Blood-brain barrier model on a microfluidic chip for
the study of tumor cell extravasation

By

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B.S., Mechanical Engineering
B.A., Economics
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Abstract

With up to 40% of cancer patients showing metastatic lesions to the brain and a 30% five-year survival rate post-diagnosis, secondary tumors to the brain are a leading cause of cancer-related deaths. Understanding the mechanisms of tumor cell extravasation at the brain is therefore crucial to the development of therapeutic agents targeting this step in cancer metastasis, and to the overall improvement of cancer survival rates. Investigating the interactions between tumor cells and brain stroma is of particular interest due to the site’s unique microenvironment. In fact, the interface between brain and blood, known as the blood-brain barrier (BBB), is the tightest endothelial barrier in humans. The presence of tight junctions between brain endothelial cells, coupled with the spatial organization of pericytes and astrocytes around the vasculature, restrict the entry of most solutes and cells into the brain. Yet, the brain constitutes a common metastatic site to many primary cancers originating from the lung, breast and skin. This suggests that tumor cells must employ specific mechanisms to cross the blood-brain barrier. While in vitro models aimed at replicating the human blood-brain barrier exist, most are limited in their physiological relevance. In fact, the majority of these platforms rely on a monolayer of human brain endothelial cells in contact with pericytes, astrocytes and neurons. While this approach focuses on incorporating the relevant cell types of the brain microenvironment, it fails to accurately replicate the geometry of brain capillaries, the barrier tightness of the BBB, and the juxtacrine and paracrine signaling events occurring between brain endothelial cells and stromal cells during vasculogenesis. To integrate these features into a physiologically relevant blood-brain barrier model, we designed an in vitro microvascular network platform formed via vasculogenesis, using endothelial cells derived from human induced pluripotent stem cells, primary human brain pericytes, and primary human brain astrocytes. The vasculatures formed with brain pericytes and astrocytes exhibit decreased cross-section areas, increased endothelial cell-cell tight junction expression and basement membrane deposition, as well as reduced and more physiologically relevant values of vessel permeability, compared to the vasculatures formed with endothelial cells alone. The addition of pericytes and astrocytes in the vascular system was also coupled with increased extravasation efficiencies of different tumor cell subpopulations, despite the lower permeability values measured in this BBB model. Moreover, an increase in the extravasation potential of metastasized breast tumor cells collected from the brain was recorded with the addition of pericytes and astrocytes, with respect to the parental breast tumor cell line. These results were not observed in metastasized breast tumor cells collected from the lung, thus validating our BBB model and providing useful insight into the role of pericytes and astrocytes in extravasation. Our microfluidic platform certainly provides advantages over the current state-of-the-art in vitro blood-brain barrier models. While being more physiologically relevant than most in vitro platforms when it comes to geometry, barrier function and juxtacrine/paracrine signaling between the relevant cell types, our model provides a robust platform to understand tumor cell-brain stromal cell interactions during extravasation.

Thesis supervisor: Roger D. Kamm
Title: Professor of Mechanical and Biological Engineering
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Finally, I would like to dedicate this thesis to my two grandfathers, Salim Haddad and Assaad Hajal, who left us early. I am thankful that progress is being made in this community to further the general understanding of diseases and push forward novel treatments where they are needed.

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Table of Contents

CHAPTER 1: INTRODUCTION................................................................................................ 7
  1.1 THE BLOOD-BRAIN BARRIER......................................................................................... 7
  1.2 METASTATIC CASCADE AND EXTRAVASATION AT THE BRAIN............................... 8
  1.3 STATE-OF-THE-ART BLOOD-BRAIN BARRIER MODELS AND EXTRAVASATION
      ASSAYS AT THE BRAIN.................................................................................................. 11
  1.4 OBJECTIVE AND THESIS OVERVIEW........................................................................ 14

CHAPTER 2: IN VITRO BLOOD-BRAIN BARRIER MICROVASCULAR NETWORK
    FORMATION ..................................................................................................................... 17
  2.1 INTRODUCTION ........................................................................................................... 17
     2.1.1 Bridging the gap: from in vivo brain models to in vitro BBB microvascular
          networks ..................................................................................................................... 17
     2.1.2 Blood-brain barrier formation and stabilization in vivo and in vitro....................... 18
  2.2 METHODS ................................................................................................................... 19
     2.2.1 Assay design ........................................................................................................... 19
     2.2.2 Cells and device preparation ................................................................................ 21
           Microfluidic device fabrication .............................................................................. 21
           Cell culture ............................................................................................................ 21
           Gel fabrication and cell seeding ............................................................................ 22
     2.2.3 Fluorescent imaging and analysis .......................................................................... 23
     2.2.4 Calculation of vessel permeability ....................................................................... 23
     2.2.5 Microvascular network metrics ........................................................................... 24
  2.3 RESULTS .................................................................................................................... 26
     2.3.1 Vessel formation and stability ............................................................................. 26
     2.3.2 Microvascular network metrics and morphology ............................................... 29
     2.3.3 Vessel permeability measurements ..................................................................... 32
     2.3.4 Tight junction protein expression and basement membrane deposition ............. 33
  2.4 DISCUSSION .............................................................................................................. 36

CHAPTER 3: TUMOR EXTRAVASATION AT THE BRAIN FROM IN VITRO BLOOD-
  BRAIN BARRIER MICROVASCULAR NETWORKS ........................................................ 37
  3.1 INTRODUCTION ........................................................................................................... 37
  3.2 METHODS ................................................................................................................... 38
3.2.1 Blood-brain barrier microvascular network formation and maintenance ............................................. 38
3.2.2 Tumor cell perfusion in the microvascular networks ........................................................................ 38
3.2.3 Tumor cell extravasation quantification ..................................................................................... 39
3.2.4 Barrier permeability measurements following tumor cell extravasation ..................................... 39
3.3 RESULTS ........................................................................................................................................ 40
3.3.1 Tumor cell perfusion and extravasation in the BBB microvascular networks .......................... 40
3.3.2 Extravasation potential of metastasized tumor cells collected from different organs ................ 43
3.3.3 Effect of tumor cell extravasation on barrier permeability ..................................................... 45
3.4 DISCUSSION .................................................................................................................................. 47

CHAPTER 4: CONCLUSIONS ................................................................................................................ 49

REFERENCES ........................................................................................................................................ 50
List of Figures and Tables

Figure 1. The Blood-Brain Barrier.......................................................... 7
Table 1. Localization and role of most notable TJ and AJ proteins at the BBB......... 8
Figure 2. The metastatic cascade............................................................ 9
Figure 3. Outlook on brain metastases .................................................. 10
Figure 4. State-of-the-art blood-brain barrier models ............................... 12
Figure 5. Current extravasation assays at the brain employing endothelial monolayers .... 13
Figure 6. Extravasation assays employing 3D self-organized HUVEC vasculatures .... 14
Figure 7. Device schematic and platform conditions.................................. 21
Figure 8. Microvascular network quantification metrics ................................ 25
Figure 9. Microvascular networks formed in the three culture conditions......... 27
Figure 10. Vessel stability over time....................................................... 29
Figure 11. Microvascular network metrics at day 7 in culture...................... 31
Figure 12. Morphological changes with the addition of PCs and ACs .............. 32
Figure 13. Permeability measurements in the three culture conditions .......... 33
Figure 14. Tight junction protein expression .......................................... 34
Figure 15. Basement membrane deposition in three culture conditions.......... 35
Figure 16. Visualization of MDA-MB-231 and A549 extravasation events ....... 40
Figure 17. Extravasation efficiencies of MDA-MB-231 over time ................. 41
Figure 18. Extravasation efficiencies of A549 over time ............................ 42
Figure 19. Extravasation potential of breast parental tumor cells (MDA-Parental) and metastasized tumor cells collected from the brain (MDA-BrM) and lung (MDA-LM) ...... 45
Figure 20. Effect of tumor cell extravasation on barrier permeability .............. 46
Chapter 1: Introduction

