DESIGN, FABRICATION AND EVALUATION OF A NOVEL MICROFLUIDIC BASED *IN VITRO* CELL MIGRATION ASSAY

by

SAI SIRISHA DHAVALA

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I dedicate this work to my family for their love, support and encouragement through all my endeavors.

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ABSTRACT

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ASSAY

Sai Sirisha Dhavala, M.S

The University of Texas at Arlington, 2013

Supervising Professor: Young-tae Kim

Microfabrication and microfluidic devices have been recognized as potential platforms for cell-based and drug screening studies. Human glioblastoma multiforme (hGBM) cells migrate inside the central nervous system (CNS) in narrow space constrictions and rarely metastasize through bloodstream. Hence, microfluidic devices consisting of narrow channels are considered as suitable models to study cell migration *in vitro*. Further the migratory capability of each individual cell could be easily obtained and quantified using such system.

This effort presents the design and development of an alternative microfluidic system that provides an integrated array of channels for screening multiple drugs simultaneously. This system is an altered version of traditional system, where a single unit is replaced with multiple units to achieve high-throughput multi-screening platform. The device is tailored to screen various types and doses of drugs simultaneously thus increasing its efficiency. This design has the advantage of using multiple types of drugs of varying concentrations to simultaneously study their effectiveness to inhibit cancer cell migration. Using this platform the migration

characteristics of cancer cells in response to various anti-cancer drugs is investigated as part of this research effort. Also, the therapeutic potential of anti-cancer drugs were evaluated quantitatively, in comparison with standard cancer migration (scratch wound) assay.

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CHAPTER 1

INTRODUCTION

Diffuse single cell infiltration into normal surrounding brain is a pathological hallmark of glioblastoma multiforme (GBM), the most common and most lethal of all primary brain tumors [1,2]. Current multimodal treatment regimens of surgery, chemotherapy and radiation yield survival rates of only 14 months [3]. However, patients ultimately die of widely infiltrative tumors that are resistant to all therapies. Infiltrating GBM cells pose the single greatest challenge to improving the prognosis for primary brain tumor patients. Therapies which effectively block cell migration could transform this fatal tumor into a local disease, one that could be effectively treated by surgery and/or high dose focal radiation.

For past three decades, mechanisms of GBM migration and invasion have relied on the use of standard *in vitro* cell migration assays (i.e., Scratch and Boyden chamber assays) [4-7]. Mechanistic insights from these studies are limited; neither the mechanical injury model (Scratch assay) nor the chemotactic gradient driven migration through an artificial ECM gel (Boyden chamber assay) accurately represents locomotion through the brain parenchyma. There is increasing evidence that tumor cells may use fundamentally different modes of migration when traversing through confined spaces compared to standard assays. Furthermore, standard glioma cell lines that have been widely used for understanding the molecular mechanism of GBM migration and evaluating drugs to block migration show no evidence of single cell infiltration; a characteristic feature of GBM. These cell lines when transplanted into the severe combined immunodeficiency (SCID) mouse brain grow as a circumscribed mass with a clear and distinct interface with normal surrounding brain tissue. *In vitro* studies have utilized two dimensional (2D) surfaces such as cell culture dishes, glass coverslips or the porous membrane for growing cells and assessing migration. The *in vitro* Scratch assay, which involves

a mechanical injury, triggers a wound healing response, which subsequently repopulates the denuded cellular gap [8]. The adhesion-dependent (mesenchymal) mode of migration involved in this wound healing response is a poor representation of *in vivo* GBM cell migration. The Boyden chamber assay requires cells under investigation to migrate through dense extracellular matrix (ECM) and narrow (5-8 μm) pores under the influence of a strong chemo gradient [9]. There is no evidence that a chemotactic gradient plays any role in tumor cell migration. In short, the mechanisms which drive tumor cell migration in standard cell migration assays are a poor representation of migration through normal brain interstitium.

The lack of faithful biological assays of GBM cell migration is a significant impediment to progress in understanding the fundamental mechanisms of GBM cell migration and developing a rational therapeutic strategy to block tumor cell migration. There is a need to develop new treatment strategies that target the pathways regulating the migrating capability of cells to overcome these shortcomings. The need to create a powerful and unique set of *in vitro* cancer cell migration assay that provide unique insight, resulting in the development of new pathways for pharmacological inhibition is the motivation for this research effort.

The rest of the document is organized as follows. Chapter 2 presents a brief background of GBM with focus on migration mechanisms and novel cancer therapeutics. Also various models that are used for cancer drug screening are reviewed. Chapter 3 presents the research statement, approach and methodology that were followed to accomplish the goals of the thesis. Chapter 4 presents the development of a blueprint and fabrication details of a new microfluidic device. Chapter 5 lists various experiments performed to test different aspects of the newly developed device. In Chapter 6, a discussion of results and a qualitative analysis is presented. Chapter 7 presents the contributions made by this thesis effort and explores some related areas for future work.

CHAPTER 2

LITERATURE REVIEW

2.1 GBM

Cancer metastasis accounts for majority of cancel related deaths owing to poor response to anticancer therapies. Metastasis involves the progression of tumor cells from primary tumor mass to distant parts of the body [10, 11].

Among metastasizing types of cancers, GBM is considered to be a highly invasive and aggressive form of primary brain tumor [1]. It is of the most malignant astrocytoma with very poor prognosis and accounts for 15-20% of all intracranial tumors and 50 to 60% of astrocytic tumors. GBM is characterized by its extensive ability to migrate and infiltrate surrounding brain tissue, thereby increasing its recurrence despite different treatment methods [12].

Cell migration through tissue plays a vital role in the progression of cancer, which involves the dissemination of tumor from primary site to distant parts of the body [13]. Cell migration modes vary depending upon individual cell or collective cell migration movements [14]. GBM cells adopt either amoeboid or mesenchymall type movement when migrating as individual cells from the tumor site. During collective migration, they propagate as clusters, cell sheets or strands.

The following section gives an overview of different GBM cell migration mechanisms.

2.1.1 Migration Mechanisms

Mesenchymal Migration:

GBM mainly adopt mesenchymal mode of migration to infiltrate the distant organs collectively [12, 15, and 16]. These cells follow five-stage migration cycle. First stage involves

cell polarization and elongation, which is facilitated by polymerization of actin to form a leading protrusion. The second stage involves the interaction of protrusions with extracellular matrix (ECM) substrates via cell adhesion receptors. This is followed by the recruitment of cytoplasmic adaptor, signaling and cytoplasmic proteins at the adhesion sites to form focal contacts. The third stage involves degradation of matrix in the front of the cell by tumor-cell associated proteases (matrix metalloproteinase and serine proteases). In fourth stage the interaction between actin filaments and contractile proteins, stabilize and contract the actin strands. The fifth and final stage consists of forward movement of cell and its progression along the substrate resulting due to contraction and shortening of actin filaments.

Amoeboidal Migration:

Amoeboidally migrating cells adopt a les adhesive and fast gliding mechanism that allow for the early detachment and rapid dissemination of the tumor. The cells utilize a crawling mechanism driven by weak interactions with the substrate and are characterized by the lack of focal contacts, stress fibers and focalized proteolytic activity. Cells adopting amoeboidal migration migrate at higher velocities than mesenchymally migrating cells due the absence of focal contacts [17].

Collective Migration:

Collective migrating cells move as connected aggregates or clusters through the presence of large number of cells at the leading edge and cell to cell interactions. The actin and integrin mediated traction of the leading cells is mainly responsible for the dissemination of the tumor. The migration of cells as collective and heterogeneous mass provides the advantage of protection from immunological response [16].

2.1.2 Clinical Cancer Management

Surgery, radiotherapy and chemotherapy are the current conventional techniques for treatment of GBM cancer. However, none of these therapies is found to effectively treat GBM

cancer [18]. The median survival rate after the treatment was found to be between 12-18 months with few cases of long term survival [19]. Methylating agent, temozolomide and anti-angiogenic agent bevacizumab are some of the effective chemotherapeutic agents currently used for GBM chemotherapy treatment. Adjuvant chemotherapy was found to increase the survival rate by 6-10% [20, 21]. However, not even the most specific hallmark-treating drugs were able to meet clinical expectations [22]. Current developments have been focused on developing therapies which could effectively block cell migration, thereby transforming this fatal tumor into a local disease, which can be effectively treated by surgery and/or high dose focal radiation. The following section reviews some of the relevant promising anti-cancer therapies developed.

2.1.3 Novel Cancer Therapeutics

Molecular targeted therapy is the one of the currently pursued idea for cancer treatment. This therapy is based on targeting the intracellular signaling pathways that regulate the growth, invasion and progression of tumor. The major advantage of this technique is its ability to selectively kill cancer cells without affecting the normal tissue. The following section focuses briefly on different types of signaling cascades and molecular pathways regulating cytoskeleton dynamics in GBM proliferation. Each pathway is illustrated briefly in the next section supported by potent drug.

Kinase Signaling Cascades

Phosphoinositide 3 Kinase

PI3K are group of enzymes that are involved in cellular proliferation, metabolism and survival [23]. PI3K signaling is frequently hyperactivated in GBM cells. mTOR (mammalian target of rapamycin), a member of the PI3K family acts a key mediator of PI3K signaling by integrating signal transduction and cellular metabolism [24,25]. mTORC2 complex promotes growth and cell motility in gliomas cells.

Recent focus has been on developing drugs that inhibit GBM by targeting PI3K pathway. PI-103 is one such potent, ATP-competitive drug. It arrests the proliferative ability of glioma cells by selectively inhibiting PI3K and mTOR [26]. Fan et al. demonstrated the efficacy of PI-103 in reducing tumor volumes in combination with s-TRAIL in a xenograft glioma model [27].

Rho-associated Protein Kinase (ROCK) Pathway:

ROCK act as major regulators of the cytoskeleton. These are activated by RhoAGTPases and modulate several cellular processes involving cytoskeletal rearrangement such as focal adhesion formation, cell motility and tumor cell invasion [28]. ROCK regulates actin dynamics by phosphorylating the proteins involved in actin filament assembly and contraction like myosin light chain (MLC) kinase and MLC phosphatase. The phosphorylation of MLC results in the shortening of actin filaments which in turn regulate membrane blebbing. Studies conducted by Zohrabian et al. confirmed the involvement of ROCK/Rho signaling in GBM cell proliferation [29].

ROCK (Y-27632) is one of the potential drug targeting ROCK pathway [30]. In *in vitro* studies, treatment with Y-27632 for 24h suppressed the migration of GBM cells and decreased the proliferation of LN-18 cells [29] and rat ascites hepatoma (MM1) cells [31].

MEK/ERK Pathway:

MEK/ERK pathway facilitates and regulates intracellular communication and various cell signaling events. The major enzyme that controls this pathway is mitogen-activated protein kinases (MAPK). MAPK activation occurs via a cascade mechanism involving several protein kinases. This pathway is initiated upon the activation and phophorylation of tyrosine kinase effector called Raf by GTPase protein, Ras. The activated Raf in turn activates another tyrosine/threonine kinase called MEK which further phoshphorylates and activates MAPKs or extracellular signal regulated kinases (ERK). This pathway functions as an on-off switch in regulating several cellular processes [32, 33].

AZD-6244 (selumetinib) is a potent drug that blocks the activity of MAPK/ERK pathway [34]. AZD in combination with standard chemotherapeutic agent (temozolomide) enhanced the anti-tumor efficacy in human xenograft models [36]. AZD-6244 is currently undergoing phase-2 clinical trials to determine its effectiveness in treating patients with BRAF mutated cancer [35].

Pathways Regulating Cytoskeleton Assembly

Microtubule Pathway:

Microtubules are cytoskeletal structures associated with many cellular processes including vesicle transport, cell motility, cell division and signaling [37]. Polymerization dynamics regulate and determine the biological functioning of microtubules. Research is focused on developing drugs that disrupt microtubule assembly by mimicking the actions of natural regulators that modulate microtubule dynamics.

Nocodazole is an anti-neoplastic drug that leads to polymerization of microtubules by inhibiting the addition of tubulin to microtubules [38, 39]. Studies conducted by Vasquez et al. demonstrated the high specificity of nocadozole to induce microtubule instability both *in-vitro* and *in-vivo* at very low concentrations [40]. Recent studies by Park et al. demonstrate the potential of nocodazole in inhibiting cancer-related kinases ABL, c-KIT, BRAF, MEK1 [39].

Actin Polymerization Inhibitor Pathway

Actin is a globular protein that participates in many important cellular pathways such as cell division, cell signaling, muscle contraction, cell motility and cell structure [41-43]. Actin filament assembly and disassembly is mediated by actin binding proteins like thymosin-β4, profilin, Arp2/3 complex, formin, fascin and capping protein. Among them, Arp2/3 complex plays a major role in the regulation of actin cytoskeleton [44].

Pholloidins, cytochalasins, CK, latruculins, jasplakinolide are some of the naturally occurring drugs that bind to actin and alter its polymerization. CK-666 is a cell permeable compound that selectively inhibits actin assembly by preventing the activation of Arp2/3 complex upon WASP

binding [45]. Latrunculin-B is a marine toxin which blocks actin polymerization by binding with monomeric actin with 1:1 stoichiometry and thereby preventing F-actin assembly [46, 47]. Spector et al. demonstrated the potential of Latrunculin-B to induce microtubule disruption in mouse neuroblastoma and fibroblast cells at low concentrations [48].

