Three-Dimensional Microfluidic Based Tumor-Vascular Model to Study Cancer Cell Invasion and Intravasation

by

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ABSTRACT

Breast cancer is the second leading cause of disease related death in women, contributing over 40,000 fatalities annually. The severe impact of breast cancer can be attributed to a poor understanding of the mechanisms underlying cancer metastasis. A primary aspect of cancer metastasis includes the invasion and intravasation that results in cancer cells disseminating from the primary tumor and colonizing distant organs. However, the integrated study of invasion and intravasation has proven to be challenging due to the difficulties in establishing a combined tumor and vascular microenvironments. Compared to traditional *in vitro* assays, microfluidic models enable spatial organization of 3D cell-laden and/or acellular matrices to better mimic human physiology. Thus, microfluidics can be leveraged to model complex steps of metastasis. The fundamental aim of this thesis was to develop a three-dimensional microfluidic model to study the mechanism through which breast cancer cells invade the surrounding stroma and intravasate into outerlying blood vessels, with a primary focus on evaluating cancer cell motility and vascular function in response to biochemical cues.

A novel concentric three-layer microfluidic device was developed, which allowed for simultaneous observation of tumor formation, vascular network maturation, and cancer cell invasion/intravasation. Initially, MDA-MB-231 disseminated from the primary tumor and invaded the acellular collagen present in the adjacent second layer. The presence of an endothelial network in the third layer of the device drastically increased cancer cell invasion. Furthermore, by day 6 of culture, cancer cells could be visually observed intravasating into the vascular network. Additionally, the effect of tumor cells on the formation of the surrounding microvascular network within the vascular layer was evaluated. Results indicated that the presence of the tumor significantly reduced vessel diameter and increased permeability, which correlates with prior *in vivo* data. The novel three-layer platform mimicked the *in vivo* spatial organization of the tumor and its surrounding vasculature, which enabled investigations of cell-cell interactions during cancer invasion and intravasation. This approach will provide insight into the cascade of events leading up to intravasation, which could provide a basis for developing more effective therapeutics.

DEDICATION

I dedicate my work to my beloved parents Mrs. & Mr. H.T Nagaraju and my brother Suraj Nagaraju

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TABLE OF CONTENTS

APPENDIX Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
ADAM12	Metalloproteinase domain-containing protein 12
ANGPT14	Angiopoietin-like 4
ANOVA	Analysis of Variance
BAEC	Bovine Aortic Endothelial Cell
BM	Basement membrane
BSA	Bovine Serum Albumin
CAF	Cancer associated fibroblasts
CAM	ChorioAllantoic Membrane
CIS	Carcinoma In Situ
COX-2	Cyclooxygenase-2
CTC	Circulating tumor cells
DAPI	4',6-diamidino-2-phenylindole
DCIS	Ductal carcinoma In situ
DCIS	Ductal Carcinoma In Situ
DI	De Ionized
DMEM	Dulbecco's Modified Eagle's Medium
DPBS	Dulbecco's Phosphate Buffered Saline
EC	Interstitial Flow
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
EGM-2	Endothelial Growth Medium
EMT	Epithelial–Mesenchymal Transition
EREG	Epiregulin
FBS	Fetal bovine serum

FGF	Fibroblast Growth Factor
GA	Glutaraldehyde
HGF	Hepatocyte growth factor
HUVEC	Human umbilical vein endothelial cell
IDC	Invasive Ductal Carcinoma
LM	Laminin
MMP	Matrix metalloprotease
MTCS	Methyltrichlorosilane
NPC	Nasopharyngeal carcinoma
PAR1	Protease-Activated Receptors-1
PDGF	Platelet-derived growth factor
PDL	poly-d-lysine
PDMS	Polydimethylsiloxane
PFA	Paraformaldehyde
PVT	PreVascularized Tumor
SDF-1	Stromal-derived factor 1
TAM	Tumor Associated Macrophages
TGF-β	Transforming growth factor beta
TME	Tumor Micro Environment
TNF -α	Tumor Necrosis Factor alpha
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular Endothelial Growth Factor A

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 GLOBAL BURDEN OF BREAST CANCER

Breast cancer is the second major cause of mortality among women in the United States. Approximately 12% of women will develop breast cancer through their lifetimes (Siegel et al. 2014). Furthermore, 3,327,552 American women were estimated to be living with breast cancer in 2014, and older women, within 55-64 age, were found to have an increased incidence rate of the disease (American Cancer Society. Breast Cancer Facts & Figures 2015-2016). Although several risk factors for breast cancer have been identified for the majority of women, it is not possible to develop causal relationships that inform efficient clinical treatment options (Lacey, Devesa, and Brinton 2002). In general, a family history of breast cancer and mutations in the genes BRCA1, BRCA2 and P53 are well established risk factors (Kelsey and Bernstein 1996). Furthermore, important risk factors for breast cancer include reproductive factors such as exposure to endogenous such as estrogens due to early menstruation, late age at first childbirth and late menopause. Additionally exposure to exogenous hormones caused by use of oral contraceptives and hormone replacement therapy can exert higher risk of developing cancer (Lacey, Devesa, and Brinton 2002).

Early cases of breast cancer were first reported by Egyptians; from then breast cancer cases have been reported for thousands of years and remain a menacing disease (Rayter 2003). One primary treatment developed to cure breast cancer is mastectomy, which is removal of the affected breast tissue using surgical procedures. However, despite effectiveness of this procedure, in the mid-1800s surgeons noted that the disease had a high recurrence rate post operations because the cancer spread to nearby regions (Rayter 2003). LeDran was one of the pioneers who suggested that breast cancer begins as a local disease and spreads to other organs, so local treatment options are not completely effective for treatment. It was later established that metastasis of cancer to secondary sites results in a high recurrence rate and poor prognosis (Rayter 2003). To better understand metastasis, there have been numerous *in vivo* animal and *in vitro* cell-based models developed to study cancer metastasis (Wang, Eddy, and Condeelis 2007). Further improvements

in the fields of microfabrication, tissue engineering, high resolution cell-imaging, and genetic/molecular pathways analysis have led to developing better diagnostic and therapeutic approaches (DeVita Jr and Rosenberg 2012).

1.2 METASTASIS

Metastasis is the primary cause of mortality in individuals with breast cancer (Nguyen, Bos, and Massagué 2009). It is a process by which tumor cells migrate from the primary tumor to one or multiple distant organs (Spano et al. 2012). Metastasis progresses through a complex multistep process termed as the "Metastatic Cascade", which involves several stages as listed below (Valastyan and Weinberg 2011) **(Figure 1.1)**:

- ***a)*** Invasion of cancer cells through surrounding extracellular matrix and stroma
- ***b)*** Intravasation into the lumen and blood vessels
- ***c)*** Surviving and transport through vasculature
- ***d)*** Lodging at distant sites
- ***e)*** Extravasation to form micro metastatic structure
- ***f)*** Surviving harsh foreign micro environment
- ***g)*** Re-initiation of proliferation at distant site

It is well known that invasion and metastasis does not just depend on cancer cells but various biochemical and biophysical factors within the local tumor microenvironment (Hu et al. 2008). During metastasis, the surrounding environment becomes activated due to cancer cells secreting several growth factors such as Transforming growth factor beta (TGF-β), Platelet-derived growth factor (PDGF), Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Hepatocyte growth factor (HGF), etc. This activated tumor microenvironment will in turn influence cancer cell polarity, migration and circulation (Wolf and Friedl 2003). Thus, before tumor cells undergoes metastasis, a suitable microenvironment conducive for tumor promotion is assembled, which leads to the formation of the secondary tumor (Kucia et al. 2005, Orimo et al. 2005).

Cancer cell invasion involves the cells disseminating from the well-defined primary tumor, entering the surrounding stroma, and then moving into adjacent tissues (Wolf and Friedl 2003). The ability of cancer cells to undergo migration and invasion allows neoplastic cancer cells to enter

blood vessels and metastasize to distant organs (Chambers, Groom, and MacDonald 2002). To invade, carcinoma cells must first overcome the basement membrane (BM), a specialized membrane surrounding epithelial tissues. Active proteolysis of BM occurs by secretion of matrix metalloproteinases (MMPs) by cancer cells (Bissell and Hines 2011). Once the carcinoma cells breakdown the BM, they reach the stroma where they encounter the stromal cells. With the association of tumor cells, stromal cells transition to a phenotype where they are commonly known as "Tumor-Associated stromal cells" (Kessenbrock, Plaks, and Werb 2010). To overcome barriers they face during invasion, cancer cells opt to a cellular process crucial to invasion known as the Epithelial–Mesenchymal Transition (EMT) (Thiery et al. 2009). During the EMT, tight and adherent junctions between the cells are dissolved and cell polarity is lost. The individual cells dissociate from epithelial cell sheets and exhibit mesenchymal cell traits which are correlated with high cell migration and invasion (Wolf and Friedl 2003). Also, there will be downregulation of epithelial markers and upregulation of mesenchymal markers. To migrate, a cell modifies its shape and interacts with the surrounding microenvironment. Specifically, the cell becomes polarized and forms a pseudopod by extending its leading edge, and the entire cell body contracts, generating motility forces that will lead to gradual forward movement of the cell (Friedl and Bröcker 2000). *In vitro* and *in vivo* studies have shown that tumor cells have diverse movement patterns, such as, they can migrate as individual cells (individual cell migration), as cell sheets, strands, or clusters (collective cell migration) (Wolf and Friedl 2003). From previous studies, it was shown that motile tumor cells originate from multicellular components, but they lose their cell-cell contacts, detaching and migrating as individual cells (van Zijl, Krupitza, and Mikulits 2011). In collective migration, the cell aggregates move as one functional unit. In contrast to individual movement, cell-cell adhesions in cell groups leads to cell assembly, forming large sized multicellular bodies (Wolf and Friedl 2003). Many tumor cells lack stop signaling in migratory events, which causes a large imbalance and drives them to invade into the surrounding stroma and migrate towards distal organs (Wolf and Friedl 2003) **(Figure 1.1 b)**.

After invading into surrounding tissue, cancer cells migrate and enter blood or lymphatic vessels, a process known as 'intravasation' (Gupta and Massagué 2006). Once, the carcinoma

cells successfully enter blood vessels, they can translocate to different parts of the body through venous and arterial circulation. Cancer cells spreading through lymphatic vessels (i.e. lymphogenous spread) is observed in humans at significant levels, but the major mechanism of cancer cell dissemination is through blood circulation (i.e. hematogenous spread) (Gupta and Massagué 2006). Furthermore, cancer intravasation can be either active or passive. Active intravasation occurs when tumor cells migrate towards the blood or lymphatic vessels following gradients of secreted chemokines. On the other hand, passive intravasation happens when blood vessels are in close proximity to the tumor and cancer cells are able to enter nearby luminar structures (van Zijl, Krupitza, and Mikulits 2011). Another important distinction is whether tumor cells intravasate into the vessels through the endothelial cell body or between endothelial cell-cell junctions (Reymond, d'Água, and Ridley 2013). Recent studies have demonstrated a large involvement of endothelial junctional in both paracellular and transcellular migration, suggesting that intravasation is facilitated by the ability of cancer cells to modify and cross the endothelial cell junction and pericyte barrier. Matrix metalloproteases (MMP-1) secreted by cancer cells modify Protease-activated receptors (PAR1) on the endothelial cells and remodel the endothelial junctions (Bergers et al. 2000). Also, Disintegrin and Metalloproteinase domain-containing protein 12 (ADAM12) induce cleavage of VE-cadherin and ang-1 receptor TIE2 which can disrupt endothelial junctions (Reymond, d'Água, and Ridley 2013). Macrophages that are present in the stroma promote intravasation by secreting EGF and Tumor Necrosis Factor alpha (TNF-α), which also induce retraction of endothelial junctions. In addition, cancer cells utilize NOTCH receptors to transmigrate through endothelial junctions (Gupta and Massagué 2006).

The mechanism of intravasation is strongly influenced by tumor-associated angiogenesis, where cancer cells stimulate the formation of new blood vessels. The alterations to the tumor microenvironment (e.g. inadequate oxygen supply (hypoxia), insufficient nutrients) result in tumor cells secreting growth factors, which lead to the formation of vascular networks (Partridge, Deryugina, and Quigley 2008). There are many mechanisms through which tumor cells induce angiogenesis (Vaupel 2004). However, VEGF is known to be one of the major factor secreted; VEGF stimulates the formation of new blood vessels, a process termed as ‘Neoangiogenesis’. In

contrast to normal vascular networks, the newly formed vasculatures are leakier and permeable due to weak inter cellular junctions and absence of pericyte lining. Permeability of endothelial cells also facilitates intravasation (Gupta and Massagué 2006). Approximately 0.01% of circulating tumor cells (CTC) will reach and form metastases at secondary sites, but it is still important to investigate the progression of intravasation to understand the mechanism in-depth and decipher the efficiency of metastasis. There are several *in vivo* and *in vitro* models created to study intravasation, and they have clarified the role of different cells, signaling pathways, and molecules that contribute to intravasation (Woodfin et al. 2011, Stoletov et al. 2010). Despite progress in identifying tumor-cell autonomous intravasation mechanisms, recent studies suggest that the tumor microenvironment also plays a major role in regulating tumor cell dissemination (Joyce and Pollard 2009). Hence, a comprehensive understanding of the underlying biological mechanisms of cancer cell intravasation, both at the intracellular and tumor microenvironment levels, is critical for identifying and developing novel targeted therapies **(Figure 1.1 b)**.

Once the cancer cells successfully intravasate, they become circulating tumor cells within the blood stream. These cells are in between the primary tumor and metastatic sites, so they are called 'Metastatic Intermediates' (Meng et al. 2004). After surviving circulation and reaching the foreign site, carcinoma cells must cross the endothelial cell lumina to reach the tissue parenchyma of the host tissue, this process is known as 'extravasation' (Valastyan and Weinberg 2011). To overcome barriers during extravasation, the carcinoma cells make the vessels more permeable and perturb the microenvironment. They achieve this by secreting different factors such as Angiopoietin-like 4 (ANGPT14), Epiregulin (EREG), Cyclooxygenase (COX-2), MMP-1, MMP-2 and VEGF, which renders the nearby vessels more permeable and assist the tumor cells during extravasation (Gupta et al. 2007, Padua et al. 2008).