1.1 The Blood-Brain Barrier

The BBB is a selective interface between microvasculature and brain parenchyma formed by the endothelial cells that line the cerebral microvessels, brain pericytes enclosed within the basal lamina, and astrocytes touching their end-feet to the abluminal side of the vessels. It is described as the tightest endothelial barrier in humans, thanks to the presence of tight junctions (TJs) and adherens junctions (AJs) between adjacent endothelial cells. Intercellular gaps measuring 3 to 6 nm were recorded between endothelial cells of the brain in mice, significantly smaller in size than the gaps measured in other endothelial cells (~10 nm). The TJs and AJs characteristic of the gaps between neighboring brain endothelial cells are known to be the main obstacles to paracellular transport across the BBB, forcing most solutes (of size larger than ~6 nm) to travel via transcellular routes by melding into cell membrane “holes”, via receptor-mediated or adsorptive-mediated transcytosis, or with the help of carriers, such as GLUT1 for glucose transport and LAT1 for amino acid transport.

Most notable components of TJ and AJ proteins at the BBB, as well as their localization and role, are listed below:
<table>
<thead>
<tr>
<th>Type of junction</th>
<th>Junction protein</th>
<th>Localization in brain endothelial cells</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tight junctions</td>
<td>Occludin</td>
<td>Membrane</td>
<td>Promote redox-sensitive processes of TJ assembly</td>
</tr>
<tr>
<td></td>
<td>Claudins</td>
<td>Membrane</td>
<td>Homodimerize with claudins on adjacent endothelial cells</td>
</tr>
<tr>
<td></td>
<td>JAMs</td>
<td>Membrane</td>
<td>Facilitate assembly of TJ components and recruit polarity complex</td>
</tr>
<tr>
<td></td>
<td>Zonula Occludens (ZOs)</td>
<td>Cytoplasm</td>
<td>Bind to claudins and JAMs via PDZ domain and to occluding via other regions to sustain TJ integrity</td>
</tr>
<tr>
<td>Adherens junction</td>
<td>VE-cadherin</td>
<td>Membrane</td>
<td>Promote actin bundling</td>
</tr>
<tr>
<td></td>
<td>N-cadherin</td>
<td>Membrane</td>
<td>Regulate angiogenesis</td>
</tr>
</tbody>
</table>

Table 1. Localization and role of most notable TJ and AJ proteins at the BBB

In addition to forming an obstacle to solutes, the BBB also prevents the passage of most cells into the brain parenchyma. However, certain circulating mononuclear cells at the brain, such as leukocytes, monocytes and macrophages, have been shown to undergo diapedesis and cross the barrier via both paracellular and transcellular routes. During inflammatory pathology, trafficking of leukocytes across the BBB is highly upregulated. In these cases, several studies suggest that transcellular diapedesis seems to be the preferential route employed by leukocytes to cross the BBB, leaving TJs and barrier function undisturbed. Brain pericytes seem to play a role in neutrophil chemoattraction and transmigration, by secreting interleukin-8 (IL-8) and matrix metalloprotease-9 (MMP-9) in response to inflammation characterized by tumor necrosis factor-alpha (TNF-α), IL-1β, or lipopolysaccharide (LPS) expression.

Although solute transport and cell migration across the BBB are highly limited, several tumor cells originating from specific primary sites metastasize preferentially to the brain. The supporting role of the BBB in metastasis formation is still not fully elucidated but several findings suggest a potential role for pericytes and astrocytes in promoting tumor cell extravasation and homing at the brain.

### 1.2 Metastatic cascade and extravasation at the brain
As cancer cells multiply uncontrollably at a specific site, a primary tumor mass forms and self-vascularizes as tumor cells start secreting pro-angiogenic factors. These primary tumors are, however, only responsible for 10% of cancer-related deaths. The remaining 90% of deaths is attributed to metastasis, the spread of cancer cells from primary tumors to surrounding tissues and distant organs.

The initial phase of the metastatic cascade involves the loss of adhesion molecules, such as E-cadherin, and cytokeratins, which leads to the detachment of carcinoma cells from the primary tumor mass and their acquisition of a motile phenotype. In the vascularized tumor microenvironment, MMP-expressing cancer cells are able to digest the basement membrane surrounding the primary tumor site to come in contact and intravasate into the circulation. The circulating tumor cells (CTCs) must then travel to distant sites or tissues via the bloodstream, while escaping immune surveillance. CTCs are able to arrest in the microvasculature of the secondary site via mechanical entrapment in the capillary beds due to size restriction or via adhesion to the vascular endothelium. A fraction of these adhered or entrapped tumor cells may extravasate through the endothelial walls and colonize the secondary site. The overall metastatic process from intravasation to colonization is highly inefficient – only about 0.01% of CTCs actually lead to the formation of tumors at the secondary site, mainly due to the failure of these single cells to escape immune surveillance and reach the distant site to initiate growth, and to the failure of early micrometastases to continue growth into potentially fatal macrometastases.

![Figure 2. The metastatic cascade. Cancer cell progression from the primary tumor site to the secondary site for colonization.](image)

While the general mechanisms underlying tumor cell extravasation have been examined both in vivo and in physiologically relevant in vitro platforms, the precise mechanisms governing extravasation at the brain, on the other hand, remain poorly understood. It has been observed that melanoma cells undergo
brain, on the other hand, remain poorly understood. It has been observed that melanoma cells undergo paracellular transendothelial migration of tumor cells across the BBB, where TJs were disrupted and barrier integrity was damaged. Transcellular migration of tumor cells at the brain is possible, given the fact that TJs seal the intercellular route, and several mononuclear cells have been shown to cross the BBB via the transcellular route. However, this phenomenon has yet to be detected either in in vivo animal models or in in vitro systems.

Gathering data from several hundreds of patients showing metastatic lesions at the brain, a few primary cancers have been identified to preferentially metastasize to the brain. Around 40-50% of non-small cell lung cancer patients have been shown to develop brain metastases, followed by breast cancer patients at ~20% and melanoma patients (~10%). These findings have raised questions regarding the specific genes and underlying mechanisms governing the behavior of cancer cells from different primary sites. Although certain genes in breast cancer have been identified to play a role in preferential brain metastatic colonization, additional studies are necessary to fully elucidate the physical mechanisms and signaling pathways activated in different primary cancer cells crossing the BBB.

