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Chemotaxis of Central Nervous System-derived Tumor Cells within Controlled Microenvironments

Jennifer Rico Varela
CUNY City College

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Chemotaxis of Central Nervous System-derived Tumor Cells within Controlled Microenvironments

Thesis
Submitted in partial fulfillment of the requirement for the degree
Master of Engineering (Biomedical)
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By Jennifer Rico Varela
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Approved by:

______________________________  ________________________________
Dr. Maribel Vazquez, Thesis Advisor                        Dr. Mitchell Schaffler, Chairman
EXECUTIVE SUMMARY

Metastasis remains the cause of 90% of deaths from solid tumors. The metastasis alters the cell microenvironment, enters the circulation, and colonizes a distant organ. Migration of brain cancer cells involves a heterogeneous microenvironment that consists of stromal cells, signaling molecules, and various extracellular matrix (ECM) configurations. The significant interactions between stromal cells and tumors cells create an environment where brain cancer cells can grow and spread. The complexity and heterogeneity of tumors and their microenvironment challenge the study of metastasis and its treatment.

In the past three decades, a group of chemoattractants and their receptors have been associated to cause cancer cell migration. Chemoattractants, such as growth factors and chemokines, are believed to be secreted by neighboring cells, diffuse and generate concentration gradients that are sensed by the central nervous system-derived tumor cells (Medulloblastoma). These cells migrate away from primary tumors to settle in the brain or the spinal column. Researchers believed that the brain tumor invasion is caused by soluble factors that stimulate directional (chemotaxis) or random (chemokinesis) tumor cell motility. Despite of studies in cell migration, the role of chemoattractants is still understudied due to the highly complex microenvironment. Previous research published on migration of breast cancer, fibroblast, and glioblastoma have demonstrated that growth factor concentration gradients play an important role in cell motility. Our research group has previously illustrated the importance of the spatial-temporal gradient profiles of chemoattractant on cell migration. However, it is not well understood the impact of gradients on cell motility, and understanding them has been a long-term focus of cell migration research.
Historically, several traditional migration assays (Boyden, Zigmond, Dunn chambers; and micropipette) have been widely used for cell migration studies. However, these assays lack the ability to maintain and control chemical concentration gradients or allow the real-time quantification of chemoattractants gradients around individual cells over time. Recently, microfluidic devices have been applied to chemotaxis studies. These microsystems can generate well-defined and stable chemical concentration gradients at low fabrication costs, small reagent volumes, and high-throughput experimentation.

This work has utilized a microfluidic platform, the Bridged μLane System, to examine the in vitro migratory response of medulloblastoma (MB)-derived cells to external signaling of Epidermal Growth Factor (EGF) and the stromal cell-derived factor 1-alpha (SDF-1). Our microfluidic system can generate defined and controlled concentration gradient fields, which are categorized into five orders of magnitude along different positions of the microchannel. Within these gradient fields, the fraction of motile cells, directionality and cell path length of MB cells under EGF and SDF-1 stimulation were determined. The cellular responses to external signaling of EGF, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF-BB), and SDF-1 via motility studies were examined as well as the relative receptor expression for EGF-R, c-MET, PDGFR-BB, and CXCR4, respectively. MB cells were observed to have the potential to travel from high to low ligand concentration gradient fields. MB-derived cells responded strongly to EGF in a dosage and gradient-dependent manner with increased EGF-R activation. In addition, high concentration gradient fields of EGF caused more cells to move longer distances in a directional dependent manner as opposed to SDF-1. This work provides evidence that EGF and its receptor play an important role in MB migration than previously documented.
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CHAPTER 1. INTRODUCTION

1.1 Characteristics of malignant pediatric brain tumors:

Medulloblastoma (MB) is the most aggressive pediatric brain tumors that accounts for about 13%-20% of all childhood tumors, with over 400 cases diagnosed in USA annually (33, 44, 49). MB arises in the posterior cranial fossa, near the brainstem and cerebellum, parts of the brain that are responsible for cardiac and breathing cycle, and motor control (movement, balance, and coordination), respectively. MB tumors are characterized by a high rate of metastasis to the brain and the spinal cord (17). Clinical studies and magnetic resonance imaging (MRI) have shown that MB spreads from the cerebellum down to the spinal column (45, 72). MB first invades meninges, or the tissue that lines the brain and spinal column, before gaining access to the cerebrospinal fluid (CSF) (51, 68, 72). There, metastasis results from tumor cells that drop into the CSF and tend to “seed” in any part of the spine. MB cells migrate on the spinal column via combination of two-dimensional (2D) movements or crawling on the surface of the spine, and three-dimensional (3D) movements or infiltrating in the spinal column causing paralysis (11, 16, 23, 43).

MB cell migration is believed to be derived from so-called cancer stem-like cells (CSCs), which are the most aggressive cell type with marked capacity for proliferation, self-renewal, and differentiation in many malignant tumors, as previously reported in in-vivo and in-vitro studies (15, 45) (see Figure 1.1). CSCs are stimulated by complex and dynamic extracellular microenvironments responsible for the heterogeneity of brain tumors (45, 47, 60, 67). As a heterogeneous disease, MB is composed of 4 subtypes characterized by unique molecular and genomic features: Wingless (WNT), Sonic Hedgehog (SHH), Group 3, and Group 4. Among these, Group 3 patients have a poor
survival rate with 5-year survival chances being less than 30% while WNT achieves an overall 5-year survival rate of 60–85% (33, 49, 60). While multimodality therapeutic approaches including surgery, radiation, and chemotherapy have significantly increased survival rates, these treatments lead to threatening side effects such as impairments in language, attention, memory, motor functions, and major endocrinal deficiencies (26, 52, 56, 62).

**Figure 1.1. Schematic of the origin of brain tumors: development gone wrong.** Adapted from (1). (A) During normal development, stem cells give rise to neurons, oligodendrocytes, and Astrocytes. Genetic alternations occur within these cells that can lead to the rise of malignant brain tumors. (B) Medulloblastoma cells originate in the cerebellum, from granule neuron precursor cells (GNPCs), upon uncontrolled activation of Sonic Hedgehog (Shh) signaling pathway.

### 1.2 Mechanisms of Medulloblastoma migration:

The migration of cancer cells within the central nervous system (CNS) is decidedly complex, affected by cellular interactions with heterogeneous extracellular matrix (ECM) as well as mixed cellular responses to concentration fields of biomolecules (1). The
migration of MB-derived cells along gradients of bimolecular concentration, i.e. chemotaxis, remains incompletely understood (1). Chemoattractants can be secreted by neighboring healthy or oncogenic cells to stimulate MB migration away from the primary tumor (1). In particular, MB migration towards the vasculature is believed to fuel high chemoresistivity and recurrence (1).

MB chemotaxis has been largely examined with respect to biomolecules that stimulate the migration in glioblastoma (GL), the most common brain tumors diagnosed in adults (28). An established body of research has demonstrated GL sensitivity to external signaling initiated by PDGF, HGF, and EGF, and to a lesser extent SDF-1 (6). PDGF inhibitors have been reported to decrease the ability of both GL and MB to cross the blood-brain barrier and target tumor cells (4, 9, 21, 27, 63), while c-MET inhibition has been widely documented in MB and GL cells that do not respond to external signaling via migration (38, 69). Further, it has been reported that overexpression of the engineered EGF-R homolog, ErbB2, can increase MB and GL migration in-vitro (7, 12, 20), while transactivation of EGF-R via PDGFR-B activity was able to enhance migration, survival, and proliferation of MB and GL cells in-vitro (4, 20). Drugs such as c-met inhibitors (SGX523) (29, 66), PDGFR inhibitors (Sunitinib) (3, 46), CXCR4 inhibitors (Plerixafor) (13, 58), EGFR inhibitors (Tarceva) (18, 25), etc., have all been extensively studied to observe MB and GL tumor growth, invasiveness and metastasis. In addition, Estradiol (17beta-estradiol) occurs in the body and brain, and mediates different functions (5). The binding of estradiol to estrogen receptor, and non-steroid receptor (e.g. EGFR) activates downstream signaling events (RAS and PI3K) (5, 20). Estrogen influences the growth and migration of MB (5, 8). Similarly, anti-CXCR4 therapies (using AMD 3100–
noncompetitive antagonist of CXCL12 binding to CXCR4) showed inhibition of MB tumors and decrease in tumor proliferation (53). Research has also shown that the binding of CXCL12 to CXCR4 induces intracellular signaling pathways initiating signals related to chemotaxis, cell survival and/or proliferation, increase in intracellular calcium, and gene transcription (53, 58). EGF has been understudied in MB tumor growth and metastasis. However, our group has shown in previous studies that EGF concentration gradients modulate chemotaxis of MB-derived cells in a dose-dependent manner via EGFR (20).

1.3 Microfluidic devices and Chemotaxis:

Microfluidic devices have been widely studied in the last two decades and have become one of the most commonly used bench top method of various fields of research (14, 30). Microfluidics started out as technique for specific applications and tool in fundamental chemical, biological and pharmaceutical research (32). Whitesides and coworkers have revolutionized this field by developing and introducing poly-dimethylsiloxane (PDMS) to the field of microfluidics (48, 55, 64). Due to the physical (transparent), mechanical (flexibility), and chemical (non-toxic and biocompatible) properties of PDMS, this material is widely used to make devices for biological applications (55). PDMS has been adopted to make microfluidic devices capable of generating concentration gradients for use in chemotaxis experiments (42, 64).

Chemotaxis is defined as the directed cell migration by soluble gradients of chemoattractants (growth factors and chemokines). It is the driving force behind cellular movements that occur during inflammatory immune responses, wound repair, angiogenesis, embryogenesis, and invasion in cancer metastasis (31, 34, 65). An
important field of biomedical studies consists in identifying chemoattractant concentration gradients that maximize cell migratory behavior. Several groups have previously used time-lapse video-microscopy to observe cell polarity, motility, and ability to detect and respond to gradients of chemoattractants. In response to uniform stimulation with chemoattractants, the cells become elongated and polarized, with clear leading and trailing ends, and their random motility increases (chemokinesis) (24, 31, 57). When cells are confronted with a chemoattractant concentration gradient, they undergo chemotaxis, since these migrate toward high concentrations of chemoattractants (See Figure 1.2) (24, 31, 40).

![Figure 1.2](image)

**Figure 1.2. Illustration of chemoattractant gradients on cell motility.** When cells sense chemoattractant gradients, the polarized ones migrate toward high chemoattractant concentration (indicated by intensity of red color). The front (F) and back (B) edges of a cell are indicated in this picture. Adapted from (31).
In the past decades, different researchers have used conventional methods to measure cell migration, but these techniques have been found limited to study chemotaxis. Thus, microfluidic devices have been implemented to study cell chemotaxis.

1.3.1 Traditional migration assays:

In the past 50 years, various conventional bioassays have been developed and utilized to examine cell migration induced by chemoattractant molecules (41, 50). However, most of these methods lack the ability to maintain and control chemoattractant concentration gradients, and do not allow real-time quantification of cell migration at the single-cell level.

1.3.1.1 Boyden Assay:

Boyden chamber assays or transwell assays have been used as a high-throughput screen of multiple ligand concentrations and incubation times since 1962 (41, 50). It is composed of a cylindrical cell culture insert (filter) installed into a well of a cell culture plate. The insert and well are separated by a porous membrane with a defined pore size (See Figure 1.3 (A)). Cells are seeded on top of the insert in serum-rich media, while serum-free media mixed with chemoattractant is placed in the well. Motile cells move through the porous membrane toward the chemoattractant, then these are stained and quantified. In this assay, chemoattractant gradients are nonlinear, transient, and difficult to determine. In addition, Boyden assays do not allow researchers to image cells in real time, limiting the scope of what the assay can accomplish (41).
1.3.1.2 Zigmond Assay:

Developed in 1972, this commonly-used migration assay allows researchers to visualize cells during migration. The Zigmond chamber consists of a slide with two closely spaced rectangular reservoirs connected by a bridge (41, 50) (See Figure 1.3 (B)). A concentration gradient is established along the length of the bridge by filling one reservoir with media and the other with a chemoattractant (61, 71). The cells attached to the chamber can be visualized using a microscope when these cells migrate towards the chemoattractant. However, several problems with this method include gradients poorly controlled over time and evaporation of reagents (open chamber) (41).

Figure 1.3. Schematic of conventional migration assays. (A) Boyden assay, (B) Zigmond chamber, (C) Dunn chamber, and (D) Micropipette assay. Adapted and modified image from (50). Arrows (in red) point to the direction of cell migration.
1.3.1.3 Dunn Chamber:
The Dunn Chemotaxis Chamber was designed to improve optical properties and long-
term stability of the gradient in 1997 (41, 70). It consists of a glass slide with two
concentric rings ground into the center of the slide with a depth half the thickness of the
slide (See Figure 1.3 (C)). The inner ring of the chamber is filled with serum-rich medium
while the outer ring chamber is filled with serum-free medium mixed with chemoattractant
(10, 50). Then, a linear diffusion gradient is developed and maintained for few hours.
However, this method does not allow to measure quantitatively the ligand gradients at
different positions over time.

1.3.1.4 Micropipette:
The micropipette assay has been used to generate chemoattractant gradients by the
release of these molecules from micropipettes (54). This method provides a localized
source of diffusible molecules for in-vivo and in-vitro studies (See Figure 1.3 (D)). This
method allows direct visualization of cells but it is very irreproducible and uncontrolled
due to the angle, size, and flow rate injected (54).

1.3.2 Types of microfabricated devices
Unlike conventional cell migration assays, microfluidic gradient devices can create
defined concentration gradients of chemoattractants. In addition, these devices allow
more efficient and quantitative evaluations of cell migration in spatial-temporal domains
of complex chemoattractant fields that better mimic in-vivo conditions (39). There are two
types of microfluidic devices: flow-based devices and non-flow based devices.
1.3.2.1 Flow-based microfluidics:

These devices create stable gradients based on controlled mixing (laminar flow) of different chemical reagents. Particularly, mixing of laminar flows occurs across the width of the microfluidic channel which allows to precisely control stable chemical concentration gradients across the channels (41). In addition, the gradient profile is relatively stable over long distances along the channel. Some examples of flow-based microfluidics are three channel microfluidic devices (19, 22, 41), in which linear gradient is generated in the absence of flow or T-channel device that monitors chemotaxis perpendicular to the direction of the fluid flow (19), were developed subsequently.

1.3.2.2 Non-flow based microfluidics:

These devices generate time-evolving or stable chemical concentration gradients in a flow-free environment. They use large chemical reservoirs, and allow chemoattractants and media to mix by free diffusion in microfluidic channels (41). Because of the small dimensions of microfluidic channels compared to the volume of chemical sources, the development of gradient over time can be controlled and gradients can be established at the equilibrium state of diffusion (41). The use of 2D or 3D substratum between the reservoirs and mixing channels, better mimic the tissue environment and thus are more physiological relevant for particular cell types (41). Some examples of flow-free microfluidics are digital microfluidics such as the free-flow electrophoresis device, which control fluid flow by applying a continuous electric field between 2 insulated plates (35).
1.3.2.3 The Bridged \( \mu \)Lane System:

Our group developed a microfluidic device, called the Bridged \( \mu \)Lane System, a user-friendly platform to study cell migration. One of the advantages of this microsystem is that it creates steady-state concentration gradients of chemoattractants along the length of the microchannel as a function of time that approximates the microenvironments generated in-vivo. In addition, it allows us to examine and predict local ligand gradients at any cell position over time. This system contains 2 layers of a silicon-based organic polymer (PDMS). The top layer is composed of a bridge channel in between two chambers while the bottom layer consist of a microchannel (cross sectional area: 100\( \mu \text{m} \times 100\mu \text{m} \)) in between two reservoirs (see Figure 1.4). The elastomeric PDMS is bonded to a glass slides which serves as a bio-compound coating used to coat surface with different extracellular matrices (e.g. matrigel, laminin, fibronectin, collagen).

Figure 1.4. Image of elastomeric PDMS microchannel (\( \mu \)Lane). The first layer of PDMS is composed of two chambers while second layer of PDMS consists of a microchannel and two reservoirs.
By controlling the bulk flow, we are able to determine different chemoattractant concentration gradients across cells, and to observe single-cell migration during experimental conditions. The bulk flow is caused by inertia during the loading of chemoattractants. Previously, our group measured this bulk flow (as laminar flow) with a low Reynolds number of less than $10^{-3}$, and a Peclet number between 10 and 50 when molecules between 6 to 10KDa are used (EGF or Dextran) (36). The large chambers’ volume along with bridge channel eliminate hydraulic pressure difference within microchannel due to chamber volume differences. Also, these large chambers’ volume maintain stable chemoattractant concentration gradients within the microchannel. The transport of chemoattractant or ligands within the microchannel is predicted by the 1-Dimensional convective-diffusion equation via computational models (36).

Previously, our group has used this microfluidic device to measure cell proliferation, survival, and migration of fibroblast cells (37), glioblastoma cells (2), retinal progenitor cells (59), and Medulloblastoma cells (20) to concentration gradients of extracellular signaling molecules. This microsystem follows into the flow-free microfluidic devices category. It does not required fluidic control tools such as pressure pumps, peristaltic pumps, or valves to maintain steady-state concentration gradients of chemoattractant molecules. The computational models along with experimental measurements allow us to measure quantitatively local chemoattractant concentration and gradients and observe cell responses to temporal ligand gradients, which greatly benefit cell migration studies unlike traditional migration assays.
1.4 Overview:

In this thesis, we will discuss the uniqueness of medulloblastoma migration and summarize the main *in-vitro* and *in-vivo* studies (Chapter 1). We will describe in detail all methodologies and materials carried out by the authors for this thesis (Chapter 2). The aim of this thesis is to identify new therapeutic targets for Medulloblastoma (MB) metastasis towards controlled microenvironments of chemotactic agents previously believed insignificant for MB migration (Chapter 3). First, the *in-vitro* cellular responses of MB-derived cells to external signaling of Epidermal Growth Factor (EGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF-BB), and the stromal cell-derived factors 1-alpha (SDF-1) will be described via motility and immunocytochemistry studies (Chapter 3). Likewise, the *in-vitro* migratory responses of MB-derived cells to similar concentrations of EGF and SDF-1, and controlled concentration gradient fields within our microfluidic device will be described in detail (Chapter 3). Lastly, in Chapter 4, a summary of main findings as well as the development of a new microfluidic system will be discussed for future investigations.
1.5 References:


CHAPTER 2. MATERIALS AND METHODS

2.1 Medulloblastoma Cell Culture:
In vitro cell culture was maintained using the human medulloblastoma (MB)-derived Daoy cell line (ATCC, Cat. No.HTB-186) \((3-5, 8)\). Daoy cells were cultured with Eagle’s Minimal Essential Medium (EMEM) (VWR, Cat. No.12001-582) supplemented with 45 mL of 10% fetal bovine serum (VWR, Cat. No.45000-734), 5mL of 1% Penicillin-Streptomycin (VWR, Cat. No.45000-650), and 10mL of 2% L-glutamine (VWR, Cat. No.45000-676). Daoy cells were detached from their substratum using Trypsin EDTA 1X (VWR, Cat. No. 45000-660). To grow intact monolayers, these cells were plated onto sterile plasma-treated polystyrene tissue culture flasks (VWR, Cat. No.BD353136), and incubated at 37°C with 95% humidity and 5% CO\(_2\). Daoy cells were maintained at a density of 1.5x10\(^6\) cells/mL. The cell medium was changed every 2 days, and cells were passaged every 3 days when reached at least 80% confluence.