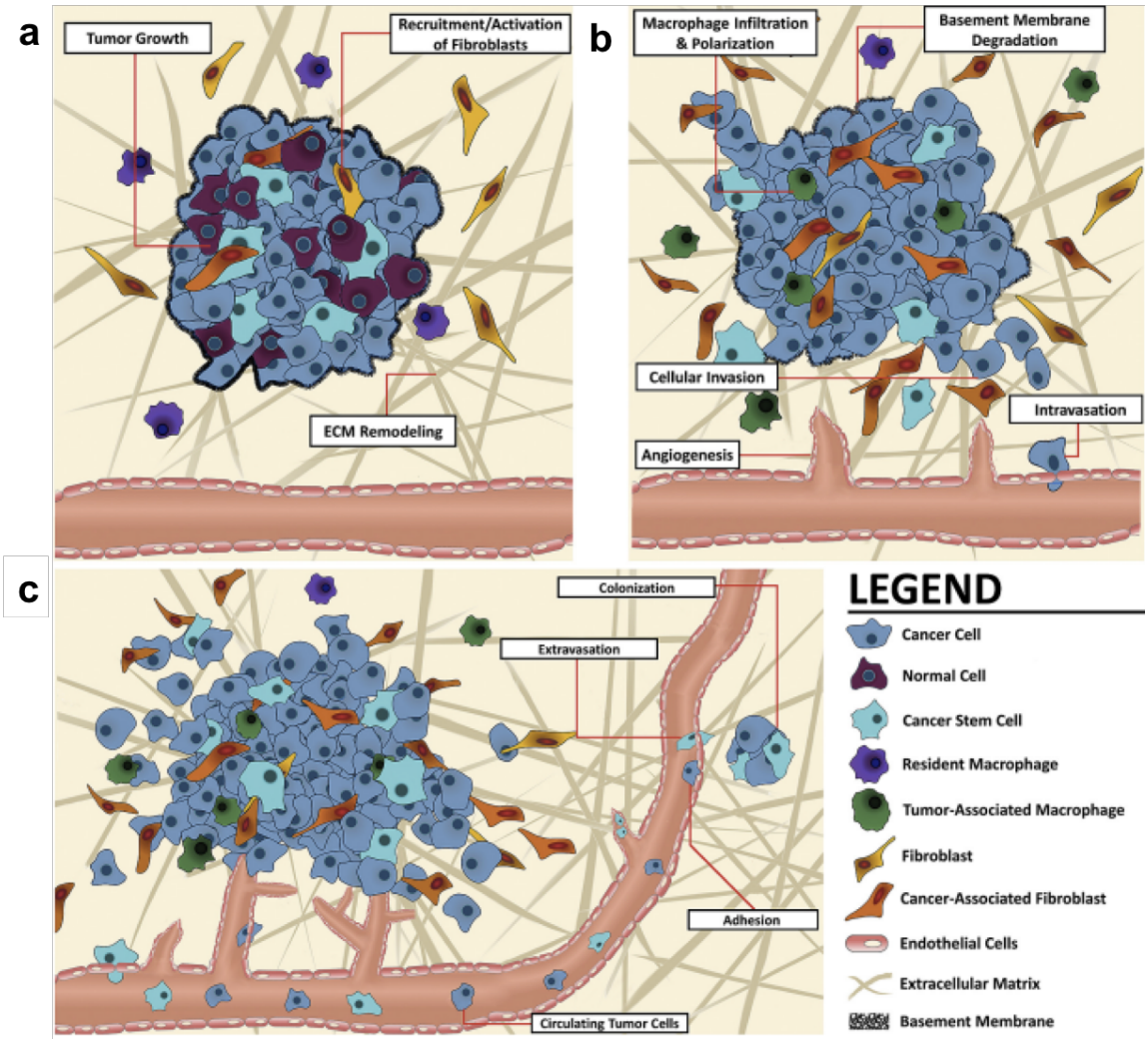

Figure 1.1: Metastatic cascade **(a)** Schematic of the metastatic cascade. Tumor growth and development **(b)** Angiogenesis, cancer cell invasion, and intravasation. **(c)** Finally, surviving cancer cells and cancer circulate through the body, attach to blood vessels, and extravasate to form secondary metastases. Adapted from Peela, Truong and Saini et al. with permission from Elsevier [Biomaterials], copyright (2017) (Peela et al. 2017)

The extravasated cancer cells must survive the foreign environment to further proliferate and form secondary cancer. Until the foreign environment is hospitable, the cancer cells are dormant and will be in the hibernating phase. Some models suggest that cancer cells adapt to the foreign environment by releasing signals that upregulate fibronectin, which leads to mobilizing 'VEGF receptor-positive hematopoietic progenitor cells' towards metastatic sites. These cells change the distant microenvironment to more hospitable sites, which are called pre-metastatic niches (Psaila and Lyden 2009). Cancer cells secrete different chemokines such as stromal-derived factor 1 (SDF-

1) which leads to formation and progression of pre-metastatic niche. Apart from the chemokines secreted by cancer cells, different stromal cells such as fibroblasts, endothelial cells and adipocytes release several growth factors which influence the tumor cell characteristics such as morphology and migration (Psaila and Lyden 2009). The ability of disseminated tumor cells to adapt to the host site and proliferate depends on their proficiency to change the foreign microenvironment to a hospitable environment (Chambers, Groom, and MacDonald 2002) **(Figure 1.1 c)**.

The cancer cells must undergo all the stages to successfully metastasize and develop a secondary tumor at distant organs. Although great advances have been made in early diagnostics and curing cancer, metastasis remains as one of the major fatal challenges. Numerous processes, signaling pathways and growth factors are involved in metastasis. However, very few molecules have been translated effectively for metastasis prevention and treatment.

1.3 TUMOR MICROENVIRONMENT

Cancer cells develop a complex surrounding microenvironment and depend on it for their survival, growth, invasion, and metastasis called 'Tumor Micro Environment' (TME) (Quail and Joyce 2013). The microenvironment is composed of both stromal cells and Extra Cellular Matrix (ECM) proteins. The basement membrane in the ECM interacts with the epithelium and is composed of collagen, laminin, glycoproteins and proteoglycans (Oskarsson 2013). The ECM maintains architecture, structure and tissue homeostasis. The surrounding stroma includes different cells like fibroblasts, endothelial cells, immune cells as well as adipocytes (Place, Huh, and Polyak 2011), and there is a bi-directional communication between the tumor cells and their surrounding stroma. It is shown that the interactions between the tumor and tumor microenvironment are critical for the disease initiation, progression, and metastasis (Quail and Joyce 2013). Several studies and observations suggest that instability of the stromal cell genome can lead to unstable epithelial cells thereby inducing carcinoma (Weber et al. 2006).

Rudolf Virchow in 1863 first proposed that there is a link between tumorigenesis and chronic inflammation. Furthermore, the infiltration of leukocytes was identified to be a key hallmark of tumors (Balkwill and Mantovani 2001). Tumor-associated macrophages (TAM) play an important

role in cancer progression; although macrophages are known as effector cells in the immune system, studies have shown that the TAM play a supporting role during cancer progression where they promote invasive characteristics in cancer cells (Biswas and Mantovani 2010).

Fibroblasts are one of the predominant cells in the connective tissues. In the TME, Cancer Associated Fibroblasts (CAFs) have increased numbers and they support the tumor by depositing ECM and maintaining homeostasis (Olumi et al. 1999). It is still not clear where CAFs arise during cancer progression, but studies suggest that they may transition EndMT (Endothelial-Mesenchymal Transition) of cells. CAFs in the TME are activated by cytokines and growth factors like Fibroblast Growth Factor (FGF), PDGF, TGF-β, monocyte chemotactic protein 1 (MCP1) and many secreted proteases. (Kalluri and Zeisberg 2006).

Tumor angiogenesis is now one of the accepted hallmarks of cancer, which is essential for nutrient and oxygen supply without which the tumor would become dormant (Hanahan and Weinberg 2011). Judah Folkman proposed that all tumors are angiogenesis dependent (Folkman 1971). Tumor angiogenesis requires multiple cells in TME like endothelial cells, pericytes, precursor cells, etc. These stromal cells are hypoxia driven (LaGory and Giaccia 2016).Cancer cells secrete different factors such as EGF, VEGF-A and FGF which activates angiogenesis. In addition to this, many other stromal cells and Mesenchymal Stem cells (MSCs) contribute to activation of endothelial cells (Weis and Cheresh 2011). *In vivo* and *in vitro* assays developed so far have been enormously helpful in elucidating the diverse mechanisms involved in the interactions of tumor cells with the surrounding microenvironment. Better relevant models that can capture the complexities of the microenvironment will give an improved understanding and may be key to combatting cancer.

1.4 MODELS TO STUDY CANCER INVASION AND INTRAVASATION

To date, there have been numerous *in vivo* animal models and *in vitro* assays to study tumor cell invasion and intravasation. Here, we present a brief overview of different experimental models, which have been utilized to investigate invasion and tumor endothelial interactions, while providing a comparison of published *in vivo* and *in vitro* experimental findings.

1.4.1. *In vivo* models:

Tumor initiation is an unscheduled hyper proliferation of cells, which occurs due to activation of either cell growth machinery or signaling pathways (Hanahan and Weinberg 2000). Throughout the process of cancer progression, there is a bi-directional relationship between tumor and its host TME (Hanahan and Weinberg 2011). *In vivo* studies have been very valuable to provide a comprehensive overview on the molecular and cellular basis of disease progression (Mak, Evaniew, and Ghert 2014). However, it is complicated to use *in vivo* models to dissect specific cell-cell interactions, and determine the cause and effect relationships as it challenging capture dynamic interactions and adaptive responses of cancer cells (Mak, Evaniew, and Ghert 2014). Additionally, visualizing the process in real-time may not be easily feasible, and it might perturb the pathophysiology of tumors as it requires specialized invasive steps. The other major challenge is using a genetically engineered mouse to incorporate human cancer cell lines, as there will be notable differences in histology (Brown et al. 2010). When using immune compromised mouse models, the major component in the TME like the immune or fibroblast cells and their role cannot be studied. In general, tumor cell intravasation within *in vivo* models is analyzed using various methods including:

a. Real-time imaging of tumor cells

b. Measuring the number of circulating tumor cells in the blood stream

c. Examining the tumor injected at the primary site and the tumor at metastatic site.

In a model developed by Xiao et al. (Xiao et al. 2015), a ChorioAllantoic Membrane (CAM) model was used to study the growth, invasion, neoangiogenesis, and metastasis by transplanting NasoPharyngeal Carcinoma (NPC) in to chick embryo. This model closely simulated the growth of carcinoma and elucidated the mechanism of invasion of NPC cells. This also helped analyzing

tumor angiogenesis, intravasation and metastasis. Tumor invasion was assessed by detecting the extent penetration of basement membrane by cancer cells. Angiogenesis was studied by analyzing the area of formed neovascular networks, while metastasis was quantified by counting the number of tumor cells in distant organs. The results showed the formation of tumor after NPC cells were inoculated into the CAM. Xiao et al. demonstrated the feasibility of applying the CAM model to visualize and evaluate invasion, and tumor angiogenesis. The limitation of this model is that the CAM model is naturally immunodeficient, hence it cannot be used to study the role of immune cells during metastasis **(Figure 1.2 a)**.

Alternatively, Zebra fish models have been used for live imaging of injected human tumor cells. The vascular system of the zebra fish is fully functional, which allows better understanding of cancer cell invasion and metastatic profile (Stoletov et al. 2010). This study showed dynamic extravasation process of cancer cells into the surrounding vasculature of the zebrafish with intravital imaging.

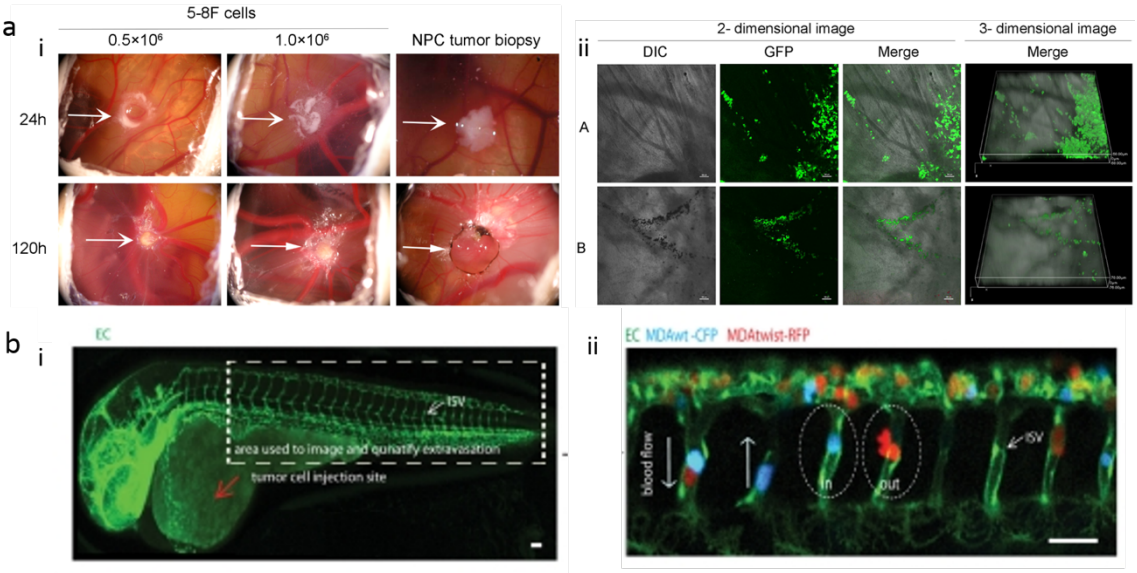

Figure 1.2: Examples of *in vivo* models to study intravasation **(a) (i)** Chorioallantoic membranes (CAMs) inoculated with NPC cells **(ii)** 3D images showing invasion of cancer cells through the basement membrane. Adapted from Xiao et al. Plos one copyright (2015) (Xiao et al. 2015). **(b) (i)** Fluorescence images of a zebrafish embryo at tumor cell injection site **(ii)** confocal images of tumor cells either inside or outside (extravasated) the vessel lumen. Scale bars: 200 μm. Adapted from Stoletov et al. with permission from Company of Biologists [Journal of Cell Science], copyright (2010) (Stoletov et al. 2010).

Using real-time intravital imaging, the authors demonstrated that process of extravasation of cancer cells involving modulation of endothelial layer by tumor cells. Additionally, the findings showed that tumor cells induce vessel remodeling rather than damaging or promoting vascular permeability at the site of extravasation. Migration of tumor cells was independent of the direction of blood flow but was dependent on β1-integrin mediated adhesion to the endothelial walls. Although this model is useful for understanding the biological process of extravasation, some of its major drawbacks include (1) The inability to visualize extravasation of tumor cells in real time, (2) The findings should be confirmed using mammalian models before using them in human clinical therapies **(Figure 1.2 b)**.

1.4.2. *In vitro* models:

Along with *in vivo* models, *in vitro* models have been also proven to be more valuable for investigating the cellular interactions and visualizing real time the dynamic interactions between the tumor and its surrounding microenvironment. The major advantages using *in vitro* models over *in vivo* models could be summarized to: high throughput, the ability to perform mechanistic studies at cellular and molecular level, low experimental costs and faster results.

1.4.2.1 Two dimensional (2D) models:

The prior studies to investigate the dynamics of cancer cell behavior such as migration and invasion as well drug screening were typically performed on microfabricated 2D models.

In this study by Kramer et al., the HT1O8O fibrosarcoma cells were seeded in a reconstituted basement membrane matrix containing Collagen, nidogen, laminin, heparan sulfate and entactin on a petri dish (Kramer, Bensch, and Wong 1986). The invasiveness of the cancer cells was examined and was compared against the normal skin fibroblasts. After 7-days incubation of cells seeded on the surface of the gel, the invasion into the matrix was examined by electron microscopy. The results demonstrated that the cancer cells proliferated and gradually migrated dissolving the matrix forming disorganized cell monolayer, electron microscopy images revealed the filipodia and lamellopodia projections from the cancer cells extending into the matrix while the normal skin fibrabalsts cells attached to matrix exhibited minimal invasion during the same period.

Boyden chamber assay is one of the well-known 2D models for intravasation. For instance, Li et al. (Li and Zhu 1999) have developed a model with modified Boyden chamber to study the migration and invasion of cancer cells. Bovine Aortic Endothelial Cell (BAEC) were used to form an endothelial monolayer and seven different cell lines both malignant and non-malignant were introduced to the chamber. Cells were radioactively labeled for visualization. The trans-migratory activity of cancer cells was correlated with the level of tumor cell induced endothelial monolayer disruption. Quantification was done by image analysis and direct visualization.

Although, the use of 2D assays has enabled addressing important biological questions and useful and important knowledge has been gained on different characteristics of cancer cells like cell motility and migration from 2D models. However, the 2D models do not depict the *in vivo* tissue structure and organization accurately, which is necessary to study cell-cell and cell-ECM interactions.

1.4.2.2 Three dimensional (3D) models:

Conventional 3D *in vitro* assays have been mainly based on spheroid or macroscale cell-laden hydrogels that have been widely utilized to perform fundamental biological studies on cancer cell invasion (Szot et al. 2013, Xu et al. 2012). However, these models do not replicate tissue-specific human pathophysiology. Recently, there have been significant initiatives in the use of microscale technologies (*i.e*. microfluidics) to develop 3D tumor models, with precise control over cell-cell and cell-soluble factor interactions for well-controlled studies on cancer cells behavior within each specific step of metastasis (van Duinen et al. 2015).

Significant progress has been demonstrated in the recent years in micro- and nanofabrication techniques (Mehrali et al. 2017, Kharaziha et al. 2016). These techniques can be applied to create 3D microenvironments to control cell-cell, cell-substrate and cell-soluble factors interactions (Park and Shuler 2003). Notably, microfabrication techniques have proven to be instrumental to control both tumor and surrounding microenvironmental factors to conduct studies at single cell level (Nikkhah et al. 2012, Peela et al. 2016, Truong et al. 2016, Peela et al. 2017).

For instance, in a set of studies by Nikkhah et al., 3D microstructures were etched on silicon surfaces and different cells like Human fibroblasts, malignant breast cells as well as normal breast

cells were cultured in these micro structures to form to identify the biomechanical signatures of cancer cells and normal cells on response to 3D topographical architecture (Nikkhah et al. 2010, Nikkhah, Strobl, Schmelz, and Agah 2011, Nikkhah et al. 2009, Nikkhah, Strobl, Schmelz, Roberts, et al. 2011). Their findings demonstrated that cancer cells adopted to the curved microengineered surfaces, while fibroblast cells stretched and normal mammary epithelial cells formed cellular sheets with tight intracellular junctions. Addition of Histone deacetylase (HDAC) inhibitor drugs interestingly imparted marked alteration is cytoskeleton of the cancer cells when interacting with 3D architecture and compared to their normal epithelial counterparts (Strobl, Nikkhah, and Agah 2010).

Other studies have utilized 3D micropatterned hydrogels or microfluidic technologies to assess cancer cell behavior within each specific stage of metastatic cascade (Peela et al. 2016). In fact, hydrogels have been proven to be excellent biomaterials for tissue engineering and disease modeling applications (Navaei et al. 2017, Navaei, Saini, et al. 2016, Saini et al. 2015, Zorlutuna et al. 2012, Navaei, Truong, et al. 2016, Cha et al. 2014).

The George group developed a 3D *in vitro* model called Pre-Vascularized Tumor (PVT) to study early events of tumor progression (Ehsan et al. 2014). In this model, spheroids of tumor cells and endothelial cells were embedded into fibrin matrix consisting of fibroblasts. This model was proven to be efficient in studying two mechanisms, vessel formation (i.e. angiogenesis) and intravasation. The study was carried out under hypoxic conditions. In the presence of tumor cells, the vessel sprouts were more irregular and shorter as compared to spheroids containing only endothelial cells and fibroblasts. Moreover, breast cancer cells intravasated into the lumens of the vessel. Notably, this effect was enhanced in hypoxia conditions. Further analysis confirmed that Slug, a marker for EMT, was highly upregulated within the hypoxic environment and played a critical role in intravasation **(Figure 1.3 a)**.

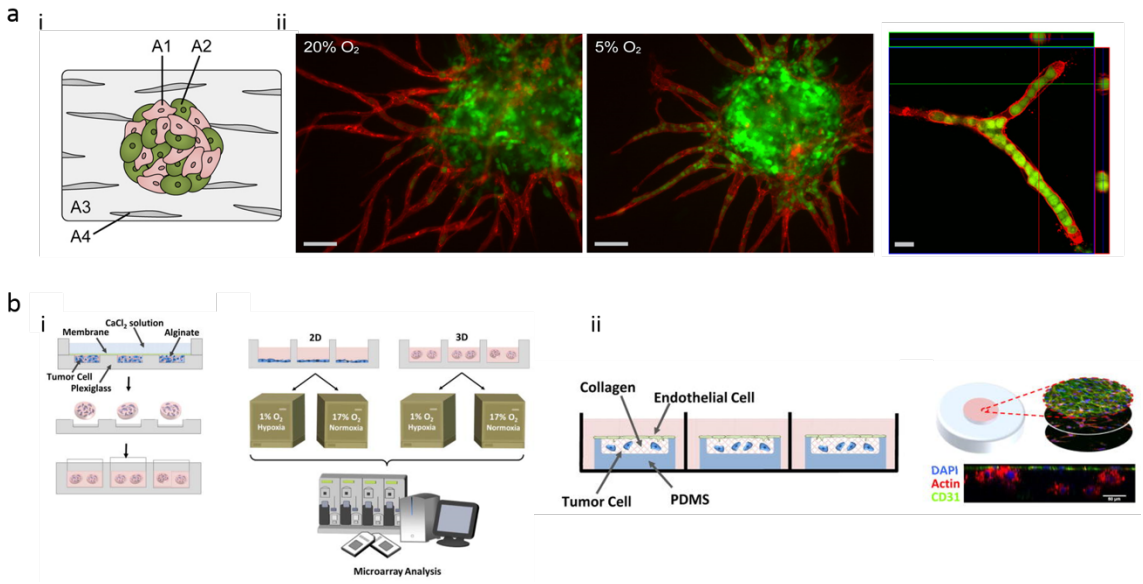

Figure 1.3: *in vitro* models to study intravasation **(a) (i)** Schematic of model showing tumor and endothelial cell embedded in fibrin matrix **(ii)** immunofluorescence images showing intravasation of tumor cells (green) into the blood vessel (red). Scale bars: 100 µm. Adapted from Ehsan et al. with permission from Royal Society of Chemistry [Integrative Biology], copyright (2014) (Ehsan et al. 2014). **(b) (i)** Schematic illustrating the fabrication of microscale alginate scaffolds. **(ii)** Representative images of a co-culture invasion assay. Adapted from DelNero et al. with permission from Elsevier [Biomaterials], copyright (2015) (DelNero et al. 2015).

In the following, we will provide a brief overview of the current existing technologies. The fischbach group developed a model to analyze the response of tumors to culture dimensionality and hypoxia (DelNero et al. 2015). They designed alginate-based model and cells were introduced as a layer to form 2D structure and 3D spheroids structures. Oxygen in culture was regulated to introduce hypoxic conditions. The extent of invasion was analyzed by counting the number of cells crossing the membrane. OSCC3 cells were cultured in 2D and 3D and under hypoxic and normoxic conditions to determine the interdependence of hypoxia and dimensionality on expression of genes. Gene transcript analysis results showed that dimensionality of culture affected a large number of genes in hypoxia than normoxia, suggesting that hypoxia response depends on whether culture conditions are performed on 2D or 3D models. Specifically, hypoxia might have triggered the cells to respond to dimensionality, or conversely, changes in dimensionality might have triggered response of cells to hypoxia. Altogether, the results suggest that there is interdependence between hypoxia and dimensionality of culture at gene expression level. As IL-8 is known to effect

inflammation and angiogenesis further investigation on the gene was performed. And results demonstrated that there was significantly higher levels of IL-8 gene expression in 3D compared to 2D culture conditions which was not dependent of oxygen concentration. These results show that there is an interdependence on dimensionality and hypoxia **(Figure 1.3 b)**.

During Intravasation, cancer cells migrate in response to gradients of chemokines (Roussos, Condeelis, and Patsialou 2011). Limitations of the above models is that cytokine gradients are not established. Thus, advances in microfluidic systems have enabled us to develop novel models which can capture different mechanistic features like cytokine gradients, fluid flow different components of the tumor as well as interactions with different cell types. For instance, Han et al. developed a 3D microfluidic model to study the effect of oriented collagen fibers on tumor cell migration across the basement membrane surrounding the vessels during intravasation (Han et al. 2016). The device was loaded with Matrigel® followed by collagen I leading to sandwich of two hydrogels. During polymerization Matrigel® volume was swollen while collagen volume shrunk developing strain into the system which led to collagen fibers to orient vertically at the interface of collagen-Matrigel®. MDA-MB-231 cells embedded within the sandwiched gel began intravasating to the gel interface (collagen- Matrigel® interface) as early as 48 h. By 144 h most of the cells invaded and crossed the Matrigel® region. On the other hand, when collagen fibers were not oriented homogenously, MDA-MB-231 cells could not invade into the Matrigel®. These results demonstrated that the fiber alignment enhanced cell–ECM interactions, where metastatic MDA-MB-231 breast cancer cells followed the direction of fiber alignment during the intravasation **(Figure 1.4 a)**.

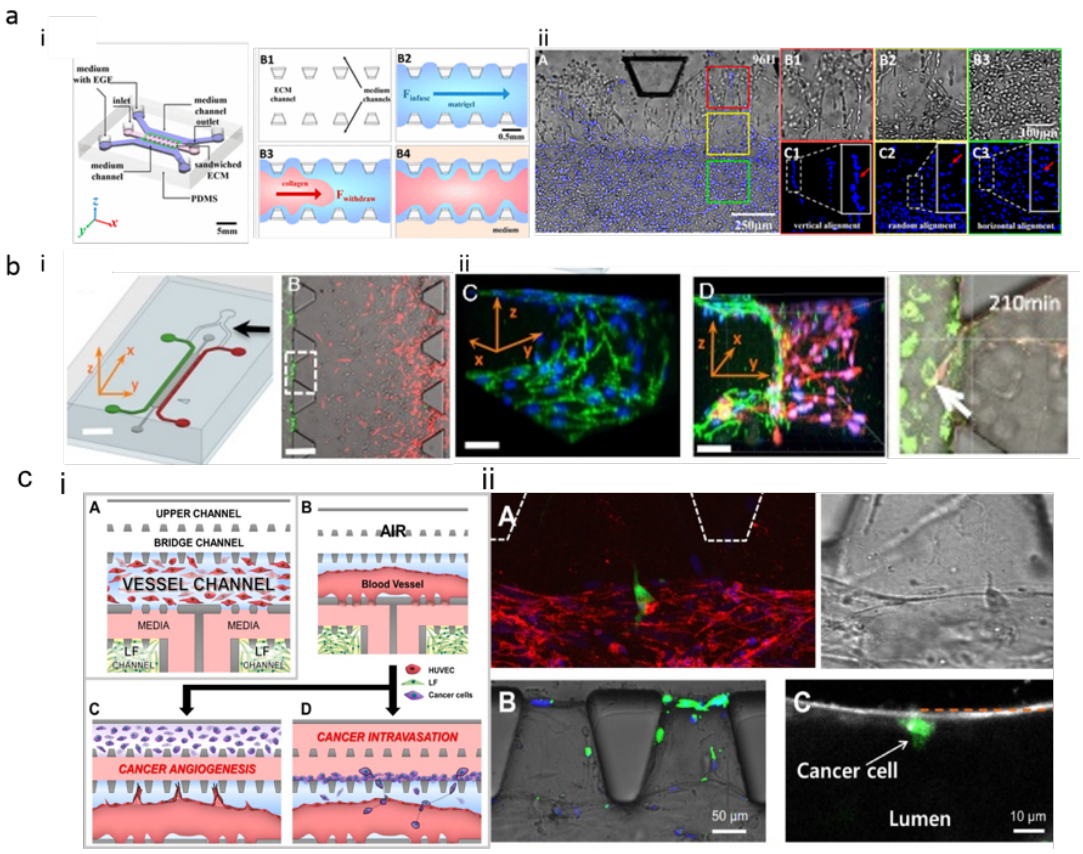

Figure 1.4: Microfluidics based models to study intravasation **(a) (i)** Schematic representation design and fabrication of microfluidic model **(ii)** Representative images depicting tumor cell intravasation into ECM due to collagen fiber orientation. Adapted from Han et al. with permission from National Academy of Sciences [PNAS], copyright (2016) (Han et al. 2016). **(b) (i)** Schematic of the microfluidic device **(ii)** Representative images showing tumor cell (red) intravasation into endothelial lining (green). Scale bars: 30 μm. Adapted from Zervantonakis et al. with permission from National Academy of Sciences [PNAS], copyright (2012) (Zervantonakis et al. 2012). **(c) (i)** Schematic of the microfluidic device **(ii)** Representative images showing tumor cell (green) intravasation into endothelial lining (red). Adapted from Lee et al. with permission from AIP Biomicrofluidics copyright (2014) (lee et al. 2014).