<table>
<thead>
<tr>
<th>Primary cancer</th>
<th>MSKCC [1*], 1994; n=210</th>
<th>Nussbaum et al., [19], 1996; n=729</th>
<th>Counsell et al. [6*], 1996; n=214</th>
<th>Lagerwaard et al. [23], 1999; n=1202</th>
<th>Stark et al. [18], 2011; n=509</th>
<th>Fabi et al. [12*], 2011; n=290</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>41%</td>
<td>39%</td>
<td>53%</td>
<td>56%</td>
<td>50%</td>
<td>---</td>
</tr>
<tr>
<td>NSCLC</td>
<td>35%</td>
<td>24%</td>
<td>---</td>
<td>---</td>
<td>42%</td>
<td>44%</td>
</tr>
<tr>
<td>SCLC</td>
<td>6%</td>
<td>15%</td>
<td>---</td>
<td>---</td>
<td>8%</td>
<td>---</td>
</tr>
<tr>
<td>Breast</td>
<td>19%</td>
<td>17%</td>
<td>13%</td>
<td>16%</td>
<td>15%</td>
<td>30%</td>
</tr>
<tr>
<td>Melanoma</td>
<td>10%</td>
<td>11%</td>
<td>8%</td>
<td>---</td>
<td>7%</td>
<td>6%</td>
</tr>
<tr>
<td>Renal</td>
<td>3%</td>
<td>6%</td>
<td>2%</td>
<td>4%</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>GI</td>
<td>7%</td>
<td>6%</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>9%</td>
</tr>
<tr>
<td>Colorectal</td>
<td>4%</td>
<td>---</td>
<td>3%</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Unknown</td>
<td>2%</td>
<td>5%</td>
<td>14%</td>
<td>8%</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Table 1 Percentages of brain metastases from different primary cancers

Figure 3. Outlook on brain metastases. Lung primary cancer cells preferentially metastasize to the brain, followed by breast primary cancer cells and melanoma cells.

The specific BBB microenvironment allows for distinct cellular and molecular interactions. It is not surprising that extravasating tumor cells interact closely with the endothelium, but also with brain pericytes and astrocytes located on the abluminal side of the vasculature. It has been observed that extravasated tumor cells at the brain maintain close contact with the abluminal side of the endothelium, where they can benefit from the protection of the BBB against anti-tumoral immune cells circulating in the brain, and the support of the basement membrane lining the vasculature. In the context of brain metastasis, two subpopulations...
of pericytes have been identified in connection with tumor cell extravasation. CD13-positive pericytes have been linked with normal BBB function and barrier integrity, while desmin-positive pericytes were found in brain metastases where BBB permeability is significantly increased. CD13+ pericytes could transform into desmin+ pericytes in the presence of tumor cells, thus impairing barrier function and promoting extravasation, however, desmin+ pericytes might have originated from their own expansion, the bone marrow or potentially endothelial cells during endothelial-mesenchymal transition. In addition, several studies suggest that astrocytes might promote cancer invasion and survival at the brain. It has been observed that astrocytes are activated in the vicinity of tumor cells, even prior to their extravasation. These reactive astrocytes, characterized by up-regulated glial fibrillary acidic protein (GFAP) expression, secrete MMP-9, promoting tumor cell extravasation and invasion into the matrix. Tumor cells have also been shown to activate the production of TNF and interferon (IFN)-α in astrocytes, which in turns supports brain metastasis via the STAT1 and NF-κβ pathways. While the exact mechanisms and cellular interactions between tumor cells and brain stromal cells have not been fully elucidated, evidence seems to suggest that brain pericytes and astrocytes play a role in promoting tumor cell extravasation. Recapitulation of tumor cell arrest and extravasation in a highly controlled and physiologically relevant brain microenvironment would then provide useful insight into the precise mechanism of action of pericytes and astrocytes, and the molecular alterations associated with tumor cell extravasation across the BBB. This assay would therefore be crucial to the development of therapeutic agents targeting this step in cancer metastasis, and to the overall improvement of cancer survival rates.

1.3 State-of-the-art blood-brain barrier models and extravasation assays at the brain

Most extravasation studies at the brain are performed in animal models. These in vivo studies allow for the investigation of specific genes and proteins involved in tumor cell extravasation at the brain. Although these models offer a highly physiologically relevant platform of study where most in vivo responses can take place, significant challenges associated with the translation of experimental results in animal brains to conclusive outcomes in human brains persist. While most in vivo models are limited to time-point data collection, real-time imaging of tumor cell extravasation at the brain has been performed in mice; however, the study required the use of immunodeficient mice, which may have perturbed the tumor cell response, and success rates were low (~50%), which resulted in low throughput experiments (Fig. 4A). Controlling for specific parameters in animal studies, as well as observing the underlying mechanisms...
In an attempt to circumvent these limitations, several groups have focused their efforts on developing in vitro platforms replicating the human BBB physiology. The most commonly used in vitro model relies on the use of a Transwell chamber, where human brain cells can be cultured in 2D in a simple high-throughput platform33-47 (Fig 4B). While these platforms offer an additional degree of physiological relevance with the incorporation of human cells from the brain, they do not allow real time imaging of the entire extravasation process and fail to fully replicate the geometry and spatial cellular organization of the BBB microvasculature.

Moving away from 2D models, assembled 3D monolayer systems have been adopted to more closely recapitulate the geometry of brain capillaries. In these systems, 3D channels are fabricated in microfluidic devices where cells can be cultured as a monolayer coating the inner cavity layer. Several groups have adopted this technique to recreate geometrically relevant platforms where brain endothelial cells coat the inner channel walls and brain stromal cells can be cultured in adjacent fluidic channels or in a gel on the abluminal side of the assembled endothelial “vessels”11,48,49 (Fig. 4C). Assembled 3D monolayer systems allow for greater control of the vessel geometry as well as the spatial organization of brain stromal cells and neurons around the vasculature. However, they fail to fully replicate 3D self-assembled vasculatures in terms of branching, diameter sizes, barrier tightness and activated signaling pathways during vasculogenesis between the relevant brain cell types50.

![Figure 4. State-of-the-art blood-brain barrier models.](image)

(A) In vivo models of mice brains employed for the study of tumor cell extravasation25,42. (B) Transwell assay model of the BBB: brain endothelial cells are cultured on a 2D membrane with astrocytes on the opposite side of the membrane, and pericytes and neurons at the bottom of the well16. (C) Assembled 3D monolayer model of the BBB: a liquid flow is used to create a cavity inside a collagen gel where brain endothelial cells are subsequently plated. Pericytes and astrocytes can be cultured prior to the addition of brain endothelial cells in order to replicate the cellular spatial organization of the BBB11.
Only a few studies of extravasation at the brain have been performed in microfluidic in vitro platforms, as opposed to in vivo animal models. Most of these assays rely on the use of a 2D monolayer of brain endothelial cells, adjacent to a layer of brain astrocytes or pericytes. These platforms validated the trends of Fig. 4, suggesting that lung (A549), breast (MDA-MB-231) and melanoma (M624) cancer cells preferentially migrated across the brain endothelium, as opposed to liver (BEL-7042) cancer cells.

These platforms have provided tremendous insight into the specific mechanisms of tumor cell extravasation, as well as the different signaling pathways activated and molecular alterations occurring during transmigration and colonization of cancer cells. For instance, Chen et al. examined the roles of tumor integrin β1 in extravasation using an in vitro platform with HUVEC microvascular networks and NHLFs in adjacent channels. While in vivo models do not allow to fully elucidate the role of β1 in the different steps of metastatic dissemination, the in vitro platform employed demonstrated that tumor cells send activated β1-rich protrusions past the endothelium to engage with subendothelial matrix, particularly laminin, via α3β1 and α6β1 integrins. Similarly, Jeon et al. demonstrated that tumor cell extravasation rates were significantly higher in the presence of human bone marrow-derived MSCs and osteo-differentiated MSCs. By investigating the effects of adenosine, known to be secreted in the skeletal muscle microenvironment, on metastasizing tumor cells in this controlled

Figure 5. Current extravasation assays at the brain employing endothelial monolayers. Lung, breast and melanoma tumor cells have been shown to preferentially cross the 2D brain endothelium adjacent to astrocytes or pericytes.
platform, it was observed that extravasation rates were reduced, confirming the anti-metastatic and protective role of skeletal muscle cells\textsuperscript{54}.