2.2 Boyden Chamber Assay for Cell Migration:
The Boyden chamber assay or transwell assay was used to measure the number of MB cells that migrated towards different nano-concentrations of EGF, HGF, PDGF, and SDF-1. After splitting a confluent flask with Daoy cells, the cell pellet was disrupted to obtain individual cells by utilizing a cell strainer (VWR, Cat. No. 102095-532) for use with 50 mL centrifuge tubes, and cells were resuspended twice through the cell strainer. Daoy cells were seeded in the upper compartment and were allowed to migrate through the porous membrane (with pore size of 8-\(\mu\)m) into the lower compartment for 6 hours at 37°C in a
5% CO₂ incubator as displayed in **Figure 2.1 (A) and (B)**. The cell culture filters (VWR, Cat. No.62406-198) were inserted into wells of a 24-well plate, and were utilized to build up the Boyden chamber assay. Once MB cells were at least 80% confluent, approximately 1×10⁶ cells/mL were seeded in 300μl of EMEM complete medium in each upper chamber, while 700μl of serum-free medium (EMEM only) was loaded in each lower chamber.

**Figure 2.1. Schematic of transwell assay.** (A) The assay consists of a filter with a porous membrane inserted into well. (B) Growth factors diffuse upwards and cause migration of cells on the underside of porous membrane. (C) Daoy cells with stained nuclei and cytoplasm on the underside of the porous membrane and imaged for migration analysis. Scale bar = 100μm

For all migratory measurements, growth factor solutions were diluted in EMEM serum-free medium using serial dilutions. These consisted of concentrations of 1, 10, 100, and 1000 ng/mL of EGF (Life Technologies Corporation, Cat. No.E3476); HGF (R&D Systems, Cat. No.2207-HG/CF); PDGF-BB (R&D Systems, Cat. No.220-BB-010); and finally SDF-1 (PreproTech, Cat. No.250-20A). After 6 hours of incubation, the membrane
between the two compartments was fixed and stained with fixative solutions I (VWR, Cat. No. B4132-11A), which stained the cell cytoplasm and nuclei as shown in Figure 2.1 (C). Residues from the fixative solutions in the inner and outer part of the filters were removed carefully by using double-tip cotton swabs (VWR, Cat. No. 10806-004) without touching the bottom surface of the porous membrane where Daoy cells were located. The 24-well plates were covered with aluminum foil and stored in the refrigerator at 40ºF. Following, the number of cells that have migrated to the underside of the membrane was determined by using the checkerboard analysis (1, 2).

2.2.1 Imaging and Analysis:
Transmitted light microscopy images of Daoy cells were obtained using an inverted microscope equipped with short and long range objectives (Nikon TE2000) and a cooled CCD camera (CoolSNAP EZ CCD Camera, Photometrics, Tucson, AZ). Daoy cells were located within five areas of the culture inserts and counted upon each filter using the checkerboard pattern (1, 3, 10) for a total of five rectangular locations. These data were used to gather representative cell counts per nano-concentration of the growth factors measured (n=12 per nano-concentration).

2.3 Immunocytochemistry of Growth Factor Receptors:
Daoy cells were plated at a concentration of 1×10^5 cells/mL in EMEM complete, on borosilicate glass well plates (Lab-Tek, Cat. No.155383). In each confocal well, 500μL of cell solution was added to fully cover the bottom of each well. Thus, Daoy cells were allowed to attach to the wells during approximately 2 hours of incubation at 37°C in 5% CO₂. Two solutions were prepared to begin immunostaining: 0.1% Triton X-100 (Sigma-
Aldrich, Cat. No.X100) in 0.1% of Bovine Serum Albumin (BSA) in Phosphate Buffered Saline (PBS), and 1% blocking solution (BSA in PBS). After Daoy cells were fully attached to each well of the confocal plates, cells were exposed to ligand concentrations, which showed the largest numbers of motile cells in the Boyden chamber assay results. Prior to the immunocytochemistry assay, Daoy cells were exposed to 5 minutes of stimulation of 100ng/mL EGF (Life Technologies, Cat. No.PGH0311), 100ng/mL SDF-1 (Life Technologies, Cat. No.PHC1364), 10ng/mL HGF (Life Technologies, Cat. No.PHG0324), and 100ng/mL PDGF-BB (Life Technologies, Cat. No.PHG0044). Then, the supernatant was aspirated and each well was rinsed 3 times with 500μL of 1X PBS (Sigma-Aldrich, Cat. No.D8537). Each well was fixed using 10% paraformaldehyde (Sigma-Aldrich, Cat. No.HT501128) for 10 minutes, and rinsed twice with 1X PBS. The samples were permeabilized using 500μL of 0.1% Triton X-100 in BSA-PBS solution for 10 minutes, then blocked for 60 minutes with 1% BSA in 1X PBS, and rinsed twice with 500μL of the same blocking solution.

Then, samples were exposed to the primary antibody for each receptor studied for 2 hours at room temperature (22°C): 5μg/mL anti-EGFR (Life Technologies, Cat. No.700308), 5μg/mL anti-CXCR4 (Life Technologies, Cat. No.35-8800), 2μg/mL anti-c-Met (Millipore, Cat. No.07-2242), and 3μg/mL anti-PDGFR (Life Technologies, Cat. No.701142). These primary antibodies were diluted in Dako Antibody diluent with background reducing components (Dako North America Inc, Cat. No.S302281-2) at a concentration of 4μg/mL. Each plate was covered with 500μL of primary antibody solution diluted in Dako reducing background solution (same volume for secondary antibody). After primary antibodies were removed, each sample was rinsed 3 times with 300μL of 1X PBS. A fluorescent
secondary anti-rabbit IgG (Millipore, Cat. No.AP132F) was for used EGFR, c-Met, and PDGFR samples, while a fluorescent anti-mouse secondary antibody (Millipore, Cat. No.MAB1976) was used for CXCR4 samples. All samples were exposed to the secondary antibody (diluted in Dako antibody diluent solution) at a concentration of 4μg/mL for 30 minutes at room temperature (22°C), and then rinsed twice with 300μL of 1X PBS. Finally, two drops of nuclear staining (Life Technologies, Cat. No.R37605) per well was added for 20 minutes at room temperature (22°C), and then the samples were rinsed twice with 300μL of 1X PBS and 700μL of glycerol were added (Life Technologies, Cat. No.15514-011) for preservation, and confocal plates were covered with aluminum foil and stored in the refrigerator at 40°F prior confocal measurements.

2.3.1 Imaging and Analysis:
Fluorescent imaging of Daoy cells was performed using Zeiss LSM 710 confocal microscope with a 63x oil immersion objective. The National Institutes of Health (NIH) ImageJ software was utilized to measure the fluorescence intensity of basal membrane receptors (EGF-R, c-Met, PDGFR-BB, and CXCR4) using an average over the entire cell area.

2.4 Bridged μLane System:
The bridged μLane is a microfluidic system utilized to image the real-time migratory responses of individual Daoy cells within microenvironments of defined gradient profiles of growth factor solutions (EGF and SDF-1). It is fabricated from Polydimethylsiloxane or PDMS via contact photolithography (1, 6, 7). In brief, the system consists of two layers of PDMS bonded to a glass slide using ozone gas exposure (see Figure 2.2). The first layer
of PDMS consists of a closed microchannel of 100μm-width and 1.3cm-length (0.1μL), which connects the source (SRR) and the sink (SKR) reservoirs (9μL each). The second layer of PDMS consists of the source (SRC) and the sink (SKC) chambers (170μL each), connected by an open bridge channel which sustains the hydrostatic equilibrium of the system (6). Both SRC and SKC are vertically and fluidically connected with SRR and SKR, respectively.

Figure 2.2. The μLane system used to measure cell migration. (A) Image of top view of the first layer PDMS bonded onto a glass slide. Two 9-nL reservoirs are connected by a microchannel of 13mm in length and 150μm in diameter. (B) Image of top view of second layer PDMS bonded to the first layer. The source and sink chambers are connected by a bridge channel.

2.4.1 Microfabrication Techniques:

Soft lithography, also known as micromolding was used to replicate the structure of Bridge μLane system using elastomeric polydimethylsiloxane or PDMS molds (Fisher Scientific, Cat. No. NC9644388). The base and curing agent were mixed at particular ratio (10:1, respectively), and then degassed using a vacuum desiccator for 40 minutes to remove bubble formed due to mixing (see Figure 2.3). When bubbles were removed from the mixture completely, the liquid PDMS was poured into an aluminum mold and allowed to
solidify in a convective oven at 85°C for 1 hour. Then, the double-layered PDMS with patterns was detached from the mold, and ready to proceed with bonding.

Figure 2.3. Microfabrication of bridged μLane system. (A) Sylgar-184, base and curing agent in liquid state, were mixed and poured into a 50-mL centrifuge tube, then placed in (B) a vacuum desiccator to remove bubbles. (C) Two aluminum cast masters were used to make the first and second layer of the μLane system. (D) The liquid-state PDMS without bubbles was poured around the aluminum mold. (E) The microchannel chambers were created by using 0.1-10μL micro pipet tips while elastomeric PDMS was inside a convection oven. (F) Elastomeric PDMS mold of μLane system prior bonding to a pre-cleaned glass slide.

