INVESTIGATING MECHANISMS OF GLIOMA CELL MIGRATION WITHIN A 3D BIOMIMETIC MICROENVIRONMENT

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This thesis is dedicated to my mother, Barbara J. Powell, and my father, Gordon R. Powell. I would also like to thank my close friends, coworkers, family and brothers for their constant support and encouragement in my professional and academic endeavors.
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ABSTRACT

Despite advances in medical practices and technologies, glioblastoma multiform continues to present as one of the most malignant forms of cancerous cerebral tumors. A rapid, infiltrative cellular phenotype characterizes glioblastoma tumors, often leading to tumor necrosis and uncontrollable vascular proliferation reflected through a malignant morphology. Histologically defined by numerous multinucleated giant cells with various morphological features, glioblastoma cells are typically characterized by their ability to aggressively proliferate, migrate, and remodel surrounding environment. These morphological features were shown to be influenced by the structure of the surrounding extracellular matrix (ECM), as well as by the presence of gradients imposed by nutrients and other components necessary for cell vitality. Little research, however, has gone into studying the effects of diffusible gradients mediating the migration of tumor masses. In this study, we developed a microfluidic device capable of incorporating multiple 3D matrices, signaling molecules, and heterogeneous cell types, for studying glioma cell migratory behavior in response to diffusive chemogradients. Results suggest that glioblastoma cell migration is strongly influenced by the stiffness of matrix microenvironment (type I collagen; 1-3 mg/mL), type (VEGF vs. EGF) and concentration (0-10 μM) of growth factor gradients, and the presence or absence of heterogeneous cells (human microvascular endothelial cells). Results from such studies not only expand our understanding of cancer cell biology, but also could lead to therapeutic drug development and screening, identification of targeting moieties on cell surfaces, and so forth.
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CHAPTER I

INTRODUCTION

Uncontrollable cellular proliferation and the formation of localized or remote tumor sites have long characterized the cancer phenotype. Progressive acquisition of organs and various vital systems by cancerous cell bodies often follows a seemingly benign growth of tumorous cell types. As these bodies continue to metastasize and proliferate, processes imperative for the continuation of life become unbearably labored and ultimately nonfunctional. Much research, therefore, has gone into understanding the mechanisms, influences, and tendencies of cancerous cells. Migratory behavior, proliferation, and the formation of tumor bodies are just a few processes that are highly influenced by the stimuli presented within both the ECM and surrounding media. Therapeutic approaches for the treatment of cancerous phenotypes, therefore, rely on a better understanding of the mechanisms underlying these biological developments.
As opposed to the conventional petri dish or two dimensional platforms, such as the Transwell Boyden chamber, a three dimensional microfluidic design offers increased potential for the enhanced study of cell behavior. A microfluidic device able to integrate several laboratory conditions upon a single chip, a few millimeters in size, offers researchers the opportunity to model the ECM microenvironment with very few reagents, high sensitivity and control of spatiotemporal resolution. Microfluidics offers a unique platform to study cell behavior and increase our understanding of the influence of various cues upon cell processes, while allowing to subtly alter the imposed microenvironment experienced by a given cellular phenotype. As will become apparent, microfluidic devices significantly simplify cell analysis by allowing for controlled cell seeding, and precise delivery of growth factors, matrices, and media. A multitude of biological cues, including the presence of cytokines, proteins, and neighboring cells, affects the biochemical and mechanical properties of cells within the ECM. One such cell type particularly affected by imposing gradients and biological signals are cancer cells. The pathophysiology of such cells inherently relies on external and internal stimuli provided from both the surrounding media and adjacent cell bodies. By allowing researchers the opportunity to regulate the transportation of fluids, growth factors, and other various soluble factors, microfluidics offers a novel approach to a field of study currently limited by the biochemical and geometrical complexity of the cell environment.
1.1 Cancer Cell Pathophysiology

Healthy tissue is composed of cells with specified sizes, growth rates, structures, and functions. Alterations to gene expression can modify the biology of normal cell phenotypes, forming metastatic tumor bodies with complex biological properties. After formation, either from modifications to cellular DNA or other biological processes, cancerous cells develop progressively, invading nearby stroma through cross talk that can regulate gene expression\(^2\). The unregulated growth of cancerous cells often leads to the formation of malignant tumors that have the ability to invade other parts of the body\(^2,3\). It should however be noted that the characteristics of tumor cells are highly specific to their location in the body. The development of normal cells into metastatic cell lines heavily influences cell processes such as genome stability, proliferation, motility, etc. It has long been a goal of cancer researchers to understand the physiology of these diseased cell lines\(^8-10\). Complex assemblies of trans-membrane proteins create networks capable of controlling cell metabolism, DNA and RNA synthesis, migration and signal processing\(^6\). Current methods used to study the interactions of these protein structures with the ECM do not allow for a full predictive model to be developed that could increase our understanding of the molecular dynamics responsible for cancer cell migration and proliferation\(^3\). The cell is a dynamic system, possessing the ability to change biological processes and features in order to survive and reproduce. These dynamical properties are implicit in the protein networks underlying cell physiology. Understanding how these networks are modified due to cancer metastasis is crucial for the development of future treatment options.
1.1.1 Cancer Cell Proliferation and Differentiation

The underlying biological mechanisms dictating the proliferation, differentiation and apoptosis of both cancerous cell lines and healthy cells is almost precisely comparable. The primary difference between the two cells inherently lies in the degree to which these biological functions occur. Cancer cells are abnormally regulated, showing an uncontrollable rate of proliferation, migration, and differentiation. The failure to regulate many of the cell-specific functions necessary for survival causes these cell types to present with an altered phenotype. As previously stated, the four cellular functions most aberrantly regulated include cellular proliferation, differentiation, chromosomal and genetic organization and apoptosis (programmed cell death).

Cellular life is heavily reliant upon on their ability to replicate within a minimum amount of time whilst constrained by the surrounding biochemical reactions imposed during the process of cell division. Healthy human cells tend to divide and replicate at a rate of once or twice per day, with the capability to constrict their proliferative capacity to appropriate times and environments. There are three factors that significantly contribute to the rate of cell proliferation: (1) the overall rate of cell division, (2) the proportion of cells undergoing division with respect to the entire culture, and (3) the rate of cell loss, either from programmed cell death or terminal differentiation. The signaling pathways influencing these factors, and ultimately cell proliferation, function in response to environmental stimuli or as a result of defects within the cell cycle. Many of these external stimuli are presented in the form of growth factors. Growth factor receptors are large, complex proteins embedded within the plasma membrane that respond only to specific growth factor molecules within the ECM. Epidermal growth factor (EGF), for
example, is one growth factor required for the transition between checkpoint stages during the cell cycle. The binding of EGF to its respective protein receptor triggers a phosphorylation cascade that ultimately regulates transcription factors necessary for cell division.

In order to sustain healthy tissue development and homeostasis, cells keep a tight regulation over the rate of division, percentage of growth, and rate of cell loss, ensuring that proliferation occurs as intended. Any alterations to these factors tend to increase cell division and tissue growth. External stimuli present within the ECM are one such reason for modifications to these critical rates of development. Interestingly, healthy cells are able to regain control over their proliferation methods once these physiological cues have been removed. Tumor cells, however, can proliferate, differentiate and migrate even in the absence of such stimuli.

The inability to regulate cell growth due to the introduction of physiologic stimuli is what classifies cells as cancerous. Under normal physiologic levels of growth factors, chemokines, etc., normal cells tend to proliferate, differentiate and undergo apoptosis (cell death) at normal rates, whereas tumor cells show an increased affinity for abnormal rates of these processes (see Figure 2).

The presence of growth factors and ECM proteins also has an effect on the differentiation, or anaplasia, of cancerous cell lines. Such differences in phenotype lead to alterations in their rate of tumor formation, degree of malignancy, and ultimately, their
sensitivity to therapeutic drugs\textsuperscript{4-6}. The biological mechanisms underlying cell differentiation are incompletely understood, but the ability of a cell to proliferate is inherently linked to the differentiation process. Biological cues intrinsically found within the cell environment act to maintain homeostasis of the proliferative and differentiating processes\textsuperscript{6}. As cell mass increases, proliferation mechanisms become restricted, leading to differentiated cell states. Most healthy cells are unable to continue proliferative processes upon differentiating. An exception to this rule is embryonic cells, as they maintain the ability to continuously proliferate, migrate and differentiate. Tumor cells also display this capability, with the added capacity to increase blood supply of local tissue\textsuperscript{14,17-19}.

Factors influencing migration and differentiation inherently lie within the ECM itself. These cues include insoluble matrix components, the identity and proximity of local cell types, as well as a number of soluble growth factors, chemokines and other molecules. The ECM plays a particularly important role in the stimulation and maintenance of differentiation for many cell lineages\textsuperscript{4,6}. Many cells have receptors spanning the plasma membrane capable of binding to specific components of the ECM\textsuperscript{7}. For example, signaling pathways activated through the interaction of the ECM and cellular membrane proteins are comparable to those signaling pathways generated through the binding of growth factors and growth factor inhibitors.

The interactions of cells with surrounding substrates such as growth factors, chemokines, and other soluble factors can be sorted into two broad categories: those that attach directly to cell surface receptors and those that bind to intracellular organelles after crossing through the plasma membrane\textsuperscript{4,7}. Molecules directly adhering to receptors
embedded within the cell membrane include growth factors such as epidermal growth factor (EGF) and vascular epidermal growth factor (VEGF), two signaling molecules that will be studied later in this paper\textsuperscript{15-17}. The signaling molecules produced by the cells themselves are also of interest. Tumor cells tend to produce signaling products that influence both differentiation and proliferation\textsuperscript{8-10}. Additionally, the inappropriate responses of these cell types to certain molecules, such as EGF or VEGF, can result in the altered differentiation and growth of neighboring cells as well\textsuperscript{10}.

1.1.2 Cell Migration

The orchestrated migration of cell bodies is necessary for the regulation and development of tissues, organs, and all other systems composing a given organism\textsuperscript{4-7}. This movement is highly controlled through cues presented from surrounding molecules, cells and ECM components\textsuperscript{4,6}. When cell migration becomes unorganized and uncontrollable, tumor bodies and metastasis can suddenly take effect, leading cells to invade both remote and localized tissues and organs\textsuperscript{6}. Often, the uncontrollable proliferation and migration of cells can be linked to systematic alterations to those cues expressed by, or presented to, the cells.

Cellular locomotion, despite numerous types of cells, can be generalized by four main processes: (1) membrane protrusion, (2) formation of attachment units, (3) translocation via generation of contractile forces, and (4) detachment of the rear membrane\textsuperscript{6}. Cancerous metastasis begins when cells enter into the lymphatic system, a gateway to blood circulation and ultimate relocation of a tumorous body\textsuperscript{4-6,18,21}. Tumor cell adhesion
and migration, following the previously stated processes, are necessary for the proliferation of disease in secondary, remote organs and tissue. Extravasation through the vessel wall leads to final adhesion and migration through endothelial tissue, although the behavior and mechanisms of which are still unclear. Intense research has gone into understanding the dynamic relationship between the molecular components of a given cell type and the surrounding environment. The mechanisms by which they work together are heavily influenced by the presence of integrin adhesion receptors. These proteins play a central role in the linkage of the intracellular cytoskeleton of cell bodies to the ECM, also serving as transducers of biochemical signals influencing the propulsion mechanism necessary for cell locomotion. Falling into this same classification for signal transduction pathways are growth factor receptors. These will be detailed in subsequent sections.

1.2 What is Glioblastoma Multiforme?

Histologically defined by numerous multinucleated giant cells with various morphological features, glioblastoma presents with a wide array of proliferative, migration, and other physiological features. These morphological features have shown to be influenced by the structure of the surrounding ECM, as well as by the presence of gradients imposed by nutrients and other compounds necessary for cell vitality. Despite progress in medical practices and technology, glioblastoma multiforme continues to present as one of the most malignant forms of cerebral tumors. Accounting for approximately 17% of all primary brain tumor types, incidence rates range around 3.2 per
100,000 people per year in the United States and Europe\textsuperscript{34}. A rapid infiltrative cellular phenotype characterizes glioblastoma tumors, often leading to tumor necrosis and uncontrollable vascular proliferation reflected through a malignant morphology\textsuperscript{15}. Glioblastoma can present in one of two ways: as an initial high-grade lesion that is highly recognizable during brain imaging, or through time-dependent development from a lower-grade precursor lesion\textsuperscript{14}. As the disease progresses, glioblastoma masses increase in size and typically contain central areas of tissue necrosis, surrounded by widespread, peritumoral vasogenic edema\textsuperscript{15,16}. The prognosis for patients diagnosed with glioblastoma cancer types is dismal. Despite treatment options, median survival times hoover around 12 to 15 months after diagnosis, with a five-year survival rate of less than 5\%\textsuperscript{34}.