**Figure 6. Extravasation assays employing 3D self-organized HUVEC vasculatures.** (A) 3D microvascular networks formed with HUVECs and perfused with breast MDA-MB-231. Cross-sectional views of the transmigration steps of a single tumor cell are shown over time\textsuperscript{54}. (B) 3D microvascular networks formed with HUVECs, with osteo-differentiated MSCs in an adjacent channel, and perfused with bone seeking breast MDA-MB-231, extravasating over time\textsuperscript{54}.

While these 3D self-organized HUVEC vasculatures offer a general, more physiologically relevant platform for the study of tumor cell extravasation than 2D assembled monolayer models, they do not fully recapitulate different organ specificities, in terms of cell types incorporated, organ-specific barrier tightness, vessel morphology, and paracrine/juxtacrine signaling\textsuperscript{54,57,58}. In the context of modelling the BBB on a chip for tumor cell extravasation studies, there remains a need for the development of 3D vasculatures \textit{in vitro}, incorporating the advantages offered by microfluidic technologies, as well as the physiological relevance pertaining to incorporated cell types, cellular interactions and localizations, barrier function and protein expression.

### 1.4 Objective and thesis overview

The objective of this project is to incorporate the unique features of the BBB in current 3D \textit{in vitro} microfluidic platforms, in an attempt to design a physiologically relevant model that can be employed for the study of tumor cell extravasation at the brain. While current brain planar models and 2D assembled monolayer systems aim to incorporate most relevant brain cell types, they fail to replicate the appropriate geometry of brain vasculature, and exhibit suboptimal barrier function. On the other hand, state-of-the-art 3D vasculature systems do not fully encompass organ-specific characteristics, in terms of vascular architecture, cellular interactions and protein expression. Most tumor cell extravasation studies are currently
monolayer systems aim to incorporate most relevant brain cell types, they fail to replicate the appropriate geometry of brain vasculature, and exhibit suboptimal barrier function. On the other hand, state-of-the-art 3D vasculature systems do not fully encompass organ-specific characteristics, in terms of vascular architecture, cellular interactions and protein expression. Most tumor cell extravasation studies are currently performed in in vivo animal models. However, these are restricted in their physiological relevance to humans, and in their ability to perform controlled, high-throughput studies. The lack of physiologically relevant 3D vascularized BBB models for the study of tumor cell extravasation may limit our understanding of the mechanisms, cellular interactions and signaling pathways involved in this step of brain metastasis formation. Our goal is then to build an in vitro BBB-specific vascularized extravasation assay that allows for high-throughput capture of extravasation events across physiologically relevant BBB vasculatures, exhibiting low permeabilities when compared to those of animal brains, and human in vivo-like vessel morphology and cellular interactions.

The first aim of this project, discussed in Chapter 2, is to establish in vitro perfusable BBB-like vasculatures that can be used for the study of tumor cell extravasation at the brain, across this barrier. This can be achieved by developing 3D microvascular networks (MVNs) composed of physiologically relevant endothelial cells and stromal cells to recapitulate the brain microenvironment. The MVNs should be stable in terms of morphology and barrier function, and should exhibit permeabilities similar to those measured in animal brains. Tumor cells must be able to flow through the vascular networks and their arrest, transmigration and colonization should be observed in the in vitro platform. The MVNs must be able to incorporate relevant brain cell types successively, in order to examine the underlying effect of each cell type on tumor cell extravasation. The platform should allow for a high level of throughput in order to produce several devices per condition (at least 15 devices per condition per experimental repeat).

Different conditions pertaining to the incorporated cell types are examined in terms of morphology, barrier tightness, and overall physiological relevance. By progressively incorporating different cell types specific to the brain, this assay determines the roles of each one of them when it comes to vascular parameters and barrier function.

In Chapter 3, the platform employed for tumor cell extravasation studies across the BBB is described. It consists of endothelial cells derived from human induced pluripotent stem cells (iPS-ECs) in juxtacrine co-culture with brain primary pericytes (PCs) and brain primary astrocytes (ACs). The platform is validated previously by tracking vessel stability over time with and without the different brain stromal cells, as well as vessel morphology, barrier tightness, EC tight junction protein expression, and basement membrane deposition after vascular stability is achieved. This assay is then used to understand the underlying mechanisms of tumor cell extravasation, including the differences in extravasation efficiencies over time.
in different vasculatures progressively exhibiting BBB features, the disparity in extravasation patterns of distinct tumor cell lines originating from various primary sites, and the correlation of preferential homing site with extravasation ability. With this platform, we aim to elucidate the mechanisms underlying extravasation across a 3D BBB vasculature in vitro, as well as shed the light on potential cellular interactions and factors that may favor extravasation at the brain.
Chapter 2: *In vitro* blood-brain barrier microvascular network formation

2.1 Introduction

2.1.1 Bridging the gap: from *in vivo* brain models to *in vitro* BBB microvascular networks

The unique microenvironment of the brain, its central role in vital functions, as well as the severity of brain disorders, have been drivers in the use and development of brain assays. In order to fully understand the complexities of the brain microenvironment and to isolate specific features, cellular interactions and signaling pathways characteristic of certain brain disorders, there is a tremendous need for engineered vascularized brain tissue models.

Currently, the majority of *in vitro* 3D vascularized assays rely on the self-organization of endothelial cells (ECs) into microvascular networks in a hydrogel. These assays can be achieved in two ways – ECs seeded into a matrix of collagen or fibrin can come in contact and self-organize into an *in vivo*-like vasculature via a process called vasculogenesis, or ECs sprout into a gel, following angiogenic stimuli, to form vessels which split into new ones via angiogenesis. The vasculatures formed through either process are mature, interconnected tubular networks with patent lumens, allowing for the perfusion of drugs, dyes and other cells in order to replicate physiological responses and perform *in vitro* studies.

In the context of tissue engineering models, most assays employ generic HUVECs for vascularization, owing to their robustness, homogenous phenotype, as well as ease of access and comparison with state-of-the-art models. To generate organ-mimicking microenvironment and investigate the role of organ-specific stromal cells in biological responses, several platforms also incorporate stromal cells in the matrix, in addition to the ECs. For instance, bone- or muscle-specific microenvironment were recapitulated by co-culturing HUVECs with osteo-differentiated MSCs or myoblasts, respectively. While these organ-mimicking microfluidic models aim to replicate closely the *in vivo* tissue, there remain limitations in the use of HUVECs for vascularization. While stromal cells play an important role in several biological events, such as the metastatic cascade, immune cell infiltration or the development of certain diseases, organ-specific endothelial cells still remain the principal barrier that cells and drugs come in contact with when circulating in the vasculature.

3D vascularized networks formed with organ-specific ECs are extremely rare in current models. Instead, a few *in vitro* platforms rely on human induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs), which can be differentiated into cells possessing endothelial properties, to develop vasculatures in...
microfluidic devices. These iPS-ECs or ESC-ECs are non-organ specific, and thus provide the advantage of being further differentiated into ECs possessing the biological characteristics and phenotype of organ-specific ECs. For instance, Lippmann et al. have generated human iPS- and ESC-derived brain microvascular endothelial cells (BMECs) by co-differentiation of neural and endothelial progenitors. Various differentiation protocols have been developed to obtain generic ECs from iPS and ESC, or organ-specific ECs from iPS-ECs and ESC-ECs. Most differentiation procedures into generic ECs rely on treatments with specific growth factors or metabolites. In order to obtain organ-specific ECs from stem cells or stem cell-derived ECs, current protocols rely on the use of growth factors and on the co-culture of iPS-ECs or ESC-ECs with stromal cells found in the microenvironment of the organ of interest. Several studies have determined a prominent role for brain pericytes, astrocytes and neurons in differentiating iPSCs and ESCs into ECs with BBB characteristics.