Myosin Pathway

Myosin II is an ATP motor protein that regulates cellular processes involving motility like growth cone motility, fibroblast locomotion and astrocyte outgrowth and contractile response. Myosin II is the major protein implicated in the progression and invasion of human carcinomas [10]. In gliomas, invasion occurs due to the generation of internal compressive forces by myosin II which causes the extrusion of cell body and nucleus through the narrow intracellular spaces of the brain parenchyma [49].

Blebbistatin is one of the drugs that inhibit myosin II ATPase activity. This regulates myosin II activity by blocking myosin heads in an aqueous cavity between nucleotide pocket and cleft of actin-binding interface [50].

2.2 Models and Assays in Cancer Research

Several *in vitro* as well *in vivo* models have been successfully implemented in drug screening. However *in vitro* assays are preferred over *in vivo* due to the following reasons:

- Relatively easy to handle
- Less expensive
- Allow for the examination and analysis during the assay
- Not subjected to any ethical concerns [7]
- Results can be reproduced
- Can be scaled to achieve high-throughput [8]

The following sections provide an overview of the current *in vitro* assays, their advantages and limitations in cancer drug discovery.

2.2.1 Scratch Assay

Scratch Assay is the commonly used technique to study cell migration and conduct drug screening assays [9]. The basic principle involved is the creation of a cell free scratch on a confluent monolayer of cells. The variability of the scratch created and damage caused to the underlying ECM limits its application in cell migration studies [7]. Further physical damage of the cells due to the scratch might lead to the release of certain factors that affect cell migration measurements [8, 51]. Moreover the technique is labor intensive and time consuming [52].

An improved and automated version of the scratch assay technique using 96-well plate has been developed by Essen Biosciences [53]. This assay provides standardized and high-throughput analysis. Use of biological matrices like collagen, have further enhanced the physiological relevance of these assay

2.2.2 Boyden Chamber Assay

Transmembrane/Boyden assay is one of the commonly used *in vitro* assays to analyze the anti-migratory effect of drugs on cell migration. The basic principle involved is the migration of cells through a porous membrane in the presence of chemical gradient [54]. The major limitations of Boyden assay are the non-linearity of gradient between compartments and the difficulty to visualize the cells migrations across the non-physiological membrane [9]. Moreover, the assay does not allow for the real-time monitoring of cell migration. The current methods are also labor-intensive, expensive and time-consuming [8].

Recent advancements in this area is the introduction of multi-transwell assays by various commercial providers (Merck Millipore, Beckton Dickinson, Neuroprobe Inc.) in 24,96 or 348

well format for high-throughput screening of migration [8]. However the technique is still limited by its inability to track the migration of each individual cell over time.

2.2.3 Microcarrier Bead Assay

This is one of the earliest *in vitro* cell based assay to study cell motility. The principle involved is the migration of cells encapsulated in microcarrier beads onto cell culture vessel surface [55, 56]. One of the major limitations is the inability to track the motility of each individual cell. Moreover, the technique is not commercially viable and does not provide high-throughput analysis [8].

One of the advancement of this assay is the integration of 3D technology with 2D through the introduction of multicellular tumor spheroids on top of culture dish in place of the conventional microcarrier beads. The 3D spheroid structure more closely mimics the physiological tissue like morphology with close cell-cell interactions making it a valuable assay to study cancer progression [57].

2.2.4 Ring Assay

This technique is based on tracking of cells migration through a circular monolayer of defined diameter [8]. Various research groups have successfully implemented this assay to study cell proliferation, cell-ECM interactions, and signal transduction [59]. One of the major limitations of these assays is low throughput and usability with non-adherent cells.

Impedance based electric fence technique, a modified version of the ring assay was developed to monitor and quantify cell motility [58].

2.2.5 Cell Exclusion Zone Assay

The principle involved is the creation of cell free zone in tissue culture wells. One of the main advantages of this assay is that migration can be monitored in real-time without fixing of cells and there is no presence of any wound-related factors as in scratch assay [51]. However,

this assay is not suitable for non-adherent cells. Improvements have been made to enhance the efficiency and output of the cell exclusion assays [8, 9].

An automated cell exclusion assay was developed by Oris labs consisting of dissolvable biocompatible gels replacing the standard stoppers [60] which provide high-throughput screening.

2.2.6 Colloidal Particle Assay

This is one of the commonly used techniques to track migratory potential of each single cell. This assay is based on the quantitative analysis of the gold colloidal particles phogocytosed by the cells. This technique provides a quantitative measure of the metastatic potential and thereby distinguishes between non-invasive and invasive cell lines [61]. However the technique is labor-intensive and requires automation [8].

Gu W. et al. replaced gold particles with colloidal CdSe/ZNS semiconductor nanocrystals (quantum dots) to measure the metastatic ability of cancer cells [62].

2.2.7 Microfluidic Assay

Recently, microfabrication and microfluidic devices have been recognized as potential platforms for cell-based drug screening studies [63]. In current research, focus has been on enhancing design of microfluidic assays. The following section provides a brief background on microfluidic devices.

2.3 Microfluidics Overview

Microfluidics are devices of micron scale that allow precise manipulation of fluids through the means of interconnected channels. The dimension of microchannels is comparable to physical scale of cells hence making it a potential platform for cell based studies [64, 65]. Following advantages are notable:

- Ease of automation

- Real-time imaging capable
- Ability to track single cell
- Reduced sample and reagent consumption
- Low fabrication cost
- Tailored to provide high-throughput

2.3.1 Material for Fabrication:

The material of the devices used for cell migration studies should possess the following properties: should be noncytotoxic and support cell growth, possess appropriate surface chemistry to promote cell attachment, optically transparent to enable real-time tracking of cell migration, compatible with standard sterilization techniques such as ethanol exposure or oxygen plasma treatment, should not leach monomers or solvents into culture media [64, 66].

Polydimethylsiloxane (PDMS) is one such material that possesses the above characteristics and is commonly used for molding microfluidic devices [66]. PDMS is a silicone elastomer that allows for the rapid transfer of patterns with high precision and fidelity. PDMS mixed with curing agent is poured into a microstructured template and cured to obtain a replica of the master mold. Some of recent efforts explored other materials like polystyrene, gelatin, hydrogels for fabricating microfluidic structures. However, PDMS is still the preferred material for microfluidic fabrication due the following reasons:

- Highly flexible and capable
- Gas-permeable
- Non-toxic to cells and biocompatible making it suitable tool for cell based assays

- Excellent optical properties and low auto-fluorescence making it suitable for observing microscopic observation
- Economical with low fabrication cost
- Binding capable to substrates like glass to create enclosed microchannels

PDMS is hydrophobic and does not wet the surface easily, limiting its use in cell culture applications. However, this limitation is overcome by plasma treating the devices to enhance surface hydrophilicity and adsorption resistance to hydrophobic compounds [66].

2.3.2 Microfluidic models for cell based studies

To overcome the limitations of conventional migration assays, new approaches based on microfluidic technology which could provide a much more realistic *in vitro* microenvironment were developed. This section presents an overview of some of the current microfluidic models employed for studying cell migration.

Smita et al. reported a microfluidic device to evaluate the migration characteristics of cancer cells in presence of chemo attractants. The device offered various advantages, some of them being: real time quantification of cell migration, stable chemokine gradients, minimal reagent consumption and cost effective platform [78].

Wan et al. designed and fabricated a microfluidic device consisting of tapered channels for evaluating migration characteristics of hGBM cells. The effect of aptamer bound to epidermal growth factor in inhibiting the proliferation and migration of hGBM cells has been demonstrated in this study. The device was integrated with ambient controlled microscope for real-time imaging of cell behavior. This design provided a framework for studies on efficacy of other inhibiting molecules [65].

K.C. Chaw et al. reported a similar system using a gradient based microfluidic device for the cell migration study. The device consisted of multiple micro channels combined with high resolution microscope imaging system for tracking cell movement effectively. The better

controlled conditions in the microfluidic system provided stable and suitable platform for multiple applications [79].

Sudo et al. reported a 3D microfluidic platform coupled with image acquisition system for evaluating cellular morphogenesis. The device was used to quantify hepatocyte cell response upon co-culturing with endothelial cells. .The platform provided capability to effectively control biochemical gradients make it more stable and suitable for cell migration studies [80].

Similar models were proposed by various authors [81, 82]. The advantages of these microfluidic based devices being (1) cells could be easily seeded in micro channels, thereby offering more suitable microenvironment for studying cell migration (2) minimized reagent consumption (3) Integration with microscopes and imaging systems enabled real-time observation. However the main limitation of these models is the ability to provide high-throughput [68].

2.3.3 High-Throughput Microfluidic Models

The conventional format of microfluidic devices is not suitable for high-throughput drug analysis. Lot of emphasis is being given for developing microfluidics that provides high-throughput [67, 68]. The current section describes few recent efforts in this area.

Microfluidics are mainly comprised of a single chamber with interconnected microchannels providing drug screening for a single compound at a time. Recent developments in this area comprise microfluidics integrated with microarrays, gradient generators and valved arrays to enhance the throughput and efficiency.

Microfluidics coupled with gradient devices are capable of generating different concentrations of a single reagent/compound, thereby increasing the screening output [64].

Various improvements have been made to microfluidic design to establish a steady and controllable gradient profile [69-72].A two layer microfluidic device consisting of gradient generator that could facilitate nine linear dilutions of varying concentrations from two input

streams was developed by Walker et al. [70]. A similar device consisting of a 10 X 10 array was developed by Hung et al. to enable long time monitoring of cells [69]. Further improvements have been made to allow for high-throughput screening [71, 72].

The main limitation of the combinatorial systems with gradient generators is inability to provide isolation of each chamber, which can lead to transfer of metabolites/reagents between chambers affecting the results of the study [64].

Microfluidics integrated with valves was developed to enable high density parallel screening with minimal reagent consumption [64]. A high density microfluidic array integrated with pneumatically activated elastomeric valves, for cell cytotoxicity testing was developed by Wang et al. [73]. The device successfully assessed the effect of toxins on the viability and morphology of three different cell lines and results were comparable to the micro titter assay.

Various modifications have been made to the existing designs to enable simultaneous testing of multiple compounds and cell types. However, the constraints imposed by the physical connections limited the throughput [64].

CHAPTER 3

RESEARCH STATEMENT AND METHODOLOGY

Current *in vitro* cell migration models do not accurately represent locomotion of cells through the brain parenchyma, which limits their application in screening of anti-migratory drugs. Development of new treatment strategies that target the pathways regulating the migrating capability of cells is warranted. Based on the need to address the limitations of existing models, a new microfluidic based assay allowing for real-time automated monitoring of cancer cell migration is implemented in this effort. An array consisting of independent microfluidic units with space constrictions for evaluating the behavior of cells under confined environment is developed. Such a cell culture array offers an efficient platform for high-throughput *in vitro* screening of anti-migratory drugs.

3.1 Research Objectives

The purpose of this thesis was to design, develop, and test a microfluidic based *in-vitro* cell migration assay. To achieve this, following objectives were identified:

- To design and develop a high-throughput microfluidic array platform for cancer migration study.
- To analyze the therapeutic potential of anti-cancer migratory inhibitors on tumor cell migration.
- To compare the newly designed assay with standard cancer migration (scratch wound) assay with specific focus on quantitative migratory behavior.

3.2 Scope of the Research

The purpose of this research was to develop an *in vitro* cell migration assay and study its application in analyzing cell motility in response to anti-cancer drugs. The central idea was to develop a microfluidic platform with spatial constrictions for studying hGBM migration *in vitro.* The goal of this thesis is not to offer solutions for specific screening problems, but rather to offer a general platform for addressing them. As a part of the current effort, the metastatic potential of the existing and approved drugs were evaluated using the migration assay. Discovering novel pharmacological inhibitors of cancer migration was also not a part of this thesis effort.

3.3 Research Methodology

In order to accomplish the objectives stated in Section 3.1, the effort was divided into following stages:

Stage 1: Existing cell based migration assays and approaches were studied and explored in detail to gain an understanding of their purpose, strengths and limitations.

Stage 2: In this stage, a blueprint of new microfluidic device for high-throughput screening was designed. Specifically the existing design was modified to allow for high-throughput based on the information collected above.

Stage 3: To fabricate the microfluidic device using design from the previous stage for design feasibility.

Stage 4: This stage involved the design of various experiments to evaluate feasibility or reliability of the device.

Stage 5: This stage involved conducting experiments to evaluate the metastatic potential of anti-cancer drugs in inhibiting hGBM migration.

Stage 6: Data and results collected from the previous stage were analyzed, compared and documented in this final stage.

CHAPTER 4

DESIGN AND FABRICATION

PDMS based microfluidic devices with compartmentalized chambers connected via microchannel provide an excellent platform for cell migration studies [63].The PDMS based microchannels provide geometries that are similar to the *in vivo* scale. The main advantage of PDMS devices is the flexibility in fabrication with varying dimensions and geometries. They provide an optically transparent surface for microscopy and optical manipulation [66]. Several research efforts were focused on developing combinatorial assays integrating microfluidic and gradient generators [69, 70]. However, the design of such devices is not suitable for performing different individual experiments [64].

