Engineering a Three Dimensional Micropatterned Tumor Model for Breast Cancer Cell

Migration Studies

by

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ABSTRACT

Breast cancer cell invasion is a highly orchestrated process driven by a myriad of complex microenvironmental stimuli. These complexities make it difficult to isolate and assess the effects of specific parameters including matrix stiffness and tumor architecture on disease progression. In this regard, morphologically accurate tumor models are becoming instrumental to perform fundamental studies on cancer cell invasion within well-controlled conditions. In this study, the use of photocrosslinkable hydrogels and a novel, two-step photolithography technique was explored to microengineer a 3D breast tumor model. The microfabrication process presented herein enabled precise localization of the cells and creation of high stiffness constructs adjacent to a low stiffness matrix. To validate the model, breast cancer cell lines (MDA-MB-231, MCF7) and normal mammary epithelial cells (MCF10A) were embedded separately within the tumor model and cellular proliferation, migration and cytoskeletal organization were assessed. Proliferation of metastatic MDA-MB-231 cells was significantly higher than tumorigenic MCF7 and normal mammary MCF10A cells. MDA-MB-231 exhibited highly migratory behavior and invaded the surrounding matrix, whereas MCF7 or MCF10A cells formed clusters that were confined within the micropatterned circular features. F-actin staining revealed unique 3D protrusions in MDA-MB-231 cells as they migrated throughout the surrounding matrix. Alternatively, there were abundance of 3D clusters formed by MCF7 and MCF10A cells. The results revealed that gelatin methacrylate (GelMA) hydrogel, integrated with the two-step photolithography technique, has great promise in creating 3D tumor models with well-defined features and tunable stiffness for detailed studies on cancer cell invasion and drug responsiveness.

DEDICATION

I dedicate my work to my beloved parents, Mrs. & Mr. Sam Mathews.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

3D	Three dimensional
2D	Two dimensional
AFM	Atomic Force Microscopy
ANOVA	Analysis of Variance
BioMEMS	Bio Microelectromechanical Systems
BM	Basement membrane
BSA	Bovine Serum Albumin
CAF	Cancer associated fibroblasts
CDM	Cell derived matrix
CI	Calcein AM
CIS	Carcinoma in situ
CTC	Circulating tumor cells
DAPI	4',6-diamidino-2-phenylindole
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco's Modified Eagle's Medium
DPBS	Dulbecco's Phosphate Buffered Saline
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ETD	Ethidium homodimer

FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GelMA	Gelatin methacrylate
GEM	Genetically engineered mouse models
HA	Hyaluronic acid
Hgbm	Human glioblastoma multiforme
HMF	Human mammary fibroblasts
H NMR	Proton Nuclear magnetic resonance
HUVEC	Human umbilical vein endothelial cell
IDC	Invasive ductal carcinoma
LM	Laminin
MAT	Mesenchymal to amoeboid transition
MEMS	Microelectromechanical Systems
MMP	Matrix metalloprotease
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PLA	Polylactide
PLG	poly(lactide-co-glycolide)
PLGA	Poly(lactic-co-glycolic acid)
PVA	poly(vinyl alcohol)
SD	Standard deviation

SDF-1	Stromal-derived factor 1
TMSPMA	3- (trimethoxysilyl) propyl methacrylate
UV	Ultraviolet
VEGF A	Vascular endothelial growth factor A

CHAPTER 1

INTRODUCTION

1.1 BURDEN OF BREAST CANCER

Breast cancer is the second highest cause of cancer related deaths in the United States. According to the National Cancer Institute, over 200,000 cases of invasive breast cancer are expected to be diagnosed in the U.S., in 2015, and nearly 20% of these individuals are estimated to succumb to it. Women in the age range of 55-64 years are seen to be more prone to die of breast cancer with the risk increasing with age. Studies have shown that in women with a family history of breast cancer, the risk increases two to three-fold. Other minor risk factors/early indicators for breast cancer include early onset of menstruation, late menopause, first full-time pregnancy at a late age, breastfeeding for less than a year, use of oral contraceptives and BRCA1, BRCA2 gene mutations (Kelsey and Bernstein 1996). However, most cases of breast cancer occur and are detected in women who have no perceptible risk factors (Kelsey and Bernstein 1996).

Breast cancer cases have been recorded for thousands of years and have primarily been treated using surgical procedures (Rayter 2003). Early cases, reported by the Egyptians, were treated by cauterizing the tissue that was afflicted by the disease, with a tool called a "fire drill". Dr. Henry LeDran, in 1757, was one of the pioneers to propose the theory that breast cancer begins as a local disease but spreads to neighboring as well as distant organs through the circulatory system (Rayter 2003). In the mid-1800s, surgeons started to keep a more detailed account of breast cancer. They noted that even after a mastectomy, there was a high recurrence rate of the disease due to its spread to nearby glands or lymph nodes (Rayter 2003). When breast cancer was observed to recur near the

surgical site, Charles Moore, cancer specialist at the Middlesex Hospital in London, established some principles that involved removal of surrounding tissues and infected axillary glands (Rayter 2003).

Metastasis of the tumor to secondary sites results in a poor prognosis of the patient, and there has not been a definitive way to detect and combat it. Thus, in recent years, there have been numerous attempts at studying breast cancer metastasis and invasion. In particular, there has been increasing focus on how the tumor microenvironment contributes to changes in cancer cell behavior and, motility (Wang, Eddy, and Condeelis 2007). Modern studies using technologies based on imaging and profiling focus on identification of molecular pathways that can lead to diagnostic and therapeutic approaches to treat breast cancer.

1.2 METASTASIS

Metastasis, the primary cause of mortality among individuals with breast cancer, is the spread of cells from the primary tumor site to distant organs (Nguyen, Bos, and Massagué 2009). There are several steps in the metastatic cascade **(Figure 1.1)** and including the epithelial-mesenchymal transition (EMT), degradation of the basement membrane, invasion of surrounding tissue, intravasation of tumor cells into the neighboring blood vessels, transportation of tumor cells through the vasculature, arresting of the tumor cells and their extravasation, and formation of the secondary tumor along with extracellular matrix (ECM) remodeling and angiogenesis (Geiger and Peeper 2009). The successful completion of each of these stages would result in metastasis and the development of a secondary tumor **(Figure 1.1 (7))**. A cancer cell might fail to complete any one of the stages

of the metastatic cascade and it would result in unsuccessful metastasis. Thus, metastasis is considered to be an inefficient process (Wong et al. 2001).
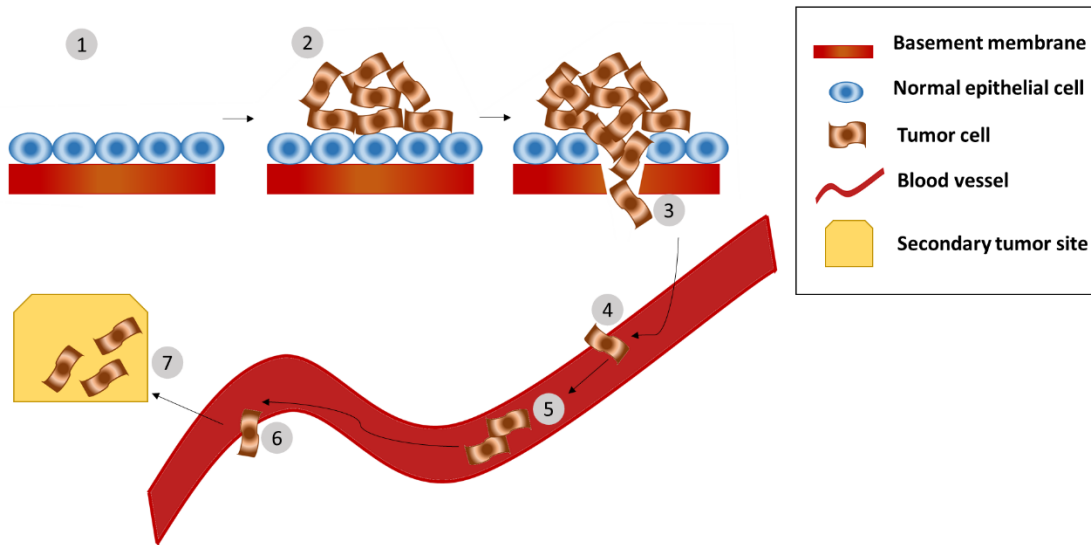
Figure 1.1: The metastatic cascade (1) Normal organ lined with epithelial cells bound by the basement membrane (2) epithelial-mesenchymal transition, (3) degradation the basement membrane and invasion into the surrounding tissue, (4) intravasation into neighboring blood vessel (5) transport through the vasculature, arrest of tumor cells, (6) extravasattion from the blood vessel and (7) formation of tumor in the secondary site.

The 'seed and soil' hypothesis that Paget proposed in 1889 based on the data collected from 735 women suffering from breast cancer states that cancer cells, "the seed", need a conducive environment, "the soil", to metastasize, thrive and form a tumor in a distant organ (secondary tumor) (Paget 1989). The conducive environment is called the premetastatic niche and promotes the invasion of the tumor cells into surrounding tissue. Initiation and development of the pre-metastatic niche is observed to originate from many different factors. Apart from factors such as the presence of stromal-derived factor 1 (SDF-1) chemokine, the pre-metastatic niche contains microenvironmental components such as fibroblasts and endothelial cells which secrete growth factors and chemokines that influence tumor cell polarity, circulation, and migration (Kucia et al. 2005, Orimo et al.

2005). Therefore, before tumor cells initiate the first step of metastasis, a receptive microenvironment assembles, which eventually promotes the formation of the secondary tumor.

Most solid tumors arise from epithelial cells, and metastasis is initiated with the cells undergoing the EMT. The EMT starts as cells lose their epithelial polarity and epithelial proteins such as E-cadherin, cytokeratins and catenin proteins get downregulated (Christofori 2006, Jechlinger et al. 2003). Furthermore, the EMT promotes metastasis by allowing cancer cell invasion with the loss of cell-cell adhesion (Perl et al. 1998), secreting matrix metalloproteinases (MMPs) which aid in degrading the proteins of the ECM (Giannelli et al. 1997), overexpressing mesenchymal proteins like N-cadherin (Hazan et al. 2000, Nieman et al. 1999), and inhibition of apoptosis (Maestro et al. 1999, Vega et al. 2004). Invasion is the second step in metastasis which will be discussed in detail in the next section, owing to its relevance to this study. Angiogenesis, a hallmark of cancer, is the process by which new blood vessels grow from pre-existing vessels. For a tumor to grow and metastasize to distant organs, it needs to be part of a vascularized network and gain access to necessary nutrients and oxygen. Tumors which do not have vascularized networks have historically not grown past 1 mm in size (Gimbrone et al. 1972, Bergers and Benjamin 2003, Kalluri 2003). In adults, angiogenesis is typically rare and strictly kept in check, but during tumor progression, vascularization is activated with the help of factors such as epidermal growth factor (EGF), vascular endothelial growth factor A (VEGF A), and fibroblast growth factor (FGF) (Bergers and Benjamin 2003, Kalluri 2003). The angiogenic switch is an important step for the tumor to grow to a disproportionate size (Bergers and Benjamin 2003).

In the next step of metastasis, cancer cells spread to distant organs by entering and getting transported through the blood vessels. In a study where metastatic breast cancer cells were injected into a mouse, it was observed that the tumor cells direct and orient themselves along the blood vessels (Li et al. 2000). Tumor cells can also enter lymph vessels passively as has been seen in a pancreatic β-cell tumor mouse model (Geiger and Peeper 2009). Large tumors spew/shed millions of tumor cells into circulation every day, but since metastasis is a very inefficient process, very few of them survive (Cameron et al. 2000). Once the cells enter the circulatory system, a large number of them might be eliminated due to anoikis; the process of programmed cell death or apoptosis caused due to loss or inadequate cell adhesion (Paoli, Giannoni, and Chiarugi 2013), due to the force of blood flow. In the first few hours after tumor cells attach to the wall of the blood vessel, extravasation occurs with the help of cytoplasmic protrusions and deformations (Tsuji et al. 2006). The cancer cells may proliferate within the blood vessels and extravasation may occur when these cells outgrow the vascular structures, destroying the vessel boundary in the process (Wong et al. 2002). Most of the cells after extravasation undergo apoptosis in the first 24 hours. Lack of adhesion cues and several other external factors may lead to anoikis of tumor cells at the secondary site. One of the factors of anoikis is the release of cytotoxic products by the surrounding cells and another factor is the presence and action of immune cells on the cancer cells. The tumor cells are seen to survive as individual, a small group or a large group of cells and remain dormant for a long period of time.

1.3 INVASION

Invasion is a subcategory of metastasis when the cancer cells must attain the ability to migrate in order to invade tissues and blood vessels. Membrane protrusions such as lamellipodia, filopodia, pseudopods and invadopods (Adams 2001) assist in the migration and invasion of the tumor cells through actin polymerization and depolymerization. Mesenchymal mode of cell migration is accomplished with a few inter-reliant steps. The first step in migration is cell polarization and elongation. Further, a pseudopod is formed at the leading edge, which attaches to the ECM substrate. Finally, the cell body contracts, pulling the trailing edge and the cell body forward by generating traction forces (Friedl and Wolf 2003). The role of integrins and focal complexes growing and stabilizing into focal contacts emerges after the cell elongates and comes in contact with the ECM (Friedl and Wolf 2003). Both integrin and non-integrin receptors play a prominent role in the formation of focal contacts and consequently, migration and invasion through the basement membrane (BM) with the help of recruited proteases (Friedl and Wolf 2003).

