Examining the relationships between tumor cell motility type, cell migration rate, & matrix pore size in a variety of 3D matrix models

[redacted]

I. Executive Summary

The migration of tumor cells from a primary tumor to the surrounding tissue and vasculature is the first step towards metastasis [14]. Recent advances have shown that in addition to increased capacities for migration, tumor cells also have the ability to use several modes of motility in order migrate through different types of surrounding tissue [18]. Currently, there is much research being done about the mechanism through which tumor cells initially become motile, the ability of tumor cells to traverse a variety of extracellular matrix (ECM) compositions, and the role of different motility types in overall migration and tumor metastasis[14]. Just as cells are known to respond differently to different biomechanical cues, different matrix compositions can significantly affect the results due to differences in fiber architecture, stiffness, and the overall physical properties of the gel [5].

This study aims to map the general relationship between cell migratory rate and ECM composition; This will be achieved by examining the effects of several *in vitro* variations of Matrigel and collagen I gels on the migratory rate of tumor cells. We have chosen to focus on the relationship between relative pore size and cell migratory rate for both types of matrix in order to establish a standard by which different compositions might be compared. We also intend to examine the effect of cell motility type on migration speed and its relationship to relative pore size, and to use two different approaches to induce the mesenchymal-amoeboid transition, which will be compared to the mesenchymal motility of the experimental control for each ECM density.

For each type of matrix, we will test the migratory rate of HT-1080 fibrosarcoma cells at a range of matrix densities. At each density, the average pore size will be measured via the mean void area method as described by Zaman *et. al.*[22]. In addition the migratory rate of the cells at each density will be measured and averaged also using the method described by Zaman *et. al*[22]. By comparing migratory rate against pore size, we hope to glean some insight on the general relationship between the two (i.e. maximum migratory rates versus relative pore size). In addition, we also intend to compare the results between different matrix compositions by aligning the data by pore size.

In order to examine the effect of motility type, we will use a broad-spectrum protease inhibitor to induce amoeboid motility by inhibiting mesenchymal motility (Wolf2003); Overall, we hope to see some correlations between motility type and pore size, and perhaps even distinguish between the effects of the various types of mesenchymal motility inhibition.

II. Background and Significance

Tumor metastasis is comprised of a series of steps which include leaving the primary tumor, entering the vasculature, escaping detection by the immune system, leaving the vasculature, penetrating the tissue to reach a target site, and finally proliferating to establish a secondary colony[16]. When the cell dissociates from the primary tumor and invades the surrounding ECM, it needs to migrate through several types of tissue before reaching the circulatory system[16].

More recently, it has been found that when the mesenchymal motility of tumors cells is inhibited by blocking proteolysis, cells are able to almost fully compensate by switching to amoeboid motility, in which cells squeeze through the matrix pores rather than enzymatically degrading the fibers[19]. This is particularly important to note in relation to the the research and development of cancer-inhibiting drugs, as blocking one mode of motility will be ineffective if cells can switch to a different form of motility that is not dependent on the molecules being inhibited[22]. In fact, clinical trials of peptidomimetic MMP inhibitors, such as Batimastat and Marimastat, showed no increase in survival rate and are no longer tested for the treatment of human cancer.[3]

A. Tumor cells migrate through different types of tissue

During metastasis, tumor cells migrate through a variety of tissues that exhibit very different extracellular matrix (ECM) fiber architecture, pore sizes, and densities [4]. When modeling 3D matrices it is important to take into account these differences when determining the composition. The three major types of ECM are dense connective tissue, loose connective tissue, and tightly packed basement membrane and their properties are described in the following section[4].

1) Dense connective tissue: Dense connective tissue forms strong, rope-like structures which provide strong connections between tissues or bone [20]. Example of dense connective tissue include tendons (muscle to bone) and ligaments (bone to bone) [20]. Dense connective tissues mainly consist of collagen type I and, as seen in Fig. 1b, contains fibroblasts as well [20][4]. Irregular dense connective tissue, shown in Fig. 1d, is characterized by collagen fibers that are not aligned in parallel but still densely packed [4].

2) Loose connective tissue: Loose connective tissue (Fig. 1c), also known as areolar connective tissue, is an elastic and mesh-like [20]. Comprised mainly of collagen, elastic fibers, and fibroblasts, loose connective tissue functions to cushion and protect organs [20]. Areolar connective tissue is characterized by irregularly aligned collagen fibers with large pore sizes [4].

3) Tightly packed basement membrane: Basement membranes (Fig. 1a) are found in the body surrounding the blood vessels and other parts of the cardiovasculature, and serve to anchor the inner endothelial lining to the outer loose connective tissue of blood vessels[20]. Composed of type III collagen, which is reticular (cross-linked) and organized as a thin, dense acellular layer[4], basement membranes also act as mechanical barriers between malignant cells in the bloodstream and the other tissues [20].

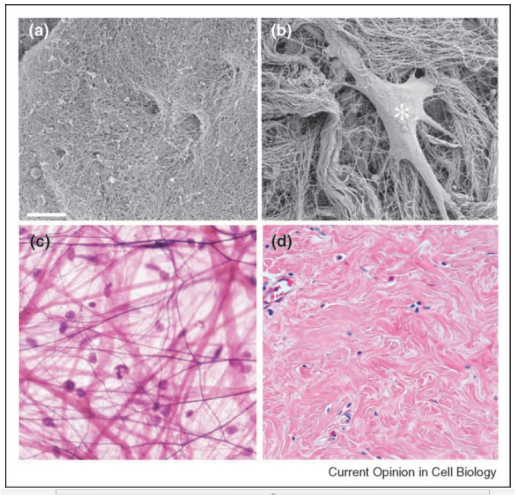

Fig. 1. **Various types of ECM** *in vivo*[4] All images are taken with scanning electron microscopy. Scale bar: $10\mu m$ a) The extremely dense basement membrane. b) The dense collagen meshwork of the dermis, in which a fibroblast (*) is visible. c) Loose areolar connective tissue from mesentery, which is stained for elastin and shows collagen fibers, elastin fibers, and fibroblasts. d) Irregular dense connective tissue from nipple skin showing irregular but densely packed collagen fibers.

B. Types of tumor cell motility and regulating mesenchymal-amoeboid transition

Active cell motility is necessary for successful invasion and metastasis of tumor cells [16] Studies have shown that tumor cells use distinct mechanisms for motility in three-dimensional matrices (Fig. 2)- collective motility, mesenchymal motility, and amoeboid motility[5]. These prespecified modes of motility are associated with cytoskeletal structure, cell-type-specific use of integrins, matrix-degrading enzymes, as well as cell-cell adhesion molecules.

1) Collective motility: Collective cell motility (Fig. 2a) involves the movement of clusters or sheets of epithelial tumor cells, due to the presence of cell-cell junctions[14]. In order for a tumor to metastasize, tumor cells must dedifferentiate and dissociate from the cluster of cells. As dedifferentiation of epithelial tumor cells proceeds, the expression E-cadherin, a transmembrane protein involved in cell-cell adhesion, is

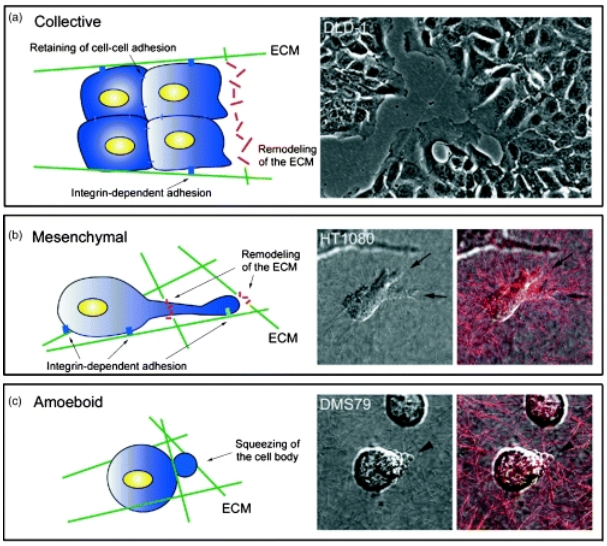

Fig. 2. **Types of Tumor Cell Motility**[21] a) In collective cell migration, cells move in sheetlike structures, retaining both cell-cell adhesion and integrin-dependent adhesion. The image shows DLD-1 (human colon adenocarcinoma) cells on a 2D substrate. b) In mesenchymal migration cells in 3D matrices release ECM-degrading enzymes, which remodel the ECM to form a path. Cells using mesenchymal migration will adhere to the substrate in an integrin-dependent manner and form membrane protrusions at the leading edge. The image shows HT1080 cells in collagen, with arrows indicating leading edge protrusions. Red indicates backscatter of collagen fibers. c) In amoeboid migration cells move within the ECM by squeezing the cell body via acto-myosin contractile force. There is little cell-substrate adhesion. The image shows DMS79 cells in collagen, with arrows indicating membrane blebbing. [21]

suppressed[11]. The cells can then separate and begin to migrate individually[21]. The loss of of E-cadherein expression results in increased tumor cell invasiveness. In fact, individuals who inherit a mutant allele for inactivated E-cadherin are at high risk of developing diffuse gastric carcinoma [11]. An estimated 10%-40% of tumors undergo the transition from collective to mesenchymal motility as a result of dedifferentiation[14].