In this chapter, the design and fabrication of a high density microfluidic array consisting of 20 individually addressable units is described. Section 4.1 presents the design of the device. Section 4.2 describes the first step of fabrication process using chrome mask template. Section 4.3 describes the process of silicon mold fabrication using photolithography. Section 4.4 describes the process of transferring pattern on silicon wafer on to PDMS. Section 4.5 presents the discussion of results.

4.1 Device Design

The basis of this design was to provide an array of multiple microfluidic units. Each microfluidic unit comprised of multiple channels with reservoirs connecting either ends of the channel lanes. The reservoir served as the cell seeding area while the channels enabled the migration of cells across the reservoirs.

4.1.1. Design Parameters

The key aspect of this design was to provide spatial constrictions of the channels, for studying the migratory behavior of cells under physically confined conditions. The presence of long micro channels would allow for precise long term monitoring of cells migration. The height differences created between seeding chamber and channels through lithographic techniques was to ensure rapid migration of cells towards the channels. Such a device could serve as an efficient *in vitro* assay for screening the anti-migratory potential of drugs.

With these objectives and previous efforts in view, following parameters have been set for feasibility. Based on size constraints, an array of 20 individual microfluidic units (A1-B10) was chosen as optimum for this design. Each unit consisted of 39 channels. Two sets of devices with varying channel widths were developed for experiment purposes. The first set of devices comprised of channels of 5 µm width, while the second were 15 µm wide. Length of each channel was set to 400 µm to allow for long term monitoring of cell migration. The diameter of both the inlet and outlet reservoir was set to 5 mm with two overlapping circles. The dimension of the entire array was chosen to be 47 (W) mm* 56 mm (L) to allow for binding onto a cover glass of 48mm *65mm size.

The device consisted of 780 channels in total, providing high-throughput for multiple screening of drugs. Two individual units were set apart by a distance of 4 mm. The cell seeding area was separated from the lanes by a distance of 50 µm to allow proximity of the cells with the channels.

Figure 1 shows the AutoCAD drawing of the design scaled to the set design parameters. Set of channels with the respective sizes are presented in Figure 2.

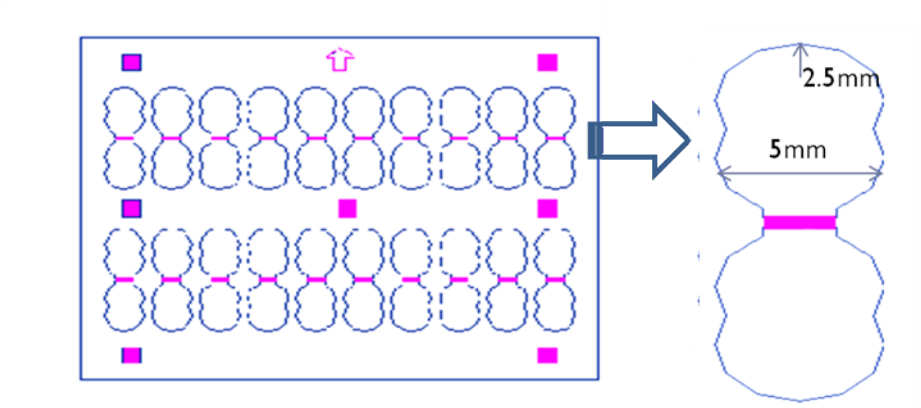

Figure 1: Schematics of the Microfluidic device

Microfluidic array consisting of 20 independent units, each unit comprising of two reservoirs bridged by 39 microchannels.

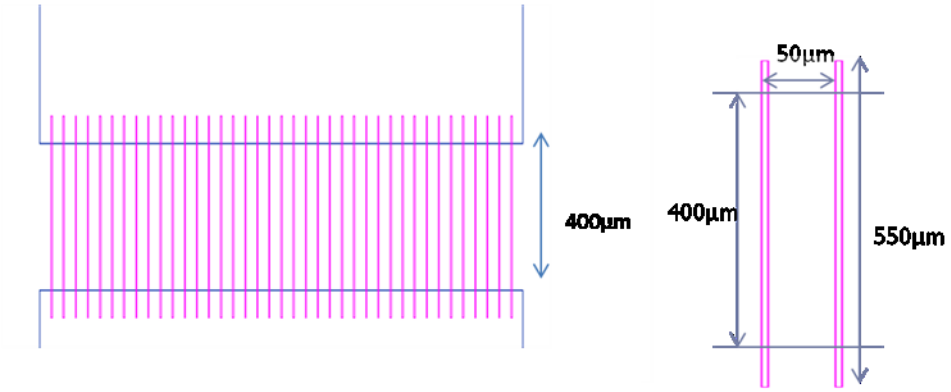

Figure 2: Longitudinal view of set of Channels

Microfluidic array with 39 microchannels of 400 µm height and 5/15 µm width

Dual layer photolithography technique (described in later section) was used to fabricate the device to achieve different heights for channels and reservoir. The height of the channels was set to either 5 µm or 15 µm, while the reservoir height was set to 100 µm.

4.2 Chrome Mask Fabrication

Chrome masks serve as templates for the preparation of microfluidic device. The desired dimensions of the chrome mask pattern were designed using AutoCAD and sent for fabrication. The masks were fabricated at Natural Science & Engineering Research Lab, University of Texas at Dallas. Since the microfluidic device required different heights for channels and reservoir, two separate masks, one consisting of microchannels and the other consisting of reservoirs were fabricated. The mask consisted of markers that allowed for accurate alignment of the two layers.

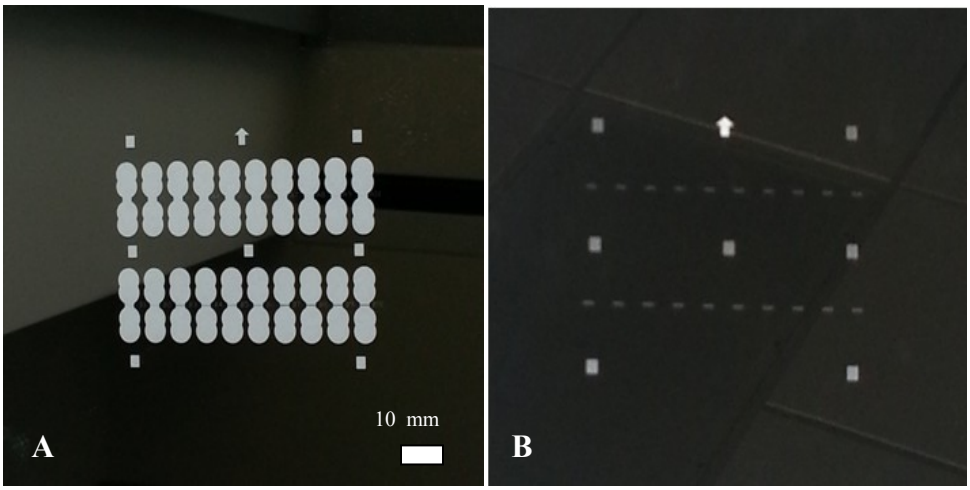

Figure 3: Chrome Masks

(A) Chrome mask consisting of reservoir (B) Chrome mask consisting of channels

4.3 Fabrication of Silicon Master Mold

Photolithography was used to optically transfer the pattern on the chrome mask onto silicon wafers that served as molds for PDMS based microfluidic device preparation. The silicon wafer was fabricated under cleanroom conditions at NanoFab lab, University of Texas at Arlington. The mold was designed in two layers; the bottom layer consisted of microchannels and top

layer consisted of reservoir. Dual layer photolithography was used to obtain different pattern heights for two sets of channels (5 μm X5 μm and 15 μm X15 μm) and the reservoir. Initially, the first layer consisting of channels was fabricated. The wafer was pretreated and dehydrated as described below. SU8 photoresist was spin coated on the wafer according to the specifications described in spin coating step in the following section. Chrome mask was mounted onto the backside aligner and held in position by the use of vacuum. The spin coated wafer was placed on a circular disk wafer holder present below the mask. The mask was exposed to UV rays to allow for crosslinking of the exposed area. After exposure the wafer was baked to initiate reactions that resulted in crosslinking of the resist. The wafer was placed in a beaker containing photoresist developer and developed to expose the design pattern.

Following flowchart summarizes the fabrication process of the microfluidic devices:

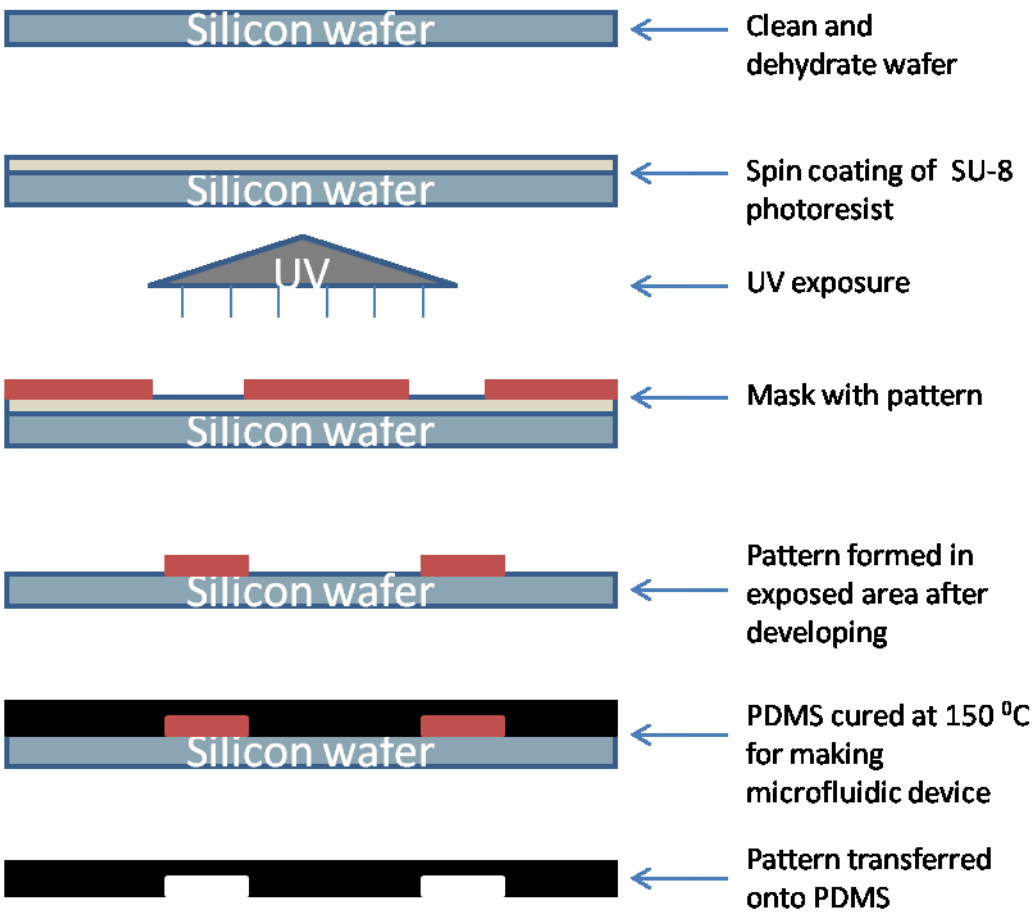

Figure 4: Schematic of Fabrication Process

The steps that were followed during the fabrication of the wafer were:

Substrate Pretreatment: The silicon wafer was cleaned with acetone and rinsed with deionized (DI) water. The wafer was then dried using nitrogen gas and dehydrated on a hot plate at 200 ^{0}C for 10 minutes to remove any adsorbed moisture content.

Spin Coating of photoresist on wafer: The type of photoresist determines the effective height of the pattern to be formed. SU-8 5 photoresist was used to design microchannels of 5 μm and 15 μm heights. SU-8 50 was used for fabricating reservoirs of 100 μm height. To achieve uniform

spread of the resist on the wafer, spin coating was done in two steps: spread cycle and spin cycle. Following steps summarizes the different spin coating parameters used:

5 µm height: Resist SU-8 5

(1) 500 rpm at 100 rpm/sec for 5 seconds.

(2) 2000 rpm at 300 rpm/sec for 30 seconds.

15 µm height: Resist SU-8 5

(1) 500 rpm at 100 rpm/sec for 5 seconds.

(2) 1000 rpm at 300 rpm/sec for 30 seconds.

100 µm height: Resist SU-8 50

(1) 500 rpm at 100 rpm/sec for 10 seconds.

(2) 1000 rpm at 300 rpm/sec for 30 seconds.