2.1.2 Blood-brain barrier formation and stabilization in vivo and in vitro

In addition to promoting iPSCs and ESCs differentiation into brain-like ECs, primary brain pericytes and astrocytes also play a significant role in BBB formation and stabilization, both in vivo and in vitro. The development of the BBB in vivo begins with angiogenesis, where pre-existing vessels sprout into the neuroectoderm producing vascular endothelial growth factor (VEGF). These sprouts already exhibit certain BBB properties, such as the expression of TJs and transporters. These barrier properties mature further as the endothelial sprouts come in contact with pericytes and astrocytes.

Specifically, pericyte-endothelial cell interactions are mediated by bidirectional signaling between transforming growth factor-β (TGF-β) and its receptor. This dictates the upregulation of endothelial cadherin-2 which in turn leads to firm adhesion between endothelial cells and pericytes. The pericytes then produce angiopoietin 1 (Ang-1) which promotes vascular development, and deposit extracellular matrix (ECM) components, contributing to basement membrane formation. Astrocytes, on the other hand, release Sonic Hedgehog (Shh) involved in development and cell division. These interactions between brain stromal cells and endothelial cells lead to the development of more advanced TJs, loss of leukocyte adhesion molecules and inhibition of transcytosis, all characteristics of a mature BBB.

The role of pericytes and astrocytes has also been investigated in vitro. In the case of 2D monolayer models, the addition of astrocytes has been shown to decrease barrier permeability and increase transendothelial electrical endothelial resistance (TEER) in the presence of flow. Furthermore, Hatherell et al. detected increased TEER values in Transwell assays where BMECs were co-cultured with astrocytes or pericytes on the abluminal side of the membrane. However, their tri-culture systems of BMECs with
both pericytes and astrocytes, culturing one of the stromal cell on the bottom of the dish and the other on the abluminal side of the membrane, yielded lower TEER values than the co-culture platforms. They concluded that juxtacrine cellular contacts between BMECs, pericytes and astrocytes are needed to induce proper barrier function in brain endothelial cells\textsuperscript{76}. In addition, Wang \textit{et al}. were able to identify \textit{in vivo}-like permeability values of $10^{-7}$-$10^{-8}$ cm/s for FITC-dextrans ranging from 4 to 70kDa in their 2D monolayer model constituted of human iPS-ECs (IMR90-4) differentiated to brain microvascular ECs, and rat primary astrocytes. In the case of 3D assembled monolayer models, both Adriani \textit{et al}. and Herland \textit{et al}. demonstrated improved barrier function when BMECs lining hollow 3D channels were co-cultured with pericytes, astrocytes, or neurons\textsuperscript{11,49}.

While these results provide useful insight into the different cellular interactions, activated signaling pathways and proteins involved in BBB development, there remain limitations in the platforms used for these studies. The aim of this project is to engineer a tightly controlled BBB model, incorporating self-organized 3D vasculatures with relevant brain cell types, and displaying physiological barrier function and morphology, for the study of tumor cell extravasation at the brain.

2.2 Methods

2.2.1 Assay design

In order to develop an \textit{in vitro} vascularized BBB extravasation assay meeting the requirements listed in Chapter 1, we employ microfluidic tools to recapitulate the brain microenvironment\textsuperscript{50}. Using a microfluidic device for cell culture applications, we generate perfusable 3D human iPS-ECs microvascular networks assembled via vasculogenesis, surrounded by brain stromal cells (pericytes and astrocytes). The procedure for HUVEC vascular network formation via vasculogenesis has been previously established and employed in our group for various assays pertaining to tumor cell extravasation with and without immune cells, interstitial flow applications, and ECM-cellular interactions\textsuperscript{34,55,80}.

In this study, we employ a vasculogenesis device based on previous designs from our group\textsuperscript{53}. The polydimethylsiloxane (PDMS) device consists of a single gel region for cell culture, lined by media channels which can all be independently filled by either gels or media. The interface between the single gel region and the media channels consists of micro-posts facing towards the central channel, allowing for surface tension-assisted filling. All the cells employed in the model are cultured in the single, central gel region where they can interact with soluble factors in the media via diffusion. A schematic of the device
used is described in Fig. 7. The single central channel is 1300μm wide and 150μm high, and the length is 8150μm. The gaps between micro-posts are 200μm.

To develop 3D vascularized networks, we use a similar vasculogenesis approach based on earlier models in our lab, in which HUVECs were seeded as a suspension in a central gel channel and normal human lung fibroblasts (NHLFs) were seeded in adjacent channels. In this project, however, the assay described employs iPS-ECs instead of HUVECs, co-cultured with brain primary pericytes and astrocytes in the same gel region instead of NHLFs in side channels. This different approach carries several advantages:

a. the contact culture of non-organ specific iPS-ECs with brain stromal cells allows for a certain degree of additional differentiation of the iPS-ECs into brain-specific ECs, as mentioned in differentiation protocols relying on co-cultures of iPS-ECs and ESC-ECs with organ-specific stromal cells.

b. the use of iPS-ECs with brain pericytes and astrocytes renders the vascular platform more physiologically relevant than generic HUVEC models when it comes to re-creating the BBB on-a-chip.

c. the platform is more suitable for brain-specific assays, such as the study of brain disorders or drug delivery, where the unique features of the BBB are required to shed light on certain mechanisms of interest.

When seeded into the central gel channel of the device, iPS-ECs spontaneously form into 3D interconnected lumens, open to the side channels in the inter-post regions. When co-cultured with brain pericytes and astrocytes, iPS-ECs form interconnected vascular networks, pericytes wrap around the vasculature and astrocytes extend their end-feet to touch the abluminal side of the vessels. The different cells cultured in fibrin gel spontaneously adopt the appropriate spatial organization to re-create the morphology and characteristic features of BBB vasculatures. The networks formed are perfusible with cells, beads and dyes from the media channels on either side of the vasculature region.
Figure 7. Device schematic and platform conditions. (A) The microfluidic device used consists of a single central gel region for cell seeding. The micro-posts separating the gel region from the adjacent media channels allow for surface tension-assisted filling. When iPS-ECs (with or without brain stromal cells) are seeded into the central channel, they spontaneously re-organize into 3D microvascular networks, perfusable with tumor cells from the media channels. (B) Three different platforms were considered: a mono-culture of iPS-ECs alone used as a baseline for comparisons, a co-culture of iPS-ECs and brain pericytes, and a tri-culture of iPS-ECs, brain pericytes and brain astrocytes to fully recapitulate the BBB microenvironment. Ratios of cell densities for the different cell types included are indicated for each platform.

2.2.2 Cells and device preparation

Microfluidic device fabrication

Microfluidic devices were fabricated using soft lithography, as described in a previous protocol. Polydimethylsiloxane (PDMS, Ellsworth Adhesives, MA) was mixed at a 10:1 ratio of base to curing agent and poured over a silicon master. The PDMS poured in the mold was incubated for at least 2 hours at 70°C before being cut from the master, punched, trimmed and autoclaved in water first and then dry autoclaved. The PDMS slabs were then incubated overnight in a 70°C oven. Once cooled, the PDMS slabs and No. 1 glass coverslips were plasma treated for 1 minutes and 30 seconds (Harrick Plasma, Ithaca, NY) and bonded together. The devices were each coated with 40μL of 1mg/mL poly-D-lysine in water (Sigma-Aldrich, Burlington, MA) and incubated at 37°C, 5% CO₂ for at least 2 hours. The devices were then washed three times with sterile water and left at 70°C for at least 24 hours before use.