One critical step in microfabrication is the bonding of the PDMS channels to a glass slide. The glass slides were chemically cleaned with Nano-Strip (VWR, Cat. No. 10135-756), and left in solution overnight. The next day, these glass slides were rinsed with deionized water and dried with a nitrogen tank (Airgas USA, Cat. No.007761). Bonding of PDMS channels to glass slides was achieved using ozone gas exposure (Electro-Technic
Product, Inc., Model BD-10A). The bottom surface of microchannel or first PDMS layer that contains microchannel was exposed to 30 seconds of ozone while the pre-cleaned glass slide was exposed to 30 seconds of ozone before bonding both surfaces together.

2.4.2 Operation and Experimental Set up of Bridged μLane System:

In order to mimic the cell microenvironment, μLanes were coated with 10-μg/mL of laminin (Becton, Dickinson and Company, Cat. No.354232), which was allowed to gel for one hour at room temperature (25°C) under sterile conditions. In 2D coating experiments, the unbound laminin was aspirated from the channels. A cell solution (1x10^6 cells/mL) was manually injected into the SRR, microchannel, and SKR using a 1-mL syringe (VWR, Cat. No.BD309659). Daoy cells were allowed to attach, then cell culture media was used to fill the SRR, microchannel, SKR, SRC, SKC, and the bridge channel (see Figure 2.4).

The bridged μLane system operates by manually inserting ligand solutions (growth factors) drop-wise into the SRC until the solution makes contact with the cell culture media solution within the bridge channel and the SKC, to initiate the molecular transport within the system via uniaxial bulk convection and diffusion (6). The small differences in the density of the reagents and in the liquid levels in the SRR and SKR generate hydrostatic pressure differences that initiate an ultra-low bulk flow within the microchannel in the first layer (6). This diminutive bulk flow facilitates the transport of ligand solutions from the SRR to the SKR, to accelerate the time required to attain a steady-state gradient profiles of ligand solutions along the 13-mm length of the microchannel.
2.4.3 Mathematical Modeling:

The bridged μLane system has been computationally modeled via finite-element analysis (FEM) in Matlab 7.7 (MathWorks, Natick, MA) and verified experimentally previously by our group (3, 6, 7). Previous experimental measurements carried out by our group with fluorescently labeled Dextran solutions (MW ~10 KDa) were added to the μLane system to measure diffusivity within the microsystem across three different channel sections over time for achieving steady-state transport (3, 6). The diffusivity (0.82 x 10^6 cm^2/sec) and the bulk velocity (0.37 μm/sec) values of fluorescently labeled Dextran, previously measured by our group (6) were utilized to solved the two-dimensional Fick’s law of convective-diffusion equation with MatLab to generate the EGF or SDF-1 concentration distribution along the length of the entire microchannel. The evolution of the gradient distribution along our microsystem (see Figure 2.5 (A)), displayed a non-linear distribution as a function of time and position across the microchannel. In addition, both EGF and SDF-1 gradient distribution is displayed after the system reached steady-state concentration distribution.

Figure 2.4. Schematic of Bridged μLane system. Schematic of the entire system, side view, showing loaded cell within SKR and SRR reservoirs, and adhered along the microchannel. Ligand solutions are loaded into the SRC, and transported to SKR to reach steady-state concentration distribution.
after 18 hours (6, 9) (see Figure 2.5 (B)). Note that the ligand concentration at 18 hours in the region of the channel closest to the SKR is approximately 63% of the starting EGF or SDF-1 concentration.

Figure 2.5. Modeled concentration gradients of bridged μ-Lane system. (A) Normalized EGF concentrations plotted against increasing distance from the source reservoir (SRR) to the sink reservoir (SKR) as a function of time (3, 6, 7, 9). Note that the ligand concentration at 18 hours in the region of the channel closest to the SRR is approximately 63% of the starting EGF concentration. (B) Normalized concentration distribution of EGF and SDF-1 along the length of microchannel. Concentration gradients were defined by five regions with different order of magnitude in the microchannel.

The initial EGF or SDF-1 concentration within the source chamber, sink reservoir, microchannel and source reservoir was set to 0-ng/mL, since no ligand solution was manually inserted. The initial ligand concentration within the source chamber was set to 100-ng/mL to reflect the sample concentration used during experiments. These boundary conditions were solved using Equation (1), as shown below (6, 7):
Where $C$ (ng/mL) is ligand concentration (EGF or SDF-1), $t$ (hours) is total time of experiment (24 hours), $u$ (μm/sec) is fluid velocity or bulk velocity, and $D$ (cm$^2$/s) is the diffusion coefficient, or diffusivity, of the ligand solution.

2.4.4 Imaging and Analysis

Transmitted light image data was analyzed using Nikon software (Nikon Instrument Element 2.30 with 6D module, Morrell Instrument Company Inc., Melville, NY). In addition, Image J was utilized to track Daoy cells and process images with Chemotaxis and Migration Tool plugin (ImageJ 1.46r). Chemotaxis parameters such as the center of mass (averaged point of all cell end points) and the maximum accumulated distance (cell path length) were generated from the tracking software. Bright field images of the microchannel were automatically captured every hour for 24 hours at every 1000 microns in the y-direction of the μLane, followed by cell tracking ($n_{cells}$~180 cells) and analysis. Also, the cell tracking software was used to develop Wind-Rose plots of cell trajectories in response to EGF and SDF-1 gradient fields, over 24 hours.

2.5 Statistical Analysis:

One-way ANOVA and Post Hoc Test (Tukey) using IBM SPSS Statistics Program (IBM Corp., Released 2011, Version 20.0. Armonk, NY) were utilized to analyze the statistical significance across growth factors. The $p$-values < 0.05 were considered statistically significant. For the immunostaining of growth factors data, the significance was
evaluated using standard deviation and unpaired student’s t-test at a 95% confidence interval using IBM SPSS Statistics Program (IBM Corp., Released 2011, Version 20.0. Armonk, NY).
2.6 References:


CHAPTER 3. EGF AS A NEW THERAPEUTIC TARGET FOR MEDULLOBLASTOMA METASTASIS

Jennifer Rico-Varela
Email: jrico00@citymail.cuny.edu

Tanya Singh
Email: tsingh09@citymail.cuny.edu

Sean McCutcheon
Email: smccutc00@citymail.cuny.edu

Maribel Vazquez
Email: vazquez@ccny.cuny.edu

1Department of Biomedical Engineering, The City College of New York, 160 Convent Avenue, ST-403D, New York, NY 10031

* Corresponding author. The City College of The City University of New York, New York, NY

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ABSTRACT

Medulloblastoma (MB) is a malignant pediatric brain tumor known for its aggressive metastatic potential. Despite the well-documented migration of MB cells to other parts of the brain and spinal column, MB chemotaxis is poorly understood. Herein, we examined the in vitro migratory and cellular responses of MB-derived cells to external signaling of Epidermal Growth Factor (EGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF-BB), and the stromal cell-derived factors 1-alpha (SDF-1). Experiments utilized transwell assays and immunocytochemistry to identify receptor activation in MB migration, and used a microfluidic platform to examine directionality, trajectory, and gradient-dependence of motile cells. Data illustrates that MB-derived cells respond strongly to EGF in a dosage and gradient-dependent manner with increased EGF-R activation, and show that high EGF gradient fields cause an increased number of cells to migrate longer directed distances. Our results provide evidence that EGF and its receptor play an important role than previously documented in MB chemotactic migration and should be considered for developing migration-target therapies against MB metastasis.

Keywords
Pediatric cancer; chemotaxis; microfluidics; gradients.
3.1 INTRODUCTION

Medulloblastoma (MB) is a family of highly-invasive tumors most commonly diagnosed in the pediatric central nervous system (7, 28, 38, 41, 50). While clinical treatments have more than doubled the overall 5-year survival rate to upwards of 60% (16, 28, 44, 52), additional therapies are needed to target the aggressive MB migration that is uncharacteristic of other brain tumors, but is a hallmark of MB recurrence, metastasis, and radioresistivity (13, 21, 24, 28, 41, 55, 61, 70). The migration of cancer cells within the CNS is decidedly complex, affected by cellular interactions with heterogeneous extracellular matrix (ECM) as well as mixed cellular responses to concentration fields of biomolecules (1, 14, 19, 60, 65). However, the mechanisms behind the migration of MB-derived cells along CNS gradients of biomolecular concentration, or chemotaxis, remain understudied and incompletely understood (14, 28). Particular complexities arise in MB, because chemoattractant fields that rouse cells away from primary tumors can be secreted by healthy or transformed distant and neighboring cells (1, 3, 9, 34, 58, 76). Further, clinical studies have illustrated that the spread of MB cells occurs predominantly to the spinal column via a combination of two-dimensional (2D) and three-dimensional (3D) movements (6, 51, 67, 77, 83). Such spreading results from tumor cells that drop into the cerebrospinal fluid and tend to seed in parts of the spine (53, 74, 77, 79, 84). As a result, the movement of MB cells on the surface of the spinal column can be examined on 2D substrata to plausibly physiologically approximate the metastatic behavior of these pediatric brain tumors.