Soft Baking of Photoresist: The spin-coated wafer was soft baked on a hot plate to evaporate the solvent and make the resist film dense. The temperature was raised gradually from 65 ^{0}C to 95 ^{0}C to provide uniform and controlled evaporation of the solvent which enhanced substrate and resist binding. Soft bake parameters that were used in this step are:

5 µm height: 1 minutes at 65 °C and 2 minutes at 95 °C

15 µm height: 2 minutes at 65 °C and 5 minutes at 95 °C

100 µm height: 10 minutes at 65 °C and 30 minutes at 95 °C

UV exposure: SU-8 photoresist is optimized for near UV (350-400 nm) wavelength. Exposure time and energy for crosslinking is dependent on viscosity of photoresist and pattern height. The exposure energy used in the process was between 300 mJ/cm^2 – 500 mJ/cm^2. Exposure to UV

light resulted in polymerization of the photoresist while the unexposed patterns remained unaffected. Following steps summarizes exposure time used:

5 μm height: Single exposure for 8 seconds.

15 μm height: Single exposure for 12 seconds.

100 μm height: Two consecutive exposures for 14 seconds.

Alignment: After the development of the first layer consisting of channels of 5 μm and 15 μm wide, a second layer consisting of reservoirs of 100 μm was fabricated over it. SU8-50 was spin coated on the wafer and then soft baked using the parameters as mentioned. After soft bake, the wafer and the mask for second layer were loaded in their respective positions in the aligner. Now the pattern on the first layer was aligned with the second layer with the help of similar alignment markers present on both the mask and wafer. After the alignment, the wafer was exposed to UV light to crosslink the desired pattern as described in next step.

Post Exposure Baking: The wafer was baked following UV exposure to selectively crosslink the exposed regions of the photoresist. The wafer was heated in two step process gradually raising the temperature from 65 ^{0}C to 95 ^{0}C to minimize the damage to the pattern caused by the unequal difference in coefficients of expansions between resist and substrate. The parameters used in this step:

5 μm height: 1 minute at 65 °C and 1 minute at 95 °C

15 μm height: 1 minute at 65 °C and 5 minutes at 95 °C

100 μm height: 1 minute at 65 °C and 10 minutes at 95 °C

Developing: The unexposed areas on the wafer were removed using SU-8 developer. The wafer was immersed in photoresist developer and then rinsed with isopropyl alcohol (IPA) followed by blow drying with nitrogen gas. If any photoresist was present the wafer turns white

upon rinsing with IPA and the resist can be removed by further developing. The wafer was finally checked under microscope to ensure removal of photoresist from the unexposed areas. Parameters used in this step:

5 µm height: 1 minute wash

15 µm height: 3 minute wash

100 µm height: 10 minute wash

Hard Baking: The wafer was baked at 125 ^{0}C on a hot plate for 30 minutes to further harden the photoresist and enhance its adhesion to the substrate. The hardened wafer was used as a mold for transferring the pattern onto PDMS.

4.4 Fabrication of PDMS Devices

This section list the materials and the steps used for fabrication of PDMS devices.

Materials: Silicon wafer with pattern, SYLGARDR® 184 elastomer kit (DowCorning), Vacuum desiccator, aluminum foil and scalpel blades.

Step 1: SYLGARD kit consisting of PDMS prepolymer and curing agent were mixed in 10:1 ratio. The PDMS mixture was then degassed by placing in a vacuum chamber (20 in Hg vacuum) for 45 min to ensure removal of air bubbles formed during the mixing process.

Step 2: The silicon wafer was covered with aluminum foil and placed on a hot plate at 150 ^{0}C. The desiccated PDMS mixture was poured on top of the mold and air bubbles formed were removed using a needle.

Step 3: After curing of PDMS, the wafer was transferred to a hotplate maintained at 70 ^{0}C for cutting of the pattern. The devices were cut using a scalpel blade and placed with the pattern facing upwards on a foil.

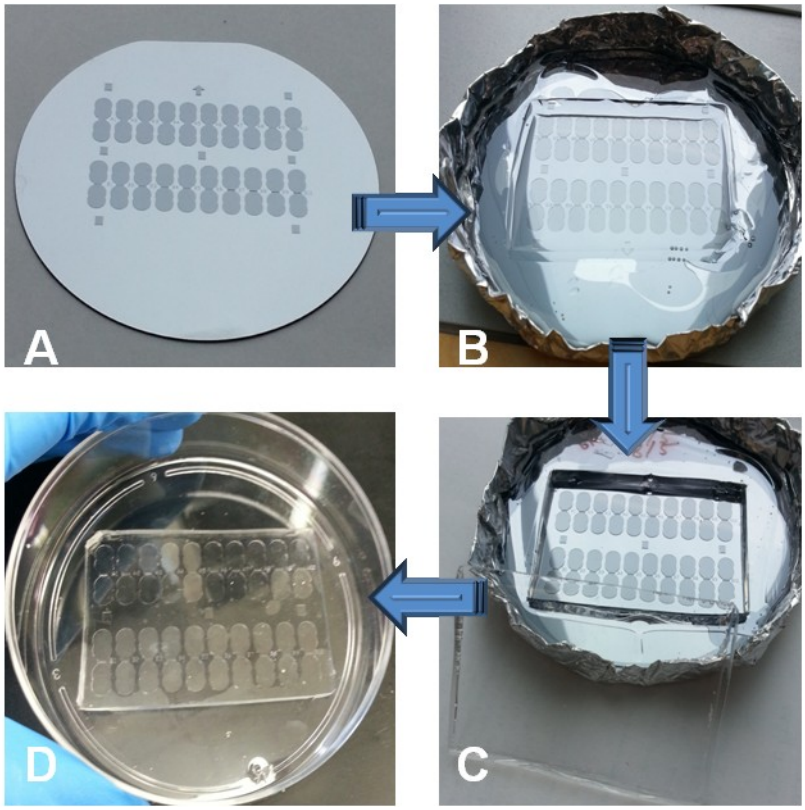

Figure 5: Process of Microfluidic Device Fabrication Using PDMS

A) Silicon wafer with patterns of photoresist. B) Mixture of pdms polymer and curing agent mixed in 10:1 ratio poured in wafer and cured at 150 ^{0}C for 5minutes. C) Cutting of device after curing of PDMS. D) Cured PDMS device

4.5 Results & Discussion

The microfluidic array consists of 20 individual units with multiple channels. Each individual unit can be considered as an independent cell culture chamber to perform multiple experiments simultaneously. The incorporation of the microchannels in the microfluidic devices provides an effective platform to culture and track the movement of each cell individually. The narrow dimensions of the channels in the device provide a better platform to understand the migration mechanism of hGBM cells in response to space restrictions.

The wafer used for preparing cell migration assay device was fabricated with required specifications and under cleanroom conditions. The device was fabricated using Dual layer photolithography with channels at a height of 5 μm and reservoir at a height of 100 μm. Alignment of the two layers is a crucial step in the process of fabrication as misalignment marks the device unusable.

Pretreatment of the wafer before initiating photolithography process is essential to obtain maximum reliability. Wafer cleaning with acetone followed by dehydration ensured the removal of micro particles, adsorbed moisture from the silicon surface and also enhanced the adhesion of resist to the wafer.

Soft baking process was very crucial to remove the solvent from the photoresist in order to obtain desired patterns. It was observed that, improper soft baking affected the sharpness of the features printed on the resist. Prolonged soft bake time at low temperatures was done which improved the resist binding to the substrate.

It was observed that optimum duration of UV exposure was required to obtain sharp and clear patterns. Single continuous exposure for 28 seconds sometimes resulted in the polymerization of surrounding unexposed areas resulting in a loss of sharp features. Over exposure of the resist film to UV resulted in patterns with negative sidewall profiles and altered dimensions while under-exposure lead to partially developed patterns. UV exposure was maintained at 2 consecutive bursts of 14 seconds each to overcome the above limitations.

Two-step contact hot plate process was used for post exposure bake of the wafer to prevent cracking of the photoresist. Rapid cooling of the wafer after post exposure bake was avoided to minimize stress on the resist.

Cell migration devices were fabricated using PDMS at 150 ^{0}C. The curing time was found to depend on the thickness and the amount of PDMS poured on the mold. The maximum curing

time for PDMS device of 15 um height was 5 minutes. During cutting of cured devices the wafer was transferred from 150 ^{0}C to 70 ^{0}C to avoid abrupt cooling of the resist. This step prevented the rapid peeling or cracking of the resist and also enhanced the quality and yield of the devices. The average yield from each wafer before peeling/ breaking of the wafer was greater than 30.

CHAPTER 5

DEVICE ASSEMBLY AND WORKING

Several *in vitro* assays have been developed to study and discover factors that can regulate the cancer migration process [8]. Microfluidics is one of the outcomes of such efforts. Microfluidic devices are considered more suitable for cancer migration studies as they closely replicate the tumor micro-environment [67].

This chapter presents the results of various experiments that were performed to test the feasibility and viability of the designed multi-unit microfluidic array. hGBM cells were seeded in the proximal reservoir and cells were tracked during their course of migration to the distal reservoir. The migration of each individual cell was tracked and measured using ImageJ. The migration behavior of hGBM cells through 5 μm as well as 15 μm channels was assessed to identify the suitable design for performing drug screening. Further dextran dye of molecular weight 40 kDa was used to test the availability of fluid within the channels.

5.1 Device Assembly

The microfluidic array was fabricated as described in Chapter 2. Briefly, dual layer photolithography was used for obtaining master template required for molding microfluidic devices. The mask was designed in two layers; the first layer consisting of channels of 5 μm height and the second layer consisting of reservoirs of 100 μm height. Two different photoresists (SU-8 5 and SU-8 50) were spin-coated on silicon wafer creating height difference between the two layers. The layers were overlapped for connecting the channels and the reservoirs and UV exposed to etch the pattern on the substrate. PDMS was then poured on the mold and cured to obtain the pattern.

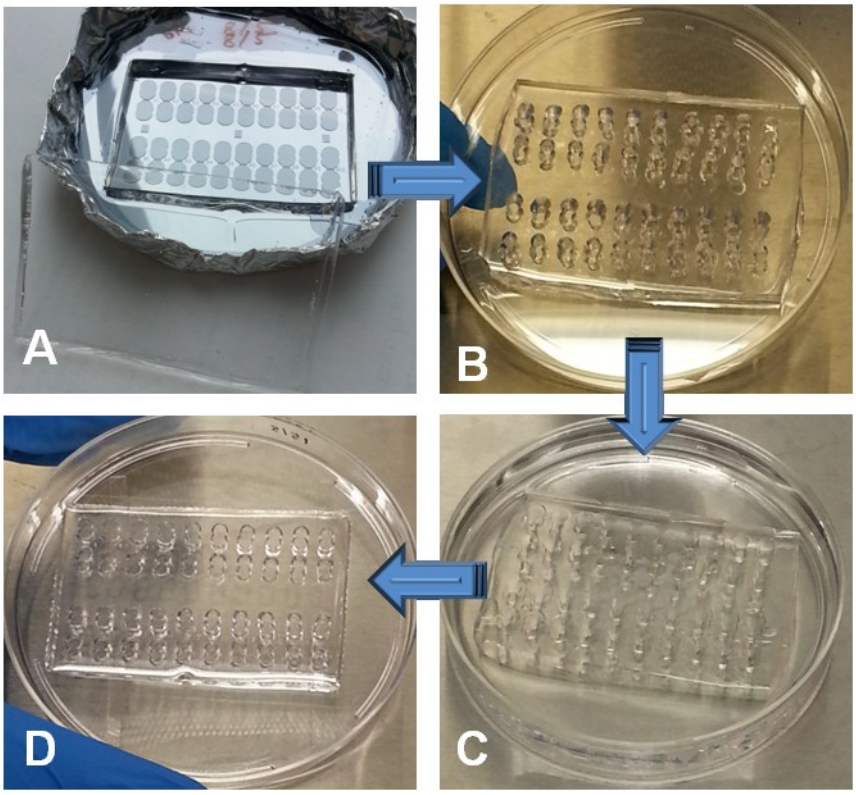

Figure 6: Schematics of microfluidic device assembly

A) Cured PDMS device cut from the wafer. B) Reservoirs of the device opened using 4 mm biopsy punches C) Punched devices were sterilized in 70% ethanol for 30min followed by rinse with DI D) Sterilized devices assembled on 50 mm cover glass.

Following steps describe the assembly of microfluidic devices on substrate:

Step 1: The fabricated PDMS based microfluidic device were placed on a flat surface and punched using 4 mm tissue biopsy punch to form the reservoirs.

Step 2: The punched devices were then cleaned using scotch tape to remove any debris followed by checking under microscope.

Step 3: Sterilization of devices - The devices were immersed in 70% ethanol for 45 minutes and transferred into biosafety cabinet. The devices were removed from ethanol and washed

with deionized (DI) water three times and then dried. Glass and spacer were also sterilized similarly.

Step 4: The dry devices were treated with oxygen plasma with pattern side facing up for 5 minutes to make the PDMS hydrophilic and then assembled immediately on sterilized glass.

Step 5: Sterile DI water was added to both proximal and distal reservoir and allowed to flow through the microchannels. The device was treated for 2 minutes in vacuum chamber followed by pipetting to remove air bubbles and provide uniform distribution of water within channels.

