devising multiple new strategies aimed at targeting the stroma and their interactions with the tumor population. This work is novel because it utilizes mathematical modeling of the tumor microenvironment to quantify these interactions and begins to identify new concepts that may disrupt the stromal influences on tumor invasion and growth.

In this study, we utilized a mathematical model to examine the complex balance of tumor phenotypic properties that may affect tumor growth. Cell proliferation in normal tissue...
under physiologic conditions is controlled by biologic constraints. However in abnormal physiologic states such as in tumors, with their abnormal vasculature and chaotic blood flow, proliferation will at times be limited by the availability of key substrates such as oxygen and glucose. Distinct tumor microenvironmental forces can exert strong selection pressures, and enhanced motility to escape this harsh environment may be selected as an optimal phenotypic property (9, 12, 33), resulting in enhanced fitness or proliferation.

Thus, our mathematical modeling predicts a coupling of proliferation and motility, as cancer cell proliferation is rewarded when enhanced motile phenotype is selected by the tumor microenvironment landscape. Here, we have validated the predictions of the adaptive tumor landscape concept experimentally by speciating cells for enhanced motility on computer simulation and in vitro 3D matrix system.

Figure 5. Motile Panc-1 cells had greater directional motility and enhanced proliferation. A, speciated cells within matrix were monitored for their cell behaviors for 12 hours. Randomly selected individual tracks were combined into a single figure. B, individual cell dynamics were measured for average velocity and directionality. M6 cells have greater velocity and directionality compared with S6 cells ($P = 0.04$ and $P = 0.001$, respectively). C, cells were tested for the growth rate by MTT assay. M6 cells had a 2-fold greater proliferation rate compared with S6 cells ($P = 0.001$). Statistical significance is indicated by an asterisk.

Computer simulation setting up an in silico system shows that phenotypic adaptation to the cover slip experiments proceeds in 2 steps. First, escape from the harsh environment under the cover slip is possible only for cells with increased motility. Second, cells that escape the cover slip enter a normal in vitro environment in which they must compete for space. In this adaptive landscape, a phenotype with high proliferation rate will be selected. Therefore, the results suggest that the invasive and proliferative phenotypes cannot be separated. In fact, increased invasiveness by itself is insufficient to change the heritable phenotype unless it also increases proliferation. That is, the Darwinian dynamics that promote invasiveness must also promote proliferation to establish increased motility as a heritable trait within the population.

Figure 6. Rho-GDI2 plays a role in regulating both cell migration and proliferation. A, the endogenous level of each gene in speciated cells was analyzed by RT-PCR after control siRNA transfection. Levels of gene expression knocked down by each siRNA confirmed depletion of targeted genes. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B, in Transwell assay, amphiregulin, Rho-GDI2, and sgII depletion made a significant reduction in the cell migration of both M and S cells ($all P < 0.001$) compared with control transfection. C, cell proliferation assay showed a significant decrease in Rho-GDI2 depleted M cells ($P = 0.02$) but had minimal effect on S cells ($P = 0.15$). Statistical significance is indicated by an asterisk.
The conclusions obtained from the computational model used in this experiment are limited by the simplifications inherent to it. For instance in this model, cell replication was limited only by the availability of metabolic substrates and pH, as well as "free space" for growth. In reality, cell-cell interactions inhibit replication in cell lines to different degrees (34), which is also likely the case for cancers in vivo. Cell growth is also dependent on growth factors produced by the stroma, which must diffuse through layers of avascular tumors before reaching the innermost layers of the tumor. We believe that these 2 limitations, however, do not compromise the conclusions drawn from these simulations. First, although actual cells are elastic and may deform under pressure allowing for regions of higher cell density and prolonged replication, these cells eventually will reach a maximum density that will induce quiescence. Computational models that have modeled the mechanical process of cell division and its importance for solid tumors have shown that these tumors have an initial exponential growth phase that later is replaced by a more linear growth (35, 36). This is what is observed in our proposed model, with only cells in the outer shell being able to replicate, thus reinforcing the natural selection for not only proliferation but also for being at the tumor-host interface. Thus highly proliferative, but poorly motile, cells will similarly become trapped in both models, although ours may underestimate the density at which this happens. Second, although this model implementation does not account for stroma-produced growth factors, the diffusion of these species is ruled by the same dynamics as the diffusion of oxygen and glucose. Thus, the cells in the inner core of the tumor would still likely become quiescent or die with a probability that increases by their distance from the tumor-host interface.

Our simulation results and in vitro studies confirm prior studies that have used evolutionary game theory to determine the outcome of a "game" played by cancer cells with different degrees of proliferative and motile strategies. Basanta and colleagues (37–39) have shown that motility is rewarded when cells are in a harsh environment (closer to the tumor inner core), whereas proliferation is the optimal strategy when cells are closer to the tumor–host interface, and that cells alternate between strategies during tumor growth. In the current model, cells are randomly seeded with different values for both phenotypes or strategies, and we observe that our model predicts the selection of cells capable of implementing both.

We also examined the evolutionary dynamics that promote invasive cancer phenotypes experimentally by speciating Panc-1 cancer cells for enhanced motility using the metabolic constraints of limited diffusion imposed by a glass cover slip. We have characterized speciated Panc-1 cells for motility/invasion, migration, proliferation, and gene expression profile. The motile Panc-1 cells have enhanced directional motility/invasion, and a nearly 2-fold greater intrinsic proliferation rate compared with stationary Panc-1 cells. This confirmed in vitro that speciated Panc-1 cells showed a coupling of enhanced motility with the resultant greater proliferation.

To characterize the molecular mechanism of microenvironment facilitated cell motility/invasion and proliferation, gene expression profiles of speciated Panc-1 cells were analyzed by microarray. The analysis revealed an essential role of Rho-GDI2 in regulating cell movement and proliferation. Depletion of Rho-GDI2 expression suppressed both motile Panc-1 cell motility and cell proliferation in vitro, suggesting that Rho-GDI2 functions as a positive regulator of tumor motility in this pancreatic cancer cell line. Rho-GDIs are guanine nucleotide dissociation inhibitors and one of the key components in controlling the biologic activities of Rho GTPases via a tightly regulated GDP/GTP cycle of cytosol/membrane alterations (40). Several studies have investigated the level of expression of Rho-GDI2 in various cancer cells compared with normal cells, revealing distinct phenotypes depending on the tumor cell context as well as the tumor stage. In human bladder cancer cells, Rho-GDI2 was found to be a negative regulator of Rho family GTPase and potential suppressor of tumor metastasis (41). In contrast, in breast cancer and ovarian adenocarcinoma, Rho-GDI2 mRNA levels were associated with tumor growth and invasiveness (42, 43). Recently, Rho-GDI2 expression was found to be positively correlated with tumor progression and metastasis in human gastric tumor tissues and cell lines (32). In this context, overexpression of Rho-GDI2 caused a significant increase in cell invasion in vitro, tumor growth, angiogenesis, and metastasis in vivo, whereas Rho-GDI2 depletion showed opposite effects.

The complex interaction of the tumor with its microenvironment is best studied in its biologic context and as a tumor "organ," with its integrated constituents and competing requirements and rewards. The use of evolutionary game theory in partnership with biologic experimentation is an attractive approach to model the complex dynamic system of the tumor microenvironment, and it may lend itself to evaluating the therapeutic impact in silico of stromal alterations in future preclinical studies. Our study shows the potential utility of an in silico system that can mimic experimental designs for predictive comparisons of experimental results.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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