2) Mesenchymal motility: During mesenchymal motility (Fig. 2b), the cell takes on an elongated morphology [14]. Mesenchymal motility is dependent on the proteolytic degradation of the ECM by proteases such as matrix metalloproteinases (MMPs), which are a family of zinc-dependent endopeptidases involved in the degradation of proteins [14]. MMPs have long been associated with tumor migration and metastasis. MMPs are upregulated in almost all types of human cancer and are associated with an aggressive malignant phenotype [3]. There are distinct structural classes of MMPs, including secreted, and membrane type MMPs (MT-MMPs) [3]. MT-MMPs covalently link to the cell membrane, while some secreted MMPs colocalize to the cell surface by binding to integrins [3]. The accumulation of MMPs at the leading edge of migrating cells results in localized proteolysis and cell migration[3].

3) Amoeboid motility: Previous studies initially showed that blocking MMPs and other proteases inhibited the invasiveness of tumor cells in both in vitro invasion models and experimental metastasis in vivo [19]. However, recent studies have found significant residual migration after the inhibition

of proteolysis degradation of the ECM and integrin-dependent adhesion, indicating the existence of another type of single cell motility [19][5]. As shown in Fig. 2c, amoeboid motility is proteolysis and integrin-independent. Rather, tumor cells squeeze through gaps in the ECM through shape changes generated by cortical filamentous actin[6]. The development of high resolution intravital imaging has shown that some tumor cells can move at high speeds (up to 4 um/min) using an amoeboid morphology in vitro [14]. By inhibiting proteolysis degradation and integrin-binding, amoeboid migration can be induced in mesenchymal migrating cells as a result of changes toward a more rounded morphology, the loss of β_1 integrin and MT1-MMP clustering interactions, and a more diffuse cortical actin distribution [19].

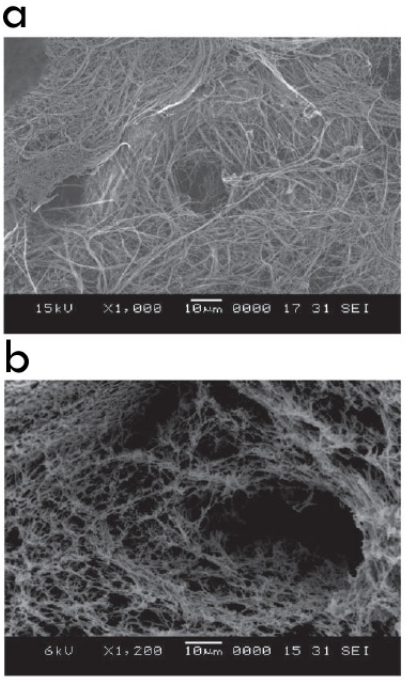

Fig. 3. **Structural differences between Matrigel and collagen I matrices**[22] Scanning electron microscopy images of a) Matrigel at 10mg/ml and b) collagen I at 2.8 mg/ml.

C. Modeling 3D ECM

Similar models for 3D ECM can induce significantly different properties in matrix composition or cellular response [4]. Additionally, ECM is a very broad term that refers to many types of different acellular tissue, so tissue context must be noted. Currently, the two main types of gels used for 3D migration studies are type-1 collagen, and Matrigel, which is a soluble basement membrane preparation consisting mainly of laminin (56%), but also containing collage IV (31%), heparan sulfate proteoglycans, and entactin (8%) [1]. Since type-1 collagen is mainly found in the loose and dense connective tissues, and Matrigel polymerizes to mimic mammalian basement membrane, both types exhibit very different matrix structures at the same resolution(Fig. 3) [22]. Additionally, there is the option of crosslinking or not crosslinking the gel, which can also affect migration [4]. For example, studies have shown that a crosslinked 3D collagen gel may prevent protease-dependent migration, while non-crosslinked collagen may not[4].

Typically collagen experiments are done at densities between 1-2 mg/ml while Matrigel concentrations are usually 10-12 mg/ml. Matrigel matrices are characterized as being much stiffer and more sterically constrained than collagen gels. In conditions of inhibited proteolysis, studies have shown that cells are unable to switch from mesenchymal to amoeboid motility in Matrigel after a certain density. [22]

D. Discrepancies in migration rate studies

Friedl *et al.* first discovered the existence of an amoeboid-type motility as a compensation mechanism in proteolysis-inhibited tumor cells when the migration rate of HT1080/MT1 cells in 3D collagen was reduced only negligibly despite complete proteolysis inhibition [19]. Subsequent studies by other groups have found that mesenchymal motility speed is generally within the range of .1-1 μm/min while amoeboid migration speed has been measured to be as fast as 4μm/min[14].

Although studies have measured migration rate relative to their particular experiments, the exact nature of the relationship between migration mode, degradation rate and pore size has yet to be mapped. We propose a comprehensive study to find this relationship by measuring migration rate of both mesenchymal-type and amoeboid-type tumor cell migration along a spectrum of pore sizes, which will be controlled through ECM density.

III. Experimental Proposal

In this study, we intend to collect the data necessary to generalize the relationship between ECM density and tumor cell migration rate. Five different ECM compositions will be tested (only Collagen I, only Matrigel, 25/75, 50/50, 75/25 ratios) at 9 different densities(from 10% to 100% in 10% intervals). Additionally, both crosslinked and non-crosslinked matrices will be tested. Since Matrigel and collagen I are used at very different density ranges, matrix density will be measured in terms of pore size in order to standardize and compare data. Since changes in matrix density affect many biomechanical properties other than pore size, such as fiber architecture and stiffness, we hope to normalize for, or at the least lessen, those effects by using the range of matrix composions.

With respect to different modes of cell motility, we will implement a broad spectrum protease inhibitor in order to induce the mesenchymal-amoeboid transition. With this data, we will be able to observe the effects of amoeboid versus mesenchymal motility in a wide range of densities and matrix compositions, and overall cell migration rates through different ECM densities.

A. Materials and Methods

1) Tumor cell type: This study will use subconfluent HT-1080 fibrosarcoma cells, which are known to have high constitutive collagenase expression and activity, allowing for visualization of fiber breakdown during migration [19].

2) Types of ECM: Given that collagen I and Matrigel are significantly different in mechanical properties, and that this study is interested in examining tumor cell migration through a variety of tissues, both collagen I and Matrigel will be tested in separate experiments.

There are a variety of crosslinking reagents including glutaraldehyde, photo-oxidation, and ultraviolet radiation, which can be used to crosslink collagen I[2]. In this study, a 0.05% glutaraldehyde solution will be used to crosslink the various collagen I/Matrigel ECM compositions, as described by Goissis *et al.*[8].

It is also important to note that as density increases, the mechanical properties of both kinds of matrices change with respect to pore size, fiber architecture, and stiffness [22]. In this study, we have chosen to quantify these changing mechanical properties in terms of pore size for the purposes of finding the basic relationship between density and migration speed. Once this data is collected, it should be possible to convert the pore sizes into relative stiffnesses, and show the same trends.

3) Varying and quantifying ECM pore size: In an attempt to cover the widest reasonable range of pore sizes, collagen I density will be varied between 0.5 - 5 mg/ml with intervals of 0.5 mg/ml, and Matrigel density will be varied between 10 - 100% with intervals of 10%. Each type of ECM will also be tested crosslinked and non-crosslinked. At every density, the pore size will be determined, and comparisons will be made by matching the various migration speed data according to pore size.

Pore size will be quantified by calculating the mean void area (μm^2) of the gel at each density, using the method described by Sieminski *et al.*[15]. The gel will be imaged using quick-freeze deep etch (QFDE), which is an electron microscopy technique involving freeze-drying sample gel slices to mica flakes [22][10]. Once the image is obtained, the images will be binarized (via IMAGEJ) and skeletonized, which allows the area covered by fibers (now represented as the dark pixels) to be calculated [22]. The mean void area, or the mean total pore area, can then be calculated by taking the reciprocal of the mean covered area of five independent gel samples [22].

Protease inhibitor cocktail

Inhibitor	Target protease	Concentration
		μM
BB-2516	MMPs[a]	100
E-64	Cysteine proteases (cathepsin B, H, L, and K)	250
Pepstatin A	Aspartatic proteases (including cathepsin D)	100
Leupeptin	Cathepsin D	2[b]
Aprotinin	Serine proteases (uPA and PA)	2.2[b]

Fig. 4. **Protease Inhibiting Cocktail**[19]

4) Regulating motility type through protease inhibitors: Recent studies have shown that MT1-MMP serves as the major proteinase necessary for tumor cell invasion via collagenolysis [13]. However, it was also found that MT1-MMP siRNA treated tumor cells still retained the ability to invade non-crosslinked collagen gels[13]. Therefore, two different methods will be used in order to induce the mesenchymal-amoeboid transition[13].

In the first approach, expression of MT1-MMP will be silenced through the use of an antisense strand of siRNA targeted against a 21-nucleotide MT1-MMP sequence, as done previously by Sabeh et.al.[13]. Briefly, the nucleotide sequence will be scrambled to generate a control siRNA sequence[13]. The siRNA oligonucleotides will be introduced into the HT-1080 cells using a nucleofector kit and electroporation[13]. Successful transfection will be confirmed using fluorescent oligonucleotides[13].

In the second approach, the gels/media will be supplemented with a broad protease-inhibiting 'cocktail' as described by Wolf *et al.*(Fig. 4) in order to target a wide range of proteolytic activity[19].

5) Measuring migration speed: As described by Zaman *et al.*., cells will be stained with CMFDA (Molecular Probes), a live cell membrane dye, and suspended in DMEM [22]. 50 μml of the cell suspension will be combined with serum-free DMEM, the appropriate concentration of either Matrigel or collagen I, and ˜1 million 1-μm sulfate Fluo-Spheres(Molecular Probes), which are detectable with 580/605nm wavelength light, and will be used to track matrix movement[22].

Each experiment will last for a duration of 6 hours, with time-lapse images of the cells and the matrix being taken at 15-minute intervals; each image will be a z-stack with 0.5-μm intervals[22]. Cells will be selected for tracking based on whether or not they move more than one cell diameter over the entire length of the experiment [22].

B. Experimental Setup

As illustrated by the overall experimental grid in Fig. 5, there will be 4 separate experiments testing a different type of 3D ECM matrix: collagen I crosslinked and non-crosslinked, and Matrigel crosslinked and non-crosslinked. For each type of gel, the migration speed will be measured at 10 different densities. Additionally, there will be 4 different types of media incorporated in the 3D gel: serum-free DMEM (control), media with broad spectrum MMP inhibitors, media with TIMP-1 (Tissue Inhibiting Metalloproteinase- inhibits secretory MMPs), media with TIMP-2 (inhibits membrane MMPs), and β_1 integrin specific mAbs.

Theoretically, all of the types of media should induce amoeboid movement except for the control, which should exhibit mesenchymal motility. Fig. 6 shows the data chart for a single experiment over a range of densities.

C. Concluding thoughts

This study aims to establish a more complete model of tumor cell migration in 3D matrices, in which we hope to provide a standard reference through which discrepancies between studies can be explained. We are choosing to focus on the specific relationship between pore size and migration rate, though there may not even be such a relationship because so many other biomechanical factors are at play. However, we are attempting to normalize these other factors by testing a range of matrix compositions as well as two different types of

Cell Migration Speed (um/min)

	Crosslinked			*Non-crosslinked*		
	Matrigel	Matrigel+ Collagen	Collagen	Matrigel	Matrigel+ Collagen	Collagen
Control						
Broad spectrum						
MT1-MMP siRNA						

Fig. 5. **Overall Experimental Grid** We will be measuring HT-1080 fibrosarcoma cell migration rate five different matrix compositions: 100% collagen I, 100% Matrigel, 25/75, 50/50, and 75/25 mixture ratios. Each of these compositions will be tested crosslinked and non-crosslinked, resulting in 10 different matrix compositions. For each type of matrix composition, the cells will be exposed to a) nothing (Control), which should result in mesenchymal motility, b) a broad spectrum protease-inhibiting cocktail, inducing amoeboid motility, and c)MT1-MMP specific siRNA, which inhibits MT1-MMP specifically.

Migration Speed in Non-crosslinked Matrigel (um/min)

	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%
Control										
Broad spectrum										
MT1-MMP siRNA										

Fig. 6. **Example Data Chart for One Experiment** For each matrix composition (e.g. non-crosslinked Matrigel), cell migration rate will be measured at 9 different densities (10%-100% for Matrigel, 0.5mg/ml - 5.0mg/ml for collagen I.

cell motility. We hope that the information gained from this study will allow future studies to choose an appropriate 3D ECM model for the particular type of behavior they wish to observe.

From this study, we intend to determine information such as the maximum migration rate, and the correlating pore size. From previous studies, we expect that smaller pore sizes usually correlate with slower migration rates, sometimes even inhibiting cell migration altogether[22]. From that information, we might guess that the maximum rate occurs when the relative pore size is roughly equal to cell size. As pore size becomes much greater than cell size, the 3D model effectively turns into a 2D model where the cell migrates along single fibers.

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