5.2 Studying Cell Migration *In vitro*

One of the main criterions for an efficient microfluidic device used for cell based assays is to support the growth and migration of cells [68]. The functionality of the microfluidic array was assessed by analyzing the ability of device to support cell growth and adhesion

Cells were seeded in both the devices with 5 μm and 15 μm channels. The cells were seeded in the proximal end reservoir of the microfluidic array and migration of cells was tracked during the course of migration towards the distal end reservoir. The device was used to study the rate of migration of cells through restricted channel space over time. Such a study would impart more knowledge on the mechanisms involved by cancer cells during migration in geometrically constricted areas and would support in betterment of treatment associated with tumor migration and metastasis.

5.2.1 Cell Culture

hGBM samples were obtained from consenting patients at University of Texas Southwestern Medical Center (Dallas, TX) with the Institutional Review Board approval. The cells were chemically dissociated with 2% papain and 2% dispase followed by trituration. The cells were suspended in serum free dulbecco's modified eagle's medium/F-12 medium

(DMEM), containing 2% B-27 supplement, 0.25% insulin-transferin-selenium-X, penicilin-streptomycin (100 units/ml and 100 µg/ml respectively) and 20 ng/ml mouse EGF. The cells were transduced with lentivirus expressing monomeric-cherry (m-cherry) fluorescent protein. The cells floating in the medium were used to cell seeding. The medium was centrifuged at 1000 rpm for 5 mins. The pellet was chemically dissociated with 1 ml trypsin-EDTA + 0.2% collagenase type II for 5 minutes at 37 °C. SBTI was added and the cells were physically triturated and centrifuged at 1000 rpm for 5 minutes. 20,000 cells from the cell pellet were used in the cell seeding area containing DMEM/F-12 + B-27 and mEGF. In this study, the substrate was pre-treated with laminin to support adhesion of hGBM cells.

5.2.2 GBM Cell Seeding:

The microfluidic devices were assembled on glass and filled with DI water as described previously. Following steps describe the hGBM cell seeding:

Step 1: Water was drained out from inlet and outlet chambers of the assembled device using a pipette and replaced with laminin to support the growth and adhesion of hGBM cells.

Step 2: The device was incubated with laminin overnight at 37 ^{0}C. The unbound protein was removed by washing the device twice with PBS. The device was filled with PBS until the seeding of cells.

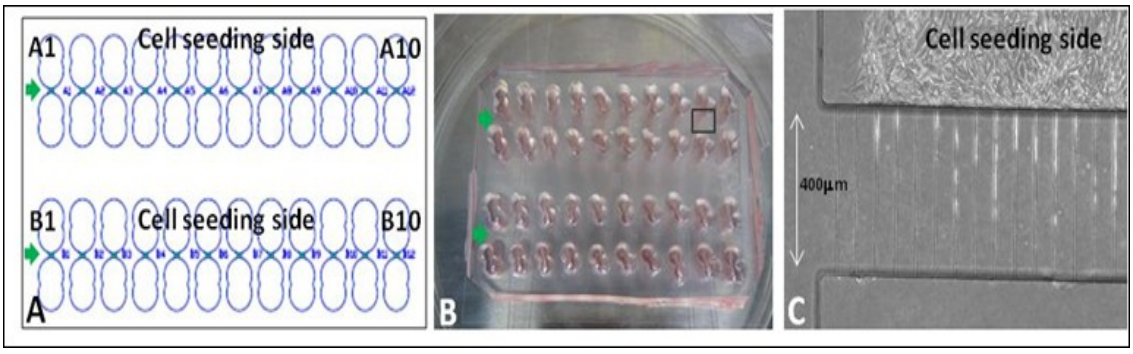

Figure 7: Cancer Cell Migration Assay

A) Two compartments (cell seeding and receiving) bridged by microchannels. B) 20,000 hGBM cells seeded in the proximal reservoir filled with tumor medium. C) GBM migration towards distal reservoir via microchannels 12h after cell seeding.

Step 3: PBS was drained out from both the inlet and outlet chambers and replaced with DMEM/F12 medium prior to cell seeding.

Step 4: hGBM cells were dissociated as described previously. 10 µl of dissociated GBM cells (20,000 cells) were seeded in the proximal reservoir. The pipette tip was placed near the channels of the device to align the cells close to the channels. The cells were incubated at 37 ^{0}C and allowed to migrate through the microchannels toward the distal reservoir.

Step 5: The migration of cells through the microchannels was tracked using a microscope. When almost all the channels were occupied by cells, the device was considered ready for performing experiments.

5.2.3 Fixing of Cells

After capturing all images, GBM cells were fixed with 4% paraformaldehyde solution (fixative), by drawing out DMEM medium from the reservoirs and filling with fixative solution. The cells were incubated with fixative for 30 minutes at room temperature followed by rinsing with PBS. The reservoirs were filled with 1X PBS and the entire device placed in a 150 mm petri dish, sealed with parafilm and was stored at 4 ^{0}C for further analysis.

5.2.4 Quantification of Migration

To characterize the rate of cell migration, cells in the microchannels were tracked over a set time frame. Images of migrated cells were taken as they migrated from proximal side to the distal side reservoir. The initial image was taken when the cell was present at the opening of the channels on the proximal reservoir side. The next set of images was taken after 5h and 24h. Cell migration distance was defined as the distance moved by the proximal end of the cell from

its initial position over a given period of time. The migration rate was calculated for single cells with respect to movement of cell body per hour. The relative migration distance was quantified as the distance moved by the cell in 5h and 24h from its initial position. Cell migration was measured using ImageJ. Figure 7C shows the migration of cells in device with 5 μm channels from proximal to distal end of the reservoir.

5.3 Drug Availability

Microfluidic device for drug screening requires the availability and distribution of drug within the microchannel. A typical device used for drug screening consists of cells occupying the channels that migrate gradually across the channels [68]. The presence of fluid in the channels even when they are occupied by cells is essential criteria of drug screening device. The fluid loaded in the reservoirs must be able to flow over the cells across the narrow channels.

In the current effort, the feasibility of the microfluidic array to permit fluid distribution through the microchannels was tested. Anti-cancer drugs generally have molecular weights around 500 Da. A suitable dye, dextran with a molecular weight of 40 kDa was used to test the availability of drug within the channels occupied by hGBM cells.

5.3.1 Device Assembly and Cell Seeding

The device was fabricated and assembled on glass as described in the previous section 5.1. Dissociated GBM (20,000 cells) were loaded in the inlet reservoirs of the device filled with DMEM/F-12 medium. When most of the channels were populated with cells, the medium was replaced with dextran dye for evaluating fluid distribution across the microchannels.

5.3.2 Dye Preparation

Fluorescein Isothiocyanate Conjugated Dextran (40 kDa FITC) was obtained from Invitrogen. 5 mg/ml stock solution of dextran in DI water was prepared and stored at -20 ^{0}C for long term use. The stock solution was then dissolved in required amount of DMEM/F-12 medium to obtain a final concentration of 20 μg/ml, which was used for drug availability study.

5.3.3 Dye Loading and Tracking

Tumor medium was drawn out from both the inlet and outlet reservoirs of the microfluidic array already occupied by cells. Dextran dye was then introduced in both the reservoirs and allowed to flow across the channels. The movement of dye through the channels was observed 3h after the initial loading. Images of the microchannels were captured using Zeiss LSM510 meta confocal microscope. The channels that were not occupied by cells were used as the control.

5.3.4 Quantification of Dye Availability

The availability and distribution of drugs within the microchannels is tested by tracking the movement of dextran dye. The presence of florescence signal confirms the availability of dye in the channels both in occupied as well as unoccupied by cells. The florescence images taken were analyzed and the florescence intensity was measured using ImageJ. The florescence intensity of dye in the channels unoccupied by cells was compared to intensity of the channels occupied by cells.

5.4 Results & Discussion

5.4.1 Device Design and Working

The designed microfluidic array consists of 20 individual units with multiple channels. The design consists of narrow channels that provide the space constriction for studying the ability of hGBM cells to undergo deformation during the migration process. The reservoirs are designed so as to minimize the use of reagents. Both the proximal and distal reservoirs together can accommodate 0.5ml of fluid. As both the reservoirs of the device are filled with medium of the same concentration; the cell migration is not gradient dependent. This migration model that provides spatial confinement in the absence of a chemical gradient could be a valuable tool to model the tumor microenvironment and hence in the discovery of novel inhibitors of cancer.

Cell seeding experiments were done to demonstrate the feasibility of the microfluidic device to support the growth and migration of cells. Cells are seeded in the proximal reservoir of the device. Generally 20,000 cells are loaded in the proximal reservoir of the device. The entire microarray typically requires 3 ml of the medium. hGBM cells were found to migrate from proximal reservoir to the distal reservoir through the microchannels. Further the cells could survive in the device without requiring medium change for a period of 3-4 days. The optimum time required for cells to migrate through the microchannels to the distal reservoir is 48h. A difference in cell morphology was observed between the cells migration through the channels and cells present in the reservoir. This suggests that the cells undergo morphological changes based on the space and geometric restriction. This can be compared to cancer cell metastasizing through the narrow spaces in the Central Nervous System (CNS). Therefore the microfluidic array can effectively used an *in vitro* platform to recreate tumor environment for performing studies related to cancer migration and inhibition.

5.4.2 Cell Migration Study in 5 μm Channels

Cells seeded in the proximal reservoir of microfluidic device with 5um channel width were tracked as they migrated through the channels toward the distal reservoir. The cells in 5μm channels exhibited an amoeboid morphology as compared to spherical morphology when present in the reservoir. The cells were found to migrate across the entire length of the channel within a maximum time span of 48hours. The migration rate of hGBM cells through the channels was observed to be 7.46μm/hr.

5.4.3 Cell Migration Study in 15 μm Channels

Cells seeded in the proximal reservoir of microfluidic device with 15 μm channel width were tracked as they migrated through the channels toward the distal reservoir. The cells in 15 μm channels exhibited a similar bipolar morphology. The cells were found to travel together as

clusters across the channel and hence it was not was not possible to track the migration of each individual cells.

5.4.4. Comparison between 5 µm and 15 µm Channels:

The cells in the 5 µm exhibited an amoeboid morphology while the cells in 15 µm exhibited a rounded-spherical morphology [Figure 8], suggesting the ability of hGBM cells to undergo morphological changes in response to space restrictions. Earlier studies conducted using microfluidic devices on cancer cells support the above results [65].Though the migration rate in 15 µm could not be determined, based on observation it could be suggested that the cells in 15 µm migrated faster compared to the cells in 5 µm channels. The device with 5 µm was more suitable for studying the migratory behavior of hGBM cells as they provide much more constrained environment than 15 µm channel device. The cells in 5 µm channels undergo deformation while travelling via channels, a mechanism observed during cancer progression and invasion [67]. Hence microfluidic array consisting of 5 µm channels was chosen for determining the inhibitory potential of drugs on hGBM migration.

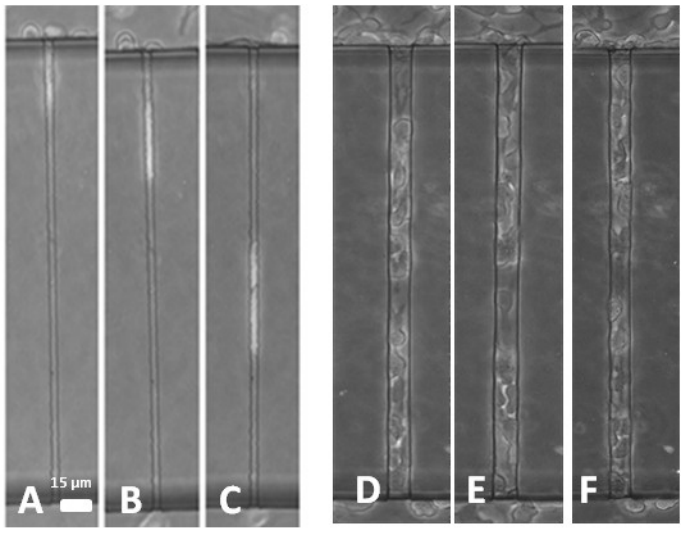

Figure 8: Comparison of GBM migration through 15 µm vs. 5 µm channels.

GBM cells were seeded in microfluidic devices with both 5 μm and 15 μm. Migration was tracked 0, 5 and 24h after the cells entered the microchannels. (A, B and C) Migration via 5 μm channels (D, E and F) Migration via 15 μm channels

5.4.5 Drug Availability:

The florescence intensity of dye in the channels unoccupied by cells was 18% higher when compared to channels occupied by cells [Figure 9]. The higher intensity of florescence in the channels unoccupied by the cells suggests that the dye can travel more readily through open channels.

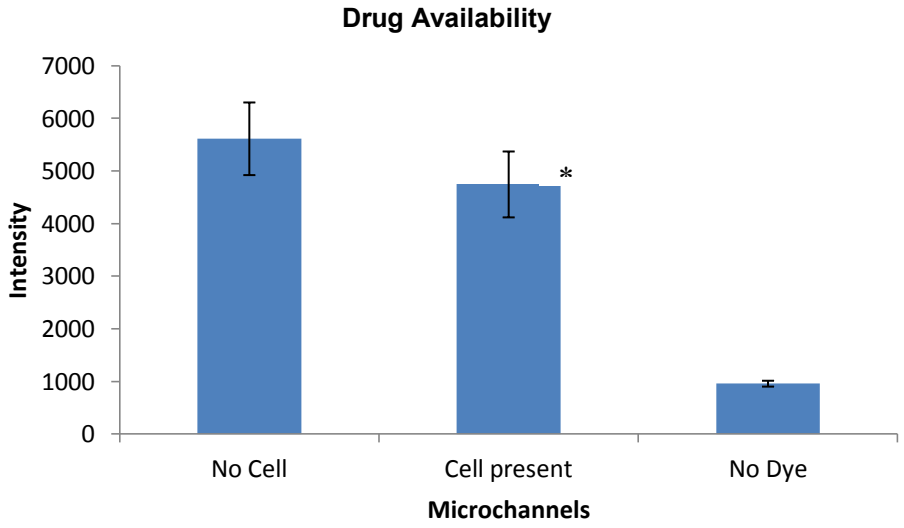

Figure 9: Dye availability in microchannels

hGBM cells were seeded in the microfluidic array. Dextran dye was loaded in the reservoirs and dye availability in the channels was observed 3h after loading. n=20 channels/condition, avg+/- SD, *p<0.05

5.5 Conclusion

A microfluidic array that supports cell growth, migration and allows the flow of fluids through the channels is demonstrated in this effort. This *in vitro* platform allows the visualization of each individual live cell migration which is restricted in current techniques like Boyden Chamber assay and Scratch assay. Microfluidic array with 5 μm channels that closely replicates tumor environment is potentially the right model for analyzing the anti-migratory potential of cancer drugs. The functionality of the device to serve as an *in vitro* platform to assess effect of pharmacological inhibitors on process of cell migration has been demonstrated successfully with this effort.

CHAPTER 6

EXPERIMENTS

6.1 DMSO Vehicle Study

Dimethyl sulfoxide (DMSO) is the commonly used drug vehicle for *in vitro* as well as *in vivo* experiments [75]. Anti-cancer drugs exhibit poor solubility in water and hence are dissolved in organic solvents [76]. An ideal vehicle for cell- based drug studies has the following qualities:

- Is free of any therapeutic value
- Is biocompatible and does not have any adverse biological effects
- Is tested for any possible effect on cell behavior to confirm its compatibility

Presence of any pleiotrophic effects is observed to show bias with the results from drug screening [77]. Numerous drugs dissolved in DMSO were shown to retain their therapeutic activities and specific properties over prolonged period of time. Several research studies have confirmed the absence of any additional effect of DMSO on hGBM migration, making DMSO the ideal choice as vehicle for this effort [42]. In this study, cell migration experiments were performed with different DMSO concentrations in the absence of drugs to evaluate the possible effects of DMSO on cell behavior. The effect of DMSO on the anti-metastatic properties of latrunculin-B on hGBM migration was analyzed.

6.1.1 Materials and Methods

Drug Preparation:

Latrunculin-B was obtained from FDA drug panel at UT Southwestern as frozen 10 mM DMSO stock solution. Latrunculin-B at concentrations ranging from 5 µm to 20 µm were

prepared by dissolving the initial stock solutions in tumor medium. The amount of DMSO present in each individual concentration was calculated and prepared.

Cell Culture:

hGBM cells were dissociated and used for seeding as described previously in section 5.2.

6.1.2 Methodology

Device Assembly and Cell Seeding

The process of microfluidic array fabrication, assembly and cell seeding were described in Chapter 2. In brief, the array was fabricated from PDMS and punched to open the reservoirs. The punched device was then sterilized and assembled on glass. Dissociated hGBM cells (20,000 cells) were seeded in the proximal reservoir and allowed to migrate via the channels. The vehicle study was initiated after the channels were populated with cells.

Experiment

In this effort, the possibility of additional effect of DMSO on Latrunculin-B drug on GBM migration was analyzed. The concentration of DMSO present in each concentration of drug is used to analyze the vehicle effect. Parallel experiments were run with the similar concentrations of drugs. Wells loaded with TM were used as the control. Four individual units consisting of at least 20 cells in the microchannels were used for each drug condition. Drugs and DMSO (only vehicle) were added 3 hours prior to the beginning of cell tracking to allow for the initiation of drug effect on cancer cells. Cell migration was then observed and photographed at 5h, 12h intervals after drug treatment. Cell migration is measured using ImageJ software with migration distance being the distance moved by the proximal end of cell in given time period. After the migration study, the cells were fixed using 4% paraformaldehyde and stored at 4 ^{0}C for further analysis.

6.2 Quantitative Comparison between Scratch Assay and Microfluidics

Scratch Assay is the conventionally used assay for analyzing the anti-migratory potential of drugs on cancer cells. Scratch assay is based on the cell migration towards an artificial gap created by scrapping a confluent cell monolayer with a fine micropipette tip [7]. Cells present along the either edge of injury respond by migrating into the gap and establish new cell to cell contact. The adhesion-dependent (mesenchymal) mode of migration involved in this wound healing response is a poor representation of *in vivo* GBM cell migration [51].

Drug screening studies were performed using scratch assay in addition to microfluidic assay to compare the effect of assay techniques on cell migration characteristics. In this experiment, the effect of nocodazole and latrunculin-B on BRAF mouse astrocytes migration was studied using scratch plate assay technique. Further, similar experiment was performed using cell migration assay and the results were compared.

6.2.1 Materials and Methods

BRAF Cell Culture

p53/pten/BRAF astrocytes were obtained from University of Texas Southwestern Medical Center. The cells were cultured and maintained in 10% FBS DMEM medium. For cell dissociation, the cell suspension was centrifuged at 1000 rpm for 5min. The pellet was chemically dissociated with trypsin-EDTA, followed by incubation at 37 ^{0}C for 5min. The cell suspension was again centrifuged at 1000 rpm for 5min. The pellet obtained was resuspended in suitable amount of 10% FBS containing DMEM/F-12 medium and cell density was determined using hematocytometer. 20,000 cells from the pellet were used for cell seeding.

Drug Preparation

Nocodazole and Latrunculin-B were obtained from FDA drug panel at UT Southwestern. Compounds were received as 500 µM and 10 mM DMSO stock solutions and were frozen until further use. Nocodazole and Latrunculin-B for the quantitative comparison study were prepared

at concentrations ranging from 0.1 μM to 5 μM by dissolving the initial stock solutions in tumor medium.

6.2.2 Scratch Assay

Twenty-four well plates were used for the scratch assay. BRAF astrocytes were dissociated as described previously. Cells were seeded in the well plates filled with tumor medium and incubated at 37 ^{0}C until monolayer formation. A scratch was made along the diameter of the well in the monolayer using a 20 μl pipette tip. The cells were washed twice with tumor medium (TM) medium to remove the detached cells. The medium was discarded from the wells and replaced with the drugs. Nocodazole and Latrunculin-B of concentrations 0.1, 0.2. 0.5,1, 2 uM and 0.2, 0.5, 0.75, 1 μM respectively were prepared as described. Each well was loaded with 1 ml of the drug. Four wells were used for each drug condition. Well loaded with TM was used as the control. The colonization of scratch by cells was tracked using the motorized microscopic stage described in the section 5.2.4. Images were taken every 2 hours until the closure of the scratch. After taking the images, the cells were fixed in 4% paraformaldehyde and stored in 4 ^{0}C for further analysis.

Quantification of cell migration

The cell migration at the wounded edges was observed at every 2h interval. Initial image was taken 3 hrs after the loading of drug. This was followed by tracking and photographing of cells at an interval of 2 hours till the closure of the scratch. The images were analyzed to determine the cell migration rate using ImageJ. Migration was defined as the advance of the scratched edges from the original position of scratch in defined time interval. The relative distance of migration was quantified as the distance advanced by the scratched edges in 5h period. The migration rate was calculated with respect to the advance of the wound edge per hour.

6.2.3 Cell Migration Assay

Assembly and Cell seeding: The device was assembled as described in the previous section 5.2. Dissociated BRAF cells were seeded in the in the inlet reservoir of the microfluidic array and allowed to migrate through the microchannels. The experiment was initiated when almost all the channels were occupied by cells. Medium was drawn out from both inlet and outlet reservoir and replaced with respective concentrations of the drugs. Four individual microfluidic units containing a total of at least 20 cells were used for each drug condition. Well loaded with tumor medium was used as the control. The cell migration was observed and tracked using microscope and images were taken. The initial image was taken 3 hours following loading of drug to allow for the initiation of drug effect. The cell migration was tracked for the same time period as the scratch assay.

Quantification

The relative rate of migration of BRAF cells in 5h following drug exposure was quantified as described earlier in section 6.1.

6.3 Drug Screening

GBM cell migration is mainly regulated by actin, microtubule dynamics, matrix-degrading proteases and protein kinases. Current efforts have focused on investigation of therapeutic drugs that target the molecular pathways regulating cytoskeleton-actin and microtubule dynamics [38, 41]. Kinase pathways which control the cellular processes have shown be to be highly upregulated in GBM [74].

In the present study, microfluidic based cell migration assay was used to investigate the effect of anti-cancer drugs on hGBM migration. The metastatic capability of the drugs modulating the functions of cytoskeleton (Nocodazole, Latrunculin-B, CK-666, Blebbistatin) and kinase signaling cascade (ROCK Inhibitor, AZD, PI-103) on hGBM migration have been evaluated.

6.3.1 Materials and Methods:

Cell migration assay was used to analyze the effect of different drugs of varying concentrations on GBM migration. The drugs used for this study are PI-103, Nocodazole, Blebbistatin, Lat-B, AZD, Rock Inhibitor and CK-666.

Drug Preparation:

The drugs CK-666, PI-103, Nocodazole, Blebbistatin, Latrunculin-B, AZD, and ROCK (Y-27632) were obtained from FDA drug panel at UT Southwestern. The compounds PI-103, AZD, Y-27632 and Blebbistatin were received as 20 mM DMSO stock solutions. CK-666, Latrunculin-B and Nocodazole were obtained as 50 mM, 10 mM and 500 µM DMSO stock solutions respectively. The stock solutions were stored at -20 ^{0}C until use. For drug screening experiments, drugs of required concentrations were prepared by dissolving the initial stock solutions in tumor medium.

Cell Culture

Primary human glioblastoma (hGBM) cells were obtained as described previously in section 5.2. GBM cells (20,000 cells) in 20 µl medium were dissociated and used for seeding in the reservoir of the microfluidic array for drug screening experiments.

6.3.2 Methodology

The PDMS devices are sterilized and assembled on glass as described previously in section 5.1. The microfluidic array was prepared for cell seeding by loading both the proximal and distal reservoirs with tumor medium. Dissociated hGBM cells were seeded in the proximal reservoir and experiments were initiated after all the channels were occupied by cells.

Drug Study

GBM cells were seeded in the inlet reservoir of the laminin coated microfluidic array filled with tumor medium. The cells were allowed to migrate through the microchannels. When almost all the microchannels are populated with cells, the device was considered to be ready for drug

screening study. Tumor medium is drawn out from both the inlet and outlet reservoirs and replaced with required concentrations of the drugs. Four individual units consisting of at least 20 cells in the microchannels were used for each drug condition. Wells loaded with DMEM (tumor medium) were used as the control. The old medium in the control wells was replaced with fresh tumor media prior to the start of drug study. Drugs were added 3h prior to the beginning of cell tracking to allow for the initiation of drug effect on cancer cells. Initial image of cells were taken after the 3h activation period. Cell migration was then observed and photographed 5 hours after drug treatment.

Quantification of Cell Migration

The movement of each individual cell from its initial position was measured using ImageJ. The position occupied by cell 3 hours following the drug treatment was considered as the initial position. The relative migration distance was quantified as the distance moved by the cell in 5h following the initiation of drug effect. After the migration study, the cells were fixed using 4% paraformaldehyde and stored in 4 ^{0}C for further analysis.

6.4 Results and Discussion

6.4.1 DMSO Vehicle Study:

Studies were conducted using the cancer cell migration assay to evaluate the effect of different concentrations of DMSO (vehicle) on hGBM migration characteristics. In this study the effect of DMSO on Latrunculin-B in inhibiting hGBM migration was analyzed. DMSO was observed to have a subtle to no effect on the migration behavior of cancer cells. The rate of cell migration after DMSO treatment was found to be similar to that of the control (only medium). Whereas Latrunculin-B considerably decreased cell migration compared to DMSO and control. Latrunculin-B showed a dose dependent decrease in cell migration. Treatment of hGBM cells with 5 μM Latrunculin-B for 5 hours, decreased cell migration by 75%, when compared to

control. While the same concentration of DMSO present in 5 µM Latrunculin-B, had negligible effect on the cell migration rate.