Similarly, the Kamm group developed a 3D microfluidic model to study tumor cell intravasation and elucidate the role of TNF-α and macrophages in regulating endothelial barrier permeability and tumor cell intravasation (Zervantonakis et al. 2012). The device had one central stromal region filled with collagen surrounded by endothelial region to left and tumor region on right. Macrophages were embedded within collagen hydrogel to study their effect on vessel permeability and tumor intravasation. This model was useful in studying the process of intravasation as it allowed real time visualization of cancer cell behavior (i.e. migration, intravasation). In the presence of macrophages,

microvessels had higher permeability. The results also showed that the presence of TNF-α resulted in increased endothelial barrier impairment, which facilitated cancer cell intravasation. To further confirm their findings, they knocked down TNF-α in the presence of macrophages and observed that intravasation levels did not decrease, suggesting that macrophages may secrete other factors, which might indeed enhanced permeability of the vessels and resulted in increased intravasation **(Figure 1.4 b).**

In another study, Noo Li Jeon group developed a microfluidic based metastasis chip to study cancer angiogenesis and intravasation (Lee et al. 2014a). Endothelial cells HUVECs (Human Umbilical Vein Endothelial Cells) and stromal cells normal human lung fibroblasts were introduced into the channels at close proximity. The vascular network formed had well formed boundaries with proper cell junctions. After 7 days in culture, cancer cells U87MG were introduced into the upper channel of the device **(Figure 1.4 c i)**. The microvessel were regarded as pre-existing at the cancer site and the cancer cells in the perivascular region which would secrete angiogenic factors to produce angiogenic sprouts. The number and coverage area of angiogenic sprouts were quantified and the results demonstrated that microvessels with cancer cells showed more angiogenic sprouts compared to microvessels without cancer cells. Further, to model intravasation, MDA-MB-231 cancer cells were introduced into the upper chamber of the device and trans-endothelial migration of cancer cells into preexisting vessels was observed. After three days in culture, several cancer intravsating into the lumens could be observed **(Figure 1.4 c ii)**.

1.5 OBJECTIVE OF THE THESIS

This thesis is aimed to investigate the role of tumor microenvironment and different biochemical factors involved during cancer cell invasion and intravasation. In particular, we developed and utilized a novel microfluidic assay to test the hypothesis that there are bidirectional interactions between cancer cells and endothelial cells during the process of invasion and intravasation. Addressing this question is not only important for understanding the molecular mechanisms, but also will validate the device as a potential tool for identifying potential targets for cancer therapy.

The first step was to create a first generation platform to optimize the process of vasculogenesis in our microfluidic model. This was achieved by incorporating HUVECs in fibrinogen hydrogel and

analyzing the vascular network formation, growth and maturation. Upon validation of the platform, VEGF level in the microenvironment was increased and endothelial network growth was characterized. Further analysis was performed to investigate the effect of increased levels of VEGF on endothelial cell permeability.

We further developed a second generation three-layer microfluidic based tumor-vasculature model to characterize tumor-endothelial interactions. To explore the relationship between endothelial barrier and cancer cell intravasation, we simultaneously investigated tumor cell invasion and endothelial vascular formation and permeability as well as intravasation. Interestingly, our assay was designed in a way to enable direct observation of tumor cells during invasion through the stroma and intravasation through the endothelial barrier while accurately controlling the organization and transport of biomolecular components. We analyzed the number and morphology of cancer cells invading through stroma. We finally assessed the influence of cancer cells on endothelial cell capillary formation and permeability and compared to endothelial mono-culture condition.

CHAPTER 2

THREE-DIMENSIONAL MICROFLUIDIC PLATFORM TO STUDY THE EFFECT OF INCREASED LEVELS OF VEGF ON VASCULOGENESIS

2.1 INTRODUCTION

Blood vessels are the most important part of the circulatory system in the human body. They enable organ functionality by initiating blood flow, transport of nutrients, oxygen supply and immune cell trafficking. Blood vessels have a layer of endothelial cells lined at their inner surface that play a major role in the functioning of the vessel, and they respond and adopt to biochemical and biophysical cues from the surrounding microenvironment (Dudley 2012). Endothelial cell phenotype can be influenced by different pathologies and disease conditions. In the case of cancer, there have been numerous *in vivo* and *in vitro* studies aimed at understanding the altered phenotype and gene expression of endothelial cells during cancer progression. For instance, Dovark group performed a mechanistic *in vivo* study to better understand the effect of cancer on vasculature (Nagy et al. 2008). Their results demonstrated the effect of VEGF on newly formed blood vessels in mouse models by studying the difference in structure and function of networks in the presence of tumor. From these studies, they found that tumor vessels differ in diameter, area and hierarchical arrangement compared to healthy vessels. Although *in vivo* studies have helped us understand the basic physiology of tumor vasculature, the set-up is complex and consequently leads to difficulties in understanding the underlying mechanisms causing these critical differences in tumor vasculature. Additionally, understanding the underlying mechanisms and elucidating different signaling pathways is critical for developing effective anti-angiogenic cancer therapies (Nishida et al. 2006). Hence, a reliable *in vitro* model to understand the process of tumor angiogenesis is still in demand. In this regard, numerous *in vitro* models, with well defined architecture and environmental factors, have been developed to delineate the precise mechanisms of tumor vasculogenesis. For instance, The Chen group developed a model to pattern functionally organized vascular networks within a microengineered platform (Chaturvedi et al. 2015). Polydimethylsiloxane (PDMS) molds were used to embed the cells to better organize the

endothelial cells into specific cords, and these formed cords were transplanted into mice to demonstrate the formation of functional networks. Their results showed that the diameter of the cords before implantation impacted the location and density of the resultant capillary networks. They also established that relevant endothelial sources such as human microvascular endothelial cells (HMVEC) and Induced pluripotent stem cell (IPSC) -derived ECs and can drive vascularization within the same system.

Microfluidic models have proven to be excellent platforms used to study tumor vasculogenesis. Significant progress has been made in the technology in past few years. Microfluidic based models have many advantages including (1) easy and excellent imaging with precise visualization of single cells, (2) easy fabrication of devices, (3) incorporation of multiple cell types with distinct spatial arrangements to study paracellular signaling and interactions (van Duinen et al. 2015). For instance, in a study by Kim et al., a microfluidic model was developed to study lymphangiogenesis (Kim et al. 2015). This study also incorporated the effect of interstitial flow (IF) on lymphangiogenesis and the results showed that the IF serves as the central regulatory cue which defines the process of sprouting. The lymphatic sprouts developed exhibited structural changes, molecular signatures, and cellular phenotypes that were similar to neovessels *in vivo*. This study specifically focused on critical role of mechanical cues that regulate lymphangiogenic sprouting.

In this study, we have used microfluidic technologies to fabricate a novel platform to recapitulate vasculogenesis. The model proposed herein was comprised of a single culture region surrounded by the media region. As the media channel is in a different compartment from the cell culture region, it facilitated the development of a gradient of biomolecules, thereby better mimicking physiological conditions. Furthermore, the tumor microenvironment was mimicked (i.e. induced environment) by spiking cell media with VEGF and assessing its effect on vasculogenesis. VEGF is known as a major growth factor secreted during cancer progression which leads to formation of new blood vessels (Nishida et al. 2006). We primarily compared the diameter and branching of vascular networks and we also assessed the permeability of microvessels. The results obtained showed that endothelial networks formed under the influence of VEGF were impaired structurally and had higher permeability.

2.2 MATERIALS AND METHODS

2.2.1 Device design and fabrication:

a) Photolithography to fabricate silicon mold:

To fabricate microfluidic devices, first a mold was created. The required design was created using CAD software. The design was then fabricated onto a transparent mask. Next, SU8-2075 (MicroChem) was spun of height 200 μm onto a silicon wafer and afterwards, the wafer with the transparent mask beneath was exposed to UV to form the mold for further fabrication of microfluidic device **(Figure 2.1 a-c)**.

b) Soft lithography for device fabrication:

The silicon wafer is hydrophobic by nature. To make the surface hydrophilic, the surface of the silicon wafer was treated with Methyltrichlorosilane (MTCS, Sigma-Aldrich). Then the Polydimethylsiloxane (PDMS, Sylgard 184 Silicon Elastomer Kit, Dow Corning) was poured onto the wafer and baked for 1.5-2 hours at 80 °C. Afterward, the casted PDMS was peeled off the wafer to retrieve PDMS molds. **(Figure 2.1 d).** The mold was then cut using blades to separate individual devices then inlet and outlet holes were made using biopsy punch.

c) Bonding:

Next step was to bond the devices onto the glass slide to form channels. To do so, first the devices and glass slides were cleaned with ethanol and nitrogen gas stream. Then treated with oxygen plasma (PDC-32G, Harrick Plasma) to make the surface hydrophilic and then the devices were bonded onto glass slide with the channel side facing down. Afterward the bonded devices were placed in oven at 80 degree Celsius overnight to secure the bond. **(Figure 2.1 f)**

d) Sterilization:

To sterilize the devices, they were first put in the liquid autoclave followed by dry autoclave and again placed in the oven at 80 degrees Celsius to completely dry the devices.

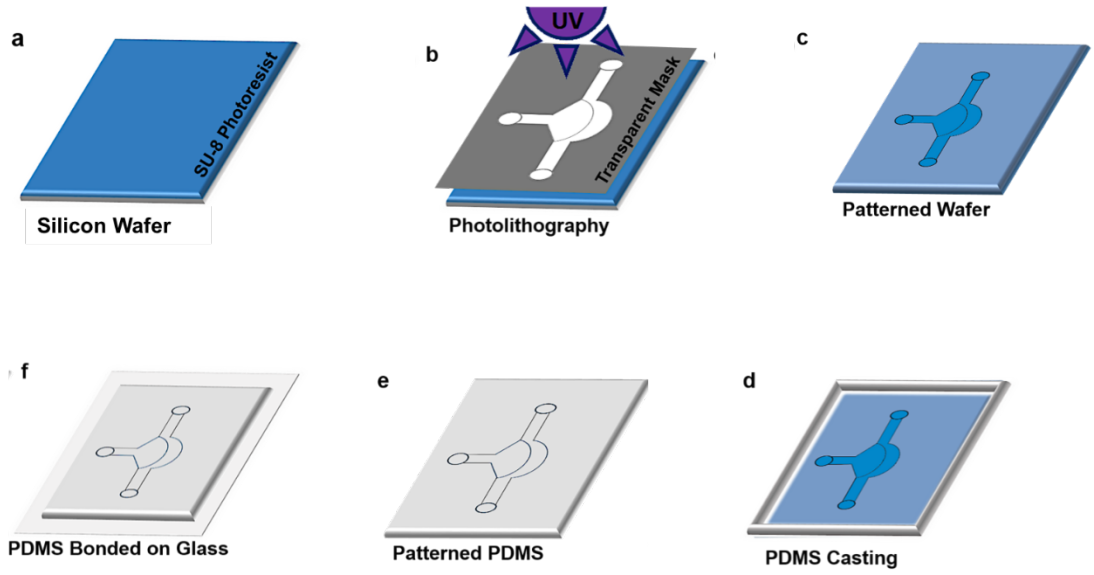

Figure 2.1: Soft lithography and microfluidic device fabrication **(a)** SU-8wafer. **(b)** UV light was exposed on the SU-8 with the mask to create desired pattern. **(c)** SU-8 with pattern of the microfluidic platform. **(d)** PDMS was poured on the wafer. **(e)** Cured PDMS with patterns. **(f)** PDMS was bonded on glass slide to create the microfluidic platform

e) Surface treatment:

The channels in the devices must be hydrophobic, to be suitable for cell culture. Hence the devices should be surface treated. To surface treat, poly-d-lysine (1 mg/mL) (PDL, Sigma-Aldrich) was injected into the cell culture region and the devices were incubated at 37 °C for 1 hour. Then they were washed with DI (De-Ionized) water once. Glutaraldehyde (0.1% (v/v)) (GA, Sigma-Aldrich) was then added and incubated for 1.5 – 2 hours at room temperature followed by washing 4-5 times with DI water. Afterward, the devices were placed in the oven at 80 °C overnight to restore hydrophobicity.

2.2.2 Cell culture:

Human umbilical vein endothelial cells (HUVEC, Lonza) were used in this study. Endothelial cells were cultured in standard Endothelial Growth Medium (EGM-2, Lonza). Cells were kept at standard physiological conditions 37 °C, 5% CO_2 humidified incubator. Media was changed every 3 days. Cells were cultured up to 70% confluency prior to passaging or to be used for experiments.

2.2.3 Vascularization assay:

Fibrinogen solution was prepared by dissolving 5mg/ml bovine fibrinogen (Sigma-Aldrich) in Dulbecco's phosphate buffered saline (DPBS, Gibco) and thrombin solution was prepared by dissolving bovine thrombin (Sigma-Aldrich) in DPBS to get 4U/ml thrombin solution. Both fibrinogen and thrombin solutions were filter sterilized (Denville scientific).

HUVECs were dissociated from culture flasks using trypsin-EDTA (Invitrogen) and were centrifuged, then suspended in fibrinogen solution (5mg/ml) to get a final concentration of 20 million cells/ml. Thrombin (4U/ml) was then added to the cell solution. Fibrinogen and thrombin solutions were added at a ratio of 1:1 to get a final concentration of 2.5 mg/ml fibrinogen and 2 U/ml thrombin. The mixture was also supplemented with aprotinin (0.15U/ml, Sigma-Aldrich). The cell solution was mixed and immediately injected into cell culture region of the devices. The devices were incubated at room temperature for 10 minutes to polymerize. After fibrin polymerization, EGM-2 media was introduced into the media channel. The devices were kept in the 37 °C, 5% CO_2 humidified incubator for 6 days in culture and supplemented with fresh media every day. To establish induced tumor microenvironment, EGM2 media supplemented with extra 50 ng/ml VEGF (Peprotech) and supplemented into the media channel every day. For control conditions, EBM-2 media without growth factors were added.

2.2.4 Immunofluorescence staining:

The cells encapsulated in the fibrin were fixed in 4% paraformaldehyde (PFA). Media from the large wells within the platform were removed and 10 µl of 4% PFA were added to the wells and a gentle negative pressure was applied to the small well to even the flow. Devices were kept in the incubator 37°C, 5% CO_2, humidified incubator for 30 minutes. After incubation, to permeabilize the cells, the samples were rinsed with PBS-glycine (100 mM glycine in PBS) 2 times with 10 minutes incubation at room temperature and washed with PBS-Tween (0.05% (v/v) Tween-20 in PBS) for 10 minutes at room temperature. Following permeabilization, to block non-specific binding of antibodies, the cells were blocked with immunofluorescence buffer (IF buffer: (0.2% (v/v) Triton X-100 + 0.1% (v/v) BSA (radioimmunoassay grade) + 0.05% Tween 20, 7.7 mM NaN3 in PBS). IF buffer plus goat serum (10% (v/v) goat serum) was added to the media channels and the devices

were incubated at room temperature for 2 hours. Afterwards, primary antibody CD-31 (10 µg/ml, DHSB) was diluted in IF buffer and centrifuged at 14K RPM for 10 minutes to ensure optimum mixing. The mixture was then added to the blocked samples and kept at 4^{o} C overnight. Devices were kept in Petri dishes and parafilmed to avoid evaporation. The next day, devices were washed with IF buffer 3 times each with 20 minutes interval at room temperature. Then the secondary antibody (Alexa Fluor® 488, Thermo Fisher Scientific) diluted in IF buffer and centrifuged at 14K RPM for 10 minutes. The mixture was added and the devices were incubated at room temperature for 3 hours. The devices were then washed once with IF buffer for 20 minutes followed by 2 times washing with PBS-Tween-20 for 10 minutes. Further, to counterstain for actin cytoskeleton and nucleus, Alexa Fluor® 488 Phalloidin (Invitrogen) (1:40) and 4′, 6-diamidino-2-phenylindole (DAPI, Invitrogen) (1:1000) were added to the devices respectively. The devices were the kept at 4^{o} C overnight. Following staining, the devices were finally washed with PBS-Tween-20 3 times each with 10 minutes intervals. Stained samples were imaged using fluorescence microscopy (Zeiss Axio Observer Z1 with the Zen Pro software suite) equipped with Apotome.2 (Zeiss) at 20X and 40X magnification. Z-stack images at 10X, 20X and 40X magnification were obtained and using the Zen software 3D images were constructed.

2.2.5 Permeability Assay:

On day 6, upon formation and maturation of endothelial networks, fluorescent dye FITC-dextran (70KDa, 2.5 µg/ml diluted in EGM2 media, Invitrogen) was used to measure the diffusive permeability of the endothelial networks. Before introducing fluorescent dextran, the integrity of the vascular networks was confirmed using phase contrast microscopy. Media from the channels were removed and 40 µl of dextran solution was added to the media channels. A slight negative pressure was applied on the other side of the channel to even the flow. Devices were placed under the microscope and the flow of dextran solution was visualized. Next, sequential images were captured using the fluorescent microscope every 15 seconds for 30 minutes. Movement of dextran into and out of the vascular networks was subsequently observed. Images were later processed and analyzed using NIH ImageJ Software. Images and t=0 and t=30 minutes were used to measure the fluorescence intensities of the vascular network in the

regions of interest. Later, permeability of the vascular networks was measured using the following equation:

$$P_d = \frac{1}{\Delta I_I}\left(\frac{\Delta I_f}{\Delta t}\right)\left(\frac{d}{4}\right)$$

P_d = Permeability

$\Delta I_i = I_i - I_b$

$\Delta I_f = I_f - I_i$

where I_b, I_i, and I_f are the background, initial, and final average intensities, respectively, Δt is the time interval between images, and d is the diameter of the imaged microvessel.

2.2.6 Imaging, Data collection and statistical analysis:

Vascular network morphology was analyzed over the course of 3 independent experiments (n=3). Each experiment had 2-3 technical replicates. Data for vascular network diameter was collected by taking phase contrast images of the samples at days 1, 3, and 5. Images were processed using NIH ImageJ software and the diameter of the vascular networks was measured by drawing a line through one point to another of the microvessels. Measure plugin of the ImageJ was used to measure the length of the line hence yielding the diameter values. The data was statistically compared with unpaired Student's t-tests using GraphPad Prism software (GraphPad Prism 6 Software).

2.3 RESULTS

2.3.1 Optimizing and characterization of vasculogenesis:

The design and dimensions of the microfluidic platform were optimized for vasculogenesis analysis. In specifics, the chip design had diameter and height of 3 mm and 200 μm respectively with micro-posts spaced evenly at 100 μm at the interface of cell culture and media channel which allowed media flow and the diffusion of growth factors through the 3D cell culture region **(Figure 2.2 a).**

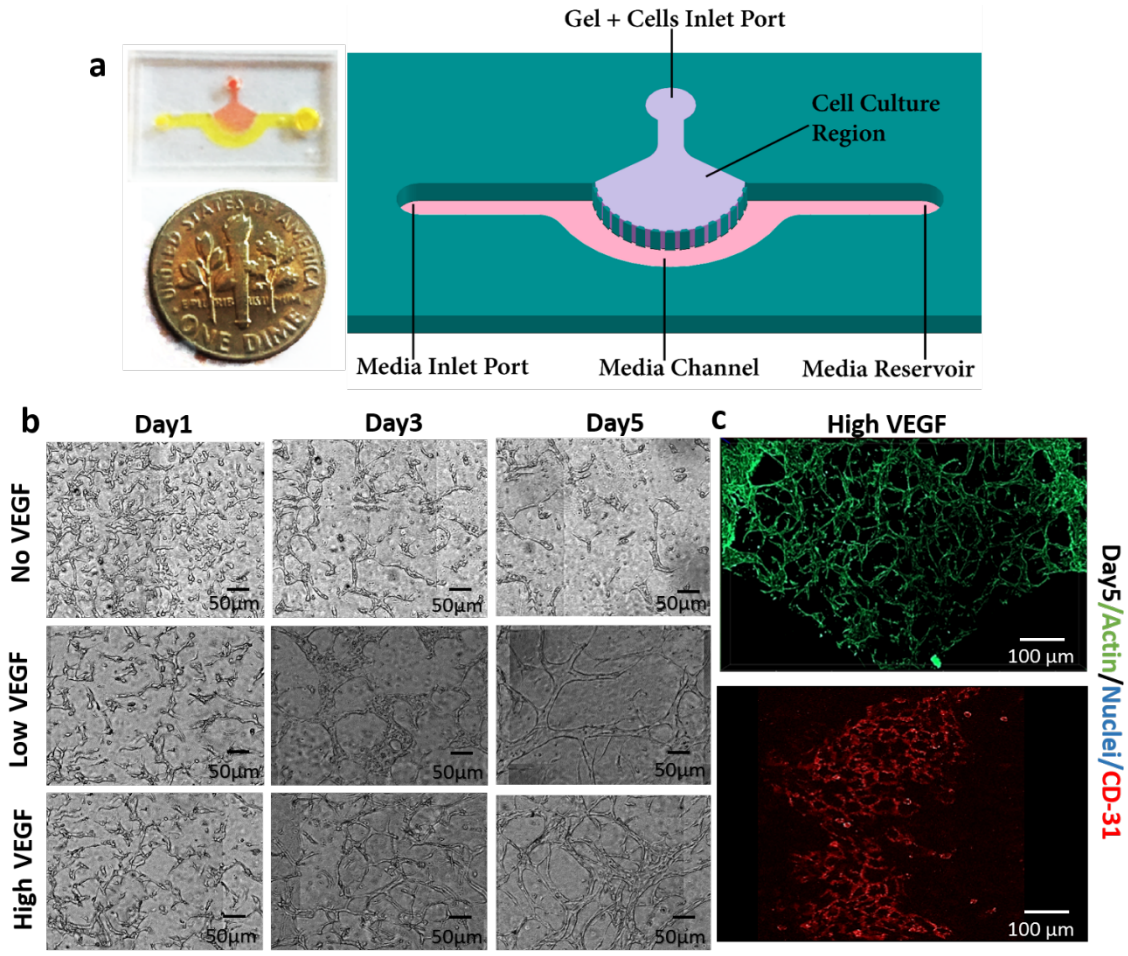

Figure 2.2: (a) Schematic representation of the microfluidic platform. **(b)** Representative phase contrast images demonstrating the growth and maturation of vascular network. **(c)** Representative Fluorescence Images of vascular networks stained with actin and CD31

HUVECs embedded in fibrin matrix were introduced into cell culture region and appropriate media was supplied for different experimental conditions. Growth and maturation of vascular networks were monitored over a culture period of 5 days. In control experimental group (No VEGF) inter cellular connections were mainly absent across the culture period. However, in low and high VEGF conditions, endothelial cells showed elongated morphology with intercellular connections from day 1. Vascular networks were developed with tube-like structures on day 3. The formed networks further matured with interconnected cellular clusters covering the entire culture region by day 5 **(Figure 2.2 b, c).**

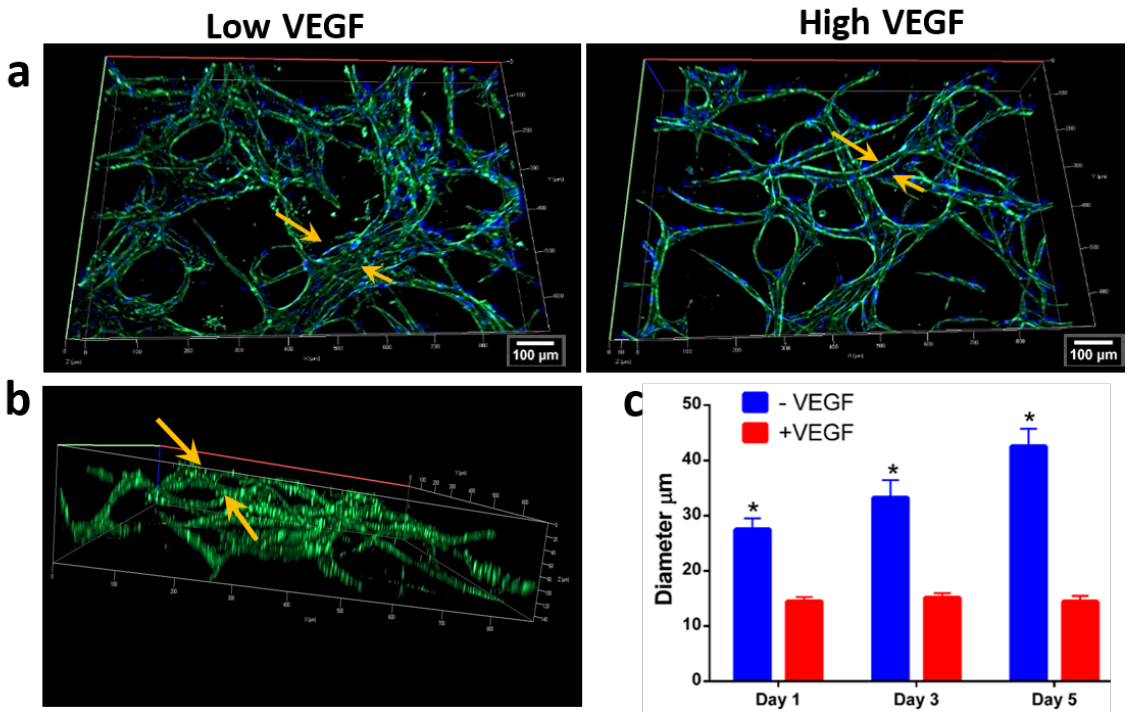

Figure 2.3: (a) Representative fluorescent images of vascular networks showing the mature networks. **(b)** Cross sectional view of the vasculature showing lumens **(c)** Diameter of the vascular network.

It is established that during tumor angiogenesis, cancer cells secrete many growth factors, importantly VEGF, which will cause the formation of underdeveloped vascular networks lacking structural stability (Nishida et al. 2006). To study this critical effect in our platform, we set up a stimulated (i.e. induced) microenvironment by supplementing the media with additional VEGF (50 ng/ml). Vascular network formation was observed in both stimulated and unstimulated conditions. The networks formed in the stimulated condition appeared to be more immature that the one in the unstimulated conditions. To confirm and quantify the extent of vascular formation, we measured the diameter of the vessels and compared stimulated against unstimulated conditions across days 1, 3 and 5, of culture. The diameter of the vessels in the stimulated conditions (19.43 ± 10.66 µm) were significantly lesser than the unstimulated conditions (37.32 ± 18.60 µm) (**Figure 2.3**). Our findings confirm that when the endothelial cells are stimulated with VEGF, it leads to formation of immature networks consistent with previous *in vivo* studies (Nagy, Benjamin, Zeng et al. 2008).

2.3.2 Permeability assay:

Permeability assays are usually used to assess the mechanistic functionality of the blood vessels (Nagy et al. 2008). It is also used for visualization and quantification of barrier function of blood vessels. Several studies both *in vivo* and *in vitro* have shown increased permeability of vascular networks in the presence of tumor cells. In other words, the vascular networks become more permeable due to the presence of VEGF secreted by tumor cells within the TME (Nagy et al. 2008). Thus, we wanted to explore whether the permeability of the vascular networks would increase in the VEGF induced microenvironment within the microfluidic 3D platform. Fluorescent dextran (2.5 µg/ml) was added to the entrance of the vascular regions and the movement of the fluorescent dye was followed to measure the permeability. Fluorescent Images of diffusion of the dextran acquired were used for analysis.

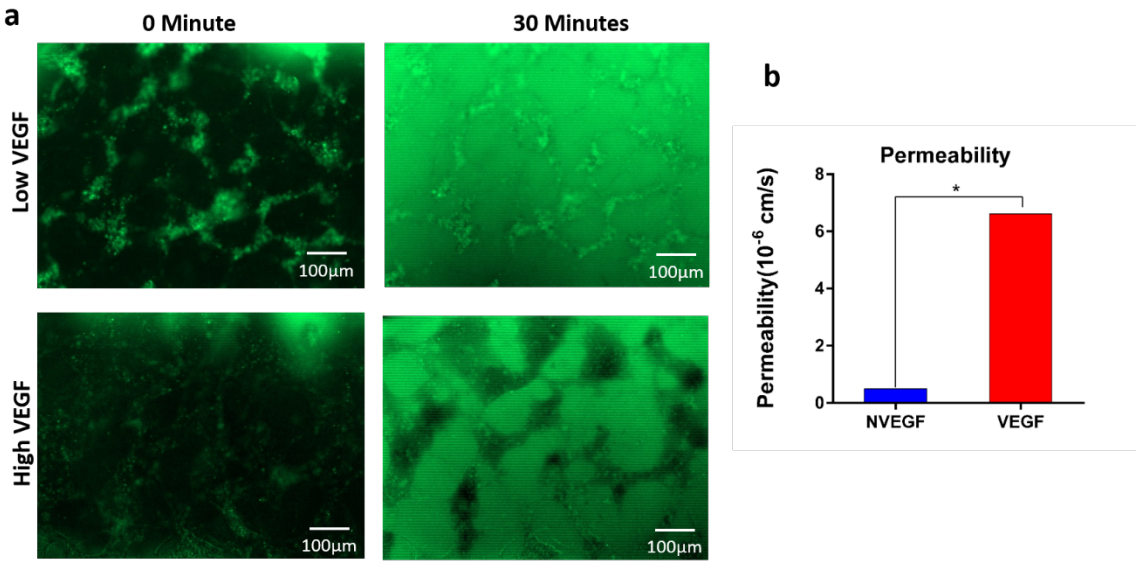

Figure 2.4: Permeability of the vascular networks **(a)** images representing the networks on 0th and 30th minute when flowing FITC- dextran. **(b)** Qualitative values of permeability of the endothelial networks.

In the stimulated conditions (increase levels of VEGF), the vascular networks were more permeable as there was enhanced leakage of dextran into the perivascular region, resulting in reduced fluorescent intensity in the vascular region. In the normal vasculature (lower amount of VEGF), due to lower permeability, fluorescent intensity was higher in the vascular region compared to the outer region **(Figure 2.4 a)**. To analyze the data, images for initial and final intensities were

acquired and analyzed using the NIH ImageJ software. The obtained initial and final optical intensities were substituted in the permeability equation and permeability values were calculated. From the obtained results, we could see that the permeability values for stimulated conditions was (6.60 cm/s) which was significantly higher compared to permeability of normal vasculogenesis setup with less amount of VEGF (0.49 cm/s) **(Figure 2.4 b).**

2.4 DISCUSSION

Tumor vasculogenesis is a process where tumor cells secrete growth factors, importantly VEGF, which results in the formation of new blood vessels (Nishida et al. 2006). The tumor vessels display abnormal structure and function, they vary in shape, size having thin walls and small, compressed lumens. Endothelial cells are poorly connected and hence, the junctions are not well formed. This ill-formed arterio-venous structure results in impaired flow of nutrients and irregular delivery of oxygen. As the tumor grows, there is excessive secretion of pro-angiogenic factors which leads to the formation of disorganized vessels further aggravating tumor due to improper supply of oxygen and nutrients leading to a vicious cycle. Impaired delivery of drug owing to ill-formed vessels limits the success rate of anti-cancer drug treatment (Potente, Gerhardt, and Carmeliet 2011). Despite significant progress, it is yet not possible to fully capture the characteristic response and function of tumor angiogenesis. Recent studies have shown success in forming perfusable blood vessels in *in vitro* assays. Still many studies rely on structural support and additional helper cells to guide the developing networks.

In this study, we present a robust novel microfluidic platform where we could optimize the 3D hydrogel and cell concentration to form functioning vascular networks with no structural support or helper cells. We spatially organized the endothelial cells in a 3D hydrogel in a cell culture compartment of the device and demonstrated the process of growth and maturation of endothelial cells to form functional vascular networks. To further understand the effect of increased levels of VEGF within the 3D microenvironment, we supplemented the media with additional VEGF to study the effect on growing endothelial cells. From the results obtained, we observed the development of vascular networks with thinner lumens and more branching. The permeability assay also revealed that, perhaps due to ill-formed endothelial junctions, the vasculature was more permeable. Our

results obtained was in accordance with the results from the previous studies and *in vivo* data (Nagy et al. 2008).

The proposed first generation platform enabled us to explore further the effect of growth factors secreted by cancer cells on vasculogenesis. Hence, in a second generation platform, we developed a three-layer microfluidic device to explore bidirectional crosstalk between the tumor and endothelial cells in a way to assess the effect of high density growing tumor on developing endothelial cell and in turn investigate the influence of the presence of blood vessels on the behavior of cancer cells in terms of invasion and intravasation. The findings of second generation device are further discussed in chapter 3.

CHAPTER 3

THREE-DIMENSIONAL MICROFLUIDIC BASED TUMOR-VASCULAR MODEL TO STUDY CANCER CELL INVASION AND INTRAVASATION

3.1 INTRODUCTION

Metastasis is one of the most important hallmarks of cancer (Hanahan and Weinberg 2011). It is a complex multi-step cascade, and there are dynamic interactions between the tumor, its surrounding microenvironment, and the vascular network during different stages of disease progression (Nishida et al. 2006). If the tumor must grow beyond 2 mm, it must have its own vascular networks. Hence, during cancer progression there is induction of tumor angiogenesis during which there is an 'angiogenic switch', which contributes to the formation of tumor-supporting vascular networks (Hanahan and Folkman 1996). It is known that these developing angiogenic vessels are abnormal and immature both structurally and functionally. Specifically, the vessels are irregular in shape, are permeable, and have underdeveloped endothelial lining and lumen (Nagy et al. 2008). These vascular networks fulfill their purpose of supplying nutrition and oxygen to the tumor and their dilated lumen and permeability will aid the invading tumor cells to escape to secondary sites (Potente, Gerhardt, and Carmeliet 2011).

Endothelial and tumor cell interactions involve bidirectional signaling. From prior studies, it has been well established that tumor cells have pro-angiogenic roles. It has been shown that MDA-MB231 cells modify the mechanical characteristics of endothelial cells by reducing the stiffness of endothelial cell junctions (Mierke 2011). In another study, it was shown that melanoma cells induce junction disassembly of endothelial cells, suggesting that initial stages of transmigration of endothelial cells is characterized by redistribution of VE-Cadherin, PECAM-1 and other endothelial junctions (Peng and Dong 2009).

More importantly, several studies also suggested that endothelial cells have an active role in regulating tumor cell invasiveness. A study by Kenig et al. suggested that invasion of glioblastoma cells often occurred due to presence of vasculature (Kenig et al. 2010). They demonstrated that co-culturing glioblastoma cells (U87) with endothelia cells (HMEC-1) increased invasiveness of

U87 cells. These studies suggest that endothelial cells attract cancer cells by secreting several factors such as SDF-1 and MMP's, which in turn influence the growth, proliferation and invasiveness of the cancer cells. Despite the significance, the majority of these studies are limited, as they do not elucidate the dynamic interactions between two cell types and the role of tumor microenvironment during intravasation.

With the advent of microfluidics, it is now possible to develop models with spatial organization of different cell types, compartmentalizing different tissues present in the native tumors and enabling precise control over the microenvironment (van Duinen et al. 2015). Microfluidics also allows for high-resolution imaging and real-time visualization of cell activity, movement, invasion, and intravasation. To date, several models have been developed utilizing microfluidic technology to study cancer metastasis. In a pioneering work, the Kamm group developed a microfluidic model containing breast cancer cells and endothelial cells separated into different channels to study cell-cell interactions and the role of macrophages on cancer cell intravasation. However, the limitation of this model was that it did not have a well-formed capillarity network and instead, the endothelial cells were seeded within the inner walls of the side channels (Zervantonakis et al. 2012). To address this limitation, the Jeon group developed a microfluidic model with well-formed vascular networks and demonstrated intravasation of MDA-MB-231 cells into the vascular networks (Lee et al. 2014b). Although this model comprised of well-formed vascular networks, cancer cells were introduced by injecting them to form an adhering layer within the wall of the device. There was no mass tumor and no gradient of biomolecules established within this system.

To addresses the limitations of the previous platforms and gain a mechanistic insight on the biological interactions of tumor and endothelial cells, we aimed to develop a model with a well-formed tumor and vascular network as well as a gradient of biomolecules to closely mimics the *in vivo* tumor microenvironment. Specifically, we have developed a novel three-layer microfluidic platform to perform integrated studies on cancer cell invasion and intravasation on a single platform. The model consists of the tumor, stroma and an endothelial layer spatially organized and composed of a different matrix and cell types. The regions within the platform, comprised of cells embedded in 3D matrix and are initially compartmentalized, however are interconnected to allow

for dynamic interactions over time. Importantly, our model allows introducing high-density cancer cell populations (20 million/ml) embedded in the collagen matrix, which better represents the 3D tumor mass inside the body. The endothelial region has high-density endothelial cells (20 million/ml) forming complex vascular networks, which are perfusable *in vivo*-like networks. Another critical feature of our model is the geometry and design of the device. Having the media channel surrounding endothelial region and having the tumor at the middle was helpful in mimicking *in vivo* environment where the blood vessels supply nutrients to the cancer cells and cancer cells migrate towards vascular networks. In addition, the presence of middle stromal layer allowed for visualizing and analyzing the cancer cells invading along with vascular development to assess cancer cells intravasation at a single cell level. We demonstrated that the number of cancer cells invading stroma was increased in the presence of endothelial network and invading cancer cells exhibited more elongated morphology suggesting that they acquire invading characteristics in the presence of endothelial network. The morphology of vascular network was also altered in the presence of tumor leading to formation of thinner and more permeable vessels. Overall, this study recapitulates interactions and signaling between cancer and endothelial cells during the process of invasion and intravasation.

3.2 MATERIALS AND METHODS

3.2.1 Device design and fabrication:

The process for device fabrication is the same as reported in section 2.2.1. The design for silicon wafer mold was as per the design for three-layer device required for intravasation assay.

3.2.2 Cell Culture:

Highly metastatic MDA-MB-231 breast cancer cells and Human umbilical vein endothelial cells (HUVEC, Lonza) were primarily used for this study. Culture of HUVECs is as explained in section 2.2.2. MDA-MB-231 cells were cultured in advanced DMEM (1X Dulbecco's Modified Eagle's Medium (DMEM) + 10% fetal bovine serum (FBS) + 1% L-glutamine + 1% 50:50 penicillin: streptomycin). Media and media supplements were purchased from Life Technologies. Cells were

kept at a standard physiological condition (humidified, 37 °C, 5% CO_2). Media was changed 3 days once. Cells were cultured up to 70% confluency prior to passage or use for the experiments.

3.2.3 Invasion and Intravasation assay:

A three-layer microfluidic device as depicted in **Figure 3.1 a** was used for this study. MDA-MB-231 transfected with tomato red were acquired for this study, kindly provided as a gift by Dr. Robert Ros group at ASU. Firstly, MDA-MB-231 cells were dislodged from the culture flasks using trypsin-EDTA and were centrifuged. MDA-MB-231 cells were then suspended in collagen I (1.5 mg/mL, Corning® Collagen I, Rat Tail,) for a final concentration of 20 million cells/ml. The cell-hydrogel solution was mixed and then injected into the tumor region of the device. The devices were then placed in the incubator (humidified, 37°C, 5% CO_2) for 20 minutes. Subsequently, collagen (1.5mg/ml) was injected into the stroma region followed by 20 minutes of incubation allowing hydrogel polymerization. Subsequently, HUVECs were disassociated from culture flasks using trypsin-EDTA and centrifuged. Fibrinogen solution (5mg/ml fibrinogen + 4U/ml Thrombin), as described in section 2.2.3 was then added to get a hydrogel solution with the final cell density of 20 million cells/ml. The mixture was then immediately injected into the vascular region of the device and the devices were kept at room temperature for 10 minutes for the fibrin to polymerize. After all hydrogel polymerization, 70:30 ratio of EGM2 bullet kit (endothelial cell media) and cancer cell media (DMEM+FBS+L-glutamine+ Penicillin+ streptomycin) was added to the media channels of each device and the platforms were kept in the incubator (humidified, 37°C, 5% CO_2) for subsequent biological studies. Media was exchanged every 24 hours throughout the cell culture period (6 days).

3.2.4 Immunofluorescence staining:

Immunofluorescent staining of CD-31, actin cytoskeleton and nucleus as based on the procedures described in section 2.2.4.

3.2.5 Permeability Assay:

Permeability assay was performed on day 6 of culture consistent with the experimental procedure mentioned in section 2.2.5 using the following equation:

$$P_d = \frac{1}{\Delta I_I}\left(\frac{\Delta I_f}{\Delta t}\right)\left(\frac{d}{4}\right)$$

P_d = Permeability

$\Delta I_i = I_i - I_b$

$\Delta I_f = I_f - I_i$

where I_b, I_i, and I_f are the background, initial, and final average intensities, respectively, Δt is the time interval between images, and d is the diameter of the imaged microvessel.

3.2.6 Imaging, data collection and statistical analysis:

Phase contrast and fluorescence images were acquired using Zeiss Axio Observer Z1 equipped with Apotome2 (Zeiss) and Zen Pro software. Throughout the cell culture period, phase contrast images were taken every day at 10X objective using 4×3 tiles. Immunofluorescent images were obtained with 10X, 20X and 40x objectives and z-stacked images were acquired to get crisp 3D images.

To quantify the number of invading cancer cells, both phase contrast and fluorescent images were utilized. Specifically, on day 2 of culture phase contrast images with 10X objective were used, while for days 4 and 6 of culture, fluorescent images of samples stained for actin and nucleus were used. Images were processed using extended particle analyzer plugin of the NIH ImageJ software and images were thresholded and the number of cells was counted. To quantify cancer cell morphology, fluorescent images of the samples on day 4 and 6 were used. Images were processed using Image J software and extended particle analyzer software plugin was used to quantify the cell morphology, specifically cell circularity, aspect ratio and roundness. To visualize the intravasation of cancer cells in the outer blood vessel capillaries, fluorescent images of tomato-expressing MDA-MB-231 cells with 20X and 40X magnification were captured.

To analyze vascular network morphology, mainly branching and diameter of the vessels, fluorescent images of samples stained for endothelial junction markers CD31 captured on days 2, 4 and 6 were used. Images were processed using the NIH ImageJ and the diameters of the vascular networks were measured drawing a line through one point to another of the vascular network.

Measure plugin of the ImageJ was used to measure the length of the line to yield the diameter values

For the entire analysis, all the values were obtained over a course of 3 independent experiments (n=3). Each experiment had 2-3 technical replicates. The data were analyzed with the unpaired Student's t-tests. Multiple comparisons tests were performed within the GraphPad Prism software (GraphPad Prism 6).

3.3 RESULTS

3.3.1 Experimental setup and specification of the fabricated platform:

The three-layer microfluidic enabled us to spatially organize the three important components of intravasation the tumor, stroma and vasculature side-by-side in different channels. The platform consisted of the inner chamber (tumor region) surrounded by chamber two (stromal region), which is surrounded by a third chamber (vascular region). The diameter of the concentric circles were 1, 2 and 3 mm and the height of these concentric regions were 200 µm, respectively. The distance between the edge of the inner region and outer region was 1 mm. All three regions were bound by trapezoidal micro-posts spaced evenly at 100 µm. The micro posts were configured to separate the regions, while also still maintaining the interactions between the regions by allowing exchange of media and movement of bio molecules throughout the platform (**Figure 3.1 a**).