Cell culture

Induced pluripotent stem cell-derived endothelial cells (iPS-ECs, Cellular Dynamics International, Madison, WI) were cultured on fibronectin coated flasks (30mg/mL fibronectin in water for 2 hours) in Vasculife (Lifeline Cell Technology, Frederick, MD) growth media, supplemented with 50mL of fetal
bovine serum (FBS) and 10mL of L-glutamine, according to the protocol developed by Cellular Dynamics International (CDI). Primary brain pericytes (PCs, ScienCell, Carlsbad, CA) and astrocytes (ACs, ScienCell) were cultured on poly-L-lysine (PLL) coated flasks (0.15μg/mL PLL in water for 2 hours). PCs were grown in pericyte media (ScienCell) and ACs in astrocyte media (ScienCell). All cells were used at passage 5.

**Gel fabrication and cell seeding**

Fibrinogen solution was prepared by dissolving 30mg of bovine fibrinogen (Sigma-Aldrich) in 5mL of PBS in a 37°C water bath for 2-3 hours. The solution was sterile filtered using a 0.2μm filter and stored at 4°C until use. iPS-ECs (CDI, WI) grown in Vasculife (Lifeline Cell Technology) growth media were lifted from the flasks using TrypLE 1X (Thermo Fisher Scientific, Waltham, MA). PCs and ACs were trypsinized for lifting. For the mono-culture model, iPS-ECs were resuspended at 10 million cells per mL in a solution of EGM-2 (Lonza, Basel, Switzerland) with thrombin (4 U/mL, Sigma-Aldrich). The cell suspension was then mixed in a 1:1 ratio with 10μL of 6mg/mL of fibrinogen to achieve a final seeding density of 5 million cells per mL when injected directly into the central gel channel of the device. In the co-culture model, iPS-ECs were resuspended at 20 million cells per mL, and PCs at 4 million cells per mL, in the EGM-2-thrombin solution, before being seeded directly into the central channel. The final concentrations for the co-culture model are 5 million cells per mL of iPS-ECs and 1 million cell per mL of PCs providing a 5-to-1 cell seeding ratio of ECs to PCs, similar to cell ratios quantified in freshly isolated mice brain capillaries. Finally, for the tri-culture model (BBB model with ECs, PCs and ACs), iPS-ECs were resuspended at 30 million cells per mL, PCs at 3 million cells per mL, and ACs at 6 million cells per mL for final seeding densities of 5 million cells per mL for the iPS-ECs, 0.5 million cells per mL for the PCs, and 1 million cells per mL for the ACs (Fig. 7B). The final seeding density of PCs was reduced in the tri-culture system (0.5 million cells per mL) when compared to the co-culture system (1 million cells per mL) to account for their increased propensity to proliferate in the fibrin gel in the presence of ACs. The seeded devices were placed in a Petri dish (which includes 5mL of water in a smaller dish for humidity) and left in the 37°C, 5% CO₂ incubator for 30 minutes until the fibrinogen gel cured completely. The different devices were filled with the same iPS-EC media, supplemented with 50ng/mL of VEGF-A for the first four days of culture (Peprotech, Rocky Hill, NJ), and placed back in the incubator at 37°C and 5% CO₂. VEGF-A was supplemented to promote vasculogenesis in the early stages of cell seeding in the microfluidic channel. From day 4 onward, Vasculife media (Lifeline Cell Technology) without any additional growth factors was used.
To perform permeability studies, a monolayer of iPS-ECs was seeded in the media channels of the device on Day 2 of the culture to prevent dye diffusion into the matrix. Briefly, after seeding the devices, the media channels were coated with 60μg/mL of human plasma fibronectin (EMDmillipore, Burlington, MA) and incubated at 37°C and 5% CO₂ for 30min. The fibronectin was then removed and iPS-ECs were perfused in one media channel side at 1.5 million cells per mL of EGM-2 (Lonza). The device was tilted to the side for 10 minutes to allow the cells to settle at the media-gel channel interface. This procedure was repeated with the other media channel side to allow for the formation a homogenous monolayer of ECs on both sides of the gel. The devices were incubated for 2 hours at 37°C and 5% CO₂ prior to changing the media and adding Vasculife (Lifeline Cell Technology) with 50ng/mL of VEGF-A (Peprotech).

2.2.3 Fluorescent imaging and analysis

To visualize microvascular network formation throughout the 7 days of culture, live devices were imaged using phase contrast microscopy (Nikon, Japan). Confocal microscopy (Olympus, Japan) was used to study and visualize end-point network morphology on day 7, at which point the devices were fixed with 4% PFA for 15 minutes and permeabilized with 0.01% Triton X-100 for 5 minutes. Devices were then blocked with 4% w/v BSA (Sigma-Aldrich) and 0.5% v/v goat serum (Gibco) in PBS overnight at 4°C. Following washing, primary antibodies were added at 1:200 in PBS and devices were incubated overnight at 4°C on a shaker. Following another wash, devices were incubated with secondary antibodies (Invitrogen, Carlsbad, CA) at 1:200 in PBS overnight at 4°C on a shaker. If needed, devices were also counterstained with Phalloidin (1:200, Invitrogen) for actin and DAPI (1:1000, Invitrogen) for nuclei. All fluorescent images were obtained via confocal microscopy and processed using the IMARIS imaging software (Bitplane, Belfast, Ireland).

2.2.4 Calculation of vessel permeability

To assess the permeability of the vasculatures formed, fluorescent tracers were perfused in the networks, based on a protocol described previously14. The media in all reservoirs was aspirated and the top reservoir on one side was injected with 5.7μL of PBS. The top reservoir on the other side was subsequently injected with 6μL of 0.4mg/mL 10kDa MW or 40kDa MW FITC-dextran (Sigma) in PBS. The volume difference on either side of the gel channel allowed for dextran convection and diffusion through the microvascular networks. The devices were then placed in the confocal microscope environmental chamber for imaging. Once equilibrium was established and the intensity inside the vessels (I_v) remained constant, fluorescent
images were captured every 3 minutes for 15 minutes. The permeability coefficient was calculated by obtaining the average intensity in a measured z-stack containing both vasculature and surrounding gel at the initial and final timepoints.

One ROI was imaged per device and n=6 devices per vasculature condition (mono-culture, co-culture or tri-culture) were selected for analysis. Prior to performing permeability studies, a monolayer of iPS-ECs was seeded in the media channels of the device, as mentioned above, to prevent diffusion of dextran into the gel matrix. Care was taken to select vessel segments in the central region of the gel channel, farthest away from the media-gel channel boundaries. Vessel permeability coefficients $P_v$ (cm/s) were computed as:

$$P_v = \frac{1}{(I_{v_{t2}} - I_{v_{t1}})} \frac{(I_{r_{t2}} - I_{r_{t1}})}{\Delta t} V A_{surface}$$

where $I_{v_{t1}}$ and $I_{v_{t2}}$ are the tissue intensities at initial ($t_1$) and final ($t_2$) timepoints, $I_{r_{t1}}$ is the initial vessel intensity, $\Delta t$ is the time interval between initial and final timepoints (seconds), $V$ is the vasculature volume in the region considered (cm$^3$), and $A_{surface}$ is the 3D surface area of the vessels (cm$^2$). All imaging was performed using a confocal microscope (Olympus) and timepoint images were analyzed using ImageJ (NIH, Bethesda, MD).