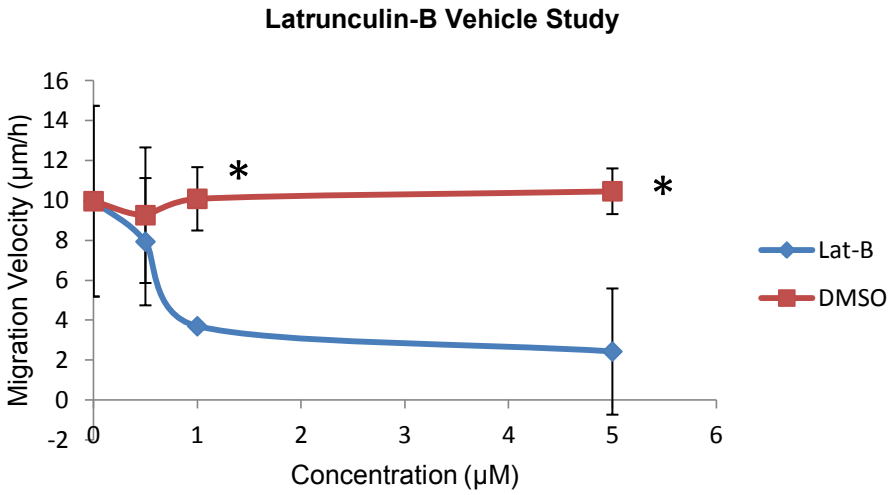

Figure 10: Effect of vehicle (DMSO) on hGBM migration.

hGBM cells cultured in microfluidic array were exposed to different concentrations of Latrunculin-B. Parallel experiments were performed with the individual doses of DMSO present in each concentration of the drug. A) Graphical comparison of the effect of drug and vehicle on cell migration following 5h treatment with Latrunculin-B.

It was observed that there was no significant effect of DMSO on cell motility even at higher concentrations. Based on the results it can be observed that Latrunculin-B considerably decreased cell migration while DMSO exhibited an insignificant effect. The above results confirm that the dose dependent decrease profile in cell migration was solely due to drug effect and not influenced by the presence of vehicle.

6.4.2 Quantitative Comparison between Scratch and Cancer Cell Assay

The anti-migratory effect of F-actin polymerization inhibitor (Latrunculin-B) and microtubule inhibitor (Nocodazole) were tested using scratch assay and compared with same cells migrating

through microchannels (Figure 11). Latrunculin-B and Nocodazole exert differential effects on cancer cell migration when assessed by scratch assay or microchannels.

0.5 µM Latrunculin-B reduced the migration speed from 9.1 to 2.7 µm/hour (71%) in the scratch assay, but this dose only reduced migration velocity by 11% in the microchannels in 5h. 0.5 µM of nocodazole inhibited migration speed by 46% in the scratch assay, while cancer cell assay performed with similar dose reduced migration by 26%. These results clearly showed that both Latrunculin-B and Nocodazole blocked glioma cell migration at significantly lower doses when evaluated by scratch assay, while the inhibition in cell migration was comparatively low with cell migration assay.

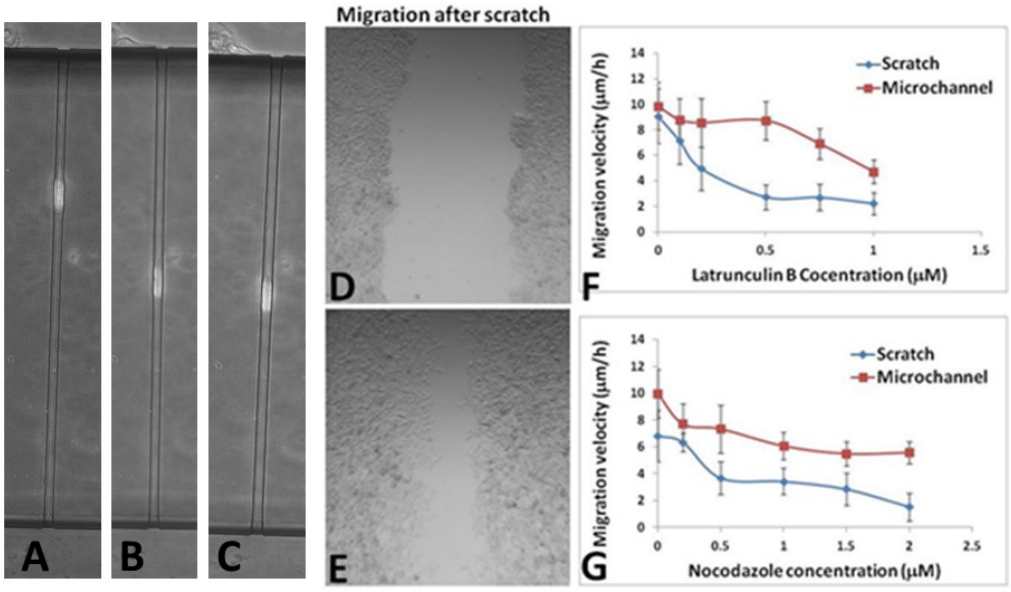

Figure 11: Comparison of cell migration between cell migration assay and scratch assay

BRAF cells were cultured in well plate as well microfluidic array were exposed to different concentrations of Latrunculin-B and Nocodazole. Comparison of Braf cell migration between microchannel (A-C) and Scratch assay (D-E). F and G) Quantitative comparison of cell

migration speed between Scratch and cell migration assay treated with Latrunculin-B (F) and Nocodazole (G). Average ± S.E.M. n=20 cells/each condition.

The above results reflect the differences in migration profile of the same drug when analyzed using both scratch assay and cell migration assay. Scratch assay does not distinguish between collective and individual migration of cells and hence is not an effective model for quantifying the migratory behavior [52]. Cancer cell migration assay allows the tracking of movement of each individual cell unlike scratch assay and hence can be considered to be more accurate and reliable. Therefore cell migration assay can be considered to be a better and efficient alternative to the scratch assay for screening of anti-metastatic drugs.

6.4.3 Drug Screening

The anti-migratory potential of Latrunculin-B, CK-666, Nocodazole, PI-103, Blebbistatin, AZD and Rock Inhibitor on GBM cell migration was assessed using the cell migration assay.

All the seven drugs showed a dose dependent decrease in migration of hGBM cells as shown in Figures 12 through 18. However, none of the seven drugs could completely inhibit migration and the degree of inhibition varied considerably among different drugs screened.

PI-103 in the concentrations ranging from 0.5 μM to 5 μM was used in the present study. A dose dependent decrease in cell migration velocity of hGBM cells was observed. Treatment of the cells with 0.5 μM of PI-103 resulted in 32% decrease in cell migration over a period of 5h when compared to control. 5 μM of PI-103 inhibited migration by 66% in 5 hour duration.

Nocodazole of concentrations between 0.5 μM and 5 μM were tested for anti-migratory potential. A concentration dependent reduction in cell migration rate was observed. 0.5 μM nocodazole decreased cell migration by 36% over 5h treatment period. While, treatment with 5 μM decreased cell motility approximately by 63%.

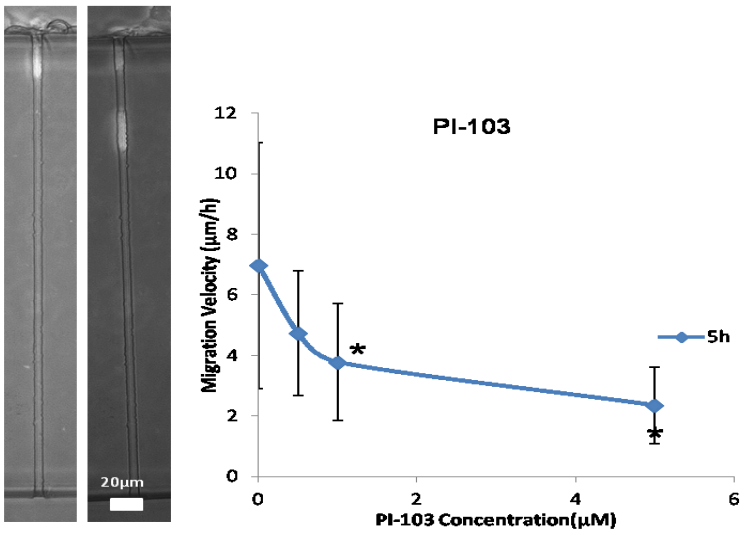

Figure 12: Effect of PI-103 on hGBM migration rate.

hGBM cells cultured in the microfluidic array were exposed to 0.5, 1 and 5 µM concentrations of PI-103 for 5h. A) Cell migration via microchannels 0 and 5h after exposure to 5.0 µM. B) Graphical representation of the variation in migration rates with drug concentration. Results represent mean±SEM of 4 independent experiments. *p<0.05 *vs*. control. Scale bar=20 µm

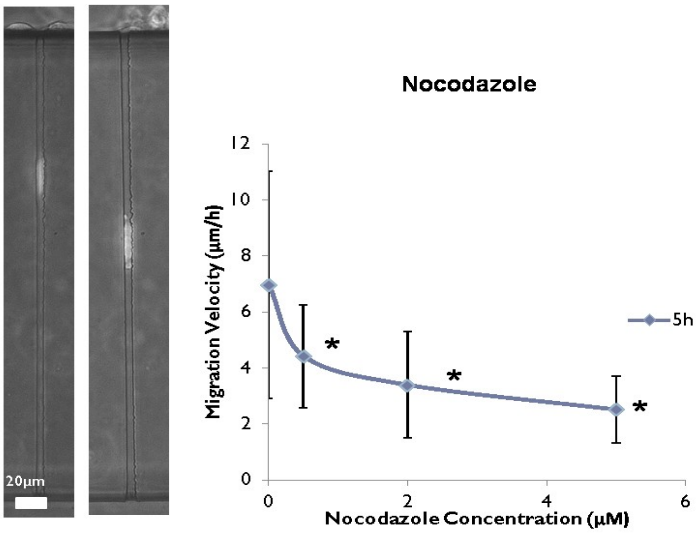

Figure 13: Effect of Nocodazole on hGBM migration rate.

hGBM cells cultured in the microfluidic array were exposed to 0.5, 2 and 5 µM concentrations of Nocodazole for 5h. A) Cell migration via microchannels 0 and 5h after exposure to 5.0 µM Nocodazole. B) Graphical representation of the variation in migration rates with drug concentration. Results represent mean±SEM of 4 independent experiments. *p<0.05 vs. control. Scale bar=20 µm

hGBM cells exposed to 5 µM CK-666 for 5 hours, inhibited cell migration by 32% when compared to control. At 20 µM, migration of hGBM cells was inhibited by 70%.

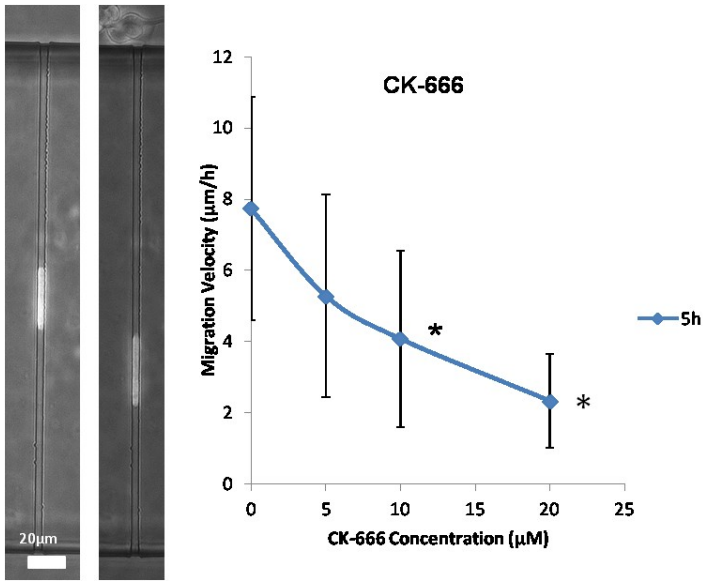

Figure 14: Effect of CK-666 on hGBM migration rate.

hGBM cells cultured in the microfluidic array were exposed to 5, 10 and 20 µM concentrations of CK-666 for 5h. A) Cell migration via microchannels 0 and 5h after exposure to 20 µM CK-666. B) Graphical representation of the variation in migration rates with drug concentration. Results represent mean±SEM of 4 independent experiments. *p<0.05 *vs.* control. The concentrations 10, 20 µM were statistically significant. Scale bar=20 µm

hGBM cells treated with 0.5 µM Latrunculin-B for 5 hours, inhibited cell migration by 19% when compared to control. At 5 µM, migration of hGBM cells was inhibited by 74%.

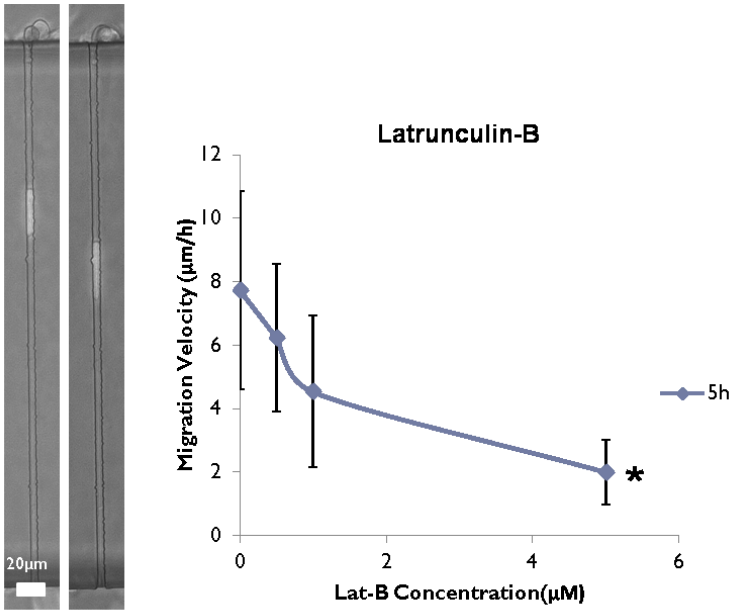

Figure 15: Effect of Lat-B on hGBM migration rate

hGBM cells cultured in the microfluidic array were exposed to 0.5, 1 and 5 µM concentrations of Latrunculin-B for 5h. A) Cell migration via microchannels 0 and 5h after exposure to 5.0 µM Latrunculin-B, B) Graphical representation of the variation in migration rates with drug concentration. Results represent mean±SEM of 4 independent experiments. *$p<0.05$ vs. control. Scale bar=20 µm

An inhibition rate of 27% in cell motility was observed when hGBM cells were exposed to 1.0 µM AZD for 5h when compared to control. Whereas at 10 µM concentration the inhibition rate of hGBM was 62%.

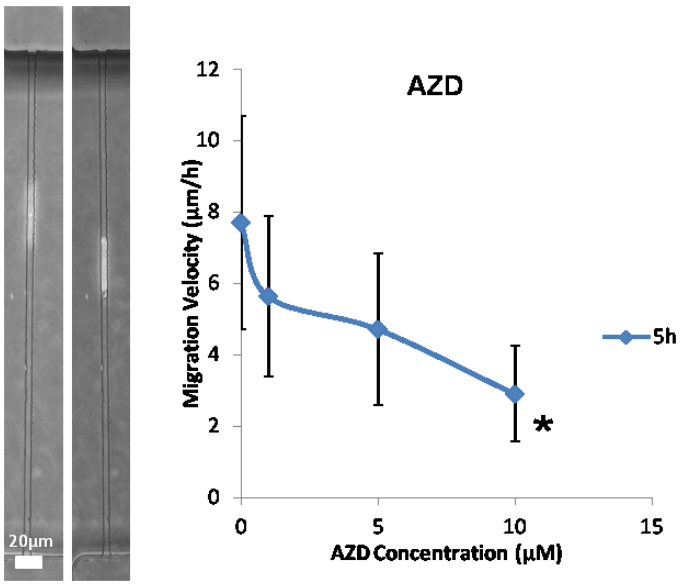

Figure 16: Effect of AZD on hGBM migration rate.

hGBM cells cultured in the microfluidic array were exposed to 1, 5 and 10 µM concentrations of AZD for 5h. A) Cell migration via microchannels 0 and 5h after exposure to 10 µM AZD. B) Graphical representation of the variation in migration rates with drug concentration. Results represent mean±SEM of 4 independent experiments. *$p<0.05$ vs. control. The concentrations 5, 10 µM were statistically significant. Scale bar=20 µm

hGBM cells treated with 1.0 µM Blebbistatin for 5 hours inhibited cell migration by 18% when compared to control. At 50 µM, migration of hGBM cells was inhibited by 69%.

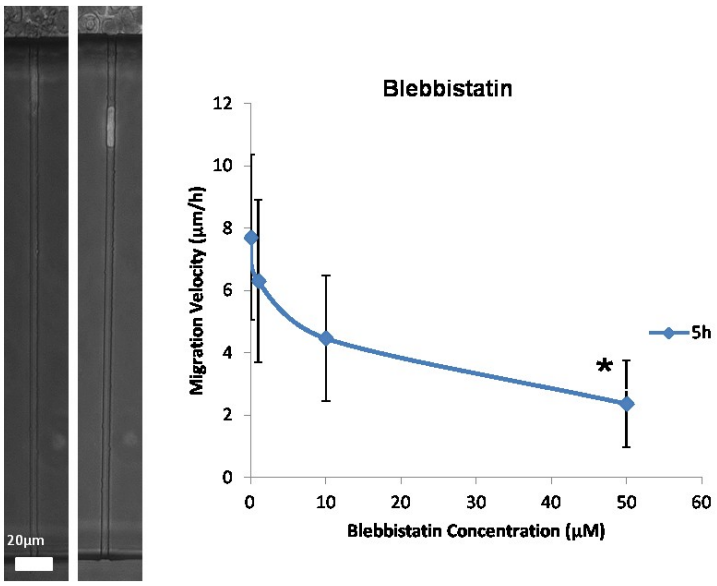

Figure 17: Effect of Blebbistatin on hGBM migration rate.

hGBM cells cultured in the microfluidic array were exposed to 1, 10 and 50 µM concentrations of Blebbistatin for 5h. A) Cell migration via microchannels 0 and 5h after exposure to 50 µM. B) Graphical representation of the variation in migration rates with drug concentration. Results represent mean±SEM of 4 independent experiments. *$p<0.05$ vs. control. The 50 µM concentration was statistically significant. Scale bar=20 µm

hGBM cells exposed to 0.5 µM ROCK for 5 hours, inhibited cell migration by 27% when compared to control. At 10 µM, migration of hGBM cells was inhibited by 57%.

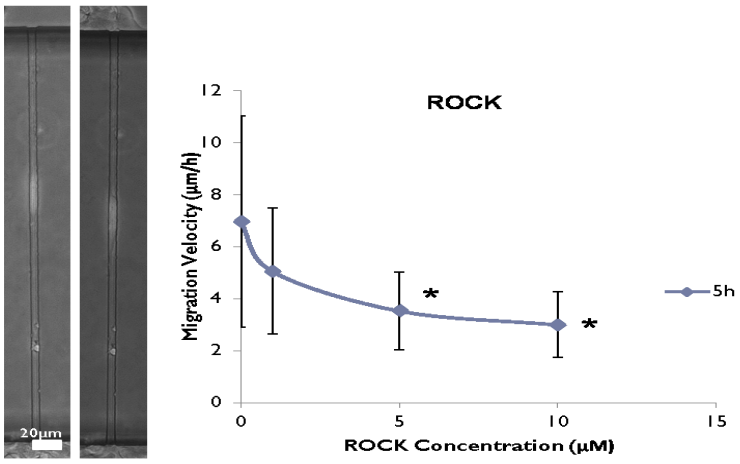

Figure 18: Effect of ROCK on hGBM migration rate

hGBM cells cultured in the microfluidic array were exposed to 1, 5 and 10 µM concentrations of ROCK for 5h. A) Cell migration via microchannels 0 and 5h after exposure to 10 µM ROCK. B) Graphical representation of the variation in migration rates with drug concentration. Results represent mean±SEM of 4 independent experiments. *p<0.05 *vs*. control. The concentrations 5, 10 µM were statistically significant. Scale bar=20 µm

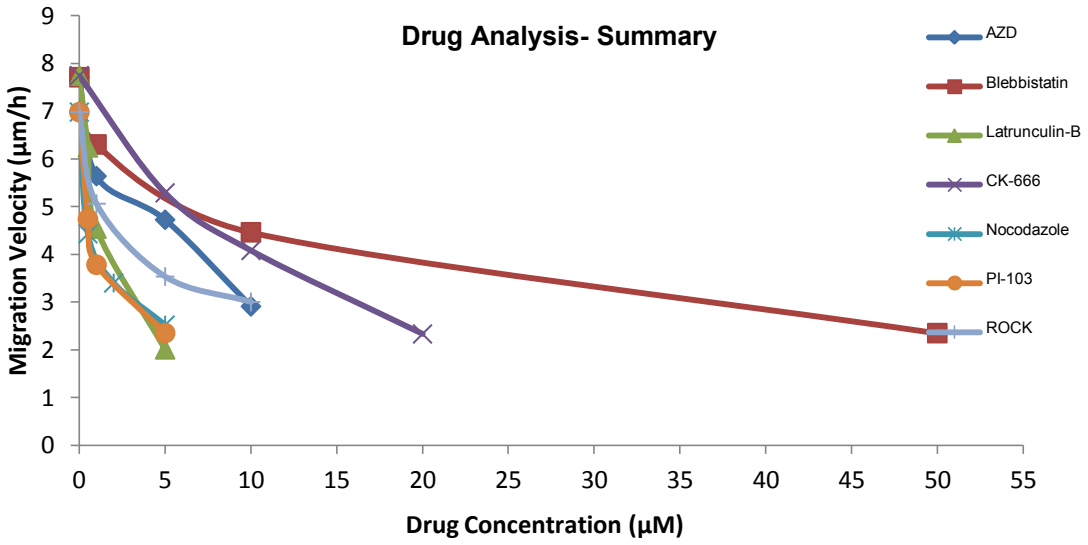

Figure 19: Drug profile summary

Table 1 presents an overview of effective dosage of selected drugs and their ability to inhibit hGBM migration.

Table 1: Effective drug concentrations

Drug	Effective Dosage	% inhibition
Latrunculin-B	5µM	74
Blebbistatin	50µM	69
CK-666	20µM	70
PI-103	5µM	66
ROCK	10µM	57
AZD	10µM	62
Nocodazole	5µM	63

From the Drug profile rates (Figure 19), it was observed that none of the seven drugs evaluated could completely inhibit the migration of hGBM cells. However, all the drugs showed a dose dependent reduction in the cell migration rate. Among the seven drugs, Latrunculin-B at 5 µM concentration was most effective in the reducing motility of cancer cells when evaluated using cell migration assay.

6.5 Conclusion

Results such as these suggest that the evaluation of prospective inhibitors of tumor cell migration need to employ ex vivo assays that require tumor cells to adopt modes of migration that they are most likely to employ *in vivo*. Cancer cell migration assay as discussed in earlier sections can serve as a better and efficient alternative to the conventional assays for the screening of anti-metastatic drugs.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

This final chapter is divided into three sections. The first section summarizes the research completed. The second section lists the contributions of this thesis and the third section outlines areas for future work.

7.1 Research Summary

As part of this research effort, a new innovative design of a microfluidic platform with spatial constrictions was proposed and developed. The device consisted of an array of 20 microchannel units and fabricated by a two layer soft lithography. Each unit was designed with two compartments (cell seeding and receiving side) bridged by 39 microchannels (either 15x15 μm (2D) or 5x5μm (3D), 400 μm length), and the entire migratory procedure was monitored in real-time. This design has the advantage of using multiple types of drugs of varying concentrations to simultaneously study their effectiveness to inhibit cancer cell migration. The migration characteristics of cancer cells in response to various anti-cancer drugs were also investigated. The metastatic potential of the existing and approved drugs were evaluated using this migration assay.

7.2 Contributions

A novel cancer cell migration assay that is more clinically relevant and suitable for high-throughput screening is the main outcome of this effort. Studying the migratory behavior of cell under spatially confined microenvironment contributes important fundamental information that is currently lacking. Following is the specific list of contributions:

- Design and Fabrication of a novel high-throughput microfluidic array platform for cancer migration study.
- Examination and Analysis of therapeutic potential of anti-cancer migratory inhibitors on tumor cell migration.
- Quantitative evaluation and comparison of the newly designed assay with standard cancer migration (scratch wound) assay.

7.3 Future Work

This thesis effort is a first step towards developing an *in vitro* assay for analyzing the migratory behavior of cancer cells. The main purpose here was to develop a high-throughput model to screen the pharmacological compounds that can inhibit cancer migration. Using this assay as base, extensions can be made to this to make it a scalable system. To make this effort complete, the following considerations could be made in future:

- A new prototype based on the current design to standardize the platform size (96 well plate) to enable automated analysis using commercial screening platforms (GE In Cell Analyzer or PE Operetta). Such a platform will enable the screening of several thousands of targeted migratory inhibitors. This provides a promising scope for the development of novel pharmacological inhibitors targeting various types of cancer cell migration and invasion.
- The current study evaluated the decrease in migration rate of hGBM cells on treatment with anti-cancer drugs. However an understanding of molecular and cellular mechanisms of GBM migration is required for the discovery of novel pharmacological inhibitors. Cytoskeletal protein expression profile during the migration process using the newly developed assay can be evaluated as a prospective option.
- Current effort was limited to hGBM cell migration behavior. This study can further be extended to renal cancer cells and lung cancer cells that metastasize to brain. Such a

study can yield information about the effect of cell type and morphology on the migratory behavior. Combining the results of these studies can provide optimum drug dosage for metastasizing brain cancer.

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BIOGRAPHICAL INFORMATION

Personal Data: Born in Visakhapatnam, on April 29, 1990, the daughter of Gowri Shankar and Ramani Dhavala. Received Bachelors of Engineering Degree in Biotechnology from GITAM Institute of Technology & Sciences, Visakhapatnam in May, 2011; graduated with first class honors. Completed the requirements for the Master of Science degree in Bioengineering at University of Texas at Arlington in May, 2013.

Experience: Currently working as Research Assistant in the Department of Bioengineering at University of Texas, Arlington. Research interests include Neuro-oncology and Neurosciences and currently pursuing research on Microfluidics for Cancer research at UTA under the guidance of Dr. Young-tae Kim.

Professional Memberships: Biomedical Engineering Students Society (BMESS)