1.2.1 Glioma Pathophysiology

The tissue network composing the mammalian central nervous system, exclusively the brain, is characterized by a soft, pliable, primarily collagenous network of neuronal tissue\textsuperscript{6}. A highly complex system of blood vessels and various other fluids help to provide the nutrients necessary for healthy tissue growth and continuous development\textsuperscript{4,6}. The deregulation of mechanisms controlling cellular proliferation, differentiation, etc., can result in the formation of brain masses and cancerous tumor bodies\textsuperscript{4,13}. Glioblastoma multiforme exists as one of the most popular forms of brain cancer. This disease model primarily presents in one of two forms: primary and secondary\textsuperscript{14-16}.

Around 60\% of all glioma cancer diagnoses are classified as primary glioblastoma multiform and predominantly show up in adults 50 years of age and older\textsuperscript{34}. These cases
present suddenly, normally without the presence of preexisting lesions. Secondary glioma is unique in that patients are usually younger than 45 years of age and unlike primary glioma, secondary classifications usually present after 4-5 years from the evolution of low-grade astrocytomas or anaplastic astrocytomas\textsuperscript{35}. As stated previously, central areas of necrotic tissue marks the development of glioblastoma multiforme, regardless of the classification\textsuperscript{15}. Cells develop rapidly, quickly forming tumorous masses that are able to easily infiltrate surrounding tissue, including preexisting fibrous tracts, to other parts of the brain due to their large density and elongated phenotypes. Anaplastic astrocytoma is the other primary form of brain cancer. Unlike glioma, cells rarely proliferate to other parts of the brain and are physiologically distinctive due to the lack of primary, hemorrhagic, necrotic tissue. Characteristics distinguishing glioblastomas are summarized in the following table\textsuperscript{36}.

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<tr>
<td>• Grade IV brain cancer</td>
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<td>• Highly invasive to other brain tissue, both remote and localized</td>
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<tr>
<td>• Gene mutations: \textit{tp53, EGFR, PTEN, 10q}</td>
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<tr>
<td>• Predominantly vascularized</td>
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<tr>
<td>• Characterized by primary, hemorrhagic necrotic tissue</td>
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**Table 1:** Glioblastoma distinguishing biological characteristics

Invasion by glioblastoma multiforme predominantly occurs along myelinated axons, vascular basement membranes and the subependyma\textsuperscript{14-16,35}. Tumor formation spreads throughout most of the frontal, parietal, occipital and temporal lobes, with migration hardly ever secularized to any one lobe\textsuperscript{4}. Much research has shown that proliferation of glioma lines is linked to the vascularization of hypoxic areas in the neuronal network,
often linked to overexpression of vascular endothelial growth factor (VEGF)\textsuperscript{33-35}. The butterfly shape that is most commonly seen during imaging is due to the spreading of the cell bodies across the corpus collosum after infiltration of the cerebral cortex and basal ganglia\textsuperscript{3,4}.

1.3 What is Bio-Microfluidics?

To properly understand the role of growth factors, introduced gradients, composition and structure of ECM, etc., \textit{in vitro}, it is imperative to be able to construct an environment that accurately portrays the setting seen by glioma cancer lines \textit{in vivo}. A multitude of conventional two dimensional and other various culturing methods have been used to study cell migration, angiogenesis, and cell-cell interactions\textsuperscript{49,62}. These various models will be discussed in further detail in the literature review section. Microfluidic platforms have broadened the field of cancer research, offering the ability to create more realistic \textit{in vitro} models\textsuperscript{79-81}. Researchers can control a variety of aspects using microfluidic devices, including the introduction of growth factors and chemokines, the diffusion rate of signaling molecules through specific matrices, and other biochemical and biomechanical factors that influence cell behavior. Microfluidic devices are versatile platforms upon which cell behavior can be studied within a three dimensional setting and real-time imaging at high resolution can be performed.
1.3.1 Benefits & Limitations

Cell behavior in response to various stimuli has previously been studied using two dimensional platforms such as the Boyden, Dunn, and Multiwell chambers\textsuperscript{76-78}. These models, however, limit researchers in the quality of imaging that can be obtained as well as in the amount of control over various factors influencing cell migration, proliferation and interaction with the surrounding matrix and other cell types. Microfluidics is able to integrate complex environmental factors affecting cell behavior, especially migration and proliferation, overcoming many of the barriers imposed through traditional methods\textsuperscript{79-81}.

One benefit to microfluidics is the ability to apply these devices to various applications. The fabrication and design of a device is individually determined, so researchers can factor in how to introduce cell types, various cues, or environmental factors. The platforms are also highly adaptable to changing conditions. Composition of the collagen matrix, for example, can be changed for different experimental protocols, as well as the concentration of growth factor or cell types used. Two dimensional Transwell chambers, on the other hand, often make changing such parameters very difficult or even impossible\textsuperscript{80,81}.

The technology provided by microfluidics also offers the ability to capture enhanced, time-lapsed imaging. Though cells are moving through a three dimensional environment, the dimensions of these scaffolds are small enough to force most movement and activity to remain on one plane of sight. Imaging is very straight forward, easily captured, and, most importantly, more accurately representative of an in vivo environment. Two dimensional platforms are limited in their ability to accurately employ forces experienced
by a cell within a 3D scaffold. Other methods, such as the Boyden chamber, make imaging very difficult as these assays disperse cells within a deep well. The activities important for imaging are often difficult to capture on the same plane.

Precise control of biochemical and biomechanical influences upon resulting cell migration is perhaps the most important benefit of microfluidic technology. The field is not only useful for cancer research, but can be applied to a vast array of studies, including the migration of neutrophils, leukemia cells, and stem cells. Precisely controlled chemical gradients can be applied, in which both the spatially and temporary controlled gradients can be controlled through the initial fabrication of the microfluidic platform. The fabrication of this technology is often inexpensive and fairly quick.

Interaction between cancer cells and both the ECM and other cell types play a critical role in the invasion of tumor bodies into surrounding and remote tissue. Many traditional methods used to study these interactions are limited by their two dimensional design, their inability to introduce more than one cell type, and their lack of control over biochemical and biomechanical cues within the microenvironment. Microfluidic platforms allow for integration of a vast array of signaling molecules and ECM compositions and concentrations with multiple cell types. Research done with these models, therefore, offers a robust and versatile opportunity to study an infinite number of conditions.
1.4 Problem Statement & Thesis Objective

Conventional treatment options for glioblastoma multiforme include surgical removal of any present tumor bodies, chemotherapy, radiotherapy, corticosteroids and anti-angiogenic therapy\textsuperscript{37,38}. While these treatments are vastly popular for a variety of different cancer types, glioblastoma presents with a highly migratory and invasive phenotype, making it harder to combat by these traditional options\textsuperscript{13-15}. The formation of new tumor sites in remote tissues outside of the originating necrotic tissue is a primary concern because of the chemotactic nature of the disease\textsuperscript{4,6}. Migration of glioma cells resulting in the formation of new tumor sites are thought to be coordinated by the presence of concentrations gradients imposed by surrounding molecules, by the interaction of glioma cells with various extracellular proteins, and through the remodeling of biomechanical structures imposed by the ECM\textsuperscript{39}.

A substantial amount of research has gone into studying the role of various biochemical and biomechanical cues on the formation, survival and proliferation of glioblastoma cells\textsuperscript{40-44}. These studies have focused primarily on the biological responses of these cell lines to various chemokines, including examining the role of brain tumor stem cells on heterogeneous cell differentiation and tumor growth. Not much research, however, has gone into studying the migration tendencies and mechanisms of these cells, particularly how their movement is effect by the composition of the ECM, surrounding cell bodies, or imposed chemical gradients.

Numerous biochemical molecules present within the ECM, including proteins inherently present there themselves, have been shown to alter glioma cellular physiology,
including differentiation, proliferation, and migration. The locomotion and invasiveness of glioblastoma cells is thought to be aided by interactions with proximate growth factors (e.g. TGF-β, EGF, VEGF, FGF)\textsuperscript{45-49}, through ECM interfaces\textsuperscript{50,51}, and integrin-receptor mediation\textsuperscript{52-55}. Our research, therefore, focuses on examining the exact roles of various growth factors, matrix compositions and different cell types on the migration, proliferation, and differentiation of glioblastoma cells.

The first part of our study seeks to elucidate the role of two different growth factors (EGF and VEGF) on the migration behavior of glioma cells through various concentrations of collagen matrix. Though collagen only composes a small amount of the brain ECM, it is a large portion of the tissues outside of the neuronal network, therefore having a strong impact on the migration of glioblastoma tumors. Studies have shown that both EGF and VEGF are produced in the brain and have a significant effect on the biological responses of surrounding cells\textsuperscript{40-43,46,48}. They are actively involved in the amplification and overexpression of genes and cell surface receptors used by glioblastoma cells during the migratory process\textsuperscript{56-59}. User-defined control over the spatio-temporal distribution of these growth factors is extremely important and will be satisfied through the appropriate engineering of a microfluidic platform. Upon determining the concentration profile that will be generated by each growth factor over time through the proposed device using a computerized modeling and simulation platform, further studies will examine the influence of these profiles on the mass migration of cancer cells, including focusing on their tendency to form tumor bodies or to alter their overall phenotype and biological responses.
The second part of our research examines the influence of various cell types on the migratory patterns and related biological responses of glioblastoma cells. After determining whether or not physiologically-relevant concentrations and gradients of growth factors can be generated and maintained \textit{in vitro} within our microfluidic device, we will assess the role of gradients imposed by human endothelial cells (HAEC). Signaling molecules secreted by ECs present a potential chemical gradient by which glioma cells respond with the co-expression of membrane receptors used during cell motility\textsuperscript{60}. Our research will examine the enhanced biological responses of glioma cells in response to potent mitogenic and angiogenic factors released by endothelial cells. These studies are anticipated to validate our hypothesis that tumor cell migration and angiogenesis is directly modulated by not only matrix composition and stiffness, but by diffusing chemical gradients released by surrounding ECM proteins and other cells. We will specifically seek to study the effects of the established gradient profiles on the angiogenesis and migration of glioma cells.
CHAPTER II

BACKGROUND

Numerous studies have been conducted researching the mechanisms underlying cell motility, proliferation, and tumor formation. Much less is known, however, about the mechanisms dictating glioblastoma migration, chemotaxis and overall invasiveness. Formulating a complete understanding of the factors influencing glioma chemotaxis, including the role of ECM proteins and concentration gradients of surrounding chemokines, is crucial to develop future treatment options. The aggressive invasiveness of cancer cells and tumor bodies are often facilitated by a number of different parameters, including the introduction of concentration gradients that are instrumental in the migration of tumor masses, the interactions of cancer cells with ECM proteins and other embedded bodies, remodeling of the ECM microenvironment because of inherent biochemical and biomechanical factors, and a vast array of other factors. These studies, however, leave much to be desired for the understanding of glioma cell migration tendencies and typically related tumor chemotaxis and invasiveness.
2.1 Tumor Formation & Tissue Necrosis

Formation of primary tumors and subsequent tumor bodies is governed by several biological and physicochemical properties of both the interstitial environment and surrounding cell bodies\textsuperscript{6}. Volume, structure, and composition of the ECM often direct distribution of tumor bodies, transport of these masses through interstitial space, metabolism and ultimate degradation\textsuperscript{4-6}. A lot of research has gone into understanding the molecular biology of cancer cells with specific interest in the biochemistry and intracellular mechanisms that prelude tumor development\textsuperscript{18,22-24}. It is important to understand the mechanisms underlying formation of primary tumors in order to understand successive growths and further progressive tumor proliferation.