Traditional cell migration studies have looked to transwell (27, 48, 81) and wound healing
assays \((36, 54, 78)\) to report numbers of cells that become motile in response to external signaling from growth factors, such as Epidermal Growth Factor (EGF) and Platelet-Derived Growth Factor (PDGF) \((4)\). However, chemotaxis is becoming more-commonly examined via the physical microenvironment of the cell, where dynamic concentration fields facilitate ligand-receptor bindings which initiate signal transduction cascades \((1, 8, 59)\). Here, precise manipulation of the cell microenvironment has been facilitated by the wide-adaptation of benchtop microfluidic devices \((26, 27, 29)\), which enable multifaceted evaluation of cell migratory behaviors in lieu of cell numbers alone. A large number of laboratories have demonstrated concentration- and concentration gradient-dependent behavior of non-cancerous cells, such as fibroblasts \((33)\), retinal progenitor cells \((69)\) and keratinocytes \((64)\), as well as tumors found in breast \((57)\), colon \((17)\) and CNS cancers \((2)\). Our own laboratory has illustrated that select populations of cells derived from CNS tumors can respond acutely via migration to ultra-low concentration gradients of select chemoattractants \((2, 14, 33)\), while others have shown greater chemotactic response with specific dosage \((3, 49, 56)\). Microfluidic analysis is, thus, well positioned to meaningfully aid in the development of migration-targeted therapies for MB via insight of migratory parameters relevant to metastasis, such as cell distance traveled, motility, gradient-sensitivity, ECM interaction and numerous others.

In this work, we examine MB migratory behavior in response to external signaling from 4 of the most extensively studied chemoattractants of CNS tumors using benchtop assays and microfluidics: Epidermal Growth Factor (EGF), Platelet-derived Growth Factor (PDGF), Hepatocyte Growth Factor (HGF), and Stromal Derived Growth Factor (SDF-1 or CXCL12). Our results illustrate that MB-derived cells exhibit gradient-dependent
behavior in EGF fields, which are able to guide MB along longer migration distances with superior directionality and increased receptor activation. These results distinguish EGF as a principal molecule with meaningful potential impact as an anti-migratory therapeutic to MB metastasis.

3.2 MATERIALS AND METHODS

Cell culture

In vitro cell culture was maintained using Daoy cell line (ATCC, Cat. No.HTB-186) (14, 60), Eagle’s Minimal Essential Medium (EMEM) (VWR, Cat. No.12001-582) supplemented with 10% fetal bovine serum (VWR, Cat. No.45000-734), 2% L-glutamine (VWR, Cat. No.45000-676) and 1% Penicillin-Streptomycin (VWR, Cat. No.45000-650). Intact monolayers were maintained and harvested cells were seeded onto sterile polystyrene tissue culture flasks (VWR, Cat. No.BD353136). MB cells were incubated at 37 °C with 5% CO₂ with cell medium changed every 2 days. Cell migration experiments were initiated with cells that were inserted into microchannel after MB cells reached 80% confluence.

Cell Migration Assay

The Boyden chamber assay (2, 14) was used to measure the number of MB cells that migrated towards different concentrations of external growth factors. This widely-used assay consists of two compartments filled with EMEM medium and separated by an 8 micron-porous membrane (VWR, Cat. No.62406-198), as shown in Figure 3.1.A. MB cells were seeded in the upper compartment and were allowed to migrate through the porous membrane into the lower compartment for 6 hours at 37 °C in a 5% CO₂ incubator.
Approximately 1x10⁶ cells/mL were seeded in 300µl of EMEM complete medium (EMEM with FBS) in each upper chamber, while 700µl of serum-free medium (EMEM only) was pipetted into each lower chamber. Lower chambers also contained concentrations of 1, 10, 100, and 1000 ng/mL of EGF (Life Technologies Corporation, Cat. No.E3476); HGF (R&D Systems, Cat. No.2207-HG/CF); PDGF-BB (R&D Systems, Cat. No.220-BB-010); and finally SDF-1 (PreporTech, Cat. No.250-20A). All growth factor solutions were diluted in EMEM serum-free medium using serial dilution. After 6 hours of incubation, the membrane was fixed and stained with fixative solutions (VWR, Cat. No. B4132-11A), which stained the cell cytoplasm and nuclei. The number of cells that migrated to the underside of each membrane was determined by using the convectional checkerboard analysis (2, 14, 82).

**Immunostaining of Receptors**

MB cells were plated at a concentration of 1x10³ cells/mL in EMEM complete, on borosilicate glass well plates (Lab-Tek, Cat. No.155383). The cells were incubated for 2 hours at 37°C in 5% CO₂ to facilitate attachment. Adhered cells were exposed to ligands at concentrations that resulted in the largest numbers of motile cells in the transwell assay results. At 37°C, each cell plate was exposed to 5 minutes of: 100ng/mL EGF (Life Technologies, Cat. No.PGH0311), 100ng/mL CXCL12 (Life Technologies, Cat. No.PHC1364), 10ng/mL HGF (Life Technologies, Cat. No.PHG0324), and 100ng/mL PDGF-BB (Life Technologies, Cat. No.PHG0044). The supernatant was then aspirated and each well was rinsed 3 times with 0.5 mL phosphate buffered saline solution (PBS), (Sigma-Aldrich, Cat. No.D8537). Each well was fixed using 10% paraformaldehyde (Sigma-Aldrich, Cat. No.HT501128) for 10 minutes, and rinsed twice with PBS. The
samples were permeabilized using a 1% solution of Triton-X (Sigma-Aldrich, Cat. No.X100) and 0.1%BSA in PBS solution for 10 minutes, then blocked for 60 minutes with 1% BSA in PBS blocking solution and rinsed twice with the same blocking solution.

Samples were then exposed to the primary antibody for each receptor studied for 2 hours at 22°C: 5μg/mL anti-EGFR (Life Technologies, Cat. No.700308), 5μg/mL anti-CXCR4 (Life Technologies, Cat. No.35-8800), 2μg/mL anti-c-Met (Millipore, Cat. No.07-2242), and 3μg/mL anti-PDGFR (Life Technologies, Cat. No.701142). Each well was rinsed 3 times with 1% BSA blocking solution. A fluorescent secondary anti-rabbit IgG (Millipore, Cat. No.AP132F) was used for EGFR, c-Met, and PDGFR samples, and a fluorescent anti-mouse secondary antibody (Millipore, Cat. No.MAB1976) was used for CXCR4 samples. All samples were exposed to the secondary antibody at a concentration of 5μg/mL for 30 minutes at 22°C, and then rinsed twice with blocking solution. Nuclear staining (Life Technologies, Cat. No.R37605) was performed for 20 minutes at 22°C, after which the samples were rinsed twice with PBS and covered in glycerol (Life Technologies, Cat. No.15514-011) for preservation. In addition, the expression of EGFR was measured at different time points of 0, 14, 22, 36, and 42 hours. The immunocytochemistry assay was performed as described above using goat-anti mouse IgG secondary antibody (Life Technologies, Cat. No. A-11005) for EGFR.

**Bridged μLane and Experimental Set up**

Our microfluidic device, the μLane, was utilized to image the real-time migratory responses of individual MB cells within microenvironments of defined EGF and SDF-1 gradient profiles. The bridged μLane system operates via a combination of uniaxial bulk
convection and diffusion to achieve controlled chemical concentration gradients over time, as described previously (2, 32, 33). This mass transfer mechanism termed as convective-diffusion has been widely-studied by several groups for bioengineering applications, to determine the transport of differently sized solutes and proteins through the walls of capillaries (11, 12, 20) and arteries (25, 30, 39, 66, 80), skeletal muscle fibers (31, 35, 45), and intervertebral discs (18, 62, 63). The two-dimensional mass transport of ligands within the microsystem was modeled via finite-element-analysis (FEM) in Matlab 7.7 (MathWorks, Natick, MA) and verified experimentally as described previously by our group (32, 33).

The framework of the μLane system consists of two layers of Polydimethylsiloxane (PDMS) (Fisher Scientific, Cat. No.NC9644388) bonded to a glass slide using ozone gas. The first layer of PDMS consists of a closed microchannel of 100µm-width and 1.3cm-length with a volume of 0.1µL, which connects two fluidic reservoirs of 9µL each, called the source (SRR) and the sink (SKR) reservoir. The second layer of PDMS consists of two chambers of 170µL each, called the source (SRC) and the sink (SKC) chamber, connected by an open, hemispherical bridge channel to maintain the hydrostatic equilibrium of the system (32, 33, 69). Both chambers are vertically and fluidically connected with both reservoirs (Figure 3.3).

The μLane system works by using the larger volumes of the SRC, SKC and bridge channel on the second layer of PDMS to generate concentration gradients within the smaller volumes of the SRR, SKR and microchannel on the first layer (14, 32, 33, 69). After inserting cells along the microchannel length, the cell culture media is used to fill the
SRR, SKR, SRC, SKC, and the bridge channel. The ligand solutions (EGF or SDF-1) are then manually inserted drop-wise into the SRC until the solution makes contact with the cell culture media solution within the bridge channel and SKC to initiate the molecular transport within the system. The small differences in the density of the reagents and in the liquid levels in the SRR and SKR generate hydrostatic pressure differences that initiate an ultra-low bulk flow within the microchannel in the first layer (32, 33, 69). This minuscule bulk flow was measured to be 0.37 μm/sec using fluorescent beads, as described previously (32). Such a low bulk flow facilitates the transport of ligand solutions from the SRR to the SKR, to accelerate the time required to attain a steady-state gradient profile of ligand solutions along the 13-mm length of the microchannel. In the absence of this bulk flow, the transport of our reagent via diffusion alone would require over 470 hours to reach a steady-state distribution within the length of the μLane instead of the 18 hours measured, as reported by our group (14, 32). We note that because the time required for the overall system to reach steady-state is much larger than the time needed for steady-state concentration gradients to be generated within the μLane system alone. Thus, the operation of the system is ‘best’ described as ‘quasi-steady-state’ (5, 10, 40, 68, 71, 73). However, because this work focuses exclusively on smaller time scales of the microchannel only, the term steady-state would be used for simplicity. Mathematical models of reagent transport within our μLane system were performed via MatLab to determine the steady-state concentration distribution as seen in Figure 3.4.A. The initial EGF or SDF-1 concentration within the SKC, SKR, microchannel and SRR was set to 0-ng/mL, as per absence of ligand. The initial ligand concentration within the SRC was set to 100-ng/mL to reflect the sample concentration used during experiments. These boundary conditions were solved using Equation (1), as shown below (32),
\[
\frac{\partial C}{\partial t} + u \cdot \nabla C = D \nabla^2 C
\]  \hfill (1)

Where \( C \) (ng/mL) is ligand concentration, \( t \) (hours) is time, \( u \) (μm/sec) is fluid velocity, and \( D \) (cm\(^2\)/s) is diffusion coefficient, or diffusivity, of the reagent molecule. Experimental validation was also performed using fluorescently labeled Dextran (~ MW 10KDa), to confirm steady-state is reached in our system after 18 hours, and is maintained for several days (14, 32).

For our experiments, the \( \mu \)Lane system was coated with 10-μg/mL of laminin (Becton, Dickinson and Company, Cat. No.354232) (14, 60), and allowed to gel for one hour at room temperature (25°C) under sterile conditions. Unbound laminin was aspirated and cells were manually seeded into the microchannel using a 1-mL syringe (VWR, Cat. No.BD309659). A cell solution (1x10^6 cells/mL) was injected into the SRR and SKR. Cells were allowed to adhere and visibly spread prior to the initiation of the experiment as illustrated in Figure 3.3.D. Finally the bridge channel was loaded with EMEM complete medium to connect the SRC and SKC, initiating the system. In this work, EGF (100-ng/mL) or SDF-1 (100-ng/mL) was individually loaded drop-wise into the SRC and allowed to reach steady state in the microchannel for 18 hours at 37°C (32), prior to imaging of MB cell migration within this precise biochemical environment; therefore, all data is collected within steady-state concentration gradient fields for 24 hours. Our group has previously showed that MB cells migrate and proliferate \textit{in vitro} until 72 hours in the \( \mu \)Lane system (14).
Statistics

One-way ANOVA and Post Hoc Test (Tukey) were used to analyze the data using IBM SPSS Statistics Program (IBM Corp., Released 2011, version 20.0. Armonk, NY). A one-way ANOVA test at a 95% confidence interval was performed for statistical significance across growth factors. The Post Hoc Test (Tukey) was performed to determine the disparity among different groups. Only p-values <0.05 were considered statistically significant. Unpaired student’s t-test at a 95% confidence interval was implemented to determine significance of relative receptor expression using IBM SPSS Statistics Program (IBM Corp., Released 2011, version 20.0. Armonk, NY).

Imaging and Processing

Transmitted light microscopy images were obtained using an inverted microscope (Nikon TE2000) and a cooled CCD camera (CoolSNAP EZ CCD Camera, Photometrics, Tucson, AZ) with a 20X objective magnification (Nikon Plant 20X, Morrell Instrument Company Inc., Melville, NY). Fluorescent imaging was performed using Leica CLSM confocal microscope and a Zeiss LSM 710 confocal microscope, both at 63x magnification with oil immersion objective. Image J was utilized to track cells and process images (Chemotaxis and Migration Tool plugin (ImageJ 1.46r) (14, 15). Fluorescence intensity was measured using an average over the entire cell area via ImageJ. Transmitted light image data was analyzed using Nikon software (Nikon Instrument Element 2.30 with 6D module, Morrell Instrument Company Inc., Melville, NY) and ImageJ (NIH) Software. Bright field images of the microchannel were automatically captured every hour for 24 hours at every 1000 microns in the y-direction of the µLane, followed by cell tracking (n_{cells}~180 cells) and analysis. Lastly, the cell tracking software was used to develop Wind-Rose plots (33, 69)
of cell trajectories in response to EGF and SDF-1 gradient fields, over 24 hours.

3.3 RESULTS

This work examined the migratory and cellular responses of MB-derived cells in response to external signaling from EGF, HGF, PDGF-BB, and SDF-1.

Motility Studies

The first set of experiments measured the average numbers of MB-derived cells that migrated toward different concentrations of EGF, HGF, PDGF-BB, and SDF-1 through transmembrane assays, as illustrated in Figure 3.1 and Table 3.1. As seen, numbers of motile cells were statistically different in response to signaling from different concentrations of EGF and HGF when compared to controls (i.e. no ligand). Further, a mid-level concentration of 100-ng/mL of EGF was observed to attract the largest numbers of motile cells overall (Fig. 3.1.B.1). By contrast, the number of MB cells that migrated in response to external signaling from concentrations of SDF-1 did not exhibit statistical difference from controls or each other (Fig. 3.1.B.4). Similarly, the number of motile MB in response to signaling from different concentrations of PDGF-BB did not display statistical differences from controls (Fig. 3.1.B.3).
Figure 3.1. Migration of MB-derived cells to different concentrations of selective chemotactic ligands. (A1) Schematic of transwell assay with motile cells attached to the underside of the porous membrane. (A2) Stained nuclei and cytoplasm of motile MB-derived cells toward different concentrations of (B1) EGF, (B2) HGF, (B3) PDGF, and (B4) SDF-1. The control groups indicate number of cells that migrated towards serum-free medium. An asterisk (*) indicates statistically significant data with p-values <0.05 against control group.

Table 3.1. Average numbers of MB-derived cells that migrated in transwell assays towards different concentrations of examined growth factors. Values were experimentally measured and are shown with mean and standard deviation. (*) denotes growth factor concentrations not measured in the current study.

<table>
<thead>
<tr>
<th>Growth Factors</th>
<th>0 ng/mL</th>
<th>1 ng/mL</th>
<th>10 ng/mL</th>
<th>100 ng/mL</th>
<th>1000 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>102 ± 5</td>
<td>*</td>
<td>156 ± 5</td>
<td>212 ± 7</td>
<td>174 ± 5</td>
</tr>
<tr>
<td>SDF-1</td>
<td>60 ± 2</td>
<td>67 ± 6</td>
<td>69 ± 14</td>
<td>72 ± 5</td>
<td>74 ± 4</td>
</tr>
<tr>
<td>HGF</td>
<td>99 ± 5</td>
<td>126 ± 10</td>
<td>160 ±13</td>
<td>122 ± 7</td>
<td>*</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>82 ± 5</td>
<td>83 ± 5</td>
<td>93 ± 8</td>
<td>100 ± 5</td>
<td>*</td>
</tr>
</tbody>
</table>
Relative Receptor Expression

The basal expression levels of the respective receptors were then compared against their activation levels upon ligand stimulation. Confocal images in Figure 3.2 illustrate differences in cellular distribution of activated receptors, as well as differences in fluorescent intensity after ligand stimulation. As seen, basal receptor expression appears to be uniformly distributed throughout the cell cytosol, with lowest intensities measured for EGF-R. Upon ligand activation, receptor expression is increased, but remains largely distributed throughout the cytosol. However, EGF-R expression is also observed to be acutely concentrated along the outer cell membrane. Analysis of fluorescence intensity then represented relative receptor expression levels upon ligand-stimulation. MB stimulated with EGF exhibited the highest increase in receptor expression for EGF-R, with a 3.5-fold increase in intensity over basal EGF-R expression measured in unstimulated controls (Fig. 3.2.A). By contrast, the expression of CXCR4, the receptor for SDF-1, was approximately 2-fold higher than its basal intensity levels in cells unstimulated with ligand (Fig. 3.2.B), while no statistical difference was measured between the activation levels of c-Met, the receptor for HGF (Fig. 3.2.C), and control cells, as well as between PDGFR-BB (Fig 3.2.D) in ligand-stimulated cells compared to controls.
Figure 3.2. Receptor activation within motile MB-derived cells. Immunocytochemistry of basal receptor expression of MB cells without ligand stimulation to (A1) EGF, (B1) SDF-1, (C1) HGF, and (D1) PDGF-BB. Receptor activation post-stimulation with (A2) EGF, (B2) SDF-1, (C2) HGF, and (D2) PDGF-BB. The expression level of receptor following ligand stimulation of (A3) EGFR, (B3) CXCR4, (C3) c-Met, and (D3) PDGFR-BB normalized to basal control levels. Scale bars are 100µm. An asterisk (*) indicates statistically significant data with p-values <0.05.
Migratory response to controlled concentration and gradient fields

The migratory behavior of MB was next examined using our microfluidic system, called the \( \mu \)Lane and shown in Figure 3.3, for real-time cell imaging and analysis. The transport of EGF and SDF-1 along the \( \mu \)Lane was modeled computationally, and verified experimentally as previously reported by our group (32). The system produced a range of concentration gradients along the microchannel length at steady-state, as shown in Figure 3.4.A (14, 33). Concentration gradients of this study are defined as the average difference in growth factor concentration (ng/mL) along the microchannel length (mm). Five orders of concentration gradient, \( G_1 \)–\( G_5 \), were delineated along the microchannel as illustrated in Figure 3.4.A: \( 10^{+1} < G_1 < 10^0 \) ng/(mL.mm), \( 10^0 < G_2 < 10^{-1} \) ng/(mL.mm), \( 10^{-1} < G_3 < 10^{-2} \) ng/(mL.mm), \( 10^{-2} < G_4 < 10^{-3} \) ng/(mL.mm), and \( 10^{-3} < G_5 < 0 \). The lowest gradient, \( G_5 \), was located near the source reservoir (growth factors only) and occupied approximately 1-mm-length of the microchannel, while the highest gradient, \( G_1 \), was located near the sink reservoir and occupied an approximate, 3-mm-length of microchannel. Concentration gradients, \( G_2 \), \( G_3 \), and \( G_4 \), occupied the remaining 9-mm-length of microchannel (distance in between the source and the sink reservoirs) with approximately 4 mm, 3 mm and 2 mm segments, respectively. Note, the core distributions of EGF and SDF-1 along the microchannel are very similar to one another given their respective molecular weights of 6.045 KDa and 7.9 KDa, and hence only one representative gradient distribution is shown. Further, cells were evenly distributed along all segments of the channel prior to the start of experiments.
Figure 3.3. The μLane system and MB migratory responses. (A) Schematic of the bridged μLane system, showing cells inserted within the sink (SKR) and source (SRR) reservoir, and adhered along the microchannel. Chemotactic agents (e.g. EGF, SDF-1) are loaded into the source chamber (SRC), and transported to SKR to reach steady-state concentration distribution. (B) Top view image of the first layer PDMS bonded onto a glass slide. Two 9-nL reservoirs are connected by a microchannel of 13mm in length and 100µm in diameter. (C) Top view image of second layer PDMS bonded to the first layer. The source (SRC) and sink (SKC) chambers are connected by a bridge channel. (D) Raw data image of motile MB cells within μLane system at (D1) source reservoir, (D2) mid channel, and (D3) sink reservoir.
Figure 3.4. Concentration distribution along μLane and number of motile cells. (A) Concentration profile of EGF and SDF-1 along 13-mm microchannel length of μLane system. Concentration gradients are identified by five orders of magnitude in the microchannel: $10^{-1} < G_1 < 10^0$ ng/(mL.mm), $10^0 < G_2 < 10^{-1}$ ng/(mL.mm), $10^{-1} < G_3 < 10^{-2}$ ng/(mL.mm), $10^{-2} < G_4 < 10^{-3}$ ng/(mL.mm), and $10^{-3} < G_5 < 0$ ng/(mL.mm). (B) Fraction of MB-derived cells observed to respond via migration to the different concentration gradient fields ($G_1$ through $G_5$) of EGF and SDF-1.
The migration of MB-derived cells in response to the different concentration gradient fields of EGF and SDF-1 were described using three parameters: (1) Fraction of motile cells, \( f \), defined as the number of cells that migrated more than two cell diameters in the \( \mu \)Lane, normalized by the total number of cells within the channel; (2) cell directionality, \( D \), defined as the percentage of cells whose net center of mass was preferably towards the positive, \( x \)-displacement or along the gradient direction; and (3) Average cell path length, \( PL \), defined as the total distance traveled by cells.

First, the average percentage of motile cells along the entire microchannel was similar for both growth factors. Table 3.2 shows that 72.82% of MB became motile in response to EGF signaling, while an average 67.3% of MB became motile in response to SDF-1 concentration fields. Values of motile fraction varied with gradient fields, \( f^{G_{1}}-f^{G_{5}} \), for both EGF and SDF-1, as shown in Table 3.3. As seen, the highest fraction of MB cells became motile when exposed to higher concentration gradient fields of EGF, \( G_{1} \) (\( f_{1}=28.3\% \)) and \( G_{2} \) (\( f_{2}=18.6\% \)), followed by decreasing percentages of motile cells within lower gradient fields of EGF, \( G_{3} \) (\( f_{3}=11.9\% \)), \( G_{4} \) (\( f_{4}=10.3\% \)) and \( G_{5} \) (\( f_{5}=3.7\% \)). Similarly decreasing fractions of cells were seen to migrate in response to concentration gradient fields of SDF-1, with highest fractions at \( G_{1} \) (\( f_{1}=18.2\% \)) and \( G_{2} \) (\( f_{2}=20\% \)), followed by significant decreases in the fraction of motile cells at concentration gradient fields \( G_{3} \) (\( f_{3}=13.8\% \)), \( G_{4} \) (\( f_{4}=8.7\% \)) and \( G_{5} \) (\( f_{5}=6.5\% \)). As shown in Figure 3.4.B, the fraction of nonmotile cells was 27.2% and 32.8%, when exposed to similar concentration gradients of EGF and SDF-1, respectively. Statistical significance was observed between concentration gradient fields of EGF while no statistical significant difference was found across the gradient fields of SDF-1.
Table 3.2. Migratory parameters of MB-derived cells along the \( \mu \)Lane system in response to concentration gradients generated by 100ng/mL of EGF and 100 ng/mL of SDF-1, respectively. Values were experimentally measured and shown as percentages, means and standard deviations. (\( n_{\text{cells}} \sim 180 \) cells for each ligand).

![Table](https://example.com/table3.2.png)

<table>
<thead>
<tr>
<th>Migration Parameters</th>
<th>EGF</th>
<th>SDF-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction of motile cells (f) [%]</td>
<td>72.8%</td>
<td>67.3%</td>
</tr>
<tr>
<td>Cell Directionality (D) [%]</td>
<td>61.6%</td>
<td>44.2%</td>
</tr>
<tr>
<td>Average Maximum Cell Path Length (PL) in ( \mu )m [Mean ( \pm ) SD]</td>
<td>264.5 ( \pm ) 67.9 ( \mu )m</td>
<td>125.5 ( \pm ) 48.6 ( \mu )m</td>
</tr>
</tbody>
</table>

Table 3.3. Migratory parameters of MB-derived cells in response to gradient fields, \( G_1-G_5 \), generated by of 100ng/mL of EGF or SDF-1 along the \( \mu \)Lane system, respectively. Fraction (f) and directionality (D) of motile cells shown as percentages of total cell numbers. Average maximum cell path lengths (PL) shown with mean and standard deviation. (\( n_{\text{cells}} \sim 180 \) cells for each ligand).

![Table](https://example.com/table3.3.png)

<table>
<thead>
<tr>
<th>Migration Parameters</th>
<th>EGF Gradient Fields</th>
<th>SDF-1 Gradient Fields</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( G_1 )</td>
<td>( G_2 )</td>
</tr>
<tr>
<td>Fraction of motile cells (f) [%]</td>
<td>28.3</td>
<td>18.6</td>
</tr>
<tr>
<td>Cell Directionality (D) [%]</td>
<td>28.3</td>
<td>15</td>
</tr>
<tr>
<td>Average Cell Path Length (PL) in ( \mu )m [Mean ( \pm ) SD]</td>
<td>281 ( \pm ) 21</td>
<td>267 ( \pm ) 40</td>
</tr>
</tbody>
</table>

Next, the average directionality of motile MB was determined by examining individual cell trajectories. The term directionality was previously introduced by our group as the ratio of the number of cells whose centroids migrated more than 80 \( \mu \)m to the total number of cells in the microchannel (33). The paths of MB cells in the presence of EGF and SDF-1
concentration gradient fields were optically tracked within the μLane system to generate the representative Wind-Rose plots shown in Figure 3.5.A. Note that although cell trajectories are each positioned at a common origin for comparison, cell paths were measured along all microchannel segments, exposed to all gradient fields G1-G5. Representative trajectories illustrate that cells migrated preferentially towards lower concentration gradients of EGF (i.e., towards the right). By contrast, SDF-1 fields resulted in MB migration that was both towards and away from lower SDF gradients without preference. The directionality of motile cells along the entire microchannel was higher for cells stimulated with EGF (61.6%) than SDF-1 (44.2%), shown in Table 3.2. As seen, cell directionality within specific gradient fields, D_{G1}^{G5}, decreased with decreasing gradient for both EGF and SDF-1, shown in Table 3.3. EGF D_{G1}^{G1} indicates that 28.3% of motile cells migrated directionally when exposed to G1 of EGF (i.e. within the first 3mm of channel length), while SDF D_{G1}^{G1} denotes that only 18.4% displayed directional migration for the same gradient field of SDF-1. The highest fraction of MB cells directionality was reported at higher concentration gradient fields of EGF, D_{G1}^{G1} (28.3%) and D_{G2}^{G2} (15%), followed by decreasing percentages for directionality within lower gradient fields of EGF, D_{G3}^{G3} (7%), D_{G4}^{G4} (8.6%), and D_{G5}^{G5} (2.7%). Similarly decreasing fractions of directionality were reported in response to concentration gradient fields of SDF-1, with highest fractions at D_{G1}^{G1} (18.4%) and D_{G2}^{G2} (10.5%), followed by decreasing fractions of directionality at D_{G3}^{G3} (9.8%), D_{G4}^{G4} (2.2%), and D_{G5}^{G5} (3.3%).
**Figure 3.5. Motility of MB-derived cells in the μLane system.** (A) Representative trajectories of cells that migrated in response to 100ng/mL of EGF and 100 ng/mL of SDF-1 stimulation. Three cell paths are shown in dashed for EGF and three in solid for SDF-1, 24 hours post steady-state. Note that concentration gradients decrease from left to right within the μLane. (B) Maximum accumulated distance of motile cells stimulated by concentration profiles generated by using 100ng/mL of EGF and 100 ng/mL of SDF-1, respectively, in the SRR of the μLane.
In addition, Wind-Rose plots display the average maximum cell path length, PL, of motile cells tracked along the entire microchannel. Using this data, 32% of motile MB migrated distances greater than 200 microns (or 20 cell-diameters) when exposed to EGF gradient fields, as seen in Figure 3.5.B. By contrast, 97.3% of cells exposed to SDF-1 gradients migrated distances less than 200 microns. MB within our μLane system in the absence of growth factors or concentration gradients (i.e. controls) displayed migration distances between 50 and 200 microns (14). As shown, increasing percentages of cells were seen to migrate in response to higher gradient fields of EGF. A larger percentage of motile MB were observed in response to G₁ fields of EGF at every distance, while only cells exposed to G₁ migrated the longest distances greater than 300 microns. In comparison, MB exposed to lower gradient fields of SDF-1 (G₃-G₅) exhibited the longest migration. Notably, zero cells were observed to migrate less than 100 microns when exposed to any EGF gradient field G₁-G₅, while zero cells were seen to migrate greater than 300 microns when exposed to any SDF-1 gradient fields G₁-G₅.