Tumor cells encapsulated in a hydrogel were loaded in to tumor region of the device. Then, hydrogel without any cells was injected into the stromal region. Next, for the vascular region, endothelial cells embedded in a hydrogel was loaded. This formed a well-defined, organized but interconnected intravasation platform. The platform had a media channel surrounding the three entities filled with the media. This allowed for diffusion of media and the creation of a gradient of biomolecules throughout the platform.

The spatial organization of the tumor-stroma surrounded by vasculature was adapted as it represents the *in vivo* organization of these components in the native microenvironment. Having the tumor region away from the media region rendered the cancer cells depleted of nutrients, which allowed for activation of cancer cells thereby making the setup more physiologically relevant.

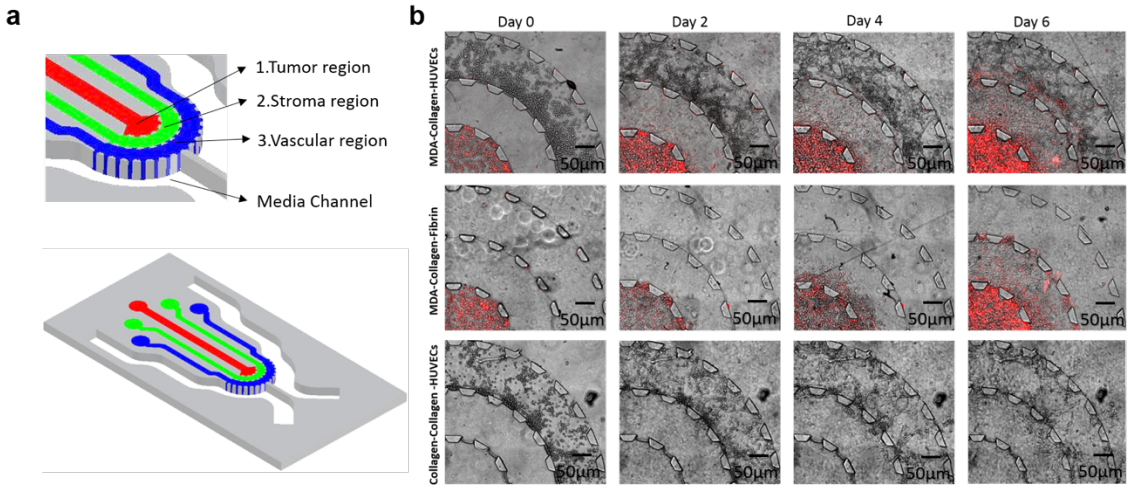

Figure 3.1: Experimental setup and modeling of intravasation. **(a)** Schematic representation of the three-layer microfluidic platform. **(b)** Experimental set up for three conditions: "+/+ tumor/vasculature", "+/- tumor/vasculature" and "–/+ tumor/vasculature" to model intravasation.

Table 1: Experimental setup for modeling intravasation

	Layer 1	Layer 2	Layer 3
1	MDA-MB-231 in Collagen	Collagen	HUVECs in Fibrinogen
2	MDA-MB-231 in Collagen	Collagen	Fibrinogen
3	Collagen	Collagen	HUVECs in Fibrinogen

3.3.2 Analysis of number of cancer cells invading the stroma:

To characterize dynamics of tumor-endothelial cell interactions, first tracking of tumor cells was performed. 3D z-stack images were acquired every 24 hours to monitor cancer cell invasion dynamics. By day 1, the cancer cells started to disseminate from the primary tumor and invaded into the stromal region. The number of tumor cells in the stroma increased exponentially over time, and by day 6, there was a significant number of cells invading the stroma while several tumor cells migrated beyond the endothelial barrier (**Figure 3.2 a, b**).

We could observe that in the presence of vascular networks, the number of cells invading the stroma was comparatively higher than the number of cells invading the stroma in control conditions, with mono-culture of tumor cells without the presence of endothelial cells. By day 2, the number of

cells in the stromal region was relatively low, therefore could be counted through the phase contrast images acquired. By day 4, the number of invading cells increased drastically. Hence, we stained the cells on day 4 and 6 with DAPI for nuclei, and certain areas of the stromal region in the device were marked as the regions of interest to count the number of cells. Fluorescent images were processed using NIH ImageJ software. Specifically, images were thresholded and the extended particle analyzer plugin was used to count the number of nuclei. We found that the number of cells invading stromal region in the presence of vasculature (570 ± 148) was significantly higher than in the control conditions (259 ± 137) (**Figure 3.2 c**). These results suggest that the endothelial cells attract tumor cells, acting as an enhancer or promoter of cancer cell invasiveness.

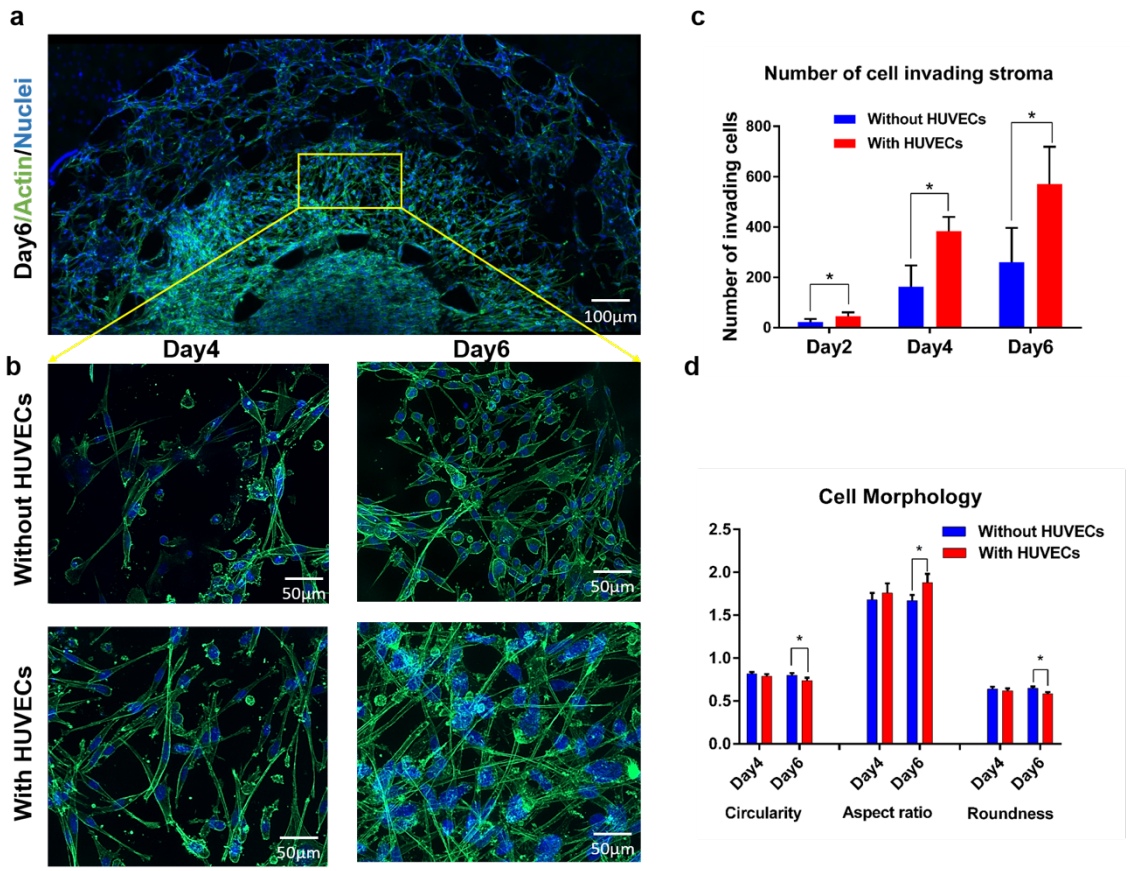

Figure 3.2: Characterization of the cancer cells invading the stroma. **(a)** Representative fluorescent image of the complete platform on day 6. **(b)** Representation of cancer cells invading the stroma on days 4 and 6 of culture. **(c)** Qualitative analysis of the number of cancer cells invading the stroma. **(d)** Qualitative analysis of the morphology of cancer cells invading the stroma.

3.3.3 Analyzing the morphology of cancer cells invading the stroma:

To further characterize tumor-endothelial cell interactions, the morphology of cancer cells invading into the stroma was analyzed. From the preliminary phase-contrast images, we could observe that the cancer cells in the presence of vascular network exhibited elongated morphologies with more protrusions (**Figure 3.2 a, b**). Observation of the immunofluorescence z-stack images of the actin cytoskeleton also confirmed the elongated morphology of the tumor cells.

To further validate our findings, we quantified cell morphology descriptors. As previously explained, the number of cancer invading the stromal region was dramatically high by day 4 and 6, which caused the cancer cells to overlap, making the characterization of cell morphology challenging. From previous studies, we learned that the nuclear shape corresponds to the morphology of the cells. In other words, when there is change in cell morphology it leads to change in shape of the nucleus (Versaevel, Grevesse, and Gabriele 2012). Hence, we used the 3D z-stack images of the nuclei, stained for DAPI, to analyze the morphology of the tumor cells.

We quantified major morphology descriptors such as circularity, aspect ratio and roundness using the extended particle analyzer plugin of the NIH ImageJ software. We limited the analysis to a constant region of interest in the stromal portion of the device. The circularity measurements showed that the cancer cells invading stroma in the presence of vascular networks were less circular (0.73 ± 0.03) than the cells in the absence of vascular networks (0.80 ± 0.02). Furthermore, the roundness of cancer cells without the presence of vasculature (control conditions) was significantly higher (0.64 ± 0.02) than the roundness of the cells in presence of vasculature (0.58 ± 0.02) at day 6 of culture. Aspect ratio, which indicates polarized length and extension of the cells, was significantly higher in the cells under the influence of developing vasculature (1881± 0.10) as compared to the control the conditions without vasculature on day 6 of culture (1.670 ± 0.066) (**Figure 3.2 d**).

Our findings suggest that MDA-MB-231 cells invading the stroma have a more elongated morphology due to the influence of endothelial cells, suggesting that endothelial cells play a major role in driving invasive characteristics of tumor cells.

3.3.4 Cancer cell intravasation:

Intravasation is the migration of tumor cells into the endothelial networks and vascular lumen by crossing the endothelial barriers. We investigated whether the presence of the endothelial network would cause highly invasive cancer cells to successfully intravasate into the surrounding vascular network. Imaging interactions of tumor and endothelial cells during intravasation is crucial for understanding the mechanism of intravasation, and our microfluidic-based intravasation platform enabled real-time high-resolution imaging of the intravasation process. To characterize the ability of cancer cells to invade the 3D endothelial network, a series of immunofluorescence z-stack images were acquired for tomato-expressing MDA-MB-231 cells, while endothelial cells were stained in green for CD31 cell-cell junction proteins. From these images, we could visualize tumor cells elongating and forming protrusions. Furthermore, tumor cells crossed the endothelial barrier and entered the endothelial lumen (**Figure 3.3 a, b**).

Furthermore, to quantify the number of intravasating tumor cells, we counted the number of cells entering the endothelial lumen. Five regions of interest (ROIs) were marked throughout the vascular region of the device, and the number of tumor cells entering the lumen was counted. The distribution of tumor cells in the regions of interest was consistent throughout the vascular region (**Figure 3.3 c**). Interestingly, we also found that in the control condition without the presence of vascular networks, MDA-MD-231 cells did not migrate into the fibrin matrix, migration was observed only when the vascular networks were present. A possible explanation for this behavior is perhaps that tumor cells do not secrete the proteases required to invade the fibrin matrix. However, further investigation is required in future studies to confirm these findings.

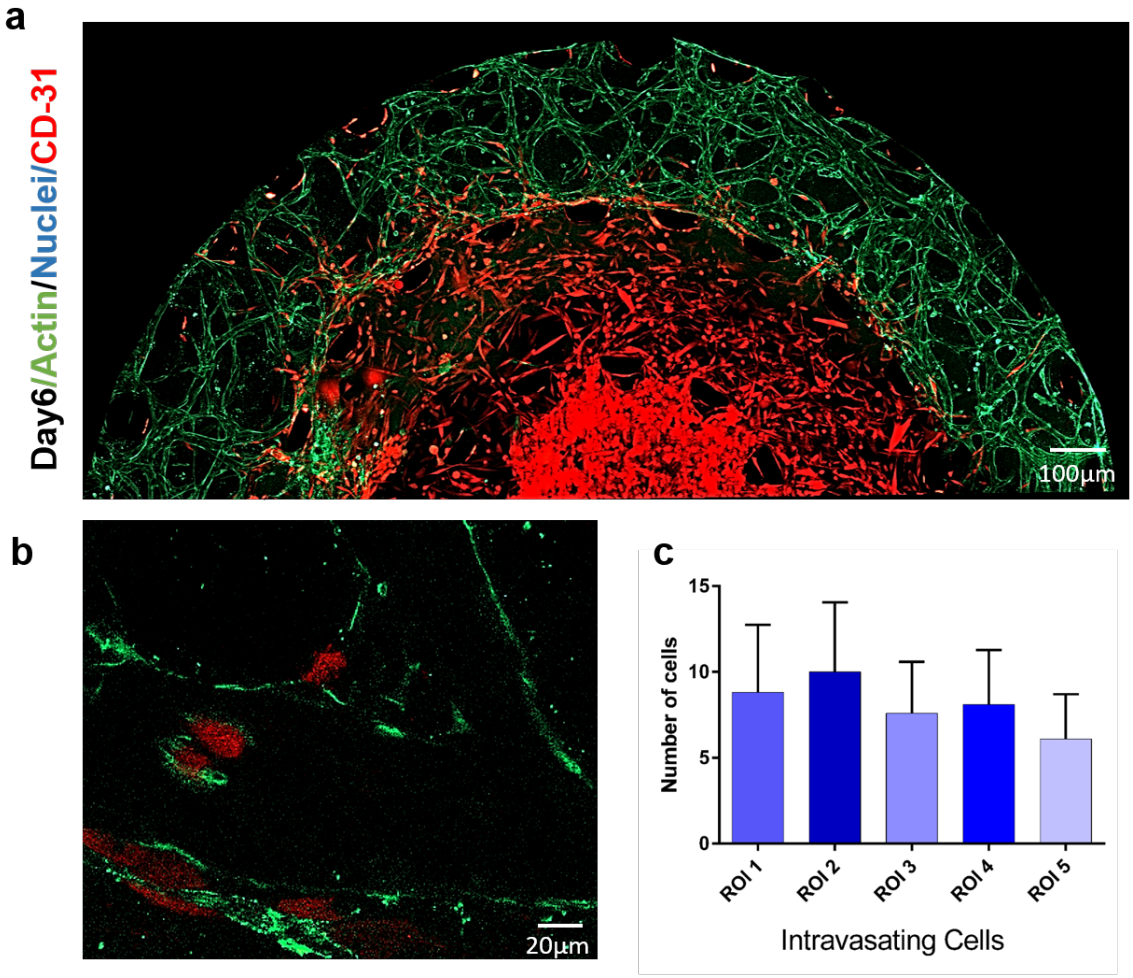

Figure 3.3: Intravasation of cancer cells into the vascular network. **(a-b)** Representative image of cancer cells intravasation into the vascular network. **(c)** Qualitative number of cancer cells in the different regions of interest within the platform intravasating into the vascular network.

3.3.5 Characterization of vascular networks:

To investigate the effects of the tumor entity and cancer cells on growth and maturation of endothelial cell networks, we studied the morphology of the formed vasculature. We observed the formation of vascular networks both in presence (+ tumor) and absence (- tumor, control) conditions. Functional microvascular networks formed were characterized by staining for CD31, cell-cell junction marker. The immunofluorescent images demonstrated the formation of well-formed, well-connected vascular networks.

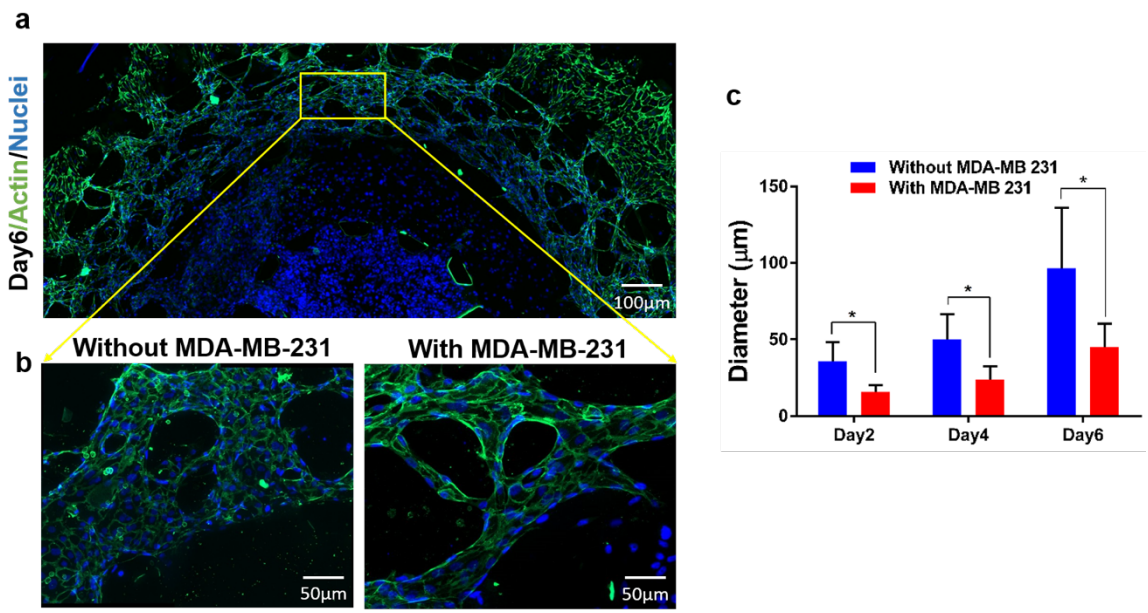

Figure 3.4: Characterizing the morphology of the vascular network **(a, b)** Representative fluorescent images of the vascular network on day 6 of culture during intravasation of cancer cells. **(c)** Quantification of the diameter of the vascular network in different conditions.

From the immunofluorescence images, we could observe the changes in the morphology of the vascular networks in the presence of the tumor cells. Vascular networks formed were analyzed by measuring the diameter of the vessels. Vascular network exhibited significantly lower diameter (41.15 ± 13.8 µm) compared to diameter of vessels in the control conditions (77.35 ± 34.26 µm) (**Figure 3.4 a-c**). These results are in agreement with the endothelial cell mono-culture studies (chapter 2), where the diameter of the developing vascular networks was reduced due to increased levels of VEGF in the microenvironment.

3.3.6 Permeability of the vascular networks:

To address the question of whether tumor cells induce endothelial permeability, we conducted permeability measurements of endothelial networks under mono-culture and co-culture with tumor cells. In presence of tumor, the vascular networks were more permeable as there was enhanced leakage of dextran into the perivascular region, resulting in reduced fluorescent intensity in the vascular region. In the absence of tumor (control condition), due to less permeability, fluorescent intensity in the endothelial network was comparatively higher. Using the plot profile plugin in the NIH Image J software, initial and final optical intensities values were calculated and values obtained

were substituted in the permeability equation. From the obtained results, we could see that the permeability value for (+) tumor setup was 0.113048 cm/s which was significantly higher compared to permeability of (-) tumor control condition 0.002160 cm/s (**Figure 3.5 a, b**). These findings suggest that tumor cells perturb endothelial function and making vasculature to be more permeable, consistent to our results obtained in mono-culture studies of endothelial cells in chapter 2. Therefore, high permeability of vascular networks might be due to tumor cells secreting biomolecules such as VEGF.

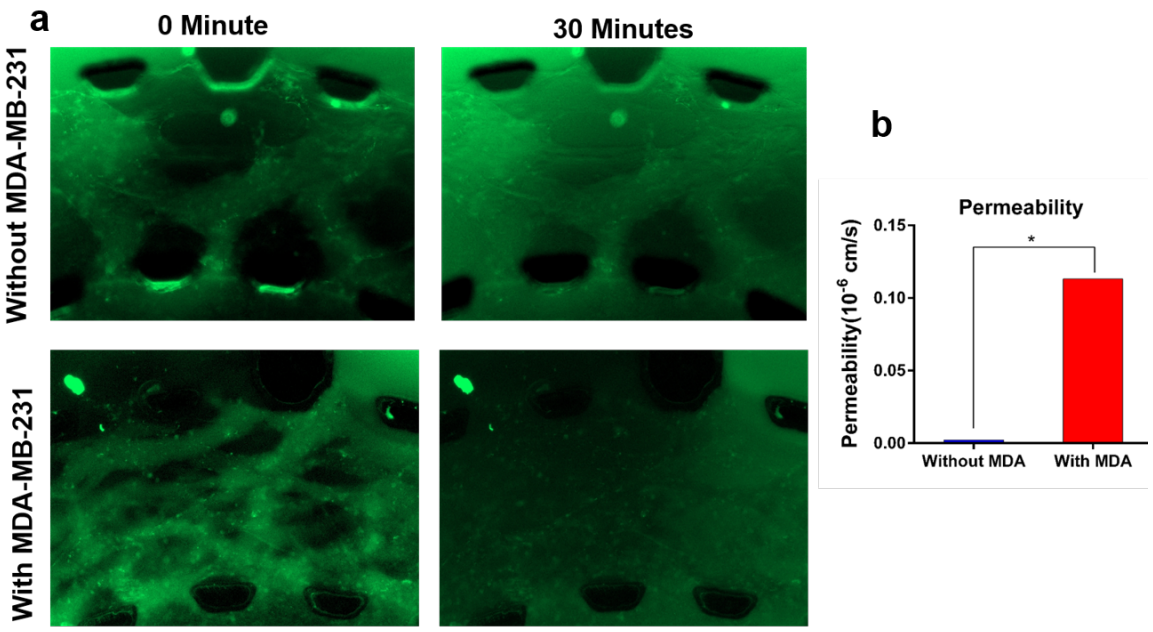

Figure 3.5: Permeability of the vascular networks **(a)** Images representing the networks on 0 and 30 minute when flowing FITC-dextran. **(b)** Qualitative values of permeability of the endothelial networks.

3.4 DISCUSSION

Despite development of numerous assays to study the process of metastasis, the mechanistic interactions between tumor and endothelial cells needs to be further studied in detail. In this regard, there is a crucial need to develop a 3D physiologically relevant *in vitro* tumor model that focuses on cell interactions, paracrine signaling and biochemical factors involved during invasion and intravasation. The creation of such models would enable us to uncover fundamental biological process involved in early stages of metastasis, namely invasion and intravsaion. Currently, a

plethora of 2 and 3 dimensional *in vitro* assays are developed to model various steps of metastasis. However, 2D platforms are unable to recapitulate the intricacies of the *in vivo* tumor microenvironment (Griffith and Swartz 2006). Moreover, most 3D models lack a distinct spatial organization of the tumor, stroma and vascular regions (Kimlin, Casagrande, and Virador 2013) to perform integrated studies on cancer cell invasion and intravasation studies a single platform. Specifically, the models that incorporated spatial compartmentalization of the tumor and stroma did not have a well-defined tumor or a well-formed vasculature.

In this work, we present a unique microfluidic *in vitro* model comprising of three distinct, but interconnected layers. This enabled spatial organization and compartmentalization of tumor, stroma and endothelial entities. Geometry and design enabled side-by-side arrangement of the tumor and stromal components in the 3D matrix, and it also incorporated diffusive barriers for transport of nutrients and growth factors. In our three-layer model, the tumor was injected into the innermost layer, surrounded by an acellular collagen based stromal region. This setup was surrounded by a third layer containing a well-formed vascular network. A media channel surrounded the endothelial region from which growth factors diffused through to the inner layers. This formed separated but interconnected platform, which allowed studies on the interactions of the cancer, endothelial and potentially stromal cells.

In our study, we assessed the effect of endothelial cells on cancer cell invasion an intravasation, our findings showed that the cancer cells significantly invaded the stromal region in high numbers in the presence of endothelial networks. We further investigated cancer cell morphology, where our analysis revealed that cancer cells in the stroma showed elongated shape with more protrusions in the presence of the vascular network as compared to cancer cells in the absence of the vascular network. These results are in accordance with previous *in vivo* studies suggesting that number of invading and circulating tumor cells increases in the presence of vascular networks (Mathur et al. 2001). These results also suggest that endothelial cells exert prometastatic characteristics where cancer cells are attracted to vascular network with a more invasive phenotype (Rosanò, Spinella, and Bagnato 2013). By day 6 in culture, the cancer cells crossed endothelial barrier and entered the vascular region through passive intravasation. The

unique ability within our platform to image the dynamics of tumor endothelial interactions enabled us to visualize in real-time the process of intravasation.

To further study the effect of tumor on structural and functional characteristics of endothelial cells, we analyzed the morphology and permeability of the formed vascular networks. Our results showed that vascular networks were thinner with reduced diameter with significantly higher permeability in presence of the tumor. The results are in accordance with endothelial mono-culture studies, completed in chapter 2, where the diameter of the vasculature decreased and permeability increased due to presence of increased levels of VEGF. Taken together the results suggest that the cancer cells secrete biomolecules such as VEGF, which modulate the morphology and the permeability of the surrounding tumor vasculature. These findings are also consistent with prior *in vivo* studies, which demonstrated increased VEGF signaling leads to more vascular leakage enabling transendothelial migration of cancer cells (Weis et al. 2004).

Therefore, our overall findings demonstrate that our developed microfluidic platform can be employed to study tumor growth, cancer cell invasion, and intravasation on a single platform to obtain mechanistic biological insights within each separate stage of the metastatic cascade within a well controlled (i.e. cell-cell and cell-ECM interactions) microenvironment.

CHAPTER 4

CONCLUSION AND FUTURE WORK

4.1 CONCLUSION

Breast cancer is one of the most common cancer in women and has high mortality rates in the U.S. Most of the breast cancer-related deaths are caused due to metastasis. Therefore, it is crucial to study the cancer cells behavior during invasion and intravasation. However, the study of intravasation is proven to be challenging due to the difficulties in establishing the invasion environment. In this study, we have microengineered a novel breast tumor microenvironment model designed to incorporate high-density tumor, stroma and well-formed vasculature. The entities were separated into different channels and these channels were composed of specific cell compositions embedded in suitable ECM-forming 3D structure. In our high throughput model, we could visualize and quantify the invasion of tumor cells into the stroma and observed increased numbers of invading cancer cells when in the presence of surrounding vasculature. Cancer cells with more protrusions and elongated morphology was observed under the influence of vasculature. Our microfluidic based model also allowed real time visualization and quantification of trans-endothelial migration of cancer cells into the vascular networks. Thereby, the developed platform enabled us to study the dynamic interactions of tumor and endothelial cells during intravasation. Furthermore, we evaluated the effect of tumor cells on growth and maturation of vascular networks. Our findings suggested that the cancer cells altered the morphology of endothelial cells by reducing the diameter and increasing the permeability of developed vasculature. As the tumor blood vessels are abnormal and hyper permeable, we hypothesize that these changes in morphology of the vascular networks and hyper permeability contribute to invasion and intravasation of cancer cells. Together, these results demonstrate the ability of our novel three-layer device to visualize and analyze the different cancer cell behaviors like migration, invasion, and intravasation. Thus, the proposed platform proves to be a valuable tool to study cancer behavior and recapitulate the interactions of tumor and endothelial cells. The platform can be further used for screening drug targets and to develop personalized medicine.

4.2 FUTURE WORK

4.2.1 Enhancing the stromal region:

In our study, we incorporated tumor and endothelial cells separated by an acellular stromal region. Owing to the complexities of breast tumor microenvironment and to recapitulate the *in vivo* characteristics, tumor associated stromal cells could be introduced into the stromal region. For example, TAMs can be introduced into the stromal region and their effect on the proliferation, migration, invasion, and intravasation of cancer cells can be analyzed. Additionally, CAFs can be introduced to study how they modulate the ECM, in terms of stiffness and architecture, to aide cancer cells in invasion and intravasation. In this regard, we could potentially increase the complexities of our platform by mixing different stromal cells and study the combined effect on morphology and behavioral changes in cancer cells.

4.2.2 Testing therapeutic targets:

As our three layer platform allows 3D spatial organization of tumor and its surrounding vasculature, the development and behavior of cancer cells mimic the *in vivo* microenvironment. An interesting study would be to test the anti-VEGF target drugs such as bevacizumab. The anti-VEGF drug can be added to the developed high density tumor and vasculature and subsequently the changes in morphology, proliferation of endothelial cells can be assessed. Furthermore, the impact of the drug on migration, invasion and intravasation of cancer cells can also be analyzed. Thus, this platform is a potential candidate for validation and screening of drugs and ultimately be used in personalized medicine.

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Figure 1.2 (b)

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Figure 1.3 (b)

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Figure 1.4 (a)

Oriented collagen fibers direct tumor cell intravasation

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Figure 1.4 (b)

Three-dimensional microfluidic model for tumor cell intravasation and endothelial barrier function

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