The development of tumor bodies begins with the progressive growth of neoplastic cells that eventually invade the surrounding stromal and interstitial space\textsuperscript{6}. Through gene expression regulated by both the up and down cross talk between cancerous and stromal cells, metastasis of these cell types are activated and promoted by growth factor signaling and nutrients supplied through simple diffusion\textsuperscript{4,6}. Until enough cells metastasize and

\textbf{Figure 3:} The stages of tumor development\textsuperscript{F3}
coagulate together to form the primary tumor, cells and the surrounding microenvironment continue to go through a cycle of unregulated growth and proliferation until pro-angiogenic agents, such as VEGF and FGF, are released to stimulate angiogenesis and eventual detachment\textsuperscript{17,18}.

Angiogenesis, or the formation of blood vessels from existing entities, is an essential process in the conversion of a benign mass into a malignant tumor body\textsuperscript{18}. This progression occurs after introduction of specific angiogenic chemical stimuli, by both the cells themselves and surrounding lymphocytes and macrophages. A structural reorganization of the endothelial cells migrated into the stroma allows for the introduction of capillaries and further blood vessels\textsuperscript{4,6,17-18}. The establishment of blood flow supplies the necessary nutrients, oxygen, etc. for the detachment and invasion of tumor masses, allowing them to attack remote organs or other distant sites in the body (Figure 3).

Tumor metastasis and ultimate invasion is the result of around 90\% of human cancer fatalities\textsuperscript{19}. The formation of metastatic colonies in remote locations presents a substantial threat, enough so that a majority of cancer research is spent trying to prevent the dissemination of tumor cells from the primary tumor site. The vascular system is the primary roadway for the relocation of epithelial cancer cells\textsuperscript{6,17}. Once in circulation, the cells travel to distant locations where they extravagate from the vascular network and take up home in surrounding tissue\textsuperscript{6,17}. Chemokines and other signaling molecules trigger cells to arrest themselves from blood circulation. Growth factors supporting tumor formation and development in new tissue also play a tangible role in the embolization of such benign tumors\textsuperscript{18}. The inactivation of cancerous suppressor genes is also crucial to
instigate the metastatic cascade of events\textsuperscript{6}. Symptoms and treatment of all the various cancerous types relies predominantly on understanding the vast mechanics, biophysics, and chemical tendencies of this diverse, complex system.

Migration of both cancerous and noncancerous cells is influenced by the concentration of imposed gradient of chemoattractants and repellents. Motility properties of cells can be quantified by their responsiveness to various chemotactic factors\textsuperscript{14-17}. It is the localized and diffusive concentration of a growth factor or other chemoattractant molecules that determines the magnitude and direction of the net movement of cells\textsuperscript{6-8}. Much research has gone into predicting migration tendencies of cells with respect to imposed concentration gradients introduced by chemokines, growth factors, etc. For these profiles to be mathematically predictable, the diffusion coefficient of the attractant through an introduced matrix must be known\textsuperscript{20}. Concentration profiles are important to satisfactorily predict the migration of a cell or cell cluster in response to both diffusive molecules and appropriate boundary conditions.

Similar to proliferation and differentiation processes, the progression of migratory responses is deregulated and strongly persistent in cancer cells and resulting tumor bodies\textsuperscript{6,17}. The concentration gradient of chemoattractants, therefore, influences the

\textbf{Figure 4:} Formation of tumor sites in remote locations\textsuperscript{4}
cancer cell phenotype more powerfully than non-cancerous cells. Upregulation of specific genes encoding cancer cell morphology and biological responses is heavily influenced by receptors specific for certain signaling molecules\textsuperscript{17-20}. The rate of diffusion of these molecules is therefore important for the response of these cell types to their environment and surrounding cell bodies.

The growth cone of axons extending into the surrounding environment are believed to guide cell movement by sampling the environment for both chemoattractants and repellents\textsuperscript{21,22}. This sampling aids in the comparison and decision-making process imperative for intracellular events dictating cell movement. This process is thought to be concentration gradient-dependent. Gradients of cytoplasmatic messengers signal the receptors necessary for the appropriate orientation of cell protrusions and fillapodia\textsuperscript{6}. Significant research has gone into understanding the effect of concentration gradients imposed by surrounding biological signals on the mechanisms influencing the growth and migration of cells\textsuperscript{23,24}. The distance of migration is thought to be increased if the concentration gradient is strong enough to reach the necessary receptors and if said receptors are not completely saturated, which consequently would lead to down-regulation of essential migratory mechanisms\textsuperscript{25}. Such processes are imperative for the formation of tumor bodies in distant tissue or organ locations (see Figure 4).

Concentration and concentration gradients of chemoattractant and chemorepellent factors vary between cells and matrices; it is thus difficult to determine the minimum concentration gradient needed for guided migration and directed growth. Chemotactic gradients vary with both time and position within a specific environment. It is therefore important to understand the factors influencing the diffusive properties of a given
molecule, including molecular weight, ECM composition and surrounding molecules or cells. If the concentration profile of a given factor can be accurately predicted, it is easier to postulate the influence and degree of guidance of such signaling molecules on the migration and migration path of a given cell or tumor body.

Concentration gradients are highly dependent on both the physical properties of the diffusing molecules and the volume, structure and composition of the extracellular space. It seems reasonable to assume that areas composed more of fluid space would offer less resistance to the transportation of key signaling molecules than areas filled with dense matrices. Accumulation of substances due to a “sink” or “reservoir” like reaction may also decrease molecule permeability and/or selective affinity for diffusion all together. It is therefore important to understand the composition of extracellular spaces and their influence on generated concentration profiles.

Tissues are mainly composed of two types of fibers: collagenous and elastic\textsuperscript{4-6}. Histological profiles of these structures show that the basic structural unit of collagenous tissue is collagen, a protein molecule cylindrical in shape that is primarily composed of three peptidic α chains wound into a tight rope forming a triple helix\textsuperscript{6,25-27}. Collagen varies in composition of the α chains, forming at least ten subsequent types with similar structures and sizes\textsuperscript{26}. Both collagen and elastic fibers are important for the total makeup of tissue due to the fact that collagen supplies the necessary tensile strength along the length of their fibers, while elastic fibers supply the elasticity\textsuperscript{6,27}. Both are inherently present within any tissue sample, but it is their concentration and volume that determines the matrix composition and permeability features of the given location within the body.
Fluid and solute movement through the interstitial space of normal tissues are believed to be governed by both the trans-capillary hydrostatic and osmotic pressure gradients\textsuperscript{27}. Tumors are unique in that they may or may not have well-defined lymphatic networks, systems necessary for the uptake of fluid filtered into the interstitial space\textsuperscript{6}. Residual fluid unable to be reabsorbed by normal mechanisms adds to the pressure within the elastic and collagenous network of cancerous ECM, ultimately leading to necrosis of local tissue and facilitation of cancerous intravasation. Local brain ECM is composed of a large amount of lecticans, Hyaluronic acid and tenascin family proteins\textsuperscript{130}. Lower amounts of collagen, fibronectin, and vitronectin are also present. Transportation of signaling molecules, including growth factors and other various biological molecules, is highly influenced by generated concentration gradients and the movement of interstitial fluids and resulting fluid pressures through these surrounding molecules\textsuperscript{26}.

The basic processes for cell migration include adhesion to the ECM, generated locomotion, and the remodeling of local tissue and fluid spaces for said movement\textsuperscript{6}. The specific mechanisms that are used to generate locomotion and remodeling of surrounding tissue are still poorly understood. The first step of cell migration, adhesion to ECM proteins, is facilitated through the presence of various receptors. The most important of these receptor types are transmembrane integrin proteins\textsuperscript{4,6}. There exists a complex relationship between the basement membrane proteins used during generated migration and the tumor cell body, however. To mediate migration of these cancerous cells, it has been hypothesized that an intricate remodeling of ECM proteins and their structures is used\textsuperscript{26}. 

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ECM stiffness and pliability have also undergone recent scrutiny for their role in the behavior of both cancerous and non-cancerous cell types\textsuperscript{28-33}. Biomechanical properties, including the influence of tensional forces imposed by the three-dimensional (3D) matrix, are thought to act in collaboration with biochemical cues\textsuperscript{6,29}. Increasing matrix stiffness and other physical properties is done primarily through the increase of collagen concentrations, promoting focal adhesion assembly and increasing migratory receptor activation\textsuperscript{6,27-29}. The increase of collagen density, however, can also impede the rate of migration and overall distribution of cell bodies. Biochemical and biophysical cues from the ECM influence a variety of cell activities, including migration, cell-cell interactions, and differentiation\textsuperscript{29}. In summary, these signals and the responses correlated with them are inherently directed by the ECM structure and composition. Understanding more of the effects of the ECM structure and organization on the gradients generated by diffusing biochemical and biomedical cues will help increase the awareness for how cells respond in both normal and cancerous environments.

Cell migration not only involves the distribution of chemotactic factors within the ECM, but also requires the proper rearrangement of the cellular cytoskeleton. Upon VEGF exposure, endothelial cells, for example, are induced to actively begin the process of phosphorylation\textsuperscript{117,118}. This process triggers the extension of filopodia necessary for the attachment of migrating cells into the surrounding substrate. Glioma cells, thought to migrate using leading edges and other similar extensions\textsuperscript{119,120}, are hypothesized to participate in active migration upon introduction of chemokines to induce a significant change in cell morphology. The hallmark invasiveness of these cell types allows them to infiltrate remote tumor locations outside of the neuronal network, aided by alterations in
morphology through interactions with signaling molecules and changes in tissue composition. Subsequently, our images taken during the migration of these cell lines were analyzed for changes in cell shape and compared across concentrations of both growth factors and collagen matrices (see Figure 15). We were interested in finding whether or not the shapes of glioma cells are influenced more heavily by the topography of the matrix through which they migrate or by the concentration of dispersing chemogradient

2.2 Microfluidics & Cancer Research

Tumor cell chemotaxis within a 3D environment is a process imperative for the metastasis of cancer and the progressive spreading to vital tissues and organs. Various models have been used to study the effects of numerous factors on the migration of cancerous cell types, including the ECM, surrounding cell bodies, and concentration profiles established by adjacent biochemical signaling molecules. Angiogenesis, cell migration, and cell-cell interactions have been studied using a multitude of conventional culturing methods, most of which are primarily done implanting two-dimensional platforms that are limited in their ability to accurately represent the in vivo environment. Recent advances in microfluidic technologies have broadened the ability of researchers to study cell responses to chemogradient, ECM structures, etc., in vitro, under more controlled environments.

Conventional culturing models include approaches such as the Teflon fence assay, the wound assay, and integrated electrical detection systems, such as the electric cell-
substrate impedance sensing (ECIS) migration assays. Cell chemotaxis, employed through these methods, occurs over a 2D platform, limiting the quantification of cell movement and overall control over the structure of the integrated ECM and generated chemogradients. Cell migration and proliferation within 3D environments behave different than those moving in two dimensions, especially in terms of morphology and interactions with other cell types and the engineered ECM.

To properly understand the role of a 3D microenvironment on cellular responses in vivo, several assays have been developed, including one of the more popular conventional methods, the Boyden chamber (Figure 5). Using this model, cells are seeded atop a porous membrane and induced to migrate through the casing under gravity. Simple quantification of the number of cells migrated is done by counting the number of bodies that appear on the other side of the membrane after a specified period of time. This approach is limited by the material that can be used to mimic the ECM and by real-time quantification of cell migration. Hydrogel scaffolds are also useful tools for the study of tumor cell migration. Assays using hydrogel scaffolds have been developed but often do not properly include various tensional and mechanical forces introduced by the natural ECM, such as fluid shear forces or properties presented through the fluid-matrix interface. Microfluidics is unique in that the technology permits
persistent control, quantification and adaptability of the various factors influencing the movement of cancerous cells and tumor masses.

Typical cell migration assays, as described previously, are unable to integrate complex factors influencing cellular behavior, including migration and proliferation. Microfluidics attempts to make in vitro platforms more adaptable to various applications, possessing the potential to study cell responses to environmental cues and biochemical signals. The phenotype of tumor cells was the focus of many early studies, many being done through the use of topographically modified surfaces. A few cell types included in these studies are neutrophils, leukemia cells, stem cells, bacteria and cancer cells. A precisely controlled chemical gradient is applied under various conditions to study responses of these cell types.

2.3 Tumor-ECM Interactions

The ECM is difficult to construct in vitro because of the complexity of the mechanical structure, imposed chemical cues, and other biological properties inherent to the diverse matrix. Microfluidic models possess the ability to control spatial and temporal biomechanical and biochemical factors presented to tumor cells through the ECM. Cancer cell migration and signaling interactions through the ECM are imperative for the invasion of tumor masses into both local and remote tissues, primarily accomplished through the incursion of surrounding vasculature. The lethal metastasis of cancerous cell bodies can then spread to discrete target organs. Existing in vitro assays have allowed significant research into the biomolecular mechanisms dictating cell migration,
proliferation, etc., but are typically limited by their control over the composition, 
structure and various other cues integrated with the ECM. Microfluidics incorporates 
novel platforms offering profound control over these conditions, therefore, providing 
more robust models for studying the three dimensional behavior of migrating tumor cells 
and cell-cell interactions involved in cancer invasion.

Regulating the constitution of surrounding chemical gradients and the passage of 
biochemical stimuli, the ECM acts primarily as a physical scaffold that binds cells and 
tissues together. Cancerous cells and tumor bodies enable controlled proteolysis and 
remodeling of the ECM network during processes of migration, proliferation, etc\textsuperscript{85}. Collagen, a main structural protein of the ECM, can be irrepressibly regulated, expressed 
and remodeled by the invasion and metastasis of tumors. Egeblad, Rasch and Weaver 
(2010) showed how the abnormal expression of these fibrils influences cellular process 
imperative for proliferation, invasion, metastasis, and therapeutic responses to treatment 
options\textsuperscript{85}. Chemical signals presented by cancerous cells and tumor masses alter the 
appearance and structure of collagen matrices to allow for easier movement through the 
scaffold and surrounding architecture. Collagen fibers, characteristically curly and 
anisotropic, alter their morphology to become more linear and progressively thicker\textsuperscript{85-87}. Increased linearity is directly related to an increase in stiffness, substantially impacting 
the concentration profiles of diffusing growth factors and ultimately, cell migration\textsuperscript{87,88}.

A microfluidic system designed to mimic the properties of the ECM 
microenvironment \textit{in vivo} must not only be compromised of the structural components 
typically composing the biological matrix, but must also present signaling molecules and 
tensional forces inherent of the natural ECM as well. Extravasation, or establishment of
secondary tumor sites within local or remote tissues, is highly regulated by the presence of biochemical messengers formed through the regulation of ECM structural molecules. Jeon et al. (2011) reported an *in vitro* microfluidic model for the study of cell-cell and cell-matrix interactions\(^{89,90}\). Their platform offers enhanced control over various parameters, including cell type and matrix composition.

A more recent study (2013)\(^90\) of the same group offers a microfluidic device for the specific study of cell migration and tumor cell intravasation. Their model consists of three independent media channels, separated by chambers into which a collagen-I scaffold mimicking the mechanical and chemical properties of the ECM can be injected. An endothelial monolayer is introduced that acts as an adhesion point for cancer cells to transmigrate upon (see Figure 6). The group was able to track the cells overtime, exploring time-dependent behavior in response to collagen matrix properties and various signaling cues. Dissemination of tumor cells from the primary tumor mass travel through the circulatory system and tend to invade remote locations throughout the body. Adhesion to distant tissue matrices is guided by the mechanical and chemical properties.
inherent within the tissue themselves. Microfluidic platforms able to integrate these various properties are immensely important for the continuation of cancer research.

2.4 Microfluidics & Gradient Profiles

ECM composition and organization are not only important for the transport of biomechanical signals impacting cell migration, but for the conveyance of biochemical cues presented through the organization and orientation of the individual components within the medium. Changes in the chemical cues originating from the ECM can lead to changes in the physical, and mechanical, properties of the matrix, inherently resulting in alterations to cell shape and behavior. Often, changes in chemical stimuli are reflected in uncontrolled proliferation and tumorigenesis as seen in disease models common for cancerous cell types. The microenvironment in which diseased cells are cultured vastly influences the biochemical cues presented to these cell bodies, both in identity and in concentration. The stiffness of the ECM, along with the porosity and presence of other masses within the matrix, is a key regulator of disease progression.

The transport of fluid and various biochemical signals is governed by the biological and physiochemical properties of both the matrix and migrating molecules. The concentration and velocity of traveling biochemical cues generates a gradient profile that in turn affects the migration of cancerous cells and tumor bodies. A 1984 article describes the influence of both the concentration and generated gradient of chemoattractants on the migration of leukocytes. In this study, researchers measured the concentration profiles of an attractant diffusing through an agarose-based assay²⁰. They use the speed and resulting
concentration profile to produce a model that can accurately predict the concentration profiles of diffusion molecules through appropriate boundary conditions. Such studies are imperative to fully understand the influence of diffusing molecules on the invasive tendencies of disease models. Further studies quantified the random motility coefficient and chemotactic coefficient of various chemotactic factors\textsuperscript{91-93}. These parameters determine the magnitude and direction of the net movement of cells within a specific environment when subjected to particular signaling molecule. Numerous studies have thus been conducted to measure the diffusion coefficient and resulting concentration profile of various chemoattractants through a multitude of different environments\textsuperscript{68,79-81}. Many of these studies are limited, however, in their two dimensional portrayal of the \textit{in vivo} environment experienced by a given cell type.

\section*{2.5 VEGF & EGF Cancer Research}

Developing tumor bodies and invasive cancerous masses are believed to migrate toward target tissue in response to a concentration gradient composed of various chemotactic factors. Vozzi, et al. used a 3D simulation COMSOL setup to simulate the concentration gradient of biomolecules such as hydrogen peroxide and lidocaine through a 1\% gelatin solution\textsuperscript{110}. They were able to determine, through changes in colored gradients over a designated time period, the velocity of the diffusing molecules inside the culture chambers. They exposed the hydrogen peroxide gradient to human endothelial cells and the lidocain concentration profile to myoblasts, fixing both cell types and staining them with DAPI in order to photograph the fluorescent response of these cell types. They
found that increasing concentrations of hydrogen peroxide and lidocaine increased the fluorescent responses of their introduced cells. This experiment, however, was ran in a 2D platform, which might not mimic the complex 3D in vivo environment.

Angiogenesis is the formation of new blood vessels, and is an important process by which new vasculature might aid the development and metastasis of cancerous tumors. This progression is highly influenced by the presence of various pro-angiogenic and anti-angiogenic factors, all of which compose an angiogenic balance for the stimulation and inhibition of a vast array of signals. One of the most important pro-angiogenic factors is vascular endothelial growth factor (VEGF)\textsuperscript{68}. This particular growth factor aids in the hyper-permeability of microvasculature necessary for the net migration of cancer cells and resulting tumors. The modulation of gradient profiles generated by the diffusion of VEGF is instrumental in the development of new therapeutic options for the treatment of glioma and other related disease models\textsuperscript{68,93-95}.

There are seven different growth factors that are all classified as members of the VEGF family: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and PIGF\textsuperscript{68}. All contain a central cysteine knot composed of eight antiparallel, dimerized cysteine residues. The stroma surrounding malignant cells originates from the vascularization of clotting plasma fibrinogen that is highly dependent on increased vascular permeability. VEGF modulates changes leading to increased capillary permeability, the mechanisms of which are still unclear. There are also specific steps involved in the transformation of benign matrices into mature collagenous tumoral stroma. These events begin with an influx of monocytes that differentiate into specified macrophages, followed by the replication of cancerous cells, the migration of macrophages, endothelial cells and
fibroblasts, and finally, the degradation of the initial matrix by tumorous stroma. VEGF and other growth factors secreted by tumor cells act as fibroblast mitogens and general chemoattractants, promoting the abundant deposition of fibrin.

Tumor cells themselves release growth factors and other chemokines that promote migration of the primary masses and subsequent related cancer cells. VEGF is a major lymph-angiogenic factor that when over-expressed within a tumor microenvironment, promotes metastasis of lymph nodes in breast cancer patients. Timoshenko et al. (2007) investigated the expression of VEGF receptors in several breast cancer cell lines and reported on the morphological effects on the cancer cells due to the chemogradient imposed by the VEGF growth factor (Figure 7). They found that tumor-derived VEGF stimulates breast cancer metastasis and enhanced migratory activity. Barleon et al. (1996) described the effect of VEGF on the induction of monocyte activation and migration, phagocytes necessary for the inflammatory process. VEGF was found to induce migration of monocytes across polycarbonate filters at varying levels of effectiveness over a vast array of doses. The mechanism by which VEGF stimulates the chemotaxis of various cells, however, is still unknown.

Constant communication between cells imperative for processes such as growth, division, migration, and even death, is carried out through a vast array of chemical signaling molecules. Most often, these stimuli include growth factors, such as VEGF as discussed previously. Growth and division are two important developments necessary for
the formation of metastatic tumors. Our research will focus on another chemotactic messenger, epidermal growth factor (EGF), a small protein used to stimulate cell growth and division. Receptors present on the surface of cells bind with EGF and relay messages to proteins inside the cells that stimulate processes needed for growth and development. Because EGF stimulates cell growth as its primary function, if used improperly, the signal can be very dangerous. Many forms of cancer alter their expression of the EGF signaling process, growing uncontrolled. Many therapeutic drugs act to block EGF receptors, such as the drug Lapatinib and Herceptin.97

The interplay between a highly-complex microenvironment and the generation of a gradient profile modulates chemoinvasion of tumor cells within a 3D tissue. Kim et al. (2013) studied the response of malignant breast tumor cells to chemical gradients established within a 3D microfluidic model61. Results found that cells increased their motility in response to the chemogradient imposed by the introduction of EGF. Cells were less directed in their movement than when subjected to pure media but their velocity and overall motility was greatly enhanced. Similar studies performed by Kim et al. (2008) and Wang et al. (2004) showed the same strong correlation between increased cell motility and introduced EGF gradient profiles98,99. It was found that increases in the density of the collagen matrix through which cells migrated helped to increase directional persistence and overall directed cell migration. These studies, however, were performed within a 2D platform, limiting their relevance to actual in vivo cellular responses.
2.6 Cell-Cell Culturing & Cancer Research

Microfluidic platforms not only allow for the introduction of various growth factors and chemokines, but for the culturing of multiple cell types as well. Cell migration and other vital processes are affected by the presence of other cell bodies, as they add to the complexity composing the diverse \textit{in vivo} microenvironment. Cell-cell interactions aid in the homeostasis of the cellular environment, controlling basic functions such as cell survival, migration, proliferation, differentiation, apoptosis, etc. The interactions resulting from multiple cell types can also be presented in a multitude of ways, including direct physical contact, diffusion of chemokines and other various stimuli, electrical signals, and transduction of mechanical signals through the surrounding cellular extracellular domain\textsuperscript{100}. In diseased tissues, the interactions of multiple cell types can stimulate the progression of uncontrollable proliferation, they can spark the destruction of cells through the early arousal of apoptosis, and they can even promote cancer metastasis.

Cells respond in a vast array of ways to the complexity of the microenvironment. The ECM is composed of both cellular and non-cellular components, making it advantageous to study cellular responses in an environment more representative of the actual \textit{in vivo} platform. Microfluidics offer a unique model for developing an infinite number of platforms for the study of cell-cell interactions. Conventional methods for studying cell-cell signaling include many 2D platforms, including the Transwell assay, 2D plating, etc. These studies are limited, however, in their ability to control cell plating and the transport of biochemical and biomechanical cues through introduced matrices. The Transwell assay, or Boyden chamber, as discussed previously, follows the net migration of cells through a porous membrane. Imaging is limited to one platform, though the cells are in a
three dimensional culture, and only two different cell populations can be studied because
of the stiff porous membrane inhibiting direct contact.

    Cells cultured under 2D conditions are not only morphologically different than those
cultured within 3D models, but their response to various signaling molecules and
concentration gradients is vastly different as well\textsuperscript{49,62}. Early studies, using conventional
2D platforms, focusing on the interaction of vascular smooth muscles cells (SMCs) and
endothelial cells (ECs), for example, suggested that ECs modulate the proliferation of
SMCs and that SMCs in turn regulate the dormant state of ECs\textsuperscript{101,102}. A 3D approach
utilizing a microfluidic platform enabled a more in-depth understanding of the process by
which the two cell types affected one another\textsuperscript{101}. Real-time monitoring and quantification
of cell migration under 3D coculture conditions in which both biochemical and
biophysical conditions were precisely controlled. EC interactions with tumor cells are
also important for consideration when studying the migratory behavior of cancerous cell
types. Angiogenic factors secreted from tumor cells during the metastasis of tumor bodies
promote the interaction of these cell types with surrounding endothelial tissue. These
interactions typically occur within the tumor microenvironment, a platform so small and
diverse that it is virtually impossible to study without an \textit{in vitro} model. Kaji et al. (2009)
used a microfluidic device incorporating two complimentary substrates on which cervical
cancer cells and human umbilical vein endothelial cells could be seeded\textsuperscript{103}. They used the
platform to study the effect of paracrine factors on cell motility of both cell types. Such
models allow for monitoring cell-cell interactions in real-time, while allowing for
accurate control of biochemical and biomechanical factors within a 3D matrix.
CHAPTER III

MATERIALS AND METHODS

3.1 Microfluidic Device Design

To improve the understanding of the biological processes influencing tumor cell migration, a three-dimensional (3D) collagen matrix was utilized within the microfluidic platform. Collagen, an important component of the connective tissue ECM, traditionally induces cell motility through the attachment of external ligands to cell surface receptors. Migration of tumor cells is heavily influenced by both the immediate concentration (i.e., steepness) and surrounding concentration gradients generated from a chemoattractant. To generate such gradients within our imposed cell environment, a microfluidic device was used that allowed for the diffusion of an attractant through the selected collagen scaffold. To enable interpretation of the collected cell migration data, the concentration profiles for chemoattractants within the 3D scaffold must be mathematically formulated, that can later be used to correlate to the behavior of these cell types in response to various concentrations of external stimuli. The proposed microfluidic device seeks to develop a platform for understanding the full structure and function of the ECM and interstitial.
environment experienced by cancerous cell lines. This device was designed by both the graduate student and PI, with CAD file design done by lab mate Mike Sawonik.

3.2 Microfluidic Device Fabrication

A microfluidic device able to integrate multiple physiological conditions upon a single chip, a few millimeters in size, was fabricated using a silicon wafer developed using photolithographic processes. The mold design was initially created using SolidWorks®, a 3D CAD design software, and then fabricated at Stanford University. Microdevices fashioned from the designed mold in our lab were made with polydimethylsiloxane (PDMS), purchased from Dow Corning (Sylgard® 184 Silicone Elastomer Kit) as this medium is easily fabricated, inexpensive, translucent in appearance, and offers little resistance to alterations in physical properties such as elasticity, gas permeability, biological inertness, etc. As this experiment studies the effect of alterations to matrix stiffness, these are important properties to consider. Before the PDMS mold was poured upon the microfluidic platform, the silicon wafer was first treated with a thin silane coating to ease device removal. Post silane-coating, the mold was cleaned using sterile, pressurized N2.
The fabrication of the microfluidic device using PDMS required awareness of all conditions present during the manufacturing process. Disparities in curing temperature, base to curing reagent ratios, or unintended surface modifications can greatly influence cell culture results. A designated elastomer base (silicone base) to curing agent (elastomer silicone agent) ratio of 10 parts base to 1 parts agent, by weight, was found to optimize the physical properties of the final PDMS. These reagents were first weighed and mixed and the mixture was agitated vigorously within a stirring cup using a glass.

**Figure 8:** Schematic of designed microfluidic device
stirring rod to release air pockets that would otherwise decrease surface tension of the final mold. These air bubbles were removed by placing the mixture into a vacuum desiccator for 20 to 30 min, after which the solution was poured over the microfluidic mold. The PDMS mixture was then cured at 65°C for a minimum of two hours.

Punching of the loading ports was the second stage of the fabrication process. There are three main compartments that needed to be extracted in order to introduce components necessary for the observation of cell migration (see Figure 8). These ports include four areas for the injection of media and/or growth factors, four areas for the injection of a collagen matrix, and one general area for the seeding of cells. After polymerizing PDMS upon the mold in by baking in a drying oven for 2-3 hours, devices were peeled from the silicon wafer, and biopsy punch sizes of various sizes (1 – 4 mm) were used to core the above-mentioned ports completely through the PDMS mold.

The media ports are large enough to ensure a constant supply of nutrients for the cells, while the collagen ports were small to avoid introducing air pockets during injection. After extracting the injection ports using the before mentioned biopsy punches, each device was then separately cut out and placed (device side up) within a petri dish for sterilization.

Figure 9: Size of microfluidic device compared to a quarter
To ensure sterility of the device for cell culture purposes, each device was first cleaned using adhesive tape to remove any debris visible to the naked eye. Then the devices were placed in a hot water bath and boiled for 45 minutes. Devices were then dried in an oven at 65°C for one hour. Finally, glass cover slips were cleaned with 70% ethanol and placed upon a clean surface for adhesion to the devices. Surface molecules of both the device and cover slides were exposed to plasma treatment (Electrotechnic High Frequency Generator) for a total of 45 sec. The plasma treated sides of the device and glass cover slip were pressed together, effectively creating a permanent bond between the two surfaces. If the surfaces were properly cleaned and excited, there was a complete seal with no air bubbles. This was critical for retention of cell media, matrices, and cell bodies during loading. After adhesion to the glass cover slides, each device was placed within a sterile petri dish until needed for experimental protocols.

3.3 Gradient Simulations in COMSOL®

A finite element analysis and simulation software package was used to predict the rate of diffusion of growth factors through the various concentrations of collagen matrices before actual experiments were performed. COMSOL® Multiphysics software (COMSOL, Inc.) offers a microfluidic module able to integrate every aspect of our experimental design, including matrix and growth factor concentration, with the actual device proposed for our studies. The module includes interfaces for studying laminar flow, the common mechanism of movement for growth factor gradients through a microfluidic platform. The 3D CAD file obtained from the design of the microfluidic
device in SolidWorks was first uploaded into the COMSOL microfluidic module.

Parameters such as diffusion coefficients and concentration of collagen-1 matrices are needed to specify the exact locations of the introduced gradients selected. All diffusion coefficients were calculated using the Einstein-Stokes equation. This formula, using the Boltzmann constant $k_B$, gives the diffusion coefficient, $D$, for a spherical particle of radius “r” within a fluid with defined dynamic velocity “$\eta$” at ambient temperature “$T$”:

$$D = \frac{k_B T}{6\pi \eta r} \quad \text{(Eq. 1)}$$

The radius of each diffusing particle is needed for calculation of the Einstein-Stokes equation. Not much literature exists, however, that defines the radius of the specific molecules (VEGF and EGF) used in these experiments. To calculate the diffusion coefficient, therefore, we used the radii for Dextran molecules of similar sizes as was found in literature$^{110-112}$. The Reynold’s number, or ratio of inertial forces to the viscosity of the surrounding medium, determines the ease with which a particle diffuses through a given liquid. The Einstein-Stokes equation determines the diffusion of a spherical particle through a fluid when the approximation of the appropriate Reynold’s number is low.

VEGF and EGF particles were found to diffuse through the matrices as shown in Table 2. Viscosity information was provided by vendors for Dextran molecules of similar sizes$^{110}$. 


Table 2: Diffusion coefficients for growth factors through 1, 2, and 3 mg/ml Collagen-1

<table>
<thead>
<tr>
<th>MW (kDa)</th>
<th>k (J/K)</th>
<th>T (K)</th>
<th>6π</th>
<th>n (kg/m*s)</th>
<th>R (m)</th>
<th>D (m^2/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1, 1, 10μm VEGF</td>
<td>19.2</td>
<td>1.38E-23</td>
<td>298.15</td>
<td>18.84956</td>
<td>3.70E-03</td>
<td>3.24E-09</td>
</tr>
<tr>
<td>0.1, 1, 10μm EGF</td>
<td>6</td>
<td>1.38E-23</td>
<td>298.15</td>
<td>18.84956</td>
<td>3.70E-03</td>
<td>2.20E-09</td>
</tr>
</tbody>
</table>

To determine the time for complete diffusion of each solute through the array of collagen matrix concentrations, we used Eq. 2.

\[
\tau = \frac{L^2}{4\pi^2D} \quad \text{(Eq. 2)}
\]

“L” is the length of the collagen chambers in the device, designed at 2.5mm. D is the diffusion coefficient found in Table 2. Results are summarized in Table 3 and discussed in further detail in Chapter 4.
3.4 Media preparation

The nutrients required to support cell survival were provided using a prepared glioma media. The media ingredients include pen/strep, L-glutamine, and 10% Hyclone cosmic calf serum (FBS). Fetal calf serum was an excellent serum for the processing of cell media because it promotes cell growth and lacks viral inhibitors. See Table 4 for the appropriate concentrations of media constituents. These were kept frozen until needed and then thawed at 37 °C prior to use. Minimum Essential Medium, Alpha 1× (Sigma-Aldrich) was used as the media base, and the above mentioned ingredients were added within a sterile hood, filter-sterilized, and stored at 4°C till further use.

<table>
<thead>
<tr>
<th>Media Components</th>
<th>Volumes Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM non-essential amino acid</td>
<td>5 mL</td>
</tr>
<tr>
<td>1 M Hepes buffer solution</td>
<td>10 mL</td>
</tr>
<tr>
<td>Minimum essential medium, Alpha 1X</td>
<td>435 mL</td>
</tr>
<tr>
<td>Pen/strep</td>
<td>5 mL</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>5 mL</td>
</tr>
<tr>
<td>FBS</td>
<td>50 mL</td>
</tr>
</tbody>
</table>

**Table 3:** Volumes of components needed to make U-87 media

3.5 Cell Culturing

Adult human glioblastoma cells (originally from ATCC, manufactured with RFP) were a gift from Dr. Joanne Belovich’s lab at CSU. They were received at passage 12, and stored in liquid nitrogen until use. Prior to performing experiments, these cells were removed from liquid N₂ and rapidly thawed in a sterile water bath at 37 °C. Cells were pipetted into a 15mL conical tube, to which 1 mL of U-87 media was added. Cells were then spun at 1200 rpm to remove DMSO solution, and resuspended in a fresh batch of
medium prepared as per protocols described above. Media and cells were pipetted a few times within a 15mL conical tube to disperse cells evenly throughout the mixture. Cells were passaged around three times before used in experimental trials. It is worth mentioning that these glioblastoma cells could be a heterogeneous mixture of cells, primarily derived from 44-year old male Caucasian glioblastoma (U-87 MG; ATCC®, HTB-14™) multiforme, and so might express markers for glial stem cells, mature glial cells (astrocytes, oligodendrocytes, star cells, etc.), and other epithelial cell populations. However, characterization of these cells with suitable markers is beyond the scope of this study.

3.6 Microfluidic Device Preparation & Cell Loading

It was crucial that one was able to consistently load cells, growth factor/media, and matrices into the appropriate channels of the microfluidic device to ensure proper analysis of the cells being studied. Unwanted settling, clumping or non-specified adhesion of cells to various surfaces can impede the necessary transport of the cells to their desired location within the device. Therefore, during the loading stage, great care was taken to load the necessary components in a slow, consistent manner with the minimal amount of pressure needed to discharge the solutions into their designated channels and chambers. The microfluidic devices were first loaded with a prepared collagen matrix, followed by introduction of the allotted growth factor/ media solution, with cells finally seeded to study their response to the given conditions.
Cell behavior in vitro is particularly influenced by the concentration of type I collagen present within the extracellular environment. Therefore, collagen scaffolds were prepared at 1, 2, and 3 mg/mL. Collagen-1 (type I, rat-tail derived; 3.84 mg/ml master batch; BD Biosciences, NJ) was used as the primary collagen solution and was always kept on ice to avoid premature polymerization. The following table (Table 5) shows the desired concentrations of components needed for the preparation of collagen gel solutions.

<table>
<thead>
<tr>
<th>Collagen-1 Solution Concentration</th>
<th>10× PBS</th>
<th>Collagen-1</th>
<th>1 N NaOH</th>
<th>DI H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mg/ml</td>
<td>10 µL</td>
<td>26 µL</td>
<td>0.6 µL</td>
<td>63.4 µL</td>
</tr>
<tr>
<td>2 mg/ml</td>
<td>10 µL</td>
<td>52.1 µL</td>
<td>1.2 µL</td>
<td>36.7 µL</td>
</tr>
<tr>
<td>3 mg/ml</td>
<td>10 µL</td>
<td>78.1 µL</td>
<td>1.8 µL</td>
<td>10.1 µL</td>
</tr>
</tbody>
</table>

**Table 4**: Concentrations of components for various compositions of Type 1 Collagen matrices

Collagen matrices were loaded into their respective designated chambers using a minimal pressure to avoid pushing the gel past the posts delineating the collagen/cell interface. The injection port was formatted with the smallest area allowing for the insertion of a 10 µL pipette tip. The tight fit ensured that no air pockets would be introduced during injection of the collagen matrix. Upon filling each of the four chambers on the separate devices, the molds were placed in an incubator to retain sterilization and left there for 30 minutes, allowing for complete polymerization of the collagen gel. Polymerization occurs from the rapid interaction of monomer units upon unfreezing; these chains link together to form three-dimensional polymer chains.
Two growth factors, VEGF and EGF, were chosen at concentrations of 0.1, 1.0, and 10 µM. These concentrations were made using serial dilutions, within glioma media, with a starting concentration of 10 µM (see Table 7). Devices which did not receive any growth factor concentration were deemed the controls. Each growth factor solution was dispensed into their appropriate loading chamber and 3 µL of cell solution containing ~3000 cells were immediately seeded into the center compartments.

<table>
<thead>
<tr>
<th>Growth Factor Concentration</th>
<th>U-87 media</th>
<th>VEGF/EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>10µM</td>
<td>1mL</td>
<td>10µL</td>
</tr>
<tr>
<td>1µM</td>
<td>900µL</td>
<td>100µL of 10µM Dilution</td>
</tr>
<tr>
<td>0.1µM</td>
<td>900µL</td>
<td>100µL of 1µM Dilution</td>
</tr>
</tbody>
</table>

Table 5: Serial dilutions used to make various concentrations of VEGF and EGF

3.7 Time-Lapse Imaging of Glioma Culture & Gradient Diffusion

The primary goal of this work is to be able to understand the stand-alone and synergistic roles of ECM characteristics and diffusing signaling molecules on glioblastoma cell behavioral functions, such as morphology and migration. Towards this end, studies were performed over 48 hour duration, with time-lapse images of cell behavior taken at designated intervals. All images were taken using a Zeiss Axiovert A1 fluorescence inverted microscope, equipped with Hamamatsu camera and image acquisition software. For the first two hours, devices were imaged at regular intervals of ten minutes. These time intervals were steadily increased to include imaging every half hour for the next two hours, every hour for the next nine hours, every six hours for eighteen hours and then at the final 48 hour time point. All images were captured using a 20× or 40× objective, for later quantification of cell shape and angles of migration.
3.8 Cell-Cell Interactions

Neighboring cells within a microenvironment could also serve as transducers of biochemical signals influencing glioblastoma cell migration and phenotype. Studies have shown that signals exchanged between neighboring cells can amplify, differentiate, and/or integrate information influencing the behavior of a particular cell type. Therefore, it is important that cell migration be viewed from the view point of interactions with surrounding cell bodies in addition to their response from imposing growth factors or other extracellular molecules. The dispersion of a cell population in relation to their proximity to other cell types greatly influences the procession of a tumor body. To study these effects, we used our microfluidic platform to culture two different types of cells. Adult human microvascular endothelial cells, HMEC cells, (Lonza; passage 5) were loaded into the chambers previously used to stock growth factor media, while glioblastoma cells were loaded in the original center compartment. Cell media was reconstituted for the endothelial cells separately by lab mate Phillip Simmers, as per vendor’s recommended protocols, and then filled on top of the cells loaded into the device. A similar technique was used to introduce the glioma media. During the duration of the experiment, both the types of media was replenished every 24 hours to ensure cell vitality. Time-lapse imaging was performed as detailed above, to document cell responses and migration patterns over a 7 day time period.
3.9 Quantification Methodology

The migratory patterns of glioma cells in response to diffusive chemogradients generated by the diffusion of both growth factors and other cell types were quantified for the following results:

- Total number of cells migrated
- Net distance of migration
- Cell speed and path of trajectory
- Persistence time & migration distance
- Cell shape morphology (CSI)

For growth factor trials, all numbers were calculated at the end of a 48 hour time period. 7 day trials were ran for glioma responses to HMEC cells, so as to be comparative to results reported in literature127-130.

The first response quantified was the total number of cells migrated into the designated scaffolds. For all trials, cells were counted from initial moment of entry into the collagen matrices over the elected time periods. Distance of migration for diffusing cells was taken from the point of initial entry into the collagen scaffolds to final point of migration at the end of 48 hours or 7 days (cell-cell interactions). Results were calculated for at least 30 cells and averaged over the given times. Distance of migration was quantified for net total distance as well, not displacement.

Averages of 30 cells were again taken to determine cell speeds and paths of trajectory, including persistence time and distance. We measured net length of diffusion and divided by the length of time needed to travel that distance to determine velocity over the time
periods in which phase contrast imaging was captured. Paths of trajectory were calculated by measuring the angle of migration from each point to subsequent locations over the course of either 48 hours or 7 days, depending on the trial being run. We recorded the time cells traveled along the same path of migration (persistence time and distance) for the length of each trial.

Finally, changes in cell morphology were calculated using a formula for cell shape index (Eq. 3). These results were again recorded for a total of 30 cells.

\[
\text{CSI} = \frac{4\pi \cdot \text{Area}}{\text{Perimeter}^2} \quad \text{(Eq. 3)}
\]

ImageJ® analysis software was used to record both the area and perimeter of cells over the designated time points. CSI values range from zero to one, with one representing a perfectly spherical particle.
CHAPTER IV

RESULTS AND DISCUSSION

Our discussion begins with the calculation of diffusion coefficients through different concentrations of collagen-1 as determined by using the Stokes-Einstein equation. Next, the experimental protocols followed previously is analyzed for the total number of cells migrated over the specified time period, distance of migration, changes in cell morphology as reflected through cell shape indices, and finally, cell speed and path of trajectory. Quantification of these parameters are analyzed for response to the growth factors VEGF and EGF, and to cell-cell interactions when glioma cells are introduced to HMEC cell lines. Finally, all results are compared quantitatively to relevant literature.

4.1 Diffusion Calculations and COMSOL®

The concentration profile generated by the migration of molecules through a particular matrix are influenced by both the morphological features of the molecules (i.e. shape, mass, etc.), their source concentration and activity, and by the topology of the surrounding environment. Many mathematical models have been constructed to
understand the influence of these various factors on the diffusion patterns of traveling cell
types and signaling molecules. By using the multiphysics software, COMSOL®, we
were able to simulate a more accurate portrayal of the dynamic gradient profile of the
tested growth factors.

COMSOL® software needs the input of two coefficients to generate the gradient
profile unique to each concentration of growth factor through the specified matrix. The
first coefficient needed is the diffusion constant, a value dependent on the radius of the
diffusing molecule and the viscosity of the molecule through the given matrix. As
described previously, the diffusion coefficient is found using the Einstein-Stokes
equation (Eq. 1). These values, when used together with the molecular weight to calculate
viscosity of these molecules, can be used to calculate the diffusion coefficient of VEGF
and EGF when diffusing through matrices of 1, 2, and 3 mg/ml collagen-1 (see Table 2).
Thus, we only need to change the diffusion coefficient and concentration of collagen to
generate a gradient profile within the COMSOL® platform.

The diffusion of growth factors through the proposed microfluidic device is shown to
generate concentration gradients that are dependent not on the concentration of growth
factor, but on the concentration of the surrounding matrix (see Figure 10). Our
experimental protocol called for the infusion of 1, 2, and 3 mg/mL collagen-1 into our
microfluidic platform. Concentrations of 0.1, 1, and 10 μM of both VEGF and EGF were
introduced, to produce a quantitative dose-responsive gradient profile. The diffusion of
these molecules is also dependent on their molecular mass. The diffusion of these
molecules through our microfluidic device were simulated using the COMSOL®
program, showing that indeed, molecular mass and concentration of the surrounding matrix influenced the concentration.

**Figure 10**: Concentration gradients generated for 10μm VEGF through 1, 2, and 3 mg/ml Collagen-1
profiles of the diffusing growth factors. Expectedly, VEGF, having a molecular weight around 19 kDa, diffused more slowly through the given concentrations of collagen than EGF, which has a molecular weight around 6 kDa. The more dense the collagen matrix, the slower the rate of diffusion.

In order to determine the exact time for complete diffusion across our microfluidic channels, we used Eq. 2, a formula found in relevant literature\textsuperscript{127-129}, to compare the diffusive times for molecules of various sizes through the collagen matrices. Results are summarized in Table 6. Using these values, we found that increases in both collagen concentration and increases in the molecular weight of the diffusing particles result in a longer time for complete diffusion. VEGF completely diffuses across a collagen-1 scaffold of 1 mg/ml, for example in a period of time just over 2.4 hours. When introduced to an increased concentration of collagen-1 at 3 mg/ml, however, the particle takes around one day to migrate through the intended scaffold. EGF travels much more quickly, due to its lower molecular weight. This growth factor diffuses across initial 1 mg/ml collagen-1 scaffolds in around 1.65 hours, while taking around 0.5 days to completely diffuse across 3 mg/ml Collagen-1.
Table 6: Times for diffusion across collagen channels for VEGF and EGF concentrations in 1, 2, and 3 mg/ml Collagen-1

Results from the COMSOL® simulations enabled approximations to be made of the growth factor concentration experienced by cells at specific distances within the collagen matrix at any time studied. One example of a diffusing profile generated by COMSOL® calculations can be found in Figure 10. Diffusing glioma cells, for example, were found to mostly migrate within the first 0.5 mm of the collagen chamber throughout all trials. Using the gradient profiles extrapolated from the COMSOL® simulations (Figure 11), 1 mg/ml Collagen-1 scaffolds, these cells experience a range of concentrations for 0.1µM VEGF ranging from 0.0025µM within the first ten minutes, to twice that concentration after 5 hours. At 24 hours, the concentration increases to around 0.008µM and to 0.0088µM at 48 hours.
Glioblastoma cells were introduced to 0.1, 1.0 and 10 μM of both VEGF and EGF chemogradients. Due to the high concentration of growth factors and the low number of cells seeded into each device (~3000), the depletion of chemogradient signaling molecules did not occur. Cell responses were quantified in terms of the overall number of

4.2 Quantification of Glioma Migration with Introduced Growth Factors

Glioblastoma cells were introduced to 0.1, 1.0 and 10 μM of both VEGF and EGF chemogradients. Due to the high concentration of growth factors and the low number of cells seeded into each device (~3000), the depletion of chemogradient signaling molecules did not occur. Cell responses were quantified in terms of the overall number of
cells migrated, distance of migration, change in cell morphology (cell shape index) and path and speed of cell trajectory. The microfluidic system presented for the study of glioma migration allowed for time-lapse imaging used to quantify these parameters.

We first demonstrated that the growth factor gradient expressed by the diffusion of both VEGF and EGF is expressed through the various concentrations of collagen matrices using the COMSOL® simulation software. Seeing that a successful concentration gradient was able to be imposed upon the cultured glioma cell lines and collagen matrices, we studied the net migration of these cell types over a period of 48 hours. A simple count of the number of cell bodies migrated into the collagen matrices was taken over the specified time period. To analyze the data collected, we will compare the total number of cells migrated into collagen matrices first across all concentrations of each growth factor individually, followed by quantification of net migration across the array of scaffold densities, and finally, the total number of cells migrated will be compared between growth factors collectively.

VEGF was found to act very strongly as a chemoattractant for glioma cells within all three concentrations of collagen networks at all concentrations of the growth factor (see Figure 12). The net total number of cells migrated into all three collagen scaffolds increased with increases in VEGF concentration. We found that no cells migrated into collagen scaffolds of 1 and 3 mg/ml with low concentrations of 0.1 μM VEGF. These results indicate that 1 mg/ml collagen-1 did not present enough of a scaffold for sufficient migration of cells with lower concentrations of attractant chemical stimuli and that the biochemical cues were too weak to entice migration into the thicker collagen networks. The numbers of migrating cells significantly decreased with increases in collagen-1
concentration (see Figure 12). There was almost a 10 fold higher response, for example, between all concentrations of VEGF in 1 mg/ml collagen-1 as compared with 2 mg/ml collagen-1.

Introduction of various concentrations of EGF to the Collagen-1 networks generated a migratory response of our glioma cells that was very similar to that produced by the diffusion of VEGF signaling molecules. Increases in EGF concentrations throughout all concentrations of Collagen-1 matrices resulted in an increased number of migrated cells. As collagen concentrations increased, however, the chemotactic ability of EGF decreased. Cells moved into the scaffolds at lower numbers with increased concentrations in Collagen. Comparing our results to those tabulated from the trials ran with VEGF concentrations (see Figure 12), the number of cells migrated into

Figure 12: Net total number of cells migrated in various concentrations of Collagen-1 scaffolds with introduced growth factor concentrations
1 mg/ml Collagen-1 was decreased by almost four fold for both 1 and 10 μM of the specified growth factors. As was seen with VEGF, no cells migrated into the collagen scaffold concentrations of either 1 mg/ml or 3 mg/ml. The lowest concentration of EGF, 0.1 μM, elicited around the same number of cells to migrate into the 2 mg/ml Collagen matrix, while both 1.0 and 10μM found roughly half the number of cells migrating as compared to VEGF. Interestingly, results showed that EGF in fact acted as a stronger chemoattractant as compared to VEGF at a concentration of 10 μM in 3 mg/ml Collagen-1. Cell numbers more than doubled for this concentration of Collagen matrix. 10 μM of EGF was found to be the only concentration able to entice cell movement, as compared to VEGF in which cells migrated into the thicker scaffold at concentrations of both 1 and 10 μM. Again, phase contrast imaging, as is seen in Figure 13, was used to capture these migratory patterns over designated time points for a duration of 48 hours. Cells are highlighted in yellow for the stated figure and as is clearly seen, the numbers significantly increase over the selected time period.

**Figure 13**: Net migration of cells into 1 mg/ml Collagen-1 with 1.0μM VEGF
Glioma cell motility within various substrates is modulated by the activation and composition of various integrins and surface receptors. An anchorage-independent response of these cells has been a central hypothesis for many research groups, focusing not only on glioma lines but on a wide array of cancerous types, including pancreatic and small lung cancer\textsuperscript{115,116}. The second area for quantification, therefore, was done for the net overall distance of migration for glioma cells within the specified concentrations of collagen in response to diffusive chemogradients generated from the random movement of growth factors. The distance of cell migration was easily quantified using time-lapse imaging performed under light microscopy using a 40\times objective (400\times magnification).

From studying the captured bright field images (Figure 14), we were able to track the total distance of migration for our studied cells types from the moment of initial entry into a given collagen network (dotted red line), to the final point of migration at the end of 48 hours (dotted yellow line). Results found that increases in collagen scaffold, across all concentrations of both growth factors, decreased the total distance of migration, results are charted in Figure 15. A similar trend for both growth factors as well was the increase in

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{Fig14.png}
\caption{Net distance of migration over 48 hours in 2 mg/ml Collagen-1 with 1.0\mu M VEGF}
\end{figure}
distance of motility with increases in growth factor concentration at the end of 48 hours. As is seen in Figure 15, cells were found to move further within the collagen matrices in response to VEGF as compared to similar concentrations of EGF for every concentration of Collagen-1. Results show a two-fold distance of migration of VEGF (at all concentrations of growth factor within all concentrations of Collagen-1) as compared to EGF, again indicating that cells react more strongly to VEGF as a chemoattractant than to EGF.

Cells were found to migrate to further distances more quickly within lower concentrations of Collagen scaffolds. VEGF, for example found cells moving to a distance of 40±0.4 µm within the first hour within a 2 mg/ml Collagen-1 matrix, while these cells took twice that amount of time within a 3 mg/ml Collagen-1 scaffold. Quantification of the total distance of migration found that cells within lower concentrations of Collagen-1 scaffolds (for all concentrations of both VEGF and EGF)

![Figure 15: Net distance of migration quantified for concentrations of VEGF and EGF within 1, 2 & 3 mg/ml Collagen-1 over 48 hours](image)
moved furthest. 2 mg/ml and 3 mg/ml Collagen-1 scaffolds saw cells moving similar distances, for 10 µM of both growth factors, just taking longer to do so as the concentration of collagen increased. No cell migration was recorded for the lowest concentrations of either growth factor in either 1 or 3 mg/ml Collagen-1 (see Figure 15).

Cells migrated in a minimal manner until around 6-7 hours after seeding, upon which the cultures were able to mass migrate into the scaffolds. A similar study performed by Ware, Wells and Lauffenburger (1998) found that the number of fibroblast cells migrating into the injected a fibronectin-coated substrata was maximum between 6 and 8 hours after incubation. Their study was performed for a maximum of 20 hours after motility parameters determined that tracking of single cell movement was non-sufficient for quantification after this length of time. Because the focus of their study was on the migratory activity of fibroblasts in response to EGF, and that their cell lines are less invasive than glioma cultures, the number of cells enticed to migrate through the introduced matrix was less effective than the highly invasive cancerous phenotype used in our current study.
Cell migration was found to be mostly linear, as will be discussed more thoroughly in later sections, and as a result, net displacement is almost directly proportional to total length of path migration. In similar studies, a cocktail of growth supplements (including VEGF) and nutrients was used to study the net migration of endothelial cells, along with their response to specific nitric-oxide inhibiting protein-kinase activity. The authors found that VEGF acted as a strong chemoattractant, enticing cells to migrate an average distance of around 200 µm. Their work, however, was not performed within a matrix designed to mirror the actual *in vivo* environment experienced by these cell types, nor did it use an invasive phenotype of cancerous cells such as glioblastoma line.

Further quantification of migratory distances was performed by dividing the chamber into which the cells traveled into five equal zones, each transversely segmented into 0.5 mm sections. While the number of cells that travelled beyond the first zone was minimal, about 12% of glioma cells originally in zone 1 (with the lowest VEGF concentration) were found to migrate into zones with higher VEGF concentrations. This number

![Figure 16: Phase contrast imaging over 48 hours for glioma cells in 2 mg/ml Collagen-1 with 1.0M EGF](image-url)
increased to around 17% for cells introduced to EGF chemogradients. Shamloo et al. (2008) found similar results when they studied the chemotactic effects of VEGF on the migration of HUVEC cell lines\textsuperscript{117}. Around 7% of their studied cells moved into zones with increased concentrations of the given growth factor, regardless of the concentration of VEGF. Their results aid in our hypothesis that wide ranges of VEGF concentrations encourage the chemotaxis of invasive cell phenotypes.

Cell morphology can drastically change in response to chemogradients generated by the diffusion of various biochemical stimuli\textsuperscript{13,15,20}. To study the influences of various chemical gradients generated by the influx of VEGF and EGF into our collagen-1 scaffolds, we tracked changes in the cell shape index (CSI). As was previously described in Eq. 4, CSI is a simple ratio of the area of the cell to the square of its perimeter. Values range from zero to one, with one representing a perfectly spherical particle. Deviations from a uniformly spherical shape reduce the numerical value of a given cell’s CSI. Expectedly, cells were found to elongate their phenotypes in response to diffusive chemogradients projected by the diffusion of both growth factors at all concentrations. Analysis of cells migrated into collagen scaffolds at all concentrations of both VEGF and EGF show an increased tendency for more elongated morphologies as collagen concentrations increase, as shown in Figure 16. Cells retained the most spherical shape, as calculated by their CSI, when in lower concentrations of Collagen-1 and when subjected to various VEGF gradients (Figure 17). When in the presence of EGF gradients, cells retained a more spherical shape with higher concentrations of collagen. This could be due to the lower diffusion times of the signaling molecules through the thicker collagen-1 scaffolds or because a lower overall number of cells migrated into the
matrices collectively. Interestingly, cells subjected to the stated concentrations of VEGF exhibited the most elongated profiles when exposed to concentrations of 1.0 µM VEGF in both 2 and 3 mg/ml Collagen-1. For comparison, similar studies using collagen matrices and introduced growth factors (specifically EGF) analyzing CSI for smooth muscle cells were ~0.45\textsuperscript{121}, ~0.2\textsuperscript{122} for human mesenchymal stem cells, and ~0.25\textsuperscript{123} for endothelial cells.

Figure 17: CSI for all concentrations of growth factors, VEGF and EGF, through 1, 2, and 3 mg/ml Collagen-1
Finally, cell migration was analyzed for speed and path of trajectory. Results showed that migration of glioma cells, both with VEGF or EGF, was progressively directed with increases in growth factor concentration. Paths of migration were averaged for 30 cells.

Table 7: Persistance time, distance and $R^2$ values for glioma cells through 1, 2, and 3 mg/ml Collagen-1 with various concentrations of growth factors

<table>
<thead>
<tr>
<th>1 mg/ml Collagen-1</th>
<th>Persistance Time (min)</th>
<th>Persistance Distance (µm)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1uM VEGF</td>
<td>0</td>
<td>0</td>
<td>0.8949</td>
</tr>
<tr>
<td>1uM VEGF</td>
<td>30</td>
<td>4.9</td>
<td>0.9957</td>
</tr>
<tr>
<td>10uM VEGF</td>
<td>10</td>
<td>20.1</td>
<td>0.998</td>
</tr>
<tr>
<td>0.1uM EGF</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1uM EGF</td>
<td>20</td>
<td>11</td>
<td>0.8061</td>
</tr>
<tr>
<td>10uM EGF</td>
<td>10</td>
<td>14.4</td>
<td>0.9628</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2 mg/ml Collagen-1</th>
<th>Persistance Time (min)</th>
<th>Persistance Distance (µm)</th>
<th>$R^2$</th>
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<tr>
<td>0.1uM VEGF</td>
<td>40</td>
<td>13.3</td>
<td>0.5571</td>
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<td>19.1</td>
<td>0.92</td>
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<td>0.9688</td>
</tr>
<tr>
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<td>1uM EGF</td>
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<td>14.1</td>
<td>0.8376</td>
</tr>
<tr>
<td>10uM EGF</td>
<td>20</td>
<td>18.1</td>
<td>0.9851</td>
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<th>Persistance Distance (µm)</th>
<th>$R^2$</th>
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<tr>
<td>0.1uM VEGF</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1uM VEGF</td>
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<td>14.3</td>
<td>0.8726</td>
</tr>
<tr>
<td>10uM VEGF</td>
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<td>57.1</td>
<td>0.98</td>
</tr>
<tr>
<td>0.1uM EGF</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1uM EGF</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10uM EGF</td>
<td>60</td>
<td>20.1</td>
<td>0.8986</td>
</tr>
</tbody>
</table>
in each case, where factors such as the random motility coefficient (RMI), persistence distance and persistence times were calculated (Table 7). First, RMI was found by referencing the $R^2$ value of the linear trend line fit to each path of migration. An $R^2$ closer to a value of one was used to define a cell moving in a strictly linear path, having no divergence. The more this value decreases, the more random the path of migration becomes. Using time-lapse imaging analysis and ImageJ® analytical tools, we found that increases in growth factor concentration increased the $R^2$ value and intuitively, directed paths of migration (see results in Table 6).

Persistence distance was quantified by tracking how long a cell continued along a specific distance of migration without severely (>10 degrees) altering the degree of movement (see Fig. 18, 19). Changes in path trajectories greater than 10 degrees indicate that cells are unable to move directly into the scaffolds; instead they are forced to find anchorage points along the walls of the device.
Figure 19: Angles of migration for glioma cells through 1, 2, and 3 mg/ml Collagen-1 in response to various concentrations of VEGF
Persistence time followed a similar analysis, quantifying the amount of time a cell spent traveling in the same direction. Migrating cells can retain a relatively unchanging direction of locomotion for minutes or hours, depending on numerous factors ranging from the concentration of diffusing chemical stimuli to the composition of the surrounding matrix. VEGF at a concentration of 10μM, for example, stimulated persistent chemotaxis over unchanging distances ranging from around 10 minutes through 1 mg/ml Collagen-1 to over an hour for 3 mg/ml Collagen-1. The less potent the growth factor and collagen matrix were, the less directed and more random the motility of our cells (see results summarized in Table 5). A 2005 performed by Harms et al., examined the efficacy of EGF in enticing long timescale directional persistence of ovarian cells through various concentrations of fibronectin-coated substrata. Their results complimented our findings, showing that addition of EGF concentration profiles increased directional persistence, along with subsequent increases in the concentration of fibronectin coating. Additionally, our research found that VEGF acted as a much stronger chemoattractant compared to EGF, enticing consistent cell motility for longer periods of time and increased distances. The randomness of migration patterns decreased with increases in concentration of both growth factors and collagen.

Cell speed was found to decrease with increases in Collagen-1 concentration across all concentrations of both VEGF and EGF. As can be seen in Figure 20, cells started with relatively high velocities at the start of each trial and quickly lost speed in an exponentially decreasing fashion. The more potent the growth factor stimuli was, the faster the cells traveled into the collagen scaffolds for both growth factors. Increases in collagen scaffold concentration decreased the speed of cell migration for both VEGF and EGF at all
concentrations as well. For example, at 10 µM VEGF, cells traveled at a speed of ~13.5±0.4 µm/h within 1 mg/ml Collagen, but the speed decreased to ~3±0.02 µm/h within 3 mg/ml collagen. Cells within 3 mg/ml Collagen-1 had the lowest changes in speed.
Figure 20: Average cell speeds through 1, 2, and 3 mg/ml Collagen-1 with various concentrations of both VEGF and EGF
4.3 Glioma Chemotaxis in CoCulture Gradients

In order to study the effects of multiple cell types upon cancer cell migration, we seeded HAEC cells in chambers opposite the loading area of the glioma line but within direct contact with the introduced collagen matrix. As was analyzed with the introduction of growth factors, cell-cell interactions were examined for the following results: total number of cells migrated into the injected matrix, distance of migration, cell shape index with respect to changes in cell morphology, and cell speed and path of trajectory. The studies were increased to 7 day trials in order to compare results with literature found with cell-cell interactions, as compared to the 48 hour time periods used to study the effects of growth factor gradients. Successful chemical gradients were found to exist upon introduction of HAEC cells in placement of growth factor stimuli, inducing invasion of glioma cells into the studied scaffolds. Time lapse, bright field imaging was again taken in order to study the invasiveness of glioma cells (Figure 21) with response to co-culturing with HAEC cells (Figure 22).

**Figure 21:** The net migration of gliomas migrated into 1 mg/ml Collagen-1 over 7 days in response to cell-cell culturing.
Seven day studies analyzing the movement of glioma cells in response to the co-culturing of HAEC lines found initial migration of HAEC lines (Figure 22), followed by the subsequent invasion of glioma cell types, into the studied scaffolds (see Figure 21). Results studying migration of these cell types within 1 mg/ml Collagen-1 matrices showed the number of glioma cells migrated into the scaffold to be comparable to the...
number of cells migrated into the same scaffold under the highest concentrations of VEGF gradients, averaging around 380 cells over the specified time period (see Figure 23). The number of glioma cells migrated into 2 and 3 mg/ml Collagen-1 significantly decreased from the initial number migrated into the first scaffold. Though a lot more glioma cells migrated into the 1 mg/ml Collagen-1 scaffold, thicker concentrations found the number of EC cells surpassing the number of U-87’s. In fact, at 3 mg/ml Collagen-1, no glioma cells migrated into the given matrix while, even though numbers were extremely low, EC cells were able to move into the scaffold. These results suggest that glioma cells respond to chemical stimuli within less dense matrices but within thicker strata necessitate much stronger biochemical signals to entice migration. EC cells, on the other hand, respond to lower concentrations of stimuli.

Precise control over the microenvironment in which our cocultured cells were studied was essential to replicate *in vivo* situations. Interstitial flow conditions and concentration gradients imposed through the stream of signaling molecules released from interacting cell types aid in the migration of diseased cell lines. As concentration profiles of these signaling molecules are increased, cells are further enticed to migrate through the surrounding stratum.
Figure 24: Average cell speeds over 7 days for U-87 & HMVECs within 1, 2, and 3 mg/ml Collagen-1
Chung et al. (2010) seeded U-87 glioma cells alongside hMVEC (human microvascular endothelial cells) to study the migratory effects of a highly invasive cancer line on surrounding non-cancerous cell bodies\textsuperscript{125}. Within a 2-3 day time period, tumor cells had formed an aggressively growing mass, sprouting through an endothelial monolayer that had formed after 24 hours. They found that the path forged by initially migrating cells was followed by subsequent tumor bodies.

As we analyzed our cells for path of migration and angles of trajectory, our results followed a similar pattern. The velocity of invading glioma cells was found to be much greater than that of the introduced HAEF cells within low concentrations of collagen, with the speed of these cell types significantly decreasing over the 7 day time period. As collagen concentrations increased, cells speeds were comparable in number within 2 mg/ml collagen, and eventually non-existent for glioma lines within the highest concentration. 3 mg/ml Collagen-1 facilitated EC cells to migrate, though slowly, at a persistent rate \(~0.15-0.10\pm0.03\ \mu\text{m/h}\). As was found with growth factor trials, increases in persistence time and distance of cell motility within our given matrices correlated directly with increases in Collagen-1 scaffolds. Cell speeds over at the end of a period of 7 days were charted in Figure 24. All biological responses of glioma cells are found to be in response to signaling molecules released from ECs which are diffusing through the 3D collagen scaffold. Similarly, U87 MG cells are also releasing some molecules which are enticing ECs.

Distance migrated into the surrounding stratum for cell-cell interactions was comparable to the results found with the introduction of high concentrate VEGF through the 1 mg/ml Collagen-1 matrix only. As can be seen in Figure 25, data shows glioma
cells moving further within 1 mg/ml Collagen-I as compared to EC cells. As the concentration of collagen increased, however, glioma cells were unable to migrate as far as the HMVEC cells. High concentrations of collagen, in fact, inhibited glioma migration while allowing for EC motility. Again, HAEF cells were found to move first through the collagen matrices, followed closely by the migration of glioma cell types. Whereas HAEF cells were found to move individually, glioma cells tend to follow initial migrating bodies in packs of three or more cells, forming clusters as they followed along predetermined paths. Distances moved through the various collagen matrices can be quantitatively compared using the table provided below:

<table>
<thead>
<tr>
<th></th>
<th>1 mg/ml Collagen-I</th>
<th>2 mg/ml Collagen-I</th>
<th>3 mg/ml Collagen-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-87 w/0.1μM VEGF</td>
<td>0 μm</td>
<td>24.4 μm</td>
<td>0 μm</td>
</tr>
<tr>
<td>U-87 w/1.0μM VEGF</td>
<td>10.2 μm</td>
<td>24.8 μm</td>
<td>15.8 μm</td>
</tr>
<tr>
<td>U-87 w/10μM VEGF</td>
<td>69.4 μm</td>
<td>39.8 μm</td>
<td>36 μm</td>
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<td>U-87 w/0.1μM EGF</td>
<td>0 μm</td>
<td>0 μm</td>
<td>0 μm</td>
</tr>
<tr>
<td>U-87 w/1.0μM EGF</td>
<td>21.6 μm</td>
<td>15.8 μm</td>
<td>0 μm</td>
</tr>
<tr>
<td>U-87 w/10μM EGF</td>
<td>27.4 μm</td>
<td>19.8 μm</td>
<td>22 μm</td>
</tr>
<tr>
<td>U-87 Glioma Cells (Cell-Cell Study)</td>
<td>54 μm</td>
<td>4 μm</td>
<td>0 μm</td>
</tr>
<tr>
<td>HAEF Cells (Cell-Cell Study)</td>
<td>20.1 μm</td>
<td>2.8 μm</td>
<td>20 μm</td>
</tr>
</tbody>
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**Table 8:** Overall distance of migration of U-87 glioma cells in response to various concentrations of VEGF, EGF and HAEF cells
Figure 25: Net total distance of migration through 1, 2, and 3 mg/ml Collagen-1 for U-87 and HMVEC cells over 7 days
Chung et al. (2010) found that introduction of EC cells to the seeding of hepatocytes within a 3D microfluidic platform caused enhanced migration of capillary-like structures from the ECs and severe alterations of the morphogenesis of the hepatocytes. Without the presence of hepatocytes, EC cells formed 2D sheet-like structures. Their morphology was significantly modified to form the capillary-like structures when introduced to traversing chemogradients imposed through the introduction of hepatocyte cultures. Such results indicate that heterotypic interactions across 3D scaffolds can induce morphological changes in multiple cell types. Using captured time-lapse images (Figures 21 & 22), we analyzed cell-cell interactions between HAEC and glioma cells for similar modifications in cell morphology by recording changes in the area and perimeter of cells. These alterations were then used to calculate changes in cell shape indices. Our results, as was found with growth factor gradients, indicates that cells (both ECs and gliomas) elongate their morphology in response to introduced cell-cell interactions (see Figure 26). Contrary to our initial hypothesis, the more concentrated the collagen matrix that glioma cells were expected to migrate through, the more spherical the cell bodies. This could be because the matrices were too thick for cells to move through, forcing them to retain their original morphology as they traveled in clusters rather than as individual
entities. EC cells significantly altered their phenotype with increases in collagen-I concentration, significantly becoming more elongated. Glioma cells, on the other hand, slightly became more rounded with 2 mg/ml Collagen as compared to 1 mg/ml. Figure 27 shows bright field images captured to study the morphological changes of glioma cells in response to signaling molecules released from EC cells over the course of 7 days.

Figure 27: Bright field images taken over the course of 7 days to study morphology changes in glioma cells in response to chemogradients generated from cell-cell culturing
CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The directed migration of glioma cells within a microfluidic platform was studied to better understand the mechanical and biochemical cues that affect motility of cancerous cell types. In this study, the chemotaxis of human glioblastoma multiform (U-87 MG) cells in response to quantified gradients of vascular endothelial growth factor (VEGF), endothelial growth factor (EGF), and adult human vascular endothelial cells (HAEC) cells was analyzed. Our proposed microfluidic device was designed and fabricated to generate stable concentration gradients of these specified biomolecules and cell types in a cell culture chamber through various concentrations of collagen matrices. Finite element simulation of the diffusion of our growth factors and cell types through the device’s geometry was accomplished using COMSOL®, a finite analysis and simulation software analytical tool. These simulations suggested stable concentration distributions over the course of 48 hours to 7 days. This device is expected to have wide applicability in the study of migration through three-dimensional matrices over an extensive array of cell
types. Glioma cells were found to chemotax towards higher growth factor concentrations of studied growth factors across the entire range of introduced concentrations (0.1, 1, 10 µM) and through all concentrations of type I collagen 3D matrices (1-3 mg/ml). VEGF acted as a stronger chemoattractant compared to EGF. Cells were enticed to move in larger quantities, to further distances, and at higher speed in response to increasing growth factor concentrations, and in more dynamically in response to VEGF as compared to EGF. The morphologies of glioma cells in response to traversing growth factor concentrations were found to elongate with increasing concentrations of the collagen matrices. Furthermore, U87 cell migratory response within HAEC cocultures was similar to that within higher VEGF concentration gradients. These results suggest that the gradient profiles generated by migrating growth factors and surrounding cell types enhance the total number of cells migrated (including distance and velocity of travel) and that gradient steepness encourages subsequent directed migration. Clinical studies have shown that the tumor ECM stiffens compared to healthy counterparts. Assuming that the stiffness of such modified ECM is comparable to that of 3 mg/mL collagen, we deduce from our coculture studies that ECs might be migrating through the dense ECM towards tumor site, initiating the onset of angiogenesis. However, further studies are needed to elucidate this mechanism in both in vitro cultures like ours, and in vivo.

5.2 Recommendations

Microfluidic devices, as established with our research, can be very helpful platforms for the future study of diseased cell types and can offer unlimited potential for advances
in therapeutic treatments of such illnesses. Allowing for specific control over the local microenvironment while reducing volume of costly reagents required for research, these devices can be instrumental for the study of various concentration profiles on the biological mechanisms governing diseased cell activities such as migration, proliferation, etc. Our research was able to successfully show the migratory effects of a range of growth factor concentrations and collagen matrices on the motility of glioma cells. While our results are congruent with our initial hypothesis, it is suggested that higher concentrations of growth factors and 3D matrix varieties be studied. The applicability of this platform would be enhanced by studying the effects of a greater number of growth factors through different matrices. Collagen Type I is only one type of structural protein present within the ECM. Future studies could incorporate other matrices, such as matrigel, hyaluronic acid or various hydrogels, to study cell migration. In addition to widening the study to further matrices and growth factor types, further research could be done using different diseased cell lines, showing the versatility of our fabricated microfluidic device. Such studies could even include pediatric glioma cell lines, allowing researchers to correlate data with what has already been presented in these studies.

Our research did not allow enough time to do elemental analysis such as live/dead assays or studies for proliferation. We did not perform staining for cell proliferation, and presumed that cells did not multiply over the 48 h culture period. Based on our understanding of cancer cell tendencies within a given matrix, it would be beneficial for these studies to be performed, aiding in the merit of any data so far collected.
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IMAGE REFERENCES