3.4 DISCUSSION

The chemotactic migration of MB-derived cells has been surprisingly understudied despite its well-known metastatic potential and aggressive invasion into the brain and spinal cord (14, 43). Our study is among the first to examine and compare the migratory responses of MB to dosage-dependent signaling from EGF, HGF, PDGF-BB, and SDF-1, the most widely-acknowledged chemoattractants of CNS tumor cells (37). The first set of experiments utilized conventional transwell assays to illustrate that MB migration was most concentration-dependent to EGF signaling. As shown in Figure 3.1, MB exhibited dosage-dependent migration in response to signaling from EGF and HGF, but seemingly
dosage-independent responses to PDGF and SDF-1 signaling. Further, EGF simulated the migration of approximately twice the number of MB cells than did PDGF, HGF, or SDF-1. Results from immunocytochemistry support the strength of MB chemotactic response to EGF signaling, as activation of its receptor, EGF-R, was two times larger than activation of other respective receptors. While strong MB chemotactic response to EGF signaling is consistent with previous findings from our group and others \((14, 42)\), it is most significant here because it is signaling from SDF-1, rather than EGF, that has been reported as the strongest MB chemoattractant \((56)\). Inhibitor AMD310, which cleaves CXCR4, has been reported to decrease MB tumor growth in mouse xenografts, chemotaxis and proliferation \((56)\). However, \textit{in vivo} use of EGF-R inhibitors such as Tarceva and Gefitinib have reported no changes in motility of cells derived from glioma \((47)\), and non-small-cell lung cancer \((23)\), and thus, were minimally used on MB. We contend that MB chemotactic response may not have been measured most meaningfully in the past, which has stymied development of anti-migratory therapies for MB metastasis. For this reason, we used microfluidic systems to more precisely study MB migratory responses using parameters relevant to metastasis.

Using the \(\mu\)Lane system, we were able to image real-time cell behavior in response to a wide range of concentrations and gradients of EGF and SDF-1, and distinguish directed-migration of chemotaxis. MB migratory behavior was observed to be concentration gradient-dependent for both EGF and SDF-1 signaling, as the fraction of motile cells decreased with decreasing concentration gradient in both cases. However, motile MB traveled longer distances within the \(\mu\)Lane in response to EGF signaling, with an average PL of 264.5 ± 67.8 µm, compared to an average PL of 125.5 ± 48.6 µm when responding
to signaling from SDF-1. Further, cell trajectories illustrated an MB directional bias towards decreasing EGF gradients not present with SDF-1 signaling, with 18.3% of cells migrating towards decreasing EGF gradients compared to 15.3% of MB in response to decreasing SDF-1 gradients. Importantly, this behavior was observed along the entire μLane length, for cells exposed to all concentration gradient fields, G₁-G₅. Here, the cell directionality compared to other methods provides insight into whether MB cells stimulated with ligand solutions (EGF or SDF-1) followed a directional migration along gradient fields. Other methods to measure cell migration include the persistence length and average velocity to determine the chemotactic sensitivity of stable gradients in 3D (22, 46, 72, 75). While the persistence length provides the ratio of the net distance traveled to the total distance, it would not report the number of cells that migrate along ligand concentration gradients, which is highly significant to studies developing migration-targeted therapies for tumors of the CNS.

This consistent MB behavior illustrates that EGF signaling from high concentration gradients initiates the most motile MB, and further enables cells to travel the longest distances. MB cells were seen to migrate towards increasing ligand concentration, which also corresponds to decreasing EGF gradient fields in our system. This response is significant because it reflects MB sensitivity to high concentration gradients (G₁-G₂), which were generated via much greater nonlinear changes in ligand concentration as compared to low gradient fields (G₃-G₅). Previous work from our group has illustrated keen abilities of MB to migrate in larger numbers in response to increasing EGF concentration via pERK signaling (14). In that work, MB cells were seen to travel out of a cell reservoir when exposed to increasing gradients and concentration. In the current
study, we now demonstrate that MB can become less motile when exposed to signaling from increasing concentration but diminishing EGF gradient fields.

Data from the current study highlights the high fraction of motile MB in response to high concentration gradients of EGF. These results have high clinical interpretation, as they point to high gradient fields and low concentration fields as optimal for MB migration. This is an in vivo scenario where paracrine signaling from neighboring cells initiate the most MB migration, such that cell displacement diminishes as cells approach the signaling source, where gradients are low and concentration is high. In addition, our findings may aid clinical development of anti-migratory therapeutics with the potential to inhibit MB metastasis along the spinal column via EGF signaling.

3.5 CONCLUSION
In summary, our results illustrate that MB migration is both concentration and concentration gradient-dependent in response to EGF signaling. Further, our findings illustrate that high gradient fields of EGF result in the largest number of motile cells, which travel long distances, and in a highly directional manner towards decreasing EGF gradients. These findings point to EGF as a viable molecule for migration-targeted therapies for MB.

3.6 ACKNOWLEDGEMENTS
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3.7 References:


CHAPTER 4. CONCLUSIONS AND FUTURE WORK RECOMMENDATIONS

4.1 Summary

In the research of the central nervous system-derived tumor cells migration described thus far, we have (1) investigated migration patterns in response to external signaling of EGF, HGF, PDGF-BB, and SDF-1 via traditional migration assays; (2) examined the basal membrane receptor expression of EGF-R, c-Met, PDGFR-BB, and CXCR4 via immunocytochemistry; (3) determined how medulloblastoma cells migrated in response to steady-state gradients of EGF and SDF-1 using a microfluidic platform. Our findings illustrate that EGF produced (a) the largest number of motile cells, (b) the highest basal membrane receptor activation (EGF-R), (c) the most directionality towards increasing gradients, and (d) the largest accumulated distance traveled.

These results have a significant clinical impact, since EGF is identified as a new target for medulloblastoma migration at low concentration and/or high concentration gradient fields of EGF. These findings might contribute to the development of anti-cancer therapeutics drugs (EGFR inhibitors or small molecules kinase inhibitors) (4, 5, 17, 18) that can impede medulloblastoma metastasis along the spinal column via EGF-R signaling.

4.2 Future Direction

In this section, we will discuss the future experiments and methodologists used to further investigate medulloblastoma migration. We propose two sets of examinations to be completed in the near future: (1) development of a microfluidic device that facilitates cell migration but with
similar characteristics as the \( \mu \)Lane, and (2) combination of different growth factors to better mimic medulloblastoma microenvironment during cell migration.

4.2.1 Development of a new microfluidic device

The \( \mu \)Lane system has been used as a quantitative platform to generate precise concentration-gradients fields of individual chemoattractants to study single-cell migration. However, we propose to modify the \( \mu \)Lane system by adding three inlets or reservoirs into the microsystem (See Figure 4.1.A). This design maintains the same dimensions as the \( \mu \)Lane system (length and area of channel) but requires pressure pumps to ensure mixing of ligand concentrations by diffusion through the microchannel. This diffusion leads to linear ligand concentration gradients at steady-state (1), which can be precisely predicted using computational models. Using this device, we will be able to observe cells responses while tracking their change in displacement along the 1.3-cm length of microchannel. This might provide another venue to investigate cell responses and dynamics to chemical gradients diffusing in a 2-Dimensional microenvironment as previously utilized by other researchers (3, 9, 11, 13, 14).

4.2.2 Combination of different growth factors

The most studied growth factors in glioblastoma metastasis are EGF, HGF, and PDGF which have been demonstrated to be involved in cell migration, proliferation, and invasion (2, 6, 7). We are interested in examine the different signal pathways triggered by these growth factors, which could affect cell functions. The mechanism by which combination of growth factors effect on cell migration is still under investigation (8, 10, 12, 15, 16).
The new version of µLane would allow us to determine the migratory behavior of medulloblastoma cells under the simultaneous stimulation of three chemoattractants to better mimic the physiological microenvironment in which these cells metastasize. This microsystem will rely on fluidic control tools such as pressure pumps to generate and maintain concentration gradients for biological experiments. Another avenue we want to continue to explore the migratory behavior of central nervous system-derived tumor cells by employing two-dimensional biomimetic extracellular matrices such as laminin and matrigel, utilizing this new microsystem to generate stable gradients of chemoattractants over time.

![Figure 4.1. Schematic of microfluidic device composed of three inlets.](image)

(A) Top view of microfluidic device with similar dimensions as µLane system (L=1.3cm; A=100x100μm). (B) Top view of microdevice displaying the loading in each inlet (three most studied chemoattractants in brain cancer). The microdevice requires pressure pumps to control fluid flow and mixing of chemoattractants.
4. 3 References

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First of all, I would like to thank my family for encouraging me in all of my pursuits and inspiring me to follow my dreams. I am especially grateful to my grandmother, mother, and cousin, for teaching me that my job in life is to learn, to be happy, and understand myself, only then I can understand others.

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