In the context of breast cancer, carcinoma in situ (CIS) begins with the neoplasm contained within the BM and in ductal carcinoma in situ (DCIS), the BM is altered even though it is intact (Kalluri and Zeisberg 2006). The transition from DCIS to invasive ductal carcinoma (IDC) includes degradation of the BM and subsequently, the "reactive stroma" as the cancer cells coming in contact with each other. The subsequent changes in gene expressions is followed by transitions like the EMT and, eventually, migration and invasion of the cancer cells (Kalluri and Zeisberg 2006).

Breast cancer cells can disseminate from the primary tumor and adopt a heterogenous morphology while invading (van Zijl, Krupitza, and Mikulits 2011). If the

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cancerous cells lose one particular migration ability, they develop an alternate migratory approach owing to the fact that cancer cells express varying degrees of proteases and integrins (van Zijl, Krupitza, and Mikulits 2011). This phenomenon of developing an alternate method of migration is called 'plasticity'. Consequently, the cancer cells can invade as individual cells, lines, sheets or clusters, which demonstrates changes such as EMT and mesenchymal to amoeboid transition (MAT) (van Zijl, Krupitza, and Mikulits 2011). Such behavior can either occur due to changes in the microenvironment like modifications in the substrate adhesiveness, cell-cell adhesions, need for ECM proteolysis or due to drug treatments such as protease, MMP inhibitors, etc. (Geiger and Peeper 2009).

The characteristics of amoeboid cell invasion are loss of polarity, limited attachment, and no remodeling of the ECM (Condeelis and Segall 2003). Amoeboid migration is faster because cells need less adhesion contacts and no ECM remodeling. Amoeboid cell invasion does not depend on proteases; it makes use of mechanical forces to displace matrix fibrils instead of completely degrading them (Sabeh, Shimizu-Hirota, and Weiss 2009). MAT frequently comes into picture when cancer is being treated with inhibitors (Wolf et al. 2003). Another form of cell invasion is collective cell invasion and its three main characteristics are maintenance of intact cell-cell junctions (Friedl et al. 2004), generation of traction force by coordination of polarity (Hegerfeldt et al. 2002) and, cytoskeletal reorganization, remodeling of the ECM and basement membrane (Wolf et al. 2007). Collective cell invasion can assume many forms such as a monolayer that invades two-dimensionally or cell strands and clusters that can invade tissues in three dimension (3D). The reduction in polarity of luminal epithelial cells in breast cancer tumors causes collective cell migration, and consequently, the cancer switches from in situ to invasive

carcinoma (Gray, Cheung, and Ewald 2010). The main requirements in collective cell migration is that the cells have to preserve their cell-cell contacts (Friedl and Gilmour 2009, Friedl et al. 2004), and the collective movement has to generate a traction force, which is majorly given by the integrins present in the leading cells (Gaggioli et al. 2007). The tumor cells, contrary to normal cells, are promoted by non-existent stop signals to the migratory events (Friedl and Wolf 2003). This lack of balance drives the tumor cells to invade the surrounding tissues and migrate to the distant organs.

1.4 COMPLEXITIES OF THE BREAST TUMOR MICROENVIRONMENT

The two leading theories on the instigation of breast cancer are "cancer stem cell hypothesis" and "stochastic model of carcinogenesis". A major concept behind cancer stem cell hypothesis is that tumors are derived from tissue stem cells or progenitor cells through a dysregulation of the self-renewal pathway (Wicha, Liu, and Dontu 2006). Owing to this property, the tumors will preserve stem cell characteristics which lead to self-renewal, differentiation and heterogeneity in the cancer cells (Wicha, Liu, and Dontu 2006). On the other hand, in the stochastic model of carcinogenesis, it is hypothesized that tumorigenesis occurs due to random mutations in the breast epithelial cells like stem, differentiated or progenitor cells (Sgroi 2010). It has been postulated that the accumulation of genomic instability in the stroma might lead to genomically unstable epithelium and consequently, neoplastic transformation (Weber et al. 2006). In addition, a number of observations in human patients have led to the postulation that mutations and, consequently, tumorigenesis can be promoted by the host microenvironment (Artacho-Cordón et al. 2012).

The components of the microenvironment **(Figure 1.2)** play a crucial role in regulating carcinogenesis (Place, Jin Huh, and Polyak 2011). The native breast

microenvironment is composed of both stromal components and ECM. The surrounding stroma includes fibroblasts, adipocytes, endothelial cells as well as immune cells (Place, Jin Huh, and Polyak 2011). The ECM that principally interacts with the epithelium is the BM which is primarily composed of collagen type IV, laminin (LM) (LM-111 and LM-332), glycoproteins (epiligrin and entactin) and proteoglycans (Oskarsson 2013). The ECM helps to maintain tissue structure and architecture as well as homeostasis of mature tissues. Initially, the tumor starts off as CIS, which is a neoplasm arising from the epithelial cells and contained within a boundary known as the basement membrane (Kalluri and Zeisberg 2006). The surrounding stroma plays a crucial part in cancer progression and researchers have tried to elucidate this role by performing various studies. In each step of metastasis, it can be seen that stromal components play an important role. For example, endothelial cells are recruited in large numbers to the tumor site and promote angiogenesis, and macrophages secrete cytokines that enhance tumor cell invasion (Khamis, Sahab, and Sang 2012). For instance, in a study where breast cancer cells were co-cultured with adipocytes, they were seen to exhibit increased invasion characteristics (Dirat et al. 2011).

Cancer associated fibroblasts (CAFs) are the most abundant component in the tumor stroma. CAFs can originate from different sources which include resident fibroblasts which get activated, mesenchymal stem cells, cells that undergo EMT and
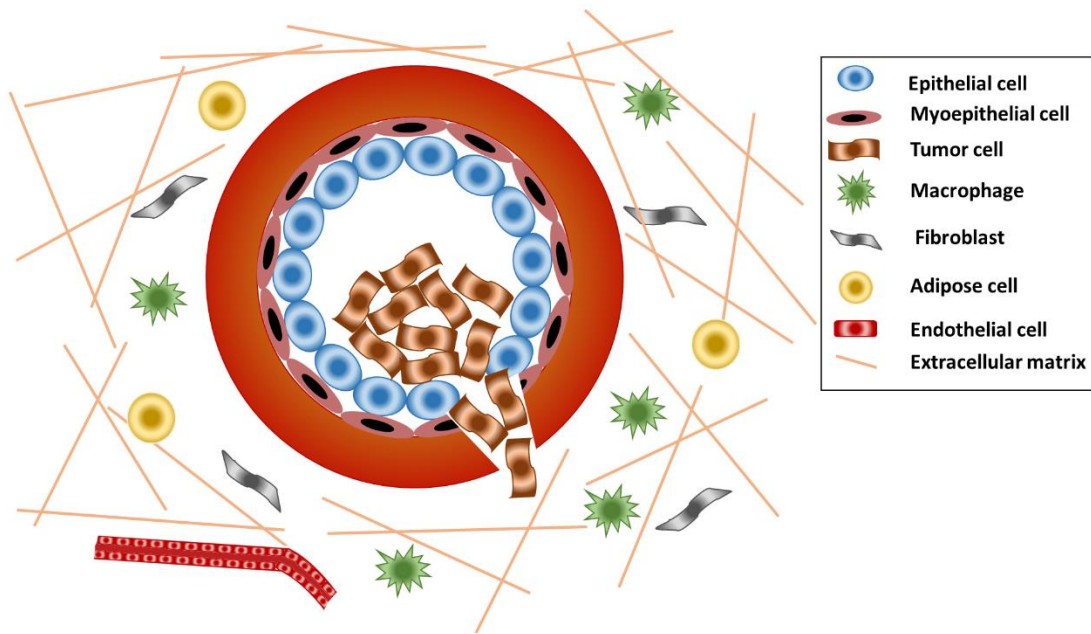
Figure 1.2: Major components of the breast tumor microenvironment

endothelial-mesenchymal transition (Mao et al. 2013). It is reported that CAFs promote

proliferation and growth of precancerous breast epithelial cells (Mao et al. 2013), induce

EMT (Hugo et al. 2012) and promote angiogenesis which are steps towards metastasis of

the cancer. Furthermore cancer cells secrete chemokine factors which recruit macrophages

and aid their intravasation into blood vessels (Tsuyada et al. 2012). There also have been

reports of carcinoma cells reversing their malignant phenotype and integrating themselves

into normal tissue when placed with normal breast epithelial cells (Bussard et al. 2010).

Cancer cells respond and redirect their development and maturation. This is evidenced by

experiments where human embryonal carcinoma cells were incorporated into the

mammary gland epithelium of a mouse, played an instrumental role in the formation of

mouse mammary gland structures (Bussard et al. 2010). Another example is where

transplanted mammary cancer cells gave rise to normal ductal structures six months after

transplantation into cleared mouse fat pads (Maffini et al. 2005). The reported results

demonstrated that CAFs and the other stromal components play a prominent role in breast cancer growth, progression and metastasis or reversion of cancer cells to normal behavior.

A plethora of mechanical forces, due to the properties of the ECM, acting on the cancer cells from outside can also alter their phenotype and consequently their migratory behavior (Artacho-Cordón et al. 2012). Collagen type I is the most dominant component in the breast ECM and plays a very important role in the developmental stages (i.e. the formation of mammary ducts) (Keely, Wu, and Santoro 1995). Matrix stiffness plays a major role in cell morphogenesis and it is seen that there is an increase in deposition of the different types of collagen (types I and III) during the formation of the tumor (Kauppila et al. 1998). A number of studies have shown that breasts with high collagen density have an increased risk of developing breast cancer (Boyd et al. 2001). Furthermore, an increase in matrix stiffness can influence integrin adhesions, increase Rho activity and lead to abnormal tissue growth and morphology (Paszek et al. 2005). For instance, it has been observed that in substrates with native tissue stiffness, mammary epithelial cells form acinus-like structures, whereas in matrices with higher stiffness, the cells lost polarity and cell-cell junction proteins (Butcher, Alliston, and Weaver 2009). This is indicative of the epithelial cell transformation, which is a step towards tumorigenesis. These findings indicate that breast microenvironment including the matrix stiffness, tissue architecture and biochemical cues plays an important role in the tumor development and disease progression.

1.5 LIMITATIONS OF CONVENTIONAL ASSAYS AND IN VIVO MODELS FOR CANCER INVASION STUDIES

In *vivo* models have been widely used in the study of breast cancer initiation, growth and progression. Based on the facet of the disease to be studied, the choice of animal model is critical. Chemically-induced rodent models (Russo and Russo 1996), human xenograft models (Clarke 1996) and transgenic mouse models (Hutchinson and Muller 2000) are some of the animal models that are extensively used. Genetically engineered mouse models (GEM) gained popularity over other models because of their ability to manipulate genes and provide seemingly accurate models for studying cancer and effects of cancer therapeutics (Van Dyke and Jacks 2002). The use of animal models are an essential stage in the preclinical phase but it is observed that they can accurately depict only the initial stages of tumor growth and progression (Van Dyke and Jacks 2002). Chemoprevention of cancer studies in GEM models are observed to have an attractive potential (Green et al. 2001, Alexander 2000). Mouse models have also been observed to have a good scope in studying and treatment of leukemia and lymphomas as opposed to solid tumors as well as in testing some of the therapies that target specific genes or pathways (Bibby 2004). Also, most of the xenograft models used in the pharmaceutical industry are human tumors placed subcutaneously into the animals and they lack the necessary host-tumor interactions. To overcome the limitations of subcutaneous transplantations, there have been advances towards orthotopic transplantations where the xenograft is placed in the physiologically relevant area of the mouse model (Bibby 2004). For example, in case of breast cancer, the xenograft would be placed in the mammary fat pads of the mouse model to mimic breast tumor development in humans. Other challenges

encountered while using *in vivo* models are the lack of control over the tumor microenvironment and difficulty in real-time imaging as well as imaging of fixed samples (Yamada and Cukierman 2007).

The majority of conventional studies on cancer migration have been conducted using two dimensional (2D) models because of the ease and convenience to set experiments. However, these models do not accurately depict the *in vivo* tissue structures required for the necessary cell-cell and cell-matrix interactions. In this regard, significant knowledge has been gained on cellular motility and migration from studies conducted on 2D surfaces. There have been exhaustive studies on the physical and molecular machinery that help movement and migration of cells (Ridley et al. 2003, Pollard and Borisy 2003, Lauffenburger and Horwitz 1996). When comparing normal cells with tumorous ones, 2D monolayer cultures might be useful in determining certain characteristics such as the replicating potential and functionalities of the cell (Khoruzhenko 2011). In breast cancer, creating a tumor model with physiologically accurate stromal components is crucial because more than 80 percent of the mammary gland is composed of the stroma (Kim 2005). Furthermore, culturing tumor cells in 2D monolayers will not promote the necessary cell-ECM interactions due to the lack of tissue structure (Kim 2005). It is seen that when normal epithelial cells are cultured on 2D substrates, they exhibit cancer cell traits (Petersen et al. 1992). The most important trait of mammary epithelial cells is their polarity which *in vivo*, helping the formation of acini structure and results in mammary morphogenesis. When cultured on 2D substrates, epithelial cells lose their polarity and cannot be seen forming any relevant physiological structures. Also, in case of focal adhesion exhibited by cells, large focal adhesions are observed in 2D culture whereas the

same cells demonstrate focal adhesions of decreased size when cultured in 3D matrices (Fraley et al. 2010). These comprehensive studies show that when culturing cells on 2D substrates, important characteristics such as chemical signaling, protein composition, and cell-cell/cell-matrix interactions are often lost or substantially inhibited (Fraley et al. 2010, Kim 2005, Petersen et al. 1992).

In vitro analysis of cancer therapeutics is a crucial step before the drugs are tested on relevant animal models. Although, 2D monolayers are the conventional assays that are currently in use for drug testing, it is evident that there is a need for a more relevant alternate model (Kunz-Schughart et al. 2004). It is crucial to eliminate the poor drug candidates in the earlier stages with the help of better designed *in vitro* assays (Kunz-Schughart et al. 2004). The need is the development of relevant *in vitro* assays that make use of human-derived cells or tissues for preclinical pharmacological testing (Mazzoleni, Di Lorenzo, and Steimberg 2009). In case of modeling breast tissue, it is necessary to have matrices that represent ECM to which the epithelial cells can attach, exhibit normal functions like proliferation, differentiation and form physiologically relevant structures like acini (Bissell, Rizki, and Mian 2003).

1.6 HYDROGEL AND POLYMERIC BIOMATERIALS TO CREATE 3D MICROENVIRONMENT FOR CANCER STUDIES

Hydrogels used in tissue engineering are polymers that have high water content, and properties that can be manipulated to mimic the ECM to provide the essential biophysical cues to the cultured cells (Seliktar 2012). For the cells to grow and respond to the microenvironment, it is important that the substrate exhibit molecular composition and mechanical stiffness similar to the native ECM. Therefore, initially, the development of 3D

tumor microenvironment was performed utilizing biomaterials derived from natural origins such as collagen, Matrigel and hyaluronic acid (HA) (Alemany-Ribes and Semino 2014). In this regard, the presence of ECM binding motifs in the biomaterial used for creating the microenvironment is crucial (Alemany-Ribes and Semino 2014). The Bissel lab performed pioneering work in creating 3D models to recapitulate normal as well as cancerous breast tissue microenvironment (Bissell, Rizki, and Mian 2003). Various studies have been conducted to study invasion and migration of tumor cells using naturally derived biomaterials (Poincloux et al. 2011, Nguyen-Ngoc et al. 2012, David et al. 2004). Apart from biomaterials such as collagen I, Matrigel and HA, another biomaterial used for modeling breast tumor *in vitro* is a silk fibroin protein called Antheraea mylitta fibroin protein which is isolated from tasar silkworm (Talukdar et al. 2011, Mira et al. 2004).

Synthetic biomaterials were developed in order to overcome certain limitations of naturally derived biomaterials such as lack of ability to pattern the matrix and manipulate stiffness (Lutolf 2009, Langer and Tirrell 2004). Polyethylene glycol (PEG) (Loessner et al. 2010), poly(lactide-co-glycolide) (PLG) (Fischbach et al. 2007), Poly(lactic acid) (PLA) and combinations like Poly(lactic-co-glycolic acid) (PLGA) (Sahoo, Panda, and Labhasetwar 2005) are some of the widely used synthetic biomaterials. Synthetic polymers have inferior cell adhesion properties as compared to their natural counterparts, however, they can be functionalized with certain ECM components in order to improve the ability of the cells to adhere (Nyga, Cheema, and Loizidou 2011). For instance, in one study, PEG functionalized with RGD peptide which is an ECM binding motif was used to culture epithelial ovarian cancer cells (Loessner et al. 2010). This study was focused on comparing the drug resistance of cells cultured on 2D versus 3D and it was observed that more cells

were viable in 3D matrix as compared to 2D substrates after the drug treatment (Loessner et al. 2010). This study validated the hypothesis that testing drugs on 2D cell monolayers might not give conclusive results for use of drugs in patients. There have been similar research work for studying cell behavior and drug testing using synthetic biomaterials including PLG scaffolds (Fischbach et al. 2007), PLGA, PLA with poly(vinyl alcohol) (PVA) (Sahoo, Panda, and Labhasetwar 2005). To create better *in vivo* models than xenografts, hydrogels can be used to inject breast cancer cells into the mammary fat pads of the mouse (Liu, Shu, and Prestwich 2007). Thus, the 3D engineered approach might provide a better representation of tumor progression than the traditional xenograft models.

1.7 MICROFABRICATED PLATFORMS TO STUDY CANCER CELL BEHAVIOR

BioMEMS (Bio Microelectromechanical Systems) is an extension of MEMS technologies used for biomedical applications. These technologies help in fabricating microscale systems with feature sizes ranging from less than 1 µm to greater than 1 cm (Whitesides et al. 2001). A subset of microfabrication techniques are found to be compatible with cells and in conjunction with biomaterials can be used for creating miniaturized platforms for fundamental biological studies and drug screening (Khademhosseini et al. 2006). Soft lithography is a set of microfabrication techniques such as microcontact printing and microfluidic patterning. Soft lithography can be used to precisely control the distribution of proteins in specific geometries on the substrate to which cells could be added or micropattern biomaterials which have cells seeded in or encapsulated in them (Zorlutuna et al. 2012, Khademhosseini et al. 2006).

Microfabricated 2D *in vitro* models have been used to study cell-cell, cell-substrate interaction as well as for drug screening (Whitesides et al. 2001). Microcontact printing, stencil and microfluidic patterning have been used to pattern cells on ECM mimicking substrates (Whitesides et al. 2001). In microcontact printing, a polydimethylsiloxane (PDMS) stamp manufactured with a chosen pattern and coated with the desired proteins is brought in contact and transferred onto the substrate based on the differences between the hydrophobicity of the surfaces (Whitesides et al. 2001). The cells that are delivered on to the surface as a solution or suspension preferentially adhere to the patterned proteins and form a 2D micropatterned platform (Whitesides et al. 2001). For instance, in a recent study, Dickinson et al. used microcontact printing **(Figure 1.3 (A))** for patterning HA and fibronectin (Fn) surfaces to study the adherence and interactions between endothelial and breast cancer cells (Dickinson et al. 2012). Some of the disadvantages of microcontact printing are protein denaturation during patterning, need for multiple stamps to create complex designs and deformation of the stamp after a number of uses (Huang 2013). The above mentioned techniques are useful only for patterning acellular substrates where the cells would preferentially adhere to the patterned surfaces (Whitesides et al. 2001, Folch et al. 2000). However, the major disadvantage associated with microcontact printing, stencil as well as microfluidic patterning is that the cells confined by the patterns are still in a 2D environment and thus, fail to express the necessary interactions that are seen *in vivo* (Park and Shuler 2003).

A 3D microenvironment is essential to recapitulate the *in vivo* tissue structures and architecture *in vitro*. 3D tumor models can vary from simple tumor spheroids to complex platforms consisting of detailed structure and multiple cell types (Nyga, Cheema, and

Loizidou 2011). The ECM provides important biochemical and biophysical cues which are essential for the development and progression of a tumor. Matrix stiffness, tissue structure and topography are some of the biophysical cues that are exhibited by the ECM and affect various functions and behaviors of the cell (Nikkhah, Edalat, et al. 2012). Tumor cells exhibit great plasticity under different conditions and 3D models have the ability to recapitulate these conditions to enable researchers to delineate and study the effect of individual microenvironmental cues on disease progression (Petersen et al. 1992, Bissell and Radisky 2001).

Micro- and nanofabrication techniques have shown great promise in the recent years in creating 3D microenvironments with structures having precise geometry (Park and Shuler 2003). Specifically, these techniques have been instrumental to precisely control factors affecting the tumor at the micro- and nanoscale. Microfabrication techniques have been able to construct 3D platforms to study the effect of surface topographies on cancer cell behavior (Nikkhah, Edalat, et al. 2012). For instance, in studies conducted by Nikkhah et al., silicon surfaces were etched to form 3D microstructures in which human fibroblasts, normal breast epithelial cells as well as malignant breast cells were cultured (Strobl, Nikkhah, and Agah 2010, Nikkhah et al. 2011, Nikkhah et al. 2010, Nikkhah, Strobl, and Agah 2008, Nikkhah et al. 2009). It was observed that the fibroblast cells stretched across the curved walls of the microstructures whereas the malignant breast cells were seen to

stretch or deform to take up the shape of
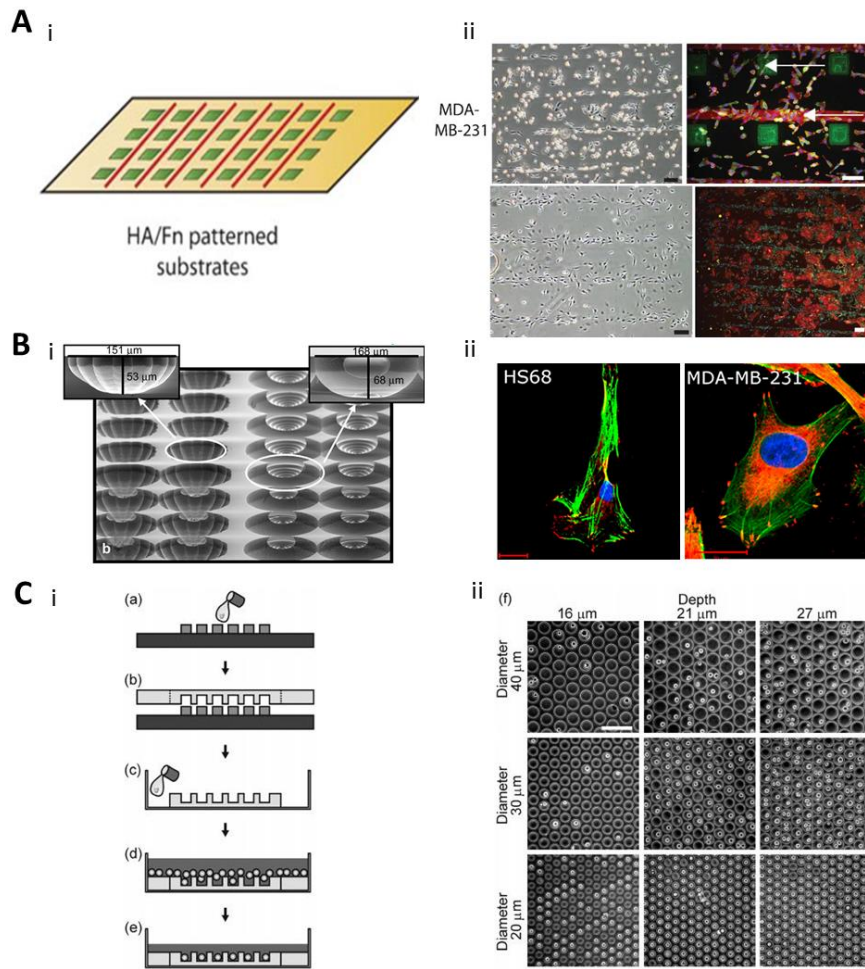
Figure 1.3: Tumor models using microfabrication techniques (A) (i) HA/Fn patterned onto the surface of substrate using microcontact printing (ii) 24 hours into cell culture showing adhesion of MDA-MB-231 cells outside HA, HA (green) and Fn/CD44 (red), nuclei (blue). Scale bars = 100 µm. Adapted from Dickinson *et. al.* with permission from Royal Society of Chemistry [Lab on a Chip], copyright (2012) (Dickinson et al. 2012) (B)(i) Silicon microchannels formed by microfabrication techniques and visualized by scanning electron microscopy (SEM) (ii) Confocal images of actin and vinculin stained cells inside the microstructures. Scale bars = 20 µm. Adapted from Nikkhah *et. al.* with permission from Springer [Biomedical Microdevices], copyright (2010) (Nikkhah, Strobl, and Agah 2008) (C)(i)(a-e) Schematic showing the procedure to create PDMS microwells using PDMS and photolithography techniques, (ii) Representative images showing individual cells trapped in microwells of varying diameters (20-40 µm). Scale bar = 100 µm. Adapted from Rettig *et. al.* with permission from American Chemical Society [Analytical Chemistry], copyright (2005) (Rettig and Folch 2005)

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the structure (Nikkhah, Strobl, and Agah 2008). In another study by the same group, co-culturing normal breast epithelial cells and breast tumor cells showed that both the cell types spread according to the geometry of the cavity. However, when the cells were treated with an anti-cancer drug, the tumor cells were seen to display a stretched morphology, comparable to fibroblasts, but the normal mammary epithelial cells did not show a change in morphology **(Figure 1.3 (B))** (Strobl, Nikkhah, and Agah 2010) . These topographies were formed in 3D but they lacked the essential ECM-like architecture that surrounds the cell and provides cues for proliferation, differentiation and migration.

Microengineered 3D cell arrays are useful in drug screening as hundreds and thousands of samples can be tested on a single chip (Torisawa et al. 2007, Nikkhah et al. 2013). For instance, in a study by Rettig et al. a large array of microwells was designed for trapping single cells (fibroblasts) and study the cellular behavior in general or their response to drugs, toxins (Rettig and Folch 2005). A master was first created using photolithography and then, the final chip was developed in PDMS using soft lithography **(Figure 1.3 (C))** (Rettig and Folch 2005). In another study, a chip with an array of multiwells was used to study the proliferation, spheroid formation, and the response of breast cancer cell line (MCF 7) and hepatoma cell line (HepG2) to four different chemical stimuli (Torisawa et al. 2007). The microarray of multiwells was created by binding an anisotropically etched silicon substrate with pyramidal holes and PDMS microchannels made with the help of soft lithography techniques (Torisawa et al. 2007).

Microfluidic technology can be used for designing single-cell, spheroid as well as co-culture assays (Wheeler et al. 2003, Kwapiszewska et al. 2014, Jeon et al. 2015). These systems have more control over the cell microenvironment as the factors surrounding the

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cells can be manipulated through the channels that deliver media and soluble factors (Marimuthu and Kim 2011). Microfluidics can also be used for isolating circulating tumor cells (CTCs) (Moon et al. 2011, Kaiser 2010), studying tumor cell
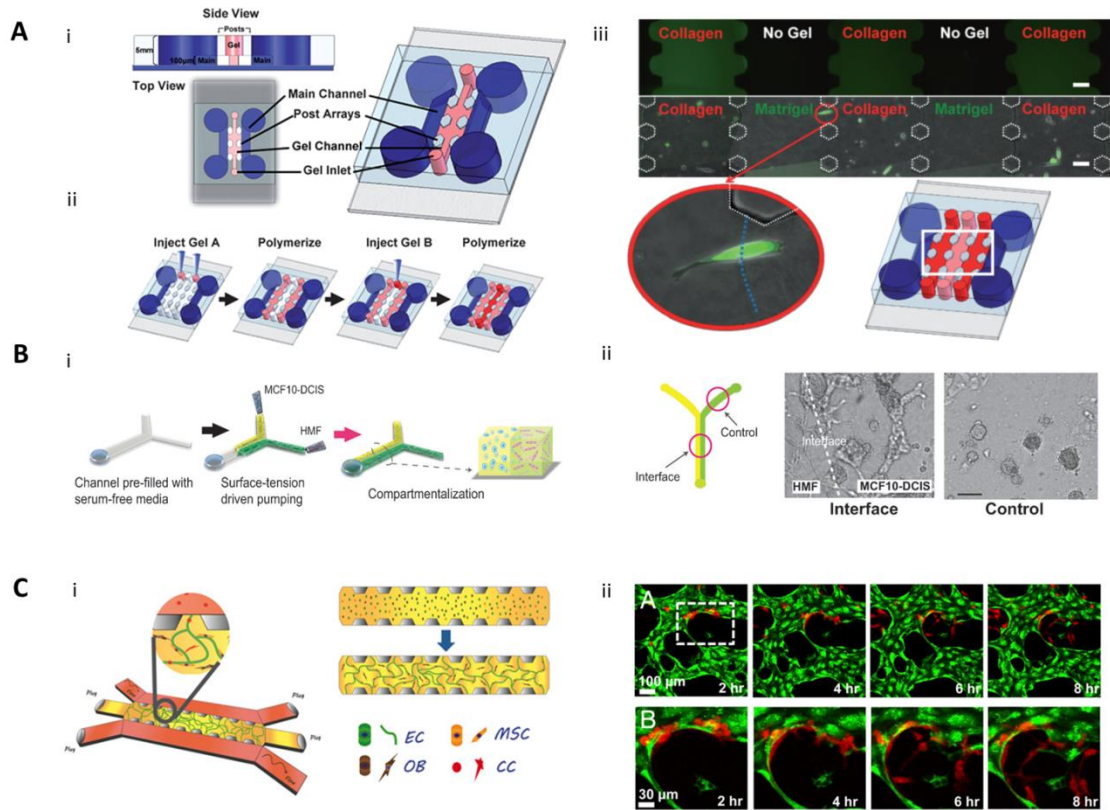
Figure 1.4: Representative microfluidic devices used in cancer studies. (A) (i and ii) Single channel microfluidic device with two parallel reservoir channels. (iii) Collagen gel with dye patterned (top row). Alternate channels patterned with collagen and Matrigel (bottom row). MDA MB 231 cells (green) cultured in collagen and the inset shows a cell crossing from collagen to Matrigel. Adapted from Huang *et. al.* with permission from Royal Society of Chemistry [Lab on a Chip], copyright (2009) (Huang et al. 2009)(B) (i)Transition of MCF-DCIS cells to IDC by compartmentalization in microfluidic device. (ii) The transition of MCF-DCIS cells into invasive phenotype is observed at the interface. Adapted from Sung *et. al.* with permission from Royal Society of Chemistry [Integrative Biology], copyright (2010) (Sung et al. 2011)(C) (i) Schematic representation showing two side channels and a gel channel in between which allowed the encapsulation of cells. (ii) (A) Cancer cells extravasating from the vascular network (B) Magnified images of the cancer cells extravasating. Adapted from Jeon *et. al.* with permission from National Academy of Sciences [PNAS], copyright (2015) (Jeon et al. 2015)

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biology (e.g. invasion) (Song et al. 2009, Huang et al. 2009), and high throughput drug screening (Zhang and Nagrath 2013, Stern et al. 2010, Kim et al. 2012) and co-culture tumor and stromal cells to study their interaction. In a study by Huang et al., a microfluidic platform was designed having an array of microposts which allowed preferential filling of hydrogels into different channels. Using this device, metastatic breast cancer cells (MDA-MB-231) and tumor derived macrophages were patterned into spatially defined geometries to study their interactions **(Figure 1.4 (A) (i and ii))** (Huang et al. 2009). It was observed that over the 7 days of culture, the macrophages invaded into the adjacent gel and not into areas where no cells were present **(Figure 1.4 (A) (iii))** (Huang et al. 2009). In another study, a compartmentalized microfluidic device was designed to study the transition of mammary epithelial cells (MCF-DCIS) from DCIS to IDC **(Figure 1.4 (B) (i)** (Sung et al. 2011). Here, MCF-DCIS cells were patterned adjacent to human mammary fibroblasts (HMF) in order to recapitulate the *in vivo* microenvironment. Results showed that close contact between stromal fibroblast cells and MDF-DCIS aided in the transition to IDC **(Figure 1.4 (B) (ii))** (Sung et al. 2011). In an alternate study, Jeon et al. created a microfluidic platform that could be used for studying the extravasation patterns of breast cancer cells, and also for drug screening applications **(Figure 1.4 (C))** (Jeon et al. 2015). Microfluidic platforms have advantages such as flexibility in device design, low number of cells and less reagents needed. Microfluidic assays can be automated and real-time analysis can be performed on cell behavior (Halldorsson et al. 2015). Some challenges associated with microfluidics are the precise control of the environment surrounding the cells including parameters like chemical gradients, composition of the medium and shear stress experienced by the cells (Zhang and Nagrath 2013). Another limitation when

working with microfluidic devices is the inaccessibility and thus, the lack of ability of manipulation of the cells using force microscopy methods such as optical tweezers and atomic force microscopy.

Self-assembling peptides and proteins can be also used to engineer 3D platforms which have precisely controlled formations at the nano-scale to develop scaffolds for culturing cancer cells (Zhang 2003). In the study by Yang et al. ovarian cancer cell lines were cultured in a 3D microenvironment formed by self-assembling RADA16-I peptide hydrogel (Yang and Zhao 2011). The three cell lines were seen to take up their respective distinct morphologies when cultured on RADA16-I scaffolds along with proliferative potential and high viability (Yang and Zhao 2011). Gelatin methacrylamide (GelMA) hydrogels have been also used for creating 3D cell cultures due to its biocompatibility and ability to be photocrosslinked. For instance in a recent study, GelMA was used for studying how the extracellular matrix contributes to the development and progression of the tumor in case of human glioblastoma multiforme (hGBM) (Pedron and Harley 2013). The concentration and the degree of methacrylation of the gel were varied to study its effect on the cultured tumor cells. The findings demonstrated proliferation and morphology of cancer cells in the different formulations of GelMA were observed and recorded (Pedron and Harley 2013).

1.8 OBJECTIVE OF THE THESIS

The objective of this thesis is to create a physiologically relevant breast tumor model which could be used to study cancer cell behavior (i.e. invasion) and ultimately, used for drug testing. The first step was to create a platform with high stiffness tumor regions and the surrounding stroma with lower stiffness. This was achieved by using

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GelMA hydrogel and photolithography techniques. After the platform was validated, three different breast cell lines, highly invasive MDA-MB-231, non-invasive MCF-7 and normal mammary epithelial cells MCF-10A, were encapsulated in GelMA, patterned using a two-step photolithography techniques and cell behavior was studied. Analyses performed included viability, proliferation, migration, quantification, and observation of morphological differences in the various regions of the 3D micropatterned platform.

CHAPTER 2

A THREE DIMENSIONAL MICROPATTERNED TUMOR MODEL FOR BREAST CANCER CELL MIGRATION STUDIES

2.1 INTRODUCTION

Metastatic dissemination of cancer cells is a highly complex and multi-step biological process starting with tumor angiogenesis (Braun and Naume 2005, Foroni et al. 2012, Friedl and Wolf 2003) and the invasion of cancer cells through the ECM toward the blood vessels (Geiger and Peeper 2009, Lu, Weaver, and Werb 2012). Cancer cell invasion through the tumor stroma is governed by diverse factors including biochemical signals and biophysical cues (Foroni et al. 2012). Despite their significance, most *in vivo* animal models present an abundance of confounding variables making it challenging to attribute specific microenvironmental cues to cellular invasion (Van Dyke and Jacks 2002). In this regard, physiologically relevant *in vitro* tumor models are crucial to understand cancer cell invasion within a native-like breast tumor microenvironment.

In the past few years, there has been a tremendous initiative to develop *in vitro* models to study cancer cell behavior in 3D microenvironments. For instance, 3D surface topographies have been widely used to study cancer cell behavior in response to various geometrical features (Lu, Weaver, and Werb 2012, Nikkhah, Edalat, et al. 2012, Nikkhah, Strobl, and Agah 2008, Nikkhah et al. 2010, Nikkhah et al. 2011). Despite their significance, these platforms lacked the capacity to alter the native-like parameters including stiffness and matrix architecture. Alternatively, a wide variety of 3D hydrogel-based matrices such as Matrigel (Kleinman and Martin 2005), fibrin (Liu et al. 2012), collagen (Jeon et al. 2013, Szot et al. 2011), and PEG (Kharkar, Kiick, and Kloxin 2013)

have shown great promise to recapitulate cancer cell invasion in a 3D matrix and assess cellular behavior in response to various biophysical and biochemical cues. Such 3D hydrogel-based matrices enable cells to retain accurate phenotype and, consequently, exhibit precise responses to microenvironmental stimuli along with cell-cell and cell-matrix interactions (Cukierman et al. 2001). Although these models have resulted in outstanding biological findings, they lack specific patterned features that would enable precise control over cellular distribution and matrix stiffness to conduct studies within biomimetic tumor architecture.

The integration of microengineering technologies and advanced biomaterials (e.g. hydrogels) has offered great promises to develop well-defined microenvironments for fundamental biological studies. These technologies are appealing since they enable tight control over the cellular microenvironment (Park and Shuler 2003). Particularly, through the use of phtotocrosslinkable hydrogels and micropatterning techniques, it is possible to generate biologically relevant constructs for tissue engineering and cancer related studies. However, there are still very few studies on the use of these types of hydrogels in the development of biologically relevant tumor models (Dickinson et al. 2012, Pedron and Harley 2013).

In this study, we explore the use of a novel, two-step photolithography technique and GelMA hydrogel to develop a highly organized micropatterned breast tumor microenvironment model. GelMA has been proven to be an excellent candidate to generate biologically relevant constructs (Nichol et al. 2010) as cells have readily adhered to, proliferated within, and migrated when encapsulated within the 3D matrix of the hydrogel (Aubin et al. 2010, Nikkhah, Eshak, et al. 2012, Schuurman et al. 2013). More importantly,

the use of GelMA enables the creation of arrays of specific cell-laden features with high precision and fidelity (Van Den Bulcke et al. 2000). Previous studies using GelMA hydrogel have been largely focused on tissue engineering and regenerative medicine applications (Aubin et al. 2010, Nikkhah, Eshak, et al. 2012), with only a few focused on cancer (Kaemmerer et al. 2014, Pedron and Harley 2013). The proposed platform, presented herein, has unique advantages through the ability to independently decouple different cell-embedded regions within the tumor model and independently tune their stiffness. Furthermore, the microfabricated model enables precise visualization of cancer cell migration within a 3D matrix in response to microenvironmental cues. In order to validate the proposed microengineered tumor model, we primarily assessed the morphology and proliferation of highly invasive human breast cancer MDA-MB-231 cells, non-invasive, tumorigenic human breast cancer MCF7 cells, and normal mammary epithelial MCF10A cells. In addition, we analyzed migration and cytoskeletal organization of the cells within different regions within the micropatterned breast tumor constructs.

2.2 MATERIALS AND METHODS

2.2.1. Synthesis of GelMA Hydrogels

GelMA preparation was completed similar to prior studies (Nichol et al. 2010, Van Den Bulcke et al. 2000). Primarily, a 10% w/v solution of type A porcine skin gelatin was prepared in Dulbecco's phosphate buffered saline (DPBS; Gibco). This solution was made at 60 °C in order to fully dissolve before proceeding to subsequent steps. Methacrylic anhydride was then added drop-wise to infuse it within the gelatin solution. The mixture was then stirred vigorously for three hours as to ensure the completion of the reaction. In order to shift the equilibrium and stop the reaction, the reaction mixture was diluted (5X)

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with warm (40 °C) DPBS. This crude prepolymer GelMA was dialyzed for one week in distilled water (replaced twice a day) using dialysis membranes (MWCO 12000-14000) at a constant temperature (40 °C) to filter out any salt byproducts created from the reaction between gelatin and methacrylic anhydride. The desired degree of methacrylation was achieved by precisely controlling the proportion of methacrylic anhydride to gelatin during synthesis (92±2% confirmed based on ^{1}H NMR). The gelatin methacrylate solution was lyophilized for one week to create a dehydrated, porous macromer, which could be preserved for future experiments.

2.2.2. Cell Culture

The invasive breast cancer MDA-MB-231 cell line, tumorigenic breast cancer MCF7 cell line, and mammary epithelial MCF10A cell line were used in this study. Cancer cells were maintained in 1X Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% 50:50 penicillin:streptomycin. Mammary epithelial cells were maintained in DMEM:F12 supplemented with 1% L-glutamine, epidermal growth factor (20ng/mL), cholera toxin (100ng/mL), insulin (10μg/mL), hydrocortisone (0.5mg/mL), and 5% horse serum. All media and media supplements were provided by Life Technologies. Cells were kept at a standard physiological condition (humidified, 37 °C, 5% CO_2), were passaged weekly, and had their media changed every three days in order to produce a controlled experimental condition.

2.2.3. Microfabrication of the Tumor Model

In order to promote adherence of the GelMA hydrogel constructs, glass slides were functionalized with 3-(trimethoxysilyl)propyl methacrylate (TMSPMA) (Sigma) as described in previous protocols (Aubin et al. 2010, Nikkhah, Eshak, et al. 2012). Subsequently, a 7 µL drop of 20% (w/v) PEG prepolymer solution included with 0.5% (w/v) photoinitiator (PI) (2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone) was placed onto cut (area: $<1cm^2$), sterilized glass slides. An untreated coverslip was placed on top of the PEG prepolymer and this arrangement was then exposed to ultraviolet (UV) light (360-480nm, 800 mW) for 50s which crosslinked to form a thin layer of PEG coating on the TMSPMA-treated glass slides.

To microengineer the tumor model, GelMA macromer was dissolved in DPBS containing 0.5% (w/v) PI. This formed a prepolymer solution, which was stored at 37 °C. Cells were encapsulated in the prepolymer solution through resuspension of pelleted cells (cell density: 6 x 10^6 cells per mL of GelMA). The tumor model was patterned by first pipetting a 15 µL droplet of cancer cell-laden GelMA onto a spacer (depth: 100 µm). A PEG-coated glass slide was then inverted on top of the spacer thereby spreading the prepolymer solution to cover the area of the glass slide and fill in the 100 µm depth of the spacer (**Figure 2.1A-B**). A photomask (designed with AutoCAD software and printed by CAD/Art Services Inc., Orgeon) was then placed on the inverted, PEG-coated glass slide and exposed to UV light for 12s (**Figure 2.1C**). Tumor models were created with three different geometrical parameters. Photomasks with an 11x11 array of translucent circles of diameters 100, 250 and 500 µm and surrounded by a black unpatterned area were used to create the high density array of tumors. The spacing between the circles was a constant 750

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µm and the height of the tumor regions were 100 µm. Upon UV exposure, the patterned glass slide was washed to remove the excess cells and stored in a petri dish filled with DPBS. Following, a 13 µL drop of pristine GelMA (no cells) was placed onto the spacer and the patterned glass slide was inverted on top of it **(Figure 2.1D-E)**. The circular constructs guided the spread of the pristine GelMA to the surrounding areas. This assembly was exposed to UV light for another 5s in order to crosslink the gel filled in between the circular constructs **(Figure 2.1F)**. Upon completion of the experiment, the micropatterned tumor model were transferred from the DPBS baths to 24-well cell culture plates with media corresponding to each cell line. Cell culture media was changed every three days over the course of the experiments.

2.2.4. Stiffness Measurements with Atomic Force Microscopy (AFM)

GelMA stiffness measurements were performed with a MFP-3D AFM (Asylum Research) placed on an inverted microscope (IX71, Olympus) (Fuhrmann et al. 2011, Physical Sciences - Oncology Centers et al. 2013, Schulz et al. 2010). A 40X objective with a NA of 0.65 (Olympus) was used to perform force measurements on the center of the circular GelMA microstructures. Large radius tip AFM probes (LRCH-750, Team NanoTec) with a tip radius of ~810nm were used **(Figure 2.1C)**. The thermal energy dissipation method (Butt and Jaschke 1995) was used to determine the spring constant of the cantilevers (~0.15 N/m). Four force-indentation measurements were taken in a 90µm^2 area at the center of 8 different circular GelMA microstructures. Alternatively, forty force-indentation measurements in a 90µm^2 area were taken on the non-patterned GelMA surface. Approach and retraction speed for all measurements was 2µm/s. A trigger force of 10nN was used for all force-indentation measurements. All measurements were done in

10X DPBS buffer solution. Young's Moduli from force indentation curves were determined using custom MATLAB routines. Force-indentation curves were analyzed using the power-law linearization method as described previously (Guo and Akhremitchev 2006) based on the Briscoe indentation model for a blunted cone with a Poisson ratio of 0.5.

2.2.5. *Cell Viability Assay*

Cell viability was assessed on day 5 using a standard Live/Dead Assay Kit (Invitrogen), which includes calcein AM (CI) and ethidium homodimer (ETD). To prepare the solution, 0.5 µl CI and 2 µl ETD were added to 1 mL DPBS. After 5 days of culture, the microenvironments were rinsed with warm DPBS and 150 µl of the CI/ETD solution was added to each well. The well plate was stored at physiological conditions (37 °C, humidified, 5% CO_2) and imaged after 30 minutes using an inverted fluorescence microscope (Zeiss Axio Observer Z1) with 10X magnification.

2.2.6. *Quantification of Cell Proliferation*

Cell proliferation was quantified through counting cell nuclei on days 0, 1, 3 and 5 of culture. The cell-laden GelMA hydrogel constructs were rinsed with DPBS and fixed with 4% paraformaldehyde (PFA) solution in DPBS. After 30 minutes, the samples were washed three times (3X) in DPBS. A 0.1% (v/v) of DAPI (4',6-diamidino-2-phenylindole) (Life Technologies) in DPBS solution was prepared and added to each well. The samples were left in DAPI contained solution for 15 minutes, and then washed 3X in DPBS. The samples were fluorescently imaged, and the number of DAPI stained nuclei were counted using ImageJ (v. 1.48) software to determine proliferation and migration of each cell line

at specific time points (Days 0, 1, 3 and 5). At least three samples were prepared for each condition within each experiment.

2.2.7. Actin Cytoskeletal Organization

To assess F-actin cytoskeletal organization, cell encapsulated hydrogel constructs were fixed with 4% PFA solution in DPBS and then permeabilized for with 0.1% Triton X-100. The samples were washed 3X in DPBS with 5-minute intervals. The cell encapsulated hydrogel constructs were then blocked with 1% bovine serum albumin (BSA) for 1 hour. A 1/40 dilution of Alexa Fluor-488 phalloidin (Life Technologies) in 0.1% BSA was added to the blocked samples for 45 minutes. The hydrogel constructs were subsequently washed 3X in DPBS. Upon F-actin staining, the cells were stained with DAPI to visualize the nuclei. The stained samples were inverted onto a glass coverslip with a droplet of ProLong Diamond Antifade solution. The cell-encapsulated hydrogel constructs were imaged using a fluorescence microscope (Zeiss Axio Observer Z1) equipped with an Apotome.2 at 20X/40X magnification. Z-stacks and 2X2 tiles of the samples were obtained and 3D images were constructed using the Zen software. Circularity of the cells was determined by using top-view images of fluorescent F-actin staining. These images of individual constructs were fed into a custom script for the ImageJ software, which compared each individual clump or each individual cell to a perfect reference circle, outputting a percent circularity value.

2.2.8. Data Collection and Statistical Analysis

Migration and proliferation data were analyzed over the course of three experiments (n=3) for each cell line. Each experiment (sample) had three replicates for a total of nine replicates per cell line at each time point (Days 0, 1, 3, 5). The data was collected within a

5X5 array of constructs in the center of each replicate. Data for the live-dead analysis had the same method of data collection in terms of experiments, sample sizes, and replicates on day 5 of culture. Data for circularity was collected by measuring the circularity of the cells within the triplicate samples of one experiment for each of the three cell types.

A one-way analysis of variance (ANOVA) was conducted, which demonstrated statistically significant differences between each group when $\alpha = 0.05$. A Bonferroni's post-hoc test was subsequently completed in order to measure statistically significant differences between individual groups. All data were presented in mean ± standard deviation (SD). Statistical analysis/data presentation were performed in Graph Pad Prism (v. 6.0).

2.3 RESULTS

2.3.1. Microfabrication and Characterization of the Tumor Model

The microengineered tumor model was developed using 5% GelMA with high (92±2%) degree of methacrylation due to its biocompatibility and reliability for photolithography applications (Nichol et al. 2010, Nikkhah, Eshak, et al. 2012, Nikkhah et al. 2010). The specific geometrical parameters of the microengineered tumor model are defined in Table 1.

Table 1: Geometrical features of the microengineered tumor model*

Shape	Depth (µm)	Diameter (µm)	Spacing (µm)**	Surface ratio construct/surrounding
Circle	100	500	750	0.536

*Visualized in Figure 2.2 A
**Spacing refers to the distance between the radii of adjacent tumor construct

The thickness of the tumor constructs was set to 100 µm due to its proven efficacy in the formation of patterned cellular constructs (Nikkhah, Eshak, et al. 2012). The
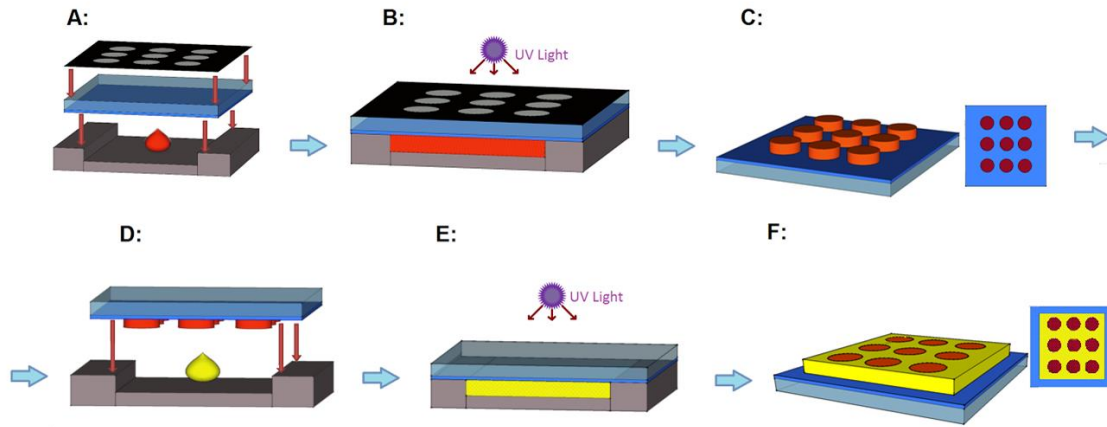

Figure 2.1: Schematic diagram depicting the development of array of the proposed tumor model. (A) A drop of breast cancer cells encapsulated in GelMA prepolymer solution was pipetted onto a spacer and a glass slide/photomask was layered on top of it. (B,C) UV light is exposed to crosslink GelMA to create an array of high stiffness circular constructs. (D) A drop of pristine GelMA prepolymer solution was pipetted onto a spacer and the micropatterned circular constructs from (C) was placed on top of it, thereby spreading the hydrogel in between the constructs. (E) UV light was exposed to crosslink the surrounding matrix. (F) Representative schematic of the final microengineered tumor model with the high stiffness tumor constructs surrounded by low stiffness matrix.

spacing and diameter of the cell encapsulated circular constructs were optimized based on a series of preliminary experiments (data not presented). After the preliminary studies with three different dimensions, it was observed that the tumorous circular regions with a diameter of 500 µm gave the best results for visualizing the dissemination and migration of the cancerous malignant MDA-MB-231 cells. The cellular constructs with 100 and 250 µm had low fidelity. Therefore, all the ensuing experiments were performed using the geometrical features defined in Table 1. Upon optimization, separate aliquots of GelMA prepolymer solution were stained with 0.01% rhodamine and 0.01% fluorescein dye to visualize the localization of hydrogel constructs after micropatterning. The developed two-

step photolithography technique, as demonstrated in **Figure 2.1**, was used to form high density array of circular constructs (red stained hydrogel) surrounded by a surrounding matrix (green stained hydrogel). In particular, the two-step process involved
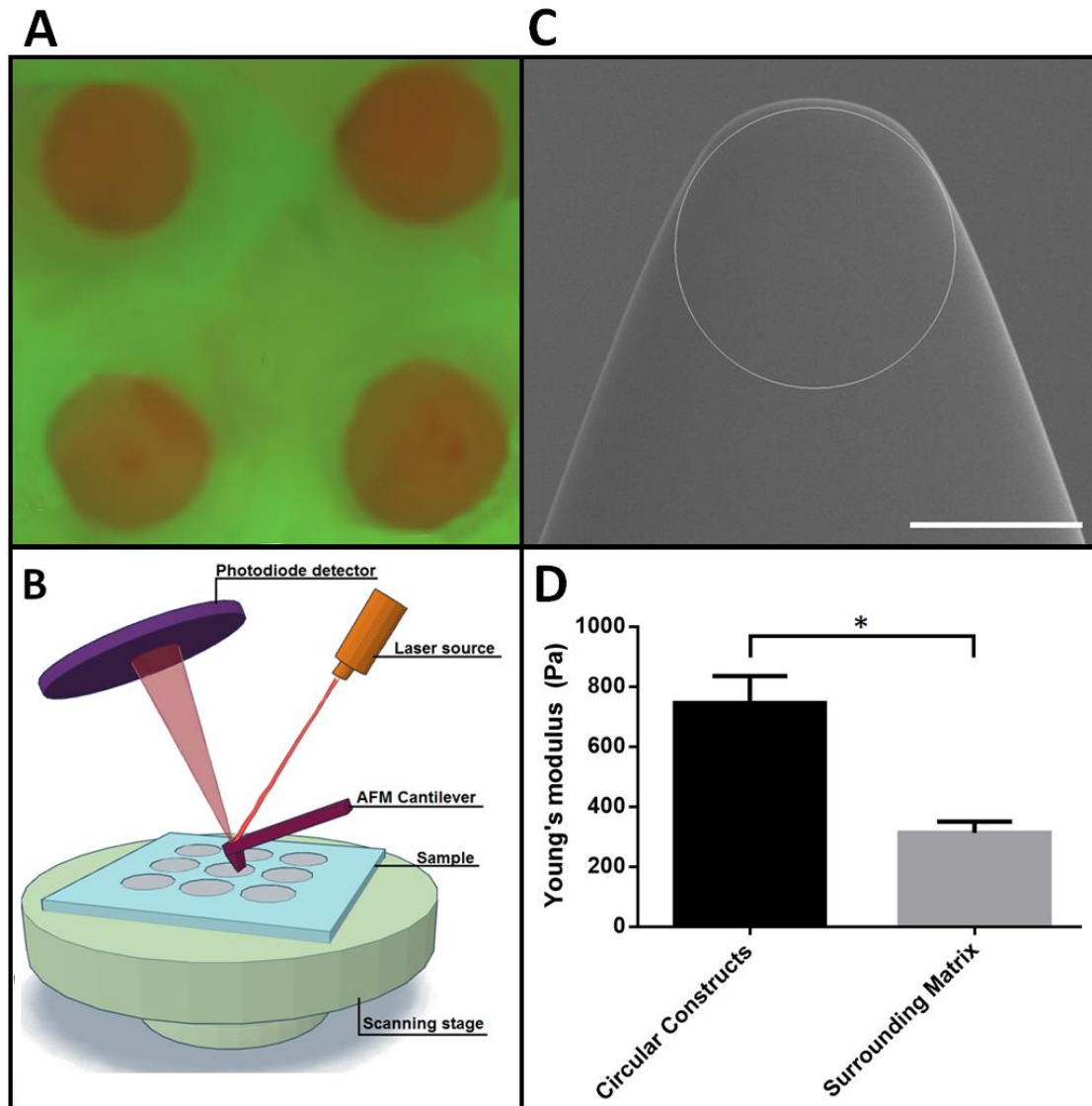
Figure 2.2: Representative fluorescence image (A) of Rhodamine B stained circular constructs and Fluorescein stained surrounding matrix. (B) Schematic diagram of the AFM setup to perform local stiffness measurements. (C) SEM image of the AFM cantilever used to probe the hydrogel (radius of tip: 810nm; scale bar represents 1 μm). (D) Mechanical stiffness of the circular constructs and the surrounding matrix reveal a Young's modulus of 748 ± 90 and 313 ± 38 Pa respectively. Data is presented in mean $\pm$ SD. (*$p < 0.05$)

fabricating the circular constructs first **(Figure 2.1 A-C)**, and, subsequently, filling in the surrounding regions by adding GelMA prepolymer in between the constructs **(Figure 2.2 D-F)**. The circular constructs were, as such, crosslinked more than the surrounding matrix. As the crosslinking time of the prepolymer solution has a direct positive correlation to the stiffness of the GelMA hydrogel (Nichol et al. 2010), we expected that this method would create cell-embedded circular constructs with stiffness that is substantially higher than the surrounding matrix to assess the capability of the proposed microfabrication technique in forming areas of differential stiffness on a single chip, the Young's moduli of the circular constructs and the surrounding regions (interstitial area) were measured by AFM **(Figure 2.2 B, C)**. These measurements revealed a stiffness of 747.8 ± 89.6 Pa within the circular constructs which was over twice as stiff as the interstitial area measured at 313.3 ± 37.5 Pa **(Figure 2.2 D)**. These data indicated the fidelity and reliability of the proposed two-step photolithography technique to create a high-density array of constructs with adjustable stiffness.

2.3.2. Cell Viability

We evaluated viability of three distinct cell types, normal mammary epithelial MCF10A cells, tumorigenic MCF7 cells, and highly invasive breast cancer MDA-MB-231 cells encapsulated within the microengineered tumor model. Representative images of the cell viability experiments **(Figure 2.3A)** demonstrated excellent cell survival upon encapsulation and the microfabrication procedure. The percent of viable cells across all the three cell types had no statistically significant difference and was within $84 \pm 5\%$ after 5 days of culture **(Figure 2.3B)**. Similarly, in previous studies, a wide array of other cell types such as ovarian cancer cells, 3T3 fibroblast cells, and human umbilical vein

endothelial cells (HUVECs), encapsulated within GelMA hydrogel, exhibited high percent

cell survival upon micropatterning(Kaemmerer et al. 2014, Nichol et al. 2010, Nikkhah,

Eshak, et al. 2012). Thus, our data confirmed that the specific parameters used to

microengineer the tumor model (the two-step, 17 second UV exposure and presence of PI

within the prepolymer solution) did not have a substantial effect on overall cell viability.
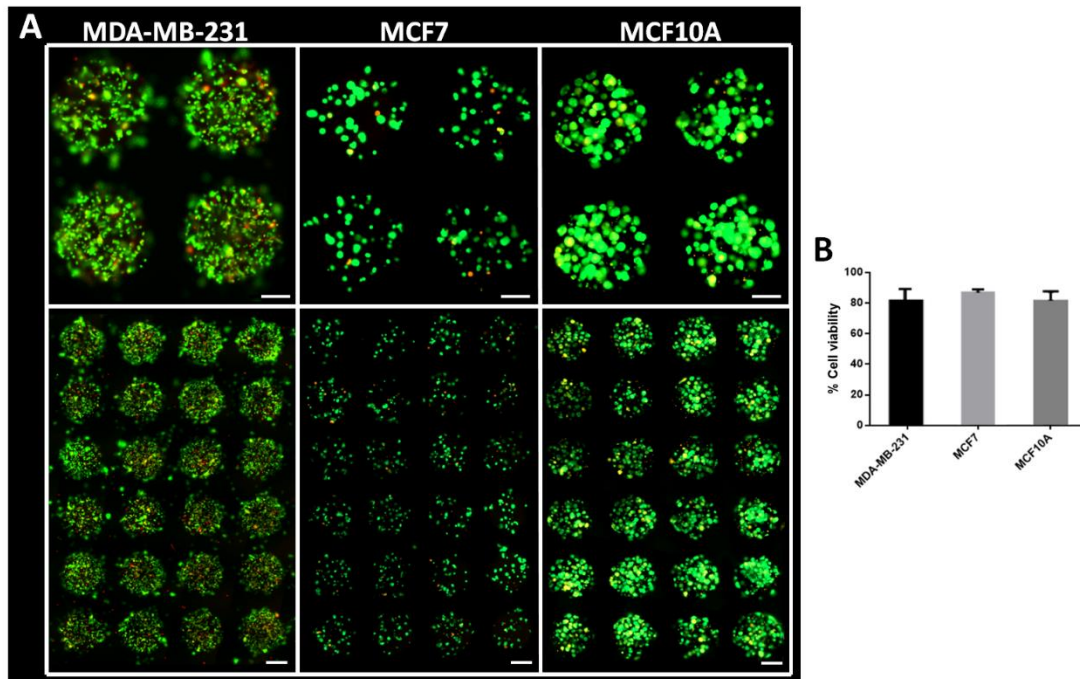
Figure 2.3: Representative fluorescence images (A) of cell-embedded tumor model stained with a live/dead assay on day 5 of culture (Live cells: green; Dead cells: red). No statistically significant difference observed between three cell types (B). Data is presented in mean ± SD. Scale bars represent 200 μm.

2.3.3. *Cell Morphology, Migration, and Proliferation within the micropatterned constructs*

Phase contrast images demonstrated that the three cell types (MCF10A, MCF7,

MDA-MB-213) were homogeneously distributed throughout the hydrogel and had a round

morphology on day 0 immediately after encapsulation within the micropatterned circular

regions. However, between days 1 and 3 of culture, the cells began to exhibit characteristics

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specific to the cell type. In particular, MDA-MB-231 cells adopted a heterogeneous morphology, both round and elongated, with higher cell density secondary to their high proliferative capacity (Nagaraja et al. 2005) **(Figure 2.4 A)**. These cells started migrating toward the outer regions of the circular constructs as early as day 3 of culture, which was further evident on day 5 of culture (Arrows, **Figure 2.4 A**; **Figure 2.5**). MCF7 cells formed clusters within and on the periphery of the constructs and
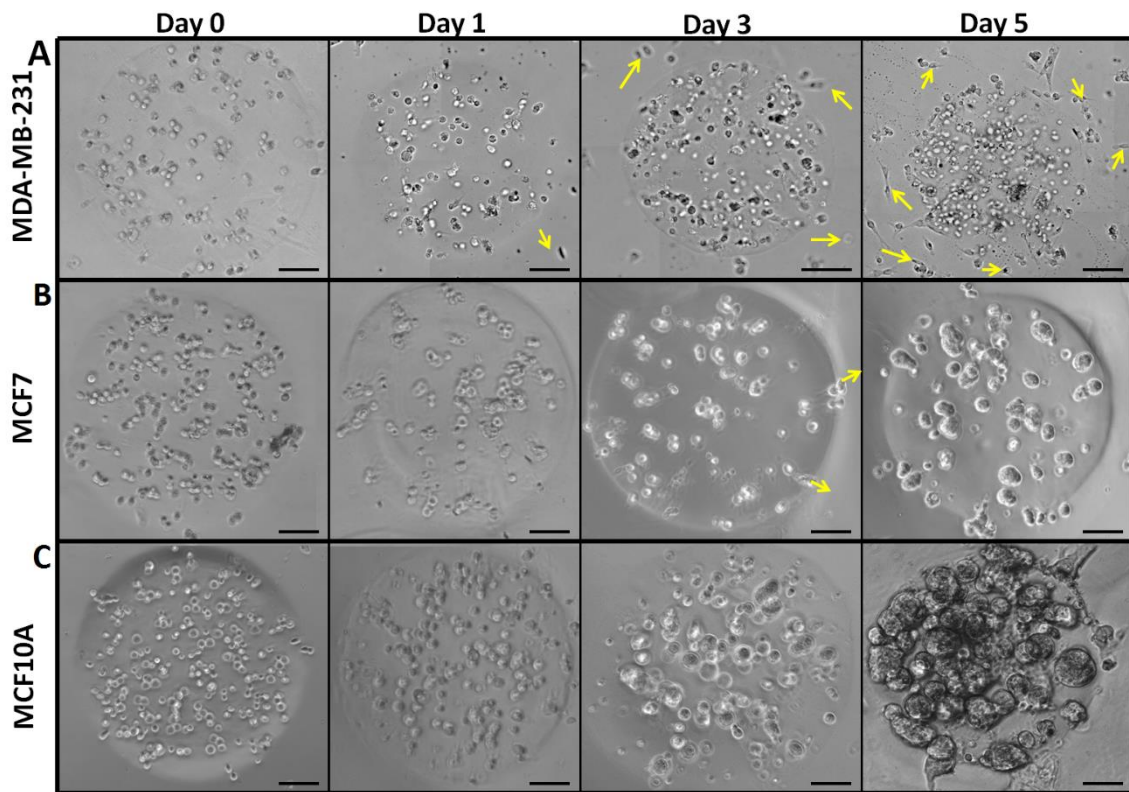
Figure 2.4: Representative phase contrast images demonstrating changes in cellular morphology. MDA-MB-231 cells spread rapidly creating a heterogeneous (spindle vs. round) morphology. Arrows point to cells that have invaded the surrounding stroma. MCF7 cells exhibited a tendency to cluster, demonstrating only weak migration on days 1 and 3 of culture and small clusters by day 5. MCF10A cells formed similar clusters by day 3 which grew bigger by day 5. Scale bars represent 100μm.

exhibited weak migratory characteristics and elongation toward the surrounding regions as early as day 1 of culture (Arrows, **Figure 2.4 B**). These cells had no indication of an

invasive phenotype by day 5 as they lost their elongated morphologies and quickly began to form clusters **(Figure 2.4 B)**. Similarly, MCF10A cells also formed cellular clusters upon day 1 of culture and demonstrated no significant migratory characteristics **(Figure 2.4 C)**. These cells maintained round morphology, while the size of the cellular clusters notably increased as a function of time.
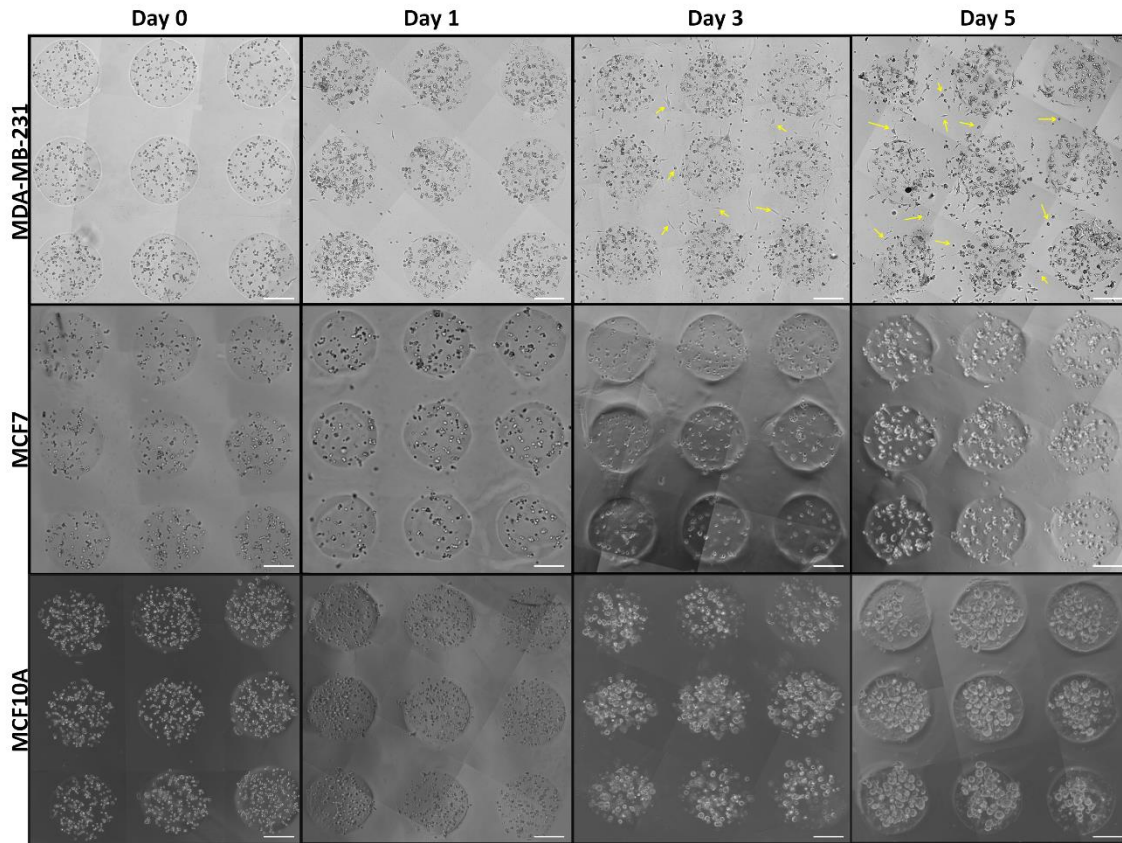
Figure 2.5: Phase contrast (3X3 tile) images of a high density array of tumor constructs demonstrating cellular morphology and migration. Scale bars represent 250 μm.

To prevent cellular attachment on glass slide and guide the migration throughout the 3D hydrogel constructs, a layer of PEG was coated onto the glass slide due to its cell-repellant properties (Nikkhah, Eshak, et al. 2012). Control experiments were conducted where the circular constructs were patterned onto glass slides with and without PEG

coating. When patterned on slides without PEG, nearly every single cell escaped from the miropatterned circular regions and migrated onto the glass slide **(Figure 2.6)**. These results indicate that, without PEG, the cells heavily adhered to and interfaced with the

Figure 2.6: Phase contrast images of a control experiment using MDA-MB-231 cells. In the presence of PEG, cells were confined within the circular constructs at all time points. Without PEG 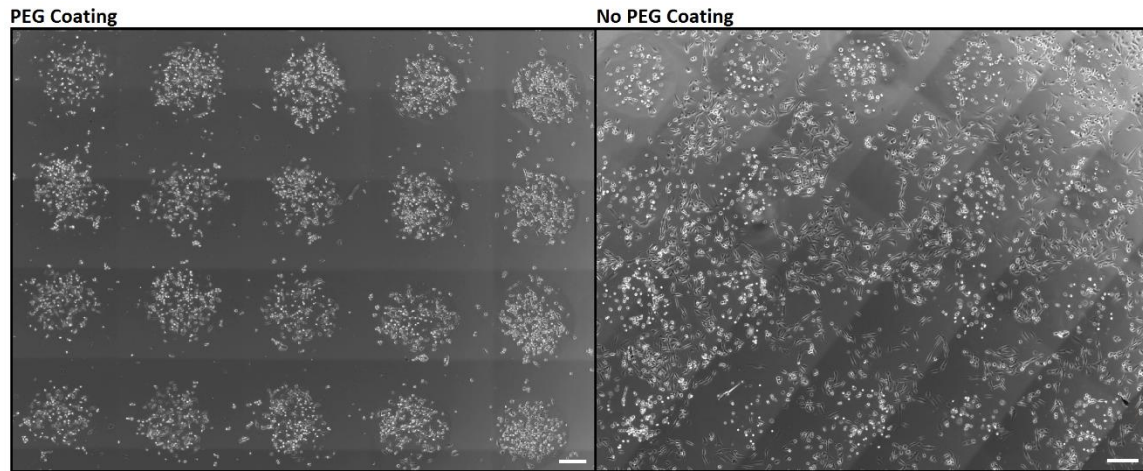coating, cells migrated down to the glass slide before diffusely migrating on the glass slide. Scale bars represent 200 μm.

glass slide. On the other hand, adding PEG coating resulted in cell-repelling properties and facilitated the migration of the cells throughout the hydrogel layer.

Consistent with phase contrast images, fluorescence images of DAPI stained cell nuclei demonstrated a significantly higher number of MDA-MB-231 cells within the circular constructs and the surrounding matrix as compared to MCF7 and MCF10A cells. Cellular clustering was also evident in DAPI stained MCF7 and MCF10A cells **(Figure 2.7 A)**. Quantitative analyses confirmed that the overall MDA-MB-231 proliferation was significantly higher compared to MCF7 and MCF10A cells within the microengineered platform **(Figure 2.7 B)**. Particularly, a similar trend was observed with respect to the number of the cells within the high stiffness circular constructs

(Figure 2.7 C). About 2.5 times more MDA-MB-231 cells disseminated from the circular areas toward the surrounding matrix by day 5 of culture as compared to MCF7 cells (12.87 ± 1.85% vs 5.16 ± 2.31%). MCF10A cells exhibited nearly no invasive characteristics toward the outer regions of circular constructs (1.08 ± 0.24% by day 5) **(Figure 2.7 D)**. However, there was still a statistically significant difference in the migration of MCF10A cells at each time point. This is due to the clumping tendency as some cells proliferated to form clusters on the edge of the constructs. Differences in migratory characteristics of the cells were further highlighted in the real time experimentations (Supplementary Movies M-1, M-2, M-3). MDA-MB-231 cells were shown to elongate at the periphery of the constructs prior to contractile motion, which guided them out of the constructs. It is also important to note that these cells demonstrated the ability to migrate between and back into constructs after initially invading the surrounding matrix (Supplementary Movie M-4). Consequently, migration data presented consists of net migration values counting only the cells that have entered and remain in the surrounding matrix by day 5 of culture.

2.3.4. *Actin Cytoskeletal Organization*

To further confirm our observations on cellular migration and gain insight into cell-matrix interactions/morphology, we performed 3D imaging of the actin cytoskeletal organization of cells embedded throughout the hydrogel layer (100 μm height). Preliminary images clearly demonstrated the cells were embedded within the hydrogel layer of the high stiffness circular constructs **(Figure 2.8A)** as well as the surrounding matrix **(Figure 2.8B)**.
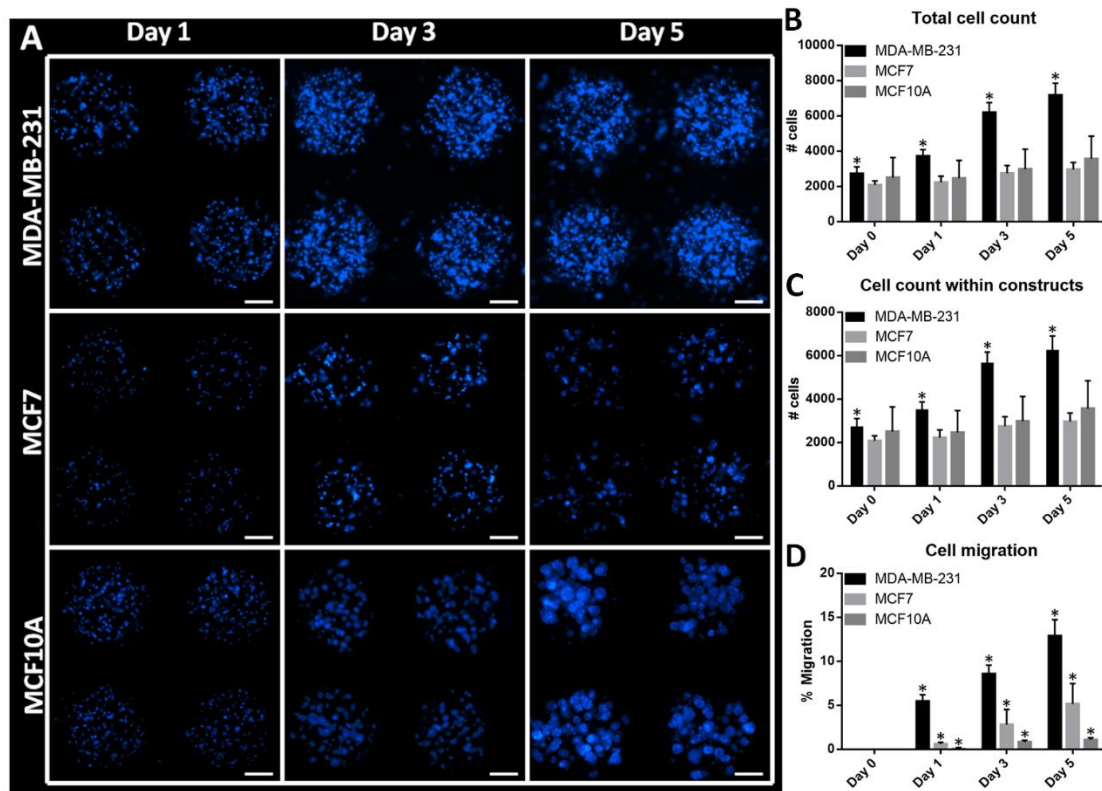
Figure 2.7: Migration and proliferation of the cells within the tumor model. Representative fluorescence images (A) demonstrating DAPI stained cell nuclei. Total cell proliferation (B), cell proliferation within the tumor region (C), and cellular invasion (D). Scale bars represent 200 μm. *$p < 0.05$ compared to the previous time point.

Using Z-stack microscopy imaging of the actin cytoskeleton, we were able to visualize the 3D structure of the cells. We observed several different structures including 3D elongated protrusions, flat protrusions and membrane blebs (**Figure 2.9**, arrows). In the representative images of the F-actin cytoskeleton, MDA-MB-231 cells particularly exhibited a wide range of invasive characteristics possessing small number of flat protrusions and many elongated 3D protrusions. In addition some cells exhibited membrane blebs as they invaded the surrounding matrix **(Figure 2.9A, D)**. A few number of MCF7 cells exhibited flat protrusions, on the periphery of the circular constructs, as demonstrated in the representative high magnification (40x) images **(Figure 2.9 E)**. In

MCF10A cells, the clustering tendency was significantly higher with no indications of protrusions. To further quantify cellular morphology, the circularity of the actin cytoskeleton was assessed within the three cell types **(Figure 2.9 G)** using a custom script for Image J software (particle analyzer module). This analysis revealed that MDA-MB-231 cells exhibited a significantly less circular morphology when compared to MCF7 and MCF10A cells, as demonstrated by the high standard deviation indicative of their heterogeneous morphology **(Figure 2.9 G)**.
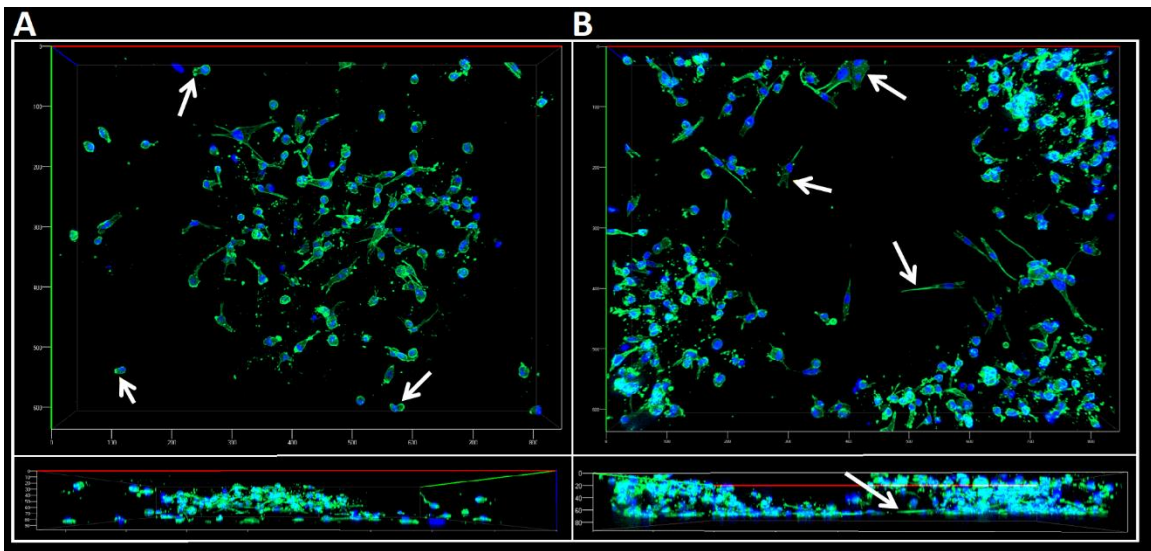
Figure 2.8: F-actin cytoskeletal organization of the cells demonstrating cells embedded within the hydrogel layer. Some MDA-MB-231 cells migrated to the glass slide and demonstrate a flat protrusions. These cells also exhibited 3D actin protrusions and membrane blebs.

2.4 DISCUSSION

The development of 3D *in vitro* breast tumor models is significant for cancer related studies, since it would enable us to perform fundamental biological analyses on metastatic processes, such as cancer cell invasion. Furthermore, biomimetic tumor models can facilitate high throughput analyses on the efficacy of various pharmaceuticals compounds on cancer cell invasion. Currently, a wide variety of 2D and 3D platforms are being used

to study breast cancer cell behavior (i.e. migration, gene expression). 2D assays do not recapitulate the complexities of the native tumor microenvironment (Griffith and Swartz 2006, Kim 2005). On the other hand, the majority of 3D hydrogel-based matrices lack organized architecture and cellular constructs, thus are limited in terms of localizing the stromal components and cancer cells within separate regions (Kimlin, Casagrande, and Virador 2013).
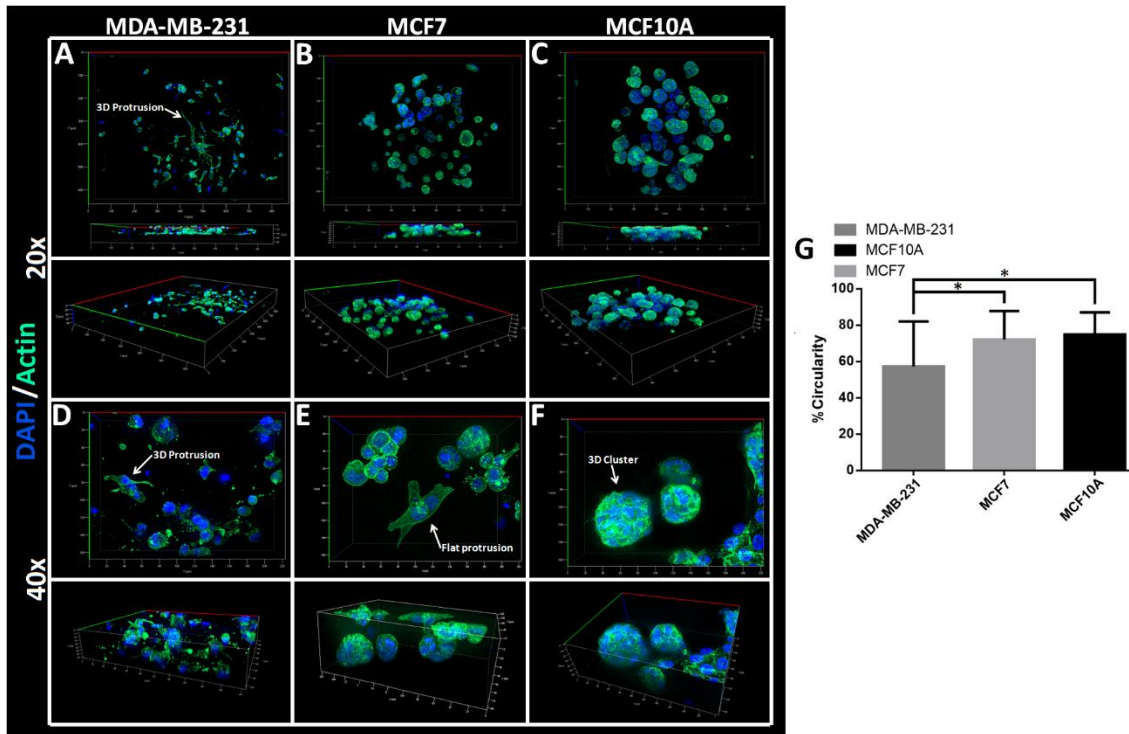
Figure 2.9: F-actin (green) and DAPI (blue) stained cell-embedded tumor model on day 5 of culture. (A-C) Representative 20X image of MDA-MB-231, MCF7, and MCF10A cells respectively. (D-F) Representative 40X images highlighting specific cell-matrix interactions. (G) Circularity amongst the three cell types. MCF10A, MCF7, and MDA-MB-231 cells had circularities of 74.9 ± 12.1%, 72.1 ± 15.7%, and 57.3 ± 24.7% respectively (*$p<0.05$).

It is now becoming more recognized that the integration of microfabrication techniques and advanced biomaterials (i.e. photocrosslinkable hydrogels) can provide a unique ability to develop highly organized cell-based constructs (Dickinson et al. 2012,

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Dolatshahi-Pirouz et al. 2014, Kimlin, Casagrande, and Virador 2013). In this regard, GelMA hydrogel is an excellent candidate for cancer related studies due to its biocompatibility and ability to create organized cellular constructs. However, the primary focus on the use of GelMA, thus far, has been centered on tissue engineering and regenerative medicine applications (e.g. formation of vascularized networks (Nikkhah, Eshak, et al. 2012)). To our knowledge, there has not been any specific study utilizing GelMA to develop microengineered breast tumor models. Furthermore, there have been no significant attempts, using hydrogel-based matrices, to localize the separate regions with tunable stiffness (i.e. circular constructs, surrounding region) within microengineered platforms. In this work, we build upon our expertise in microfabrication technology by creating a novel, two-step photolithography technique to develop a 3D highly organized breast tumor microenvironment. GelMA has been demonstrated to be a biocompatible matrix for encapsulation with a vast array of cell types including 3T3 fibroblasts, endothelial cells, aortic valvular interstitial cells, and glioma cells (Aubin et al. 2010, Benton et al. 2009, Nikkhah, Eshak, et al. 2012, Pedron and Harley 2013). Consistent with previous studies, our work also confirmed that breast cancer and mammary epithelial cells had around 85% viability, indicating that the two-step photolithography technique along with the UV exposure and the presence of a PI had minimal effect on overall cell survival. Furthermore, the crosslinking time of the GelMA (12s) resulted in a Young's modulus of about 750 Pa. This is consistent with previous studies where a crosslinking time of 60s yielded a Young's modulus of 3.5-4 kPa (Nichol et al. 2010) indicating that the stiffness of the GelMA might be linearly dependent on the crosslinking time.

An innovative aspect of our study was independently patterning 3D high stiffness circular constructs surrounded by an interstitial area of lower stiffness (surrounding regions). Matrix stiffness demonstrates a physiologically relevant condition and has consequently been heavily studied in collagen, polyacrylamide, and Matrigel hydrogels (Kraning-Rush and Reinhart-King 2012, Zaman et al. 2006). Furthermore, several studies have focused on seeding the cells on hydrogel sheets (Kraning-Rush and Reinhart-King 2012) with different stiffness rather than encapsulating them within the 3D matrix. In this regard, our model provides a distinct advantage, as we are able to independently modulate the stiffness of the matrix within distinguished regions in the microengineered tumor model. As such, we can assess the specific effects of matrix stiffness on breast carcinoma progression *in vitro* within a 3D model. In our model, it was demonstrated that some MDA-MB-231 cells were highly populated in the higher stiffness circular constructs. Although significant number of cells initially invaded within the surrounding regions of lower stiffness, but real time analysis demonstrated that some cells gained an affinity to move back into the high stiffness circular areas (Supplementary Movie M-4). Such behavior indicates the tendency of cancer cells to migrate within the stiffer regions. Using our proposed microengineering technique, our future studies will be focused on switching the stiffness of the circular constructs and the surrounding matrix.

The proposed micropatterned tumor model also shed unique insight on cancer cell morphology. MDA-MB-231 cells adopted highly invasive characteristics with a mixture of round and spindle like morphologies. Specifically, the cells that migrated down on the glass slide formed flat protrusions, which was substantially different than the morphology exhibited by the cells embedded within the 3D gel (Arrows, **Figure 2.8B**). This bi-modal

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display of migratory morphology demonstrates that the mechanism for MDA-MB-231 cells migration was heavily influenced by substrate interactions (2D vs 3D). Particularly, cells migrating through the hydrogel formed 3D protrusions or membrane blebs (Arrows, **Figure 2.8A**; Supplementary Movie M-5). These observations were consistent with the heterogeneous, 3D morphology of migrating cells cultured in Cell Derived Matrix (CDM) and Matrigel (Petrie and Yamada 2012, Poincloux et al. 2011). In order to fully guide the migration of the cells through the 3D hydrogel gel, further modifications (i.e. concentration of PEG,) are required to further enhance cellular repellency of PEG layer. MCF7 and MCF10A cells rapidly clustered as early as day 2/3 that only grew bigger by day 5. In fact, there are numerous studies that have utilized various biochemical signals (i.e Cyclic AMP) or the co-cultures with CAFs in order to produce stimulate growth of morphologically accurate cellular clusters similar to acinar structures (Krause et al. 2010, Nedvetsky et al. 2012). Within GelMA hydrogel, these cells formed clustered without the need for any biochemical stimuli, which further validates our model by confirming that it can readily recreate *in vivo* like morphologies.

The proposed photocrosslinkable hydrogel along with the two-step photolithography technique can be used to create tumor microenvironment models that have significant applicability in terms of modeling a physiologically relevant diseased condition. Specifically, matrix stiffness can be modified, cellular composition and organization can be tweaked, and biochemical stimuli can be added to the environment in an organized manner. The microenvironment remains to be a high-density, quantifiable, and morphologically accurate model regardless of the study. This has significant applicability in terms of high-throughput drug testing, the development of personalized

medicine, as well as in fundamental studies of cancer biology. In the future, we plan to build upon this microenvironment by conducting detailed studies on effects of matrix stiffness on migration/morphology and the introduction of stromal components within the tumor model.

CHAPTER 3

CONCLUSION AND FUTURE WORK

3.1 CONCLUSION

Breast cancer is one of the most commonly diagnosed cancers and results in a high mortality rate in U.S. women. Therefore, it is crucial to study the behavior of cancer cells and ultimately, their response to anti-cancer drugs. In this study, we created a tumor model using a novel, two-step photolithography technique and photocrosslinkable gelatin hydrogel. A unique aspect of our model was the compartmentalization of two distinct regions juxtaposed to each other with differential stiffness. In particular, we developed high density array of cell embedded high stiffness circular regions surrounded by low stiffness areas. We validated the model by encapsulating three cell types separately in order to investigate migratory behavior, cell viability, and cell morphology. High cell survival (~85%) as compared to previous studies was observed regardless of the cell type. Interestingly, a bimodal display of morphology was displayed in MDA-MB-231 cells as they elongated with flat protrusions on glass slid while exhibited 3D protrusions or membrane blebs when invading the surrounding hydrogel matrix. These cells were highly populated at the high stiffness circular constructs. In addition, 3D cellular clusters were observed in both MCF7 and MCF10A cells. These morphologically accurate structures were formed without the use of any biochemical stimuli, which demonstrates the versatility of GelMA in creating a biomimetic tumor microenvironment. The proposed platform could be potentially used for future studies of cancer cell behavior, high-throughput drug screening, and the development of personalized medicine. Some of the other advantages of our approach relative to microfluidics is the ease of fabrication, high density arrays which

can be used for high throughput studies, and the increased accessibility which can be used for various probing techniques such as AFM, optical tweezers, etc.

3.2 FUTURE WORK

3.2.1 Co-culture of cells in the 3D microenvironment

In our study, we used one cell-line encapsulated within the GelMA hydrogel and patterned into circular tumorous regions. Owing to the complexity of the breast tumor architecture, and in order to recapitulate the *in vivo* microenvironment, other cell types of the breast stroma can be introduced into this model. For example, CAFs can be introduced into the stromal region surrounding the tumors. The effect of CAFs on the proliferation and migration of the three breast cell lines can be observed and analyzed. Addition of endothelial cells to study the capillary formation and effect of those capillaries on the migration of the breast cell lines is another facet which can be looked into. Another interesting study could be mixing the different stromal components in the ratio observed *in vivo* and analyzing their effect on the migration and morphological changes in breast cancer cell lines.

3.2.2 Effect of varying stiffness of the tumor constructs with respect to the surrounding stroma

Mechanical force exerted by the surrounding environment is a crucial component in determining the fate of cancer cells, their transition into a tumorous phenotype, invasion and migration through the neighboring tissue. We studied the effect of high stiffness tumorous regions surrounded by a lower stiffness stroma on the migration and morphology of three different breast cell lines. An interesting study would be to look at the effect of

varying stiffness on the migration trajectory of the breast cancer cell lines in 3D. This would give insight to the cancer cell migratory patterns in case of changing stiffness.

3.2.3 Addition of anti-cancer drugs to the 3D tumor microenvironment

Cancer cell behavior in the developed platform is seen to mimic that in the *in vivo* microenvironment. An interesting study would be the addition of an anti-cancer drug to the cell encapsulated in high density array of tumors. Drugs can be added to assess the changes in proliferation and migration of the cancer cells. Morphological changes and cytoskeletal reorganization in the tumor cells can also be analyzed in response to the anti-cancer drug. Thus, this platform could be a potent candidate after appropriate calibration and validation to be used for drug screening and ultimately, in personalized medicine.

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APPENDIX A

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Figure 1.3 (A)

Title: Patterning microscale extracellular matrices to study endothelial and cancer cell interactions in vitro

Author: Laura E. Dickinson,Cornelis Lütgebaucks,Daniel M. Lewis,Sharon Gerecht

Publication: Lab on a Chip

Publisher: Royal Society of Chemistry

Date: Sep 4, 2012

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Figure 1.3 (B)

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Licensed content author	Mehdi Nikkhah,Jeannine S. Strobl,Raffaella De Vita,Masoud Agah
Licensed content date	June 2010
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Figure 1.3 (C)

Title: Large-Scale Single-Cell Trapping And Imaging Using Microwell Arrays

Author: Jacqueline R. Rettig, Albert Folch

Publication: Analytical Chemistry

Publisher: American Chemical Society

Date: Sep 1, 2005

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Figure 1.4 (A)

Figure 1.4 (B)

Title: Transition to invasion in breast cancer: a microfluidic in vitro model enables examination of spatial and temporal effects

Author: Kyung Eun Sung,Ning Yang,Carolyn Pehlke,Patricia J. Keely,Kevin W. Eliceiri,Andreas Friedl,David J. Beebe

Publication: Integrative Biology

Publisher: Royal Society of Chemistry

Date: Dec 7, 2010

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Figure 1.4 (C)

Human 3D vascularized organotypic microfluidic assays to study breast cancer cell extravasation