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Combinative *in vitro* studies and computational model to predict 3D cell migration response to drug insult

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Abstract

The development of drugs to counter diseases related to cell migration has resulted in a multi-billion dollar endeavor. Unfortunately, few drugs have emerged from this effort highlighting the need for new methods to enhance assays to study, analyze and control cell migration. In response to this complex process, computational models have emerged as potent tools to describe migration providing a high throughput and low cost method. However, most models are unable to predict migration response to drug with direct application to *in vitro* experiments. In addition to this, no model to date has attempted to describe migration in response to drugs while incorporating simultaneously protein signaling, proteolytic activity, and 3D culture. In this paper, we describe an integrated computational approach, in conjunction with *in vitro* observations, to serve as a platform to accurately predict migration in 3D matrices incorporating the function of matrix metalloproteinases (MMPs) and their interaction with the Extracellular signal-related kinase (ERK) signaling pathway. Our results provide biological insight into how matrix density, MMP activity, integrin adhesions, and p-ERK expression all affect speed and persistence in 3D. Predictions from the model provide insight toward improving drug combinations to more effectively reduce both speed and persistence during migration and the role of integrin adhesions in motility. In this way our integrated platform provides future potential to streamline and improve throughput toward the testing and development of migration targeting drugs with tangible application to current *in vitro* assays.

Introduction

Cell migration plays a vital role in several key biological processes including development, wound healing, and disease progression (1). The migration process has also been the target of drug development in treating inflammatory diseases and cancer (2). It is therefore paramount to understand how certain drugs influence migration on the cellular and sub-cellular level. Unfortunately, while migration has been studied extensively for several decades, there remains an incomplete picture of the process of drug action. This is because migration is seldom predictable, often differing between cell types and microenvironmental

conditions (3, 4). To meet these challenges, a multitude of *in vitro*, *in vivo*, and computational approaches have been designed to assay cellular response to changes in extracellular matrix (ECM) content, drug insult, or signaling pathway modulations. However, to truly understand the process, and ultimately predict how drugs will influence this process, multifaceted platforms need to be created that are able to combine the various interdependent aspects that govern migration.

The most commonly studied modulators of migration are protein signaling pathways. However, migration is controlled not only by protein signaling, but the surrounding ECM as well. Even a simple change from planar two-dimensional (2D) culture to three-dimensional (3D) matrices can substantially alter cell behavior (5, 6). The interplay between cell and ECM, especially in 3D matrices, is mediated by matrix metalloproteinases (MMPs) enzymes that are responsible for cleaving matrix fibers (7). While the general function of MMPs suggests that their role is exclusively in proteolysis, they are also involved in several signaling cascades including the Extracellular signal-related kinase (ERK) pathway, especially MT1-MMP a transmembrane bound MMP (8). The involvement of MT1-MMP in the ERK pathway has suggested that MMPs may contribute more to migration than simply cleaving matrix fibers, however, the role of this and other MMPs in migration is not fully understood. Intertwined between the ECM, the ERK pathway, and MT1-MMP are $\beta 1$ integrins, the primary adhesive integrin to collagen (9). These transmembrane proteins mediate a plethora of signaling responses including ERK activation, act as mechano-sensors to guide contractility, and co-localize with MT1-MMP at the leading edge of migrating cells (10, 11). The relationship between 3D culture, proteolytic processors such as MMPs, integrin mediated adhesions, and the signaling pathways they modulate, represents a clear example of the complexity of migration and the need for platforms to encompass all of these facets simultaneously. The goal for such an approach is to understand and ultimately predict how cells behave in different environments or in the presence of certain drugs. While meeting these challenges may be accomplished through *in vitro* studies, these assays can be very time consuming, expensive, and limited to current culturing techniques. Fortunately, computational models represent a robust and efficient means to inform *in vitro* techniques.

Migration models represent a wide array of computational techniques to describe specific processes such as cell protrusion, up to the movement of entire cell sheets. Currently, many models have focused on the physical process of migration, studying the actin network, cell protrusions, and adhesion characteristics (12-14). Models have even begun to address the role of 3D culture, the cell-ECM network, and proteolysis in migration (15). However, there is a lack of robust and scalable models that can connect proteolysis, protein signaling, integrin adhesions, and the 3D ECM network together to ultimately predict migration in response to drug insult. In addition to this, many models are based on a phenomenological framework and are not directly relatable to any tangible *in vitro* system. These deficiencies can lead to limited capacities of the models in capturing complex *in vivo* behavior (16, 17). Here we aim to formulate a model, symbiotically with *in vitro* experimental work, to serve as a multifaceted platform for predicting migration while incorporating all of MMPs, the ERK signaling pathway and 3D matrix architecture.

Our approach provides a simple methodology to synergistically predict cell migration in 3D matrices in response to drug insult using both *in vitro* results and a computational model. While previous models exist to predict responses to cancer therapeutics (18, 19), there have been almost no attempts to study migration speed and persistence on the single cell level in response to drug insult. The integrative approach described here is able to predict migration behavior in a variety of matrix densities and drug insults, with direct applicability to corresponding *in vitro* data. Our results contribute further to the knowledge base of how matrix density, MMP activity, integrin adhesions, and p-ERK expression all independently influence migration, specifically speed and persistence. We also describe how this system is capable of providing insight into drug development by using the model to perform predictions of drug combinations to more effectively ablate speed and persistence in 3D. Finally, we highlight how our model is capable of providing basic knowledge of the phenomenological mechanisms of migration. This represents an integrative and customizable methodology that can directly compliment and inform future *in vitro* assays in drug development.

Results

Matrix density, proteolytic activity, p-ERK expression all interdependently drive migration in 3D collagen matrices

In vitro experimental data was collected to serve as the basis for model assumptions. 3D experiments with cells cultured in collagen matrices demonstrated that increasing matrix concentration decreased both cell speed and persistence in 3D collagen. Migration speed and persistence was also decreased from the blocking of proteolytic activity via Marimastat and knockdown of MT1-MMP (Figure 1A,B). MT1-MMP knockdown was confirmed via western blot and RT-PCR analysis. Zymograms on conditioned media treated with siRNA showed that knockdown of the protein also led to a decrease in MMP-2 activation (Figure 2). Since MT1-MMP is a known activator of MMP-2, this provides further evidence toward efficient MT1-MMP knockdown.

Decreases in speed due to Marimastat or MT1-MMP knockdown were very similar. However, Marimastat treatment, while depressing persistence to a lower value in the 1.75 and 3 mg/ml matrix densities, did not abolish persistence to the extent of MT1-MMP knockdown (Figure 1B). Unlike Marimastat, MT1-MMP siRNA was also responsible for knockdown of p-ERK activity (Figure 1E).

p-ERK expression was also effected by the drugs U0126 and Y27632. U0126 decreased p-ERK expression (Figure 1G) and was able to ablate persistent movement in 3mg/ml samples while leaving cell speed intact (Figure 1C,D). This relationship was observed in control cells as well as those cells cultured with siRNA to MT1-MMP. Interestingly, the loss of persistent movement via treatment with siRNA to MT1-MMP was rescued upon treatment of Y27632 (Figure 1D). This rise in persistence was accompanied by the re-emergence of p-ERK expression (Figure 1F). In addition to rescuing persistent motion, Y27632 decreased cell speed at 3mg/ml, (Figure 1C). These results were later used to make assumptions and optimize the model.

Optimized model faithfully followed trends observed in *in vitro* experiments

The model was created and optimized based on assumptions established from the data as described in the methods. It was then validated to ensure it was able to recreate these relationships. The effects of several model inputs on p-ERK expression or the driving variables were tested. Each model parameter was found to have successfully altered their intended variables in a linear fashion as outlined by the model assumptions (Figure 3).

Next, the different relationships between the input variables and output speed and persistence were examined. The results showed that the model followed the general trends displayed by the data in figure 1. Marimastat was able to decrease speed along with MT1-MMP knockdown. While MT1-MMP knockdown was also able to significantly decrease persistence, Marimastat did not exhibit as strong of a response (Figure 4A-D). Changes in drug concentrations through the model also followed trends displayed by the data. While an increase in the concentration of Y27632 led to a decreased cell speed, the effect of U0126 on speed was negligible (Figure 4E,F). Increases in concentration for both drugs were able to control persistence. Y27632 was able to rescue persistence from MT1-MMP siRNA treated samples (Figure 4G). Finally, U0126 was able to ablate persistence with increasing concentration (Figure 4H).

After all model relationships were expressed appropriately by the model, initial tests were performed to confirm its general action. Both speed and persistence were probed as a function of lattice spacing and azimuthal angle. Since neither of these terms is expressed in the terms of equation 3 for cell speed they should not perturb speed output from the model. In contrast, the azimuthal angle as well as lattice spacing (which like azimuthal angle also contributes to the allowable range of lamellipodial contacts) should directly influence the directionality of movement. As anticipated, both angle and lattice spacing have no effect on cell speed but influence the persistence (Figure 5A,B). As the allowable angle is decreased cells follow a more directed path and persistence increases. Similarly, as lattice spacing is increased cells are given fewer adhesion options within the allowable azimuthal angle projection and therefore stochastic variability is lessened leading to a more direct and persistent motion. Cell tracks are also able to visually depict the difference in control and MT1-MMP samples to show the model is performing as intended. Control tracks exhibit much more persistent movement as displayed through the increased displacement after 3 hours (Figure 5C,D). The adherence of these principle relationships stemming from variable optimization allowed next for the testing of the model in its ability to recapitulate migration responses as well as test its predictive capacity.

Optimization of variable relationships allowed the model to match the previous migration data in 3 matrix densities under conditions of siRNA and Marimastat treatment. Both speed and persistence of the optimized model closely mimicked the experimental results with minimal error (Figure 1A,B). The model was also able to match the results from migration experiments for samples treated with drugs Y27632 and U0126 in 3 mg/ml for both control and siRNA treated samples. (Figure 1C,D).

Model accurately predicted speed and persistence in response to drugs in 3D

The model was then used to predict migration in other scenarios without changing any model parameter relationships. Migration predicted by the model for control and siRNA treated cells in the presence of Y27632 or U0126 at a new matrix density of 1.75 mg/ml was performed and validated via experimental procedures. The same was done for the predictions made at 1.75 mg/ml and 3 mg/ml for cells treated with both Marimastat and U0126. The predicted speed and persistence matched our values recorded from cells migrating under those conditions *in vitro* (Figure 6).

To ensure that predictions at the 1.75 mg/ml density were valid protein expression was also observed. The protein expression patterns of p-ERK under the predicted conditions remained consistent with initial observations depicted in figure 1. Marimastat, MT1-MMP knockdown, Y27632 and U0126 all retained their effects on p-ERK as observed at 3 mg/ml. This confirmed that the relationships and assumptions based on signaling data were still applicable in the predicted scenarios and that no significant signaling changes would contribute to major alterations in behavior when progressing from 3 mg/ml to 1.75 mg/ml cell culture (Figure 7).

Integrin-ECM adhesions do not solely govern migration of HT-1080 cells in 3D collagen

To model the behavior of cells under the influence of integrin inhibitor 4B4, the concentration of integrins and p-ERK content were reduced significantly as informed from previous works discussed in the methods. As expected, predictions of speed and persistence from the model dropped well below control values for unperturbed cells at 3 mg/ml. The prediction of persistence due to 4B4 treatment matched well with experimental techniques (Figure 8E). As predicted from a phenomenological standpoint, this corresponded with a decrease in p-ERK activity in the presence of 4B4 (Figure 8A). However, experimental data revealed that 4B4 treatment did not decrease cell speeds to the extent predicted in the model (Figure 8D). In order to further bolster the *in vitro* migration data obtained via 4B4 treatment, migration of cells was also tested after siRNA knockdown of $\beta 1$ integrins (Figure 8B-D). While the speed for integrin knockdown samples did not exactly match that of the 4B4 group, the two experimental data sets both showed that blocking of integrins did not decrease speed as the model predicts. Additionally, it was observed that integrin knockdown and blocking did not decrease speed to the lowest values seen during data collection. The lowest values of speed belonged to those cells treated with both siRNA against MT1-MMP and Y27632 in which proteolysis and integrin mediated contractility were inhibited. These data along with the predicted results from the model invite discussion as to the role of integrin-ligand interactions and motility.

Discussion

Understanding and predicting how drugs will influence migration is vital to the development of treatments against disease. While *in vitro* studies are commonly used to screen for drug efficacy, these results can be greatly augmented using computational techniques. Unfortunately, computational approaches to study and predict migration fail to incorporate simultaneously the roles of the prominent migration effectors proteolytic activity, 3D

matrices, integrin adhesions, and protein signaling. Our results bridge this gap in our understanding and present a methodology for the prediction of migration response to drugs using both *in vitro* and computational techniques. Our model accomplishes this while also addressing the role of signaling proteins, the proteolytic drivers MMPs, integrin-ligand interactions, and the influence of matrix density.

Our approach resulted in the accurate prediction of migration response to drug from different matrix concentrations and alterations to protein signaling pathways. The results from both arms of the study also provided additional knowledge into how matrix density, proteolytic activity, integrin adhesions and p-ERK influence speed and persistence. Specifically, our experimental and computational results showed that p-ERK expression is required for persistent migration in HT-1080 cells in 3D matrices, however, downregulation of this protein alone does not affect speed. We also conclude that matrix density can alter both speed and persistence of cells, and drugs that alter the proteolytic processing of the matrix such as Marimastat can alter speed and persistence without inhibiting p-ERK. This suggests that while p-ERK is a direct modulator of persistence, cellular directionality is also inherently a function of matrix architecture. Not only is this result observable via *in vitro* experiments but recapitulated in our model in which adhesion lattice spacing had a direct effect on persistent migration. Changes in proteolytic activity that disrupt matrix remodeling therefore harness potential to ablate persistence without influencing any signaling of p-ERK. In this way, cell migration is tunable through both physical interactions with the matrix as well as signal processing. It is this duality between cell-ECM interactions that cannot be ignored, and is addressed by our platform, when exploring how cells migrate.

Results from our experimental and modeling data also suggest that matrix adhesions mediated via $\beta 1$ integrins do not fully govern motility in 3D. Our model was able to capture this result while predicting speed and persistence of cells in the presence of an integrin $\beta 1$ inhibitor. While our model was able to correctly describe the trend in cell speed during integrin inhibition, the predicted speed was much lower than our experimental data. If migration was in fact solely dependent on integrin-collagen ligand based contractility, our experimental data would match what our model produced. However, our results indicate that extensive blocking, and knockdown of integrins reaching in excess of 90% depletion of the coding mRNA, are still incapable of fully depressing cell speed. This conclusion must however be met with skepticism regarding the actual amount of integrin inhibition and knockdown that is present during the entirety of our collection of cell movements. Indeed, it is very possible that integrin recycling could potentially decrease the load of 4B4 inhibition of integrins (20). The transient nature of integrin knockdown events also breeds caution when speculating the actual numbers of integrin-ligand interactions that are present at any given time. It is entirely possible that our model can and will predict accurate speeds in response to integrin inhibition given the exact extent to which inhibition occurs *in vitro*.

Nevertheless, our data confirms that lower migration speeds for these cells are possible when both contractile force mediated by integrins and proteolytic capacity are hindered together. This suggests that in 3D both of these cellular processes play an additive role in motility. Neither is fully responsible for motility. Indeed, during periods of amoeboid migration, in which cells primarily utilize actin-myosin contractile forces in order to

progress, cells are thought to rely less upon integrins and in extreme cases of amoeboid motion adopt a form of integrin-independent migration (21, 22). It is by these mechanisms that the cells observed in this study may continue to migrate despite integrin blocking and downregulation. This suggests that other factors must be included outside the realm of a simple integrin driven contractile force mechanism (perhaps an integrin independent actin-myosin contractility upon loss of integrin expression) when attempting to fully characterize cellular motility.

Our conclusions on this subject are validated through other work observing cells migrating in 3D networks in the presence of integrin inhibitors. Wolf et. al. has shown that while speeds of cells decrease in 3D upon integrin inhibition, further losses in speed can also be seen when MMP activity is also abrogated (23). Furthermore, Zaman and colleagues has reported that integrin inhibition potentiates a preference for softer matrices in order to achieve maximal migration speed, suggesting that proteolysis becomes ever more important in the absence of optimally acting integrins in 3D (24). Stemming from this observation we conclude that our model, while performing more accurately using parameters previously optimized is still capable of producing phenomenologically relevant migration trends without prior knowledge of a drug's effect on the current system. In this regard we are able to use the information collected from our model to make inferences about how cells migrate in 3D.

From a computational perspective we showed that cell migration can faithfully be recapitulated using a force based system as we describe while treating both speed and persistence as independent entities. In the formulation of the model, there exists no direct relationship between the two and our simulations support this notion. Whether cellular pathways also treat these two important entities as fully independent of one another is highly unlikely given the interdependent nature of biological signaling processes. However their relative detachment may prove to be a significant characteristic that requires further research and remains an important observation rendered possible by the model.

Perhaps more importantly our predicted results, validated via time lapse migration, show that while U0126 is only able to ablate cell persistence, the combination with Marimastat can produce a state in which both speed and persistence are decreased significantly. Targeted ERK inhibition remains an active chemotherapeutic strategy (25). Our model shows that the particular inhibitor U0126 does not affect migration speed, but the combination with MMP inhibitor Marimastat can help augment this treatment. This type of prediction represents the potential this model can bring to drug discovery.

Limitations of the model may stem from variations within cell lines, and other culture conditions. While the model was made to predict drug response at varying matrix densities, more complex matrices with an unclear compositional structure (e.g. Matrigel) may show deviation from the model. Nonetheless, this model provides a novel approach to characterize and predict migration behavior in a non-generalized manner. An additional limitation of the model stems from the structural framework regarding the relationship between the contractile migration force and matrix rigidity. Previous research has established that cells utilize integrins to act as mechano-sensors and as such are able to match contractile force

upon matrix fibers with the inherent stiffness of the ECM (26). The relationship between cell contractile force and ECM stiffness has also been shown to be logarithmic in nature, with traction forces reaching an asymptotic maximum upon very stiff substrates (27). This behavior of cells has been incorporated into several models of migration and particular for cells in 3D matrices (27-29). The underlying characteristics that a variable contractile force imparts are that of a bidirectional relationship between substrate stiffness and motility. At low substrate stiffness, the contractile force is insufficient to propel cell migration. As the stiffness of the ECM increases, cells are then able to utilize myosin-actin machinery to increase speeds and contractile force. However, in the regime of high substrate stiffness contractile force no longer increases with the stiffness and migration speeds decrease. The hypothesis for this behavior is that stagnant contractile forces can no longer compete with the opposing forces of an increasing viscous force in higher density gel environments, and the inability to release rear adhesions due to large focal adhesion size and strength.

Models that include this relationship between contractility and matrix stiffness are able to capture the biphasic nature of motility in regard to substrate rigidity. Several models that include contractility of cells as an ECM dependent quantity have shown that intermediate substrates offer the optimal environment for motility (24, 27-30). In contrast, our model simplifies the relationship between contractility and matrix environment by allowing it to remain a static quantity and consider viscosity and lattice spacing as the main influences of the ECM on cell migration. The result of such assumptions is the loss of the biphasic response to ECM stiffness, with softer substrates always offering less resistance and therefore a greater cell speed. However, this simplification allows our model to more easily develop a relationship between the drug Y27632 and contractility by allowing it to remain a constant force in the model. Moreover, the current form of the model does not necessitate biphasic behaviors as suggested by the data. Indeed, for our purposes simplifying the role of ECM in migration to lattice spacing and viscosity was sufficient to recreate the behaviors and trends displayed in the data suggesting that in this instance varying contractility with matrix rigidity was not necessary to fulfill our goal. Future iterations of this or other models using our framework could, however, include this behavior during optimization and be better equipped to scan a greater range of matrix stiffness. Such an example of the ability for one to include a variable contractile force to achieve the biphasic migration response to matrix stiffness is seen in Figure S1.

In an effort to simplify our approach, further assumptions were made outside of those addressed and justified in the methods section. These mainly revolve around the structure of the model space including the surrounding matrix and the cell itself. For our purposes the matrix was defined as a homogenous lattice of discretized adhesion points. In reality the matrix is composed of a network of cross-linked and bundled fibers. Other models have incorporated these intricacies in 3D networks and therefore achieve migration directions dependent on the orientation and remodeling of these fibers instead of stochastic migration events (31-33). Cellular based assumptions include static uniform adhesion distributions, constant cell shape, and a lack of viscoelasticity expressed from the cell body. Models that include the dynamic nature of adhesions are able to describe cell polarization, directionality, and the biphasic response of migration to adhesion-ligand concentrations (29, 30). Meanwhile other models have incorporated the non-uniform shape and mechanical

properties of the cell to allow for mechano-sensing during migration to recreate haptotactic tendencies (27, 28, 33). While such specific cellular and matrix components are absent in our model, our data shows that the simplifications taken do not impact the ability for recapitulation of behavioral tendencies or the accuracy of migration prediction. In fact, the interdisciplinary approach described here highlights how migration predictions are possible without incorporation of detailed mechanisms when relevant *in vitro* data is used to optimize the system. However, this also does not prevent any future iterations of this approach to consider and integrate such detailed cellular and matrix components to increase the relevancy to any specific application.

Recent work continues to reveal an increasingly interconnected relationship between cells and the extracellular matrix (34, 35), it is therefore important to develop tools to probe cell behavior in a high throughput manner. Current computational approaches perform admirably however; they often neglect multiple facets of the migration process. Models that are constructed from phenomenological systems may also have little applicability and predictability in real *in vitro* settings. The overarching benefit of this platform stems from its ability to incorporate cell signaling, changes in the ECM, integrin adhesions, and proteolytic activity governed by MMPs to predict migration response to drug in 3D. This structure allows for prediction in a wide variety of drug concentrations, siRNA treatments, and matrix concentrations, that work synergistically with and are directly applicable to an *in vitro* data set.

As our knowledge of migration becomes increasingly complex, our platforms to study it must also adapt and evolve. The framework described here represents a step toward systems to study migration as set of connected, interdependent processes. To our knowledge, a model to allow for prediction of migration in 3D matrices using signaling data together with attention to MMPs, integrin adhesions and proteolysis has not yet been undertaken. Future iterations of this model may be adopted to predict migration response to drugs to probe for patterns worthy of further *in vitro* analyses.

Conclusions

We have presented an integrated technological method combining both *in vitro* and computational approaches in a simple manner to predict migration response to drug in 3D matrices. Our platform is able for the first time to incorporate 3D culture along with the action of proteases as well as signaling pathway function. The results show that matrix density and proteolytic blocking are capable of influencing migration speed and persistence. We also have pinpointed p-ERK as an exclusive modulator of migration persistence, unable to affect the speed of HT-1080 cells in 3D. By integrating both *in vitro* and computational approaches, the formulated model was then able to provide further knowledge to the action of anti-migratory drugs by predicting the ablation of both speed and persistence through the combination of Marimastat and U0126. In this case our prediction was able to inform us of the added benefit of adding Marimastat to U0126 treated cells to induce a drop in speed, which U0126 alone is incapable of inhibiting. The ability of the model to predict at several matrix densities also showed that this affect was achievable in both 1.75 and 3 mg/ml matrices. Finally, our model was able to test phenomenological trends produced via integrin

inhibition. Our results indicated that integrin mediated contractility was not solely responsible for migration speed in 3D. When applied appropriately this integrative, innovative method has potential to greatly increase throughput for studying migration response to changing matrix and drug conditions.

Methods

Cell culture, and preparation of 3D matrices and reagents

HT-1080 cells were obtained from American Type Cell Culture (ATCC) and propagated in Eagle's Minimum Essential Media (EMEM). For culture in 3D collagen gels, cells were cultured within matrices formed from rat tail collagen type I as previously described (36). Briefly, collagen gels were formulated via a mixture of collagen derived from rat tail and maintained in acetic acid (BD Biosciences), neutralizing buffer (100mM Hepes in 2× PBS, pH 7.3) and complete media containing cells. Collagen was added to gel mixtures in an appropriate volume as to achieve desired concentrations and an equal volume of neutralizing solution was added to offset the acidity of the collagen and maintain pH balance. The remaining volume needed to reach desired collagen concentrations within gels was supplied via complete media and a corresponding cell population to achieve 200,000 cells/mL. The solution was then cast in well plates and incubated at 37°C to allow for polymerization for 1 hour. After gelation, an equal volume of media was added to the apical surface of gels.

Proteolytic activity was inhibited using the broad based MMP inhibitor Marimastat (10μM). Expression of p-ERK and its effect on migration was probed using the MEK inhibitor U0126 (10μM) and p160ROCK inhibitor Y27632 (10μM). Integrin β1 inhibition was accomplished using the 4B4 anti-CD29 antibody clone (10 μg/mL).

siRNA transfection

Sequences of siRNA targeting the RNA sense strands are as follows: MT1-MMP, 5'-UCCAGGGUCUCAA AUGGCAACA UAA-3' targeting nts 571-599, and integrin μ1, 5'-GAUGGGAAACUUGGUGGCAUUGUUU-3' targeting nts 1080-1104. The nucleotide sequences were scrambled to generate control sequences for MT1-MMP: 5'-GGCGGGUGAGGAAUAACCAAGUGAU-3' and Integrin μ1: 5'-GAUAAAGGUUCGGUGUUACGGGUUU-3', respectively. Cells were transfected using Lipofectamine 2000 after having been embedded in gels overnight according to manufacturer protocol.

Quantification of cell migration

Cells were tracked using the cytoplasmic dye Cell-Tracker Orange CMRA and embedding the stained cell population within collagen matrices as previously described. Cells were allowed to attach for 6 hours before imaging. In order to test migration in the presence of drugs media on the apical surface of gels was changed an hour prior to imaging to contain either Marimastat (10μM), MEK inhibitor U0126 (10μM), or p160ROCK inhibitor Y27632 (10μM). Cells were then tracked using a Leica DMI6000B confocal microscope and imaged with an Imagem EM-CCD Camera (Hamamatsu). Images from each well were taken every 15 minutes for 16 hours. All image stacks were analyzed using Imaris version 7.2.3

(Bitplane). Cell speeds and displacements from raw position data were obtained from recorded cell tracks using MATLAB. Speed was calculated as the mean displacement over time between each time step. Persistence time was determined via curve fitting data of average mean square displacement versus time to the following equation:

$$MSD = 2S^2P \left(t - P \left[1 - e^{\left(\frac{-t}{P} \right)} \right] \right) \quad \text{Equation 1}$$

Where *MSD* is the average mean square displacement of all cell tracks, *S* is the average speed of tracks, *P* is the persistence time, and *t* is the time lag (37).

Immunoblotting

In order to assess changes in protein expression, cells were analyzed via western blot as previously described (36). All samples treated with siRNA were cultured within transfection media containing lipofectamine and siRNA mixture for 24 hours before changing media. At this juncture media was changed to either complete media or complete media containing one of Marimastat (10μM), U0126 (10μM) or Y27632 (10μM) and allowed to incubate for 24 more hours before lysis and sample collection. Samples not treated with siRNA were cultured for 24 hours post seeding using complete media without transfection materials. Media was then changed identically to those samples treated with siRNA and allowed to incubate for another 24 hours before lysis.

Immunoblotting of samples treated with 4B4 integrin inhibitor were processed separately. These cells required pretreatment with 4B4 antibody prior to being embedded into collagen gels. Therefore, these cells were either transfected or not transfected on collagen coated flasks for 24 hours. They were then trypsinized and suspended in control media or media containing 10 μg/mL 4B4 antibody for 45 min before being embedded within collagen matrices. Cells were then harvested at the appropriate time interval identically to all other samples.

RT-PCR Analysis

siRNA mediated knockdown of MT1-MMP and integrin β1 was verified by RT-PCR. Briefly, total RNA was isolated and purified using TRIzol Reagent and PureLink RNA Mini Kit (Life Technologies). RT-PCR was performed using one step SYBR Green RNA-to-CT 1-Step Kit (Applied Biosystems) and ABI 7300 Real-Time PCR System (Applied Biosystems).

Zymography

Cells were incubated in serum free media for 24 hours and conditioned media harvested from the apical surface of cells or gels. Samples were concentrated via ultracentrifugation using a 10 kDa cutoff filter and subsequently mixed with 4× zymography loading buffer before being analyzed via zymographic technique described previously (38).

Model Formulation

Cell migration speeds and persistences were simulated using a force based model based on the work of Zaman et. al. 2006 (24), and implemented using MATLAB v7.10.0

(MathWorks). Spheres with radius R approximated single cells, and their centroid placed within a 3D lattice of discrete equidistant points. Distance of lamellipod extension was generated using an exponential random variable with the mean set in the range of previously established values (39-41). Direction of the extension was a semi-random process in which direction was chosen in the y and z axis at random. The x direction was a bounded random variable, controlled by input parameters affecting the azimuthal angle at which the protrusion vector can deviate from the x-axis (θ). After a lamellipodial vector was determined, it attached to the nearest lattice point in the matrix within the allowable angle θ . If no point existed, the lamellipod did not attach and the cell did not migrate during that time iteration.

Cell motility was calculated using a set of force equations incorporating the force of the contraction by the cell, F_c and the viscous force applied on the cell by the matrix, F_v . Summing the forces acting on the cell and assuming the shape of a cell to be a sphere in a low Reynolds number setting at constant velocity gave the equation:

$$\Sigma F = \frac{A [L] sa [I] C}{K_d} - 6\pi\eta Rv = 0 \quad \text{Equation 2}$$

where A is avogadro's number, $[L]$ is the concentration of matrix ligand at the site of the focal adhesion, sa is the surface area of the focal adhesion, $[I]$ is the concentration of integrins at the site of the focal adhesion, C is the contractile force imparted on the matrix by a single integrin-actin connection, K_d is the integrin-collagen dissociation constant, η is the viscosity of the matrix, R is the radius of the cell and v is the instantaneous velocity. The first term in the equation is representative of the contractile force F_c , while the second term is the viscous force, F_v . Solving the equation for v allowed for the determination of the instantaneous velocity for the migration step.

$$v = \frac{A [L] sa [I] C}{K_d 6\pi\eta R} \quad \text{Equation 3}$$

Values for the constants in the equation that remain unaltered, $[L]$, $[I]$, sa , K_d , and R , were estimated from previous publications. The value of η for each matrix density was also estimated via previous work (47). Velocity was the result of a stochastic Gaussian process where v was the mean and $0.4v$ was the standard deviation to match the variance seen in experiments. The cell was then displaced in the direction of the lamellipod at a distance equal to the velocity times the change in time.

At each time step, which corresponded to 15 minutes to match with our experimental techniques, cell positions were tracked. Instantaneous speed was calculated by taking the vector magnitude and dividing it by the time interval. An average speed was then determined for each cell track and repeated for 1000 cell tracks to obtain the total average speed. For determining persistence, first the mean square displacement from position data was calculated via the equation:

$$MSD(t) = \frac{\sum_i r_i^2(t - \tau)}{N} \quad \text{Equation 4}$$

where $r^2 = x^2 + y^2 + z^2$, t is the time, τ is the time lag and N is the total number of displacement vectors for that time interval. Tracks were simulated for the equivalent of 3 hours (16 time points) and the total number of tracks for any given time point N was 1000. The persistence was then calculated via fitting to equation 1. This method for measuring persistence was repeated 10 times and the average from 10 curve fitting algorithms was taken.

Model Optimization and Predictions

The model was constructed to describe and reproduce the acquired data in which the only input variables would be matrix density, MT1-MMP concentration, and concentration of drugs Marimastat, U0126 and Y27632. To allow these variables to influence the speed and persistence of the model, they were related to the variables in the model that drive speed and persistence, termed the driving variables. The driving variables of the model were the angle θ , matrix viscosity η , contractile force, and lattice spacing. By establishing relationships between the input variables and the driving variables, the input variables were then able to control the output speed and persistence. To relate the input variables to the driving variables of the model, linear relationships were constructed between the two sets based on assumptions from the data and other works.

Model assumptions based on interpretation of experimental data—From our data we recorded observations and that informed our model. These interpretations, while preliminarily backed by data, are not absolute claims but rather informal relationships we will use to manufacture the model. These assumptions are summed in Table 1.

The data showed that increasing matrix concentration decreased cell speed and persistence. The properties of the matrix that influence speed and persistence most likely stem from matrix fiber density, which has the potential to influence both the apparent viscosity as well as the pore size (38, 47). This hypothesis is supported by the drop in speed and persistence seen when proteases were inhibited via Marimastat which reduces proteolytic matrix remodeling and can affect matrix pore size and fiber density (38). Therefore we assume that increasing matrix density decreases speed by increasing the viscosity of the matrix and decreases persistence by decreasing lattice spacing.

Results for cells cultured with Marimastat showed that it decreased speed *and* persistence of cells. From our previous assumption that matrix density can affect speed and persistence through its viscosity and fiber density, we hypothesize that Marimastat influences speed and persistence in a similar way, by controlling fiber density and viscosity by blocking proteolytic activity and disrupting matrix remodeling. In a way, adding Marimastat translates to moving from a lower to higher density matrix. From this, the assumption is made that Marimastat decreases speed and persistence by increasing the viscosity of the matrix and decreasing the lattice spacing, similar to the action of moving from a sparse to dense matrix.

The knockdown of MT1-MMP via siRNA showed a similar, but different result than treatment with Marimastat. siRNA decreased MMP-2 activity (Figure 2B), reducing total proteolysis similar to Marimastat. However while speed values for cells cultured with Marimastat and siRNA MT1-MMP were similar, the persistence values were much lower for those cells with MT1-MMP knockdown. Cells treated with siRNA against MT1-MMP also showed reduced p-ERK expression. In order to fully understand out how MT1-MMP knockdown effects migration, the changes in p-ERK expression and proteolytic blocking due to siRNA against MT1-MMP need to be examined separately. The drugs U0126 and Y27632 shed insight into the relationship between MT1-MMP knockdown, p-ERK expression, and proteolysis in speed and migration.

In siRNA treated cells, Y27632 rescued p-ERK expression and persistence to the level of control cells. From this data we observe that MT1-MMP knockdown cells cannot decrease cell persistence without decreasing p-ERK. They can however continue to decrease speed. Cells cultured with siRNA MT1-MMP and treated with Y27632 still decreased speed compared to control cells treated with Y27632 alone. These data contribute to the assumption that p-ERK correlates with persistence. When p-ERK is knocked down, persistence falls, however in samples when this protein is preserved, the migration is persistent. This is further supported by the decrease in persistence seen when treated with U0126 a potent MEK inhibitor which prevents p-ERK expression. Changes in p-ERK do not, however, lead to changes in speed. These trends are observed in control and siRNA treated samples. From this we assume that MT1-MMP knockdown cells reduce persistence by reducing p-ERK expression.

How then does MT1-MMP knockdown lead to changes in cell speed? Changes in proteolytic processing and matrix remodeling are the likely culprit. This is supported by the fact that MT1-MMP knockdown cells that express p-ERK due to Y27632 treatment still have lower cell speed compared to control cells treated with Y27632. Since this decrease in speed cannot be due to any changes in p-ERK expression, we therefore make the assumption that siRNA against MT1-MMP decreases speed by increasing the viscosity of the matrix. We also assume that while Marimastat is able to affect lattice spacing by blocking proteolysis, MT1-MMP knockdown cannot match this effect because MT1-MMP is bound to the membrane, and does not block as many MMPs as Marimastat. For the basis of our model only we therefore conclude that siRNA knockdown of MT1-MMP does not affect lattice spacing.

Our final model assumption is that Y27632 decreases cell speed by decreasing cell contractility. The main function of this drug is the selective inhibition of p160ROCK which mediates cell polarity, actin organization, and stress fiber formation. Addition of this drug decreased speed at 3 mg/ml and in other works has been described to directly ablate contractile force (48). With these assumptions, we were able to create the framework for the model.

Establishing relationships between input and driving variables—Establishing the relationships between input and driving variables was done by finding the values of the driving variables such that the model output fit the experimental data for each condition

(matrix concentration, drug concentration, etc). First, a set of values for the driving variables were established such that the model data fit experimental data for control cells in 3mg/ml gels. The condition was then varied (i.e. matrix concentration changed, drug added etc.) and the appropriate driving variable or variables were scanned until a value(s) was found that minimized the error between the model output and the data. Which driving variable was scanned as the conditions were altered was based on the assumptions of the model. Using the optimized values for the driving variables in each scenario together with the known values for the input variables (known from experiments), linear relationships between the input variables and the driving variables they influence were constructed (Figure 3). An example is described below. Optimization revealed that matrix density was not linearly related to lattice spacing and, therefore a second order polynomial was used to approximate the relationships. Since some inputs influence the driving forces indirectly through p-ERK expression, the linear relationship between p-ERK and the driving variable of the angle was first constructed for all conditions. Linear relationships were then appropriately defined between the input values and p-ERK to achieve the desired control of the driving variables and outputs.

Example of linear optimization between input and driving variables

1. Set of driving variables to reproduce model output at 3 mg/ml established
2. Condition changed: Marimastat introduced at 10 μ M
3. From assumption 1 in Table S1, Marimastat is related to both lattice spacing and η .
4. From equation 3 in methods, η influences speed. From model simulations described in Figure 5B, lattice spacing influences persistence.
5. Both η and lattice spacing are scanned until the speed and persistence from the model matches that from experimental data.
6. The original values for η and lattice spacing correspond to 0uM Marimastat. The new values for η and lattice spacing correspond to 10uM Marimastat.
7. Linear relationship is constructed between Marimastat and lattice spacing and Marimastat and η . This is seen in Figure 3A,B.

Model Predictions—After optimizing the linear relationships between input and driving variables, prediction was then performed for cells in the presence of Y27632 or U0126 administration at a matrix density of 1.75 mg/ml. A second prediction was also made at both 3 mg/ml and 1.75 mg/ml matrices in which the drugs Marimastat and U0126 were used in combination. A final prediction was then performed to test the predictive capacity of the model to a foreign drug not introduced to the model during the optimization period. This study was performed to show that the model is also able to make inferences about the phenomenological aspect of migration after sufficient optimization with *in vitro* data sets.

In this prediction, the model was manipulated to address the effect of decreasing concentration of available integrin on the cell surface due to the integrin β 1 blocking antibody 4B4. Due to the fact that β 1 integrins comprise the majority of integrin subunit combinations that adhere to collagen, we assumed that this antibody effectively blocks all

integrins associated with migration that are present in the model. Previous research has indicated that concentrations of this antibody at 10 $\mu\text{g/mL}$ correspond to approximately 95% blocking of $\beta 1$ integrins (24). In our model we therefore decreased the concentration of integrin to 5% of its initial value upon treatment with 4B4. Integrin $\beta 1$, the primary subunit for collagen binding, has also been implicated in the ERK pathway with integrin-collagen binding leading to increases in p-ERK activity (49). We therefore, upon insult with the antibody 4B4 decrease our value of p-ERK to 5% of its original value. After these changes, our model was made to predict the outcome of speed and persistence in the presence of 4B4 treatment. This simulation was performed at 3 mg/ml collagen density. Subsequent experiments were then performed as outlined previously to validate all model predictions. Migration data was collected to compare to the predicted results from the model. In addition, protein expression data in each of the prediction cases was obtained to ensure that the signaling patterns used to develop the model still held under the predicted conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Insight Statement

Cell migration is an important process in disease and the subject of numerous drug development efforts. Unfortunately, current *in vitro* and computational methods fashioned to understand migration and develop drugs have yielded minimal results. To address this, we have developed and applied an integrative computational model that synergistically with *in vitro* results serves as a platform to predict migration in 3D matrices in response to drug. Our model accurately reproduced *in vitro* behavior and provided biological insight via prediction of drug combination to further depress migration of cells. Our system represents a novel technological method to test migration response to drugs in a high throughput manner with direct implications toward improving *in vitro* testing.

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Figure 1. Top: Cell speed and persistence as observed from experiments and model simulations. Experimental data shows a decrease in cell speed and persistence as matrix density increases. Further losses in cell speed are evident during MT1-MMP knockdown and treatment with MMP blocker Marimastat. While depressing speed similarly, MT1-MMP knockdown was also able to decrease persistence with greater significance than Marimastat (A,B). Treatment with Y27632 showed a concatenate decrease in speed in both control and MT1-MMP knockdown samples. This is in direct contrast to the effect of U0126, which had

no effect on cell speed (C). Both drugs also contributed differently to persistence in 3 mg/ml. Y27632 was able to rescue persistence from MT1-MMP knockdown samples while leaving control samples unaffected. Meanwhile, drug treatment with U0126 ubiquitously decreased persistence in both control and MT1-MMP samples (D). The model was able to recreate the trends seen from experiments at different matrix concentrations for speed (A) and persistence (B). Optimization of the model was also able to capture the changes in speed (C) and persistence (D) when cells are treated with either U0126 or Y27632 at 3 mg/ml. Error bars represent standard deviation. Bottom: Western blot analysis of p-ERK expression at 3 mg/ml. Western blot results combined with migration data suggested that p-ERK expression is directly correlated to persistence in 3D. While both MT1-MMP and Marimastat were shown to influence the speed and persistence of cells at 3 mg/ml, only knockdown of MT1-MMP depressed p-ERK expression (E). When treated with Y27632, samples treated with siRNA recovered their p-ERK expression, which correlated with a recovery in persistence as well (F). U0126 was able to ablate p-ERK expression similarly to MT1-MMP knockdown, causing decreases in persistence as shown earlier (G).

Figure 2.

Quantification of siRNA mediated MT1-MMP knockdown using RT-PCR, western blotting, and gelatin zymography. Target siRNA was able to induce >85% knockdown in MT1-MMP RNA as normalized to 18s rRNA (A). MT1-MMP expression was significantly reduced compared to control samples and those cultured with a scramble siRNA. In addition, knockdown of MT1-MMP led to a decrease in the active form of MMP-2 during zymographic analysis (B).

Figure 3.

Relationships between input variables and various driving variables of the model formulated through parameter optimization to experimental data. The depicted relationships are linear in nature and follow the assumptions made in the methods section. An increase in Marimastat concentration resulted in an increase in matrix viscosity (A), and a decrease in matrix lattice spacing (B). An increase in MT1-MMP concentration (decrease in siRNA against MT1-MMP) resulted in a decrease in viscosity (C) and an increase in p-ERK expression (D). Y27632 was able to rescue p-ERK expression in samples with low MT1-MMP expression. It does not have an effect for those samples in which p-ERK is natively expressed and MT1-MMP concentration is not altered via siRNA (E). Increasing amounts of the drug Y27632 resulted in a decrease in contractility (F), which is shown to lead to a decrease in speed (H), as modeled theoretically in equation 3 and outputted here. According to the phenomenological framework of the model, contractility should have no bearing on persistence time, which is reproduced from the model as well (I). Finally, an increase in

U0126 resulted in a decrease in p-ERK expression as seen in the data (G). Together these data indicate that the model is adhering to assumptions made from data analysis throughout its predictions.

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Figure 4.

Relationship between several model parameters and the output speed and persistence from model simulations. As desired, the relationship trends remained consistent with the assumptions of the model, and when applicable, were similar to results seen *in vitro*. MT1-MMP concentration showed a positive correlation with both speed (A) and persistence (B), matching the trends seen *in vitro*. Similarly, an increasing Marimastat concentration led to a minor decrease in persistence (C) and a much more dramatic decrease in speed (D). Increasing Y27632 concentration was able decrease cell speed (E), while U0126 had no effect (F). Y27632 was also able to rescue persistence in samples treated with siRNA (G). Finally, increasing U0126 concentration led to decreases in speed (H). These trends are identical to those seen *in vitro* giving credence to the accuracy and value of the model. Error bars represent standard deviation.

Figure 5.

Speed, persistence and position output to assess the performance of the model after optimization. Model persistence was intended to be governed by the azimuthal angle and lattice spacing, while speed should be indifferent to these variables (Speed is controlled by the terms in equation 3). Model output confirmed that speed was largely unaffected by either spacing or the azimuthal angle (A) while persistence dependent on the degree of the angle, and lattice spacing (B). Cell tracks representing a control cell and one treated with siRNA against MT1-MMP produced expected results with the control cells following a much more persistent path and with larger displacement (D).

Figure 6.

Speed and persistence predictions from the optimized model, validated via experimental techniques. Predictions were made for the administration of drugs Y27632 and U0126 at the untested matrix density of 1.75 mg/ml for speed (A) and persistence (B). Data from subsequent *in vitro* experiments validate the accuracy of these predictions. A second prediction at both 1.75 mg/ml and 3 mg/ml was then performed for the combination of drugs Marimastat and U0126. The model results of speed (C) and persistence (D) match extremely well to experiments. Error bars represent the standard deviation.

Figure 7.

Western blot analysis of p-ERK expression at 1.75 mg/ml. Expression of p-ERK was tested to confirm that the relationships between p-ERK and its effectors were maintained at the lower matrix density of 1.75 mg/ml. Marimastat and MT1-MMP (A), Y27632 (B), and U0126 (C), offered the same relationships with p-ERK as previously described, outlined in the model, and seen at 3 mg/ml in Figure 2. The combination of Marimastat and U0126 did not change the relationship with either drug to p-ERK expression. Marimastat was still unable to affect p-ERK, while U0126 decreased its expression. When combined, U0126 was able to depress p-ERK (D).

Figure 8.

Changes in protein expression and migration characteristics in the presence of integrin $\beta 1$ inhibitor 4B4. As predicted from phenomenological models of integrin signaling, treatment with 4B4 at 10 $\mu\text{g/mL}$ was able to decrease p-ERK expression in cells within 3 mg/ml collagen gels (A). Knockdown of integrin $\beta 1$ via siRNA is confirmed to reduce mRNA content >90% (B) and results in substantial loss of protein expression at 3 mg/ml (C) as compared to scramble sequence. Decreasing the concentration of integrins in the model almost completely abrogates speed, however this is not seen experimentally with 4B4 treatment and siRNA knockdown of integrin $\beta 1$ which maintain significant cell speeds (D). In agreement to p-ERK downregulation from (A), decreasing the p-ERK concentration in the presence of 4B4 in the model allows for the accurate prediction of persistence at 3 mg/ml as compared to integrin inhibited and knockdown samples tested *in vitro* (E).

Table 1

Summary of assumptions based on experimental data used to formulate the basic underlying relationships of the model.

Variable	Assumption	References
Matrix Density	Inversely proportional to lattice spacing and directly proportional to	(38, 47)
p-ERK Expression	Proportional to the angle , or in other words persistence	Figure 1D,G
MT1-MMP Expression	Proportional to p-ERK expression and inversely proportional to	(8,24,50-52)
Marimastat Concentration	Inversely proportional to lattice spacing and directly proportional to	(38, 53)
Y27632 Concentration	Directly proportional to	(54-56)
U0126 Concentration	Inversely proportional to p-ERK expression	(57)