### 2.2.5 Microvascular network metrics

Several parameters were employed to quantify vascular network formation and stabilization. During the first seven days of culture until stabilization, the average vessel branch length, the number of branches per device and the average lateral diameter of the microvascular networks were computed in each culture condition (mono-, co-, and tri-culture). At day 7, once the networks had formed and were stable, the number of branches per vascularized area, the average transverse diameter, and the average cross-section area of the vessels were computed, in addition to the parameters listed above. For each metric quantified, n=15 devices were considered per culture condition.

A branch is defined as the portion of a vessel between two intersecting points (Fig. 8A depicts 7 branches). Since most vessels are oriented in a plane parallel to the glass substrate, lateral (parallel to the glass) and transverse (perpendicular to the glass) diameters can be computed. Lateral diameters are obtained as the ratio of the projected lateral vessel area to the total branch length per ROI. Transverse diameters are computed using the 3D vessel volume and the surface area of the vessels in 3D (Fig. 8B describes the difference between lateral and transverse diameters for each culture condition). During stabilization, images
were captured using phase contrast microscopy (Nikon, Japan) to compute the parameters listed above. At day 7, devices were fixed and stained, and z-stack images obtained via confocal microscopy (Olympus, Japan) were used to quantify the different vascular parameters with ImageJ (NIH, MD). The equations used to compute the diameters and average cross-section area of the vessels are listed below:

\[ A_{\text{lateral}} = D_{\text{lateral}} L_{\text{branch}} \]

\[ A_{\text{surface}} = \pi \sqrt{\frac{D_{\text{transverse}}^2 + D_{\text{lateral}}^2}{2}} L_{\text{branch}} \]

\[ V = \frac{\pi D_{\text{lateral}} D_{\text{transverse}} L_{\text{branch}}}{4} \]

Using the three equations above for 2D lateral and 3D surface areas, as well as 3D volume, the lateral and transverse diameters, as well as the cross-section area can be computed as described below:

\[ D_{\text{lateral}} = \frac{A_{\text{lateral}}}{L_{\text{branch}}} \]

\[ D_{\text{transverse}} = \sqrt{\frac{D_{\text{lateral}}^2}{A_{\text{surface}}^2 D_{\text{lateral}}^2 - 1}} \]

\[ A_{\text{cross-section}} = \frac{\pi D_{\text{transverse}} D_{\text{lateral}}}{4} \]

**Figure 8. Microvascular network quantification metrics.** (A) Depiction of a vessel skeleton containing 7 branches. (B) Schematic of an elliptical vessel cross-section where the major axis (horizontal) refers to the lateral diameter and the minor axis (vertical) refers to the transverse diameter.
2.3 Results

2.3.1 Vessel formation and stability

Three different platforms were employed for the study of BBB-like microvascular network formation. In the mono-culture model, iPS-ECs alone reorganized into microvascular networks as early as 5 days after seeding, and remained stable until day 7. Networks were fixed and stained on day 7 with anti-CD31 for iPS-ECs, anti-platelet derived growth factor receptor (PDGFR)-β for PCs, and anti-glial fibrillary acidic protein (GFAP) for ACs (Abcam, MA). Image analysis reveals networks spanning over the entire gel region with wide lumens and elliptical vessel cross-sections (Fig. 9A). In the co-culture model, iPS-ECs formed stable and perfusable microvascular networks on day 7, while PCs spontaneously wrapped around the vessels on their abluminal side, as they would in the brain in vivo. Confocal imaging shows a high degree of branching and narrower lumens when compared to the mono-culture case (Fig. 9B). Via image analysis, it can be observed that the microvascular networks formed in the tri-culture platform possess a BBB-like morphology, similar to the one shown in Fig. 1. iPS-ECs spontaneously reorganize into highly interconnected vasculatures with the narrowest lumens of all three culture conditions. PCs coat the abluminal side of the vessels, while ACs are observed to stretch their end-feet to touch several branches of the networks (Fig. 9C).
Live, daily tracking of the same devices for each culture condition was performed using phase contrast microscopy (Nikon, n=15 devices for each culture condition). In the first 6 days of culture, the average branch length steadily increased in all three conditions from 80μm on day 2 to ~160-170μm on day 6. However, on day 7 in the case of the co-culture and tri-culture conditions, the average branch length remained constant at ~160-170μm, while that of the mono-culture condition kept increasing to 210μm. At the same time, the average number of branches per device decreased over time. At day 6, this number of branches stabilized for the co-culture and tri-culture conditions and remained equal to an average of ~60 branches per device at day 7. This was not observed in the mono-culture condition, where the average number of branches per device kept decreasing from day 6 to day 7, reaching a final average value of 30 branches per device. These observations suggest that PCs and ACs, when co-cultured with iPS-ECs, can promote increased vascular branching, while reducing branch length, to achieve a more stable and physiologically relevant vasculature. Finally, the average lateral diameter was computed using live devices as well. The tri-culture condition exhibited the lowest average lateral diameter of all conditions (~35μm), followed by the co-culture condition with an average lateral diameter of 60μm. The mono-culture platforms exhibited very large lumens with an average lateral diameter of 125μm. This confirms that both PCs and ACs are required to achieve capillary-like vasculatures with narrower lumens (Fig. 10).
Figure 10. Vessel stability over time. (A) Average branch length increases in all three culture conditions and stabilizes at day 6 for the co- and tri-culture systems. It increases further in the mono-culture condition until day 7. (B) The average number of branches per device decreases in all conditions and stabilizes at day 6 for the co- and tri-culture models. It decreases further in the mono-culture model until day 7. (C) The tri-culture condition exhibits the lowest average lateral diameter, followed by the co-culture condition and mono-culture condition with the highest lateral diameter. (D-F) Phase contrast microscopy images of networks in the mono-, co-, and tri-culture systems respectively, at days 3, 5 and 7. Lateral diameters are shown to increase over time, at a faster and higher rate for the mono-culture system compared to the tri-culture one. Average number of branches decreases over time. These observations all suggest higher interconnectivity and branching, as well as narrower vessels in the tri-culture model. The vasculatures formed with PCs and ACs are more physiological in terms of morphology.

2.3.2 Microvascular network metrics and morphology

At day 7, once vessel stability was reached in most culture conditions, devices were fixed and stained, and z-stack images were obtained using confocal microscopy (Olympus). The stacks were analyzed using ImageJ (NIH) to compute the average branch length, the number of branches per vascularized area, the
average lateral and transverse diameters, as well as the average cross-section area in all three conditions at day 7. The average branch length decreased significantly as the PCs were added, and further when both PCs and ACs were seeded with the iPS-ECs (Fig. 11A). On the other hand, the number of branches per vascularized area increased significantly with the addition of PCs and ACs at day 7, confirming increased branching and vessel interconnectivity in the presence of the two brain stromal cells (Fig. 11B). In addition, the average lateral diameter was significantly smaller in the tri-culture system (~60μm), when compared to the co-culture system (~90μm) and the mono-culture system (~140μm). However, when computing the average transverse diameters in the three culture conditions, it was observed that these averages were not statistically significantly different when the PCs were added alone or with ACs to the iPS-ECs in the gel (~40μm for all three models, Fig. 11C). These results suggest that vessel cross-sections go from an elliptical morphology in the mono-culture model, where the major ellipse axis is parallel to the glass to a more circular morphology in the tri-culture system where the average lateral diameter approaches the transverse one. Finally, vessel cross-sections are significantly reduced with the addition of PCs alone and further with the addition of PCs and ACs, indicating narrower lumens and more capillary-like vasculatures in the tri-culture model (Fig. 11D).
Figure 11. Microvascular network metrics at day 7 in culture. (A) Average branch length decreased when PCs and ACs are added to the culture. (B) The number of branches per vascularized area increased significantly in the tri-culture model, indicating increased branching and junctions. (C) The average lateral diameter is reduced with the addition of PCs and ACs. However, the transverse diameter remains constant in all three culture conditions, indicating a shift towards more circular vascular networks with the PCs and ACs. (D) The average cross-section area is also significantly reduced with the addition of PCs and ACs. (E) Cross-section images of vascular networks in the three different culture conditions (iPS-ECs alone, iPS-ECs + PCs, and iPS-ECs + PCs + ACs). The horizontal arrows represent the lateral (parallel to the glass) diameters, and the vertical arrows represent the transverse (perpendicular to the glass) diameters.

Confocal z-stack images were reconstituted in 3D to observe vessel morphology, prior to fixing on day 7 and staining iPS-ECs with anti-CD31 in the three different culture conditions. Using Imaris, vasculatures formed in the mono-culture system exhibited poor branching and low number of junctions. The vessels covered most of the gel channel volume and their cross-sections were all elliptical (Fig. 12A). The co-culture model exhibited more interconnected vascular branches with a higher number of junctions. The cross-section areas of the vessels were more circular than the ones in the mono-culture model, and vasculature coverage of the gel channel volume was reduced (Fig. 12B). The tri-culture vessels exhibited a morphology closest to that of capillaries. The number of branches per vasculature area was the highest achieved, and networks were highly interconnected. The lumens were the narrowest and most circular in cross-section (Fig. 12C).
Figure 12. Morphological changes with the addition of PCs and ACs. (A) Mono-culture networks exhibit large, elliptical lumens and poor vascular interconnectivity. The vessels cover most of the gel volume. (B) The addition of PCs leads to smaller and more circular lumens. Interconnectivity is improved and the vasculatures exhibit a higher number of junctions and branches. (C) Tri-culture networks have the most capillary-like structure. Branching is increased and lumen size is decreased with the addition of both PCs and ACs. Lumens adopt a more circular cross-section and vasculatures cover a lower percentage of the gel channel.

2.3.3 Vessel permeability measurements

To examine and quantify barrier tightness in the different culture conditions, the vasculatures were perfused with two different sizes of FITC-Dextran (10kDa and 40kDa). In all three conditions, the vasculatures had patent, perfusable lumens, devoid of localized leaks (Fig. 13A). With both solute sizes, barrier permeability decreased significantly with the addition of PCs, and even further with the addition of both PCs and ACs. When perfused with 10kDa dextran, diffusive permeability decreased from \((9.22 \pm 1.88) \times 10^{-7}\) cm/s (mean \(\pm\) SD) in the mono-culture system, to \((5.57 \pm 1.21) \times 10^{-7}\) cm/s in the co-culture system, finally reaching a permeability value of \((3.42 \pm 1.26) \times 10^{-7}\) cm/s in the tri-culture system (Fig. 13B). Similarly, when perfused with 40kDa dextran, barrier permeability dropped from \((7.06 \pm 2.07) \times 10^{-7}\) cm/s in the mono-culture model, to \((3.59 \pm 0.29) \times 10^{-7}\) cm/s in the co-culture model, reaching a final value of \((2.74 \pm 0.20) \times 10^{-7}\) cm/s for vasculatures of iPSC-ECs with PCs and ACs (Fig. 13C). The permeability computed using 40kDa dextran were collectively lower than the ones computed with 10kDa dextran, due to the larger size of solute crossing the vessel walls, and its lower speed. The permeability values reported were measured for vasculatures on day 7 and for \(n=6\) devices in each culture condition. These reported values are similar to permeability values obtained in rat cerebral microvessels, both for the 10kDa and 40kDa dextran solutes. In rat, \((3.1 \pm 1.3) \times 10^{-7}\) cm/s was measured for 10kDa dextran for \(n=7\) rats considered (\(p=0.701\) when compared with our in vitro tri-culture BBB model permeability values for 10kDa dextran),

32
and \((1.9 \pm 1.1) \times 10^{-7}\) cm/s was quantified for 40kDa dextran for \(n=10\) rats considered \((p=0.228\) when compared with our \textit{in vitro} tri-culture BBB model values for the same dextran molecular weight\). These results confirm the physiological relevance of our model and reveal the barrier tightening properties of PCs and ACs.

![A] Mono-culture

![B] Co-culture

![C] Tri-culture

**Figure 13. Permeability measurements in the three culture conditions.** (A) Perfusion of mono-culture, co-culture and tri-culture vessels with 40kDa dextran reveals patent lumens void of localized leaks. (B) Average permeability values computed in each culture condition when vessels were perfused with 10kDa FITC-dextran. (C) Average permeability values computed in each culture condition when vessels were perfused with 40kDa FITC-dextran. Data in graphs is represented as mean ± SEM, with \(n=6\) total devices over 3 experiments for each culture condition. Statistical significance was tested with student's t-test (*\(p<0.05\), **\(p<0.01\), ***\(p<0.001\)).

### 2.3.4 Tight junction protein expression and basement membrane deposition

TJs are strongly expressed at the border of adjacent brain ECs. These elaborate junctional complexes impose additional barriers to transport, forcing most solutes to cross the BBB via transcellular routes, rather than paracellular ones. To verify the presence of tight cell-cell junctions, we examined the expression of ZO-1 and Claudin 5 TJ proteins (1:50, Abcam) in all three culture conditions. Both proteins were expressed...
by the iPS-ECs lining the vessels in each condition. The fluorescent intensity of both ZO-1 and Claudin 5 was amplified as the PCs and ACs were progressively added to the culture. The tri-culture platform exhibited a strong ZO-1 fluorescent signal at the borders of adjacent ECs when compared with the co- and mono-culture systems. This increase in co-localization with the cell-cell borders was not as pronounced in the case of Claudin 5 (Fig. 14).

Figure 14. Tight junction protein expression. (A) ZO-1 is detected in all three culture conditions with increased expression as PCs and ACs are progressively added to the culture. In the tri-culture system, ZO-1 is more localized to the borders of adjacent endothelial cells and its expression is enhanced as shown by the increased in fluorescent intensity. (B) Similarly, Claudin-5 is expressed in all three platforms but its fluorescent intensity is improved in the presence of PCs and ACs. Scale bars are 30µm.

Basement membrane deposition is indicative of vessel maturation, when the ECs are able to secrete their own matrix to support the vasculature. To investigate this aspect of vessel stability, the expression of laminin, collagen IV (Abcam) and heparan sulfate proteoglycan 2 (HSPG, Santa Cruz Biotechnology, Dallas, TX) basement membrane proteins was examined via immunostaining in each culture condition. All platforms expressed each one of the three basement membrane proteins investigated. Although further protein analysis is needed, the fluorescent intensity of the three basement membrane proteins was observed to increase as the PCs and ACs were progressively added to the platform. The tri-culture platforms displayed the highest fluorescent intensities of basement membrane proteins (Fig. 15, Tri-culture images).
for Collagen IV, Laminin and HSPG2). This suggests that PCs and ACs play an important role in promoting proper basement membrane deposition around the vasculature.

Figure 15. Basement membrane deposition in three culture conditions. (A) Collagen IV is detected in the perivascular extracellular matrix surrounding the vessels for the mono-, co- and tri-culture models. (B) Laminin is also observed in the extracellular space surrounding the vessels in the three platforms employed. (C) HSPG2 is detected in the extracellular matrix surrounding the vessels for the mono-, co- and tri-culture platforms used. The fluorescent intensities of all three basement membrane proteins is amplified as the PCs and ACs are progressively added to the system. The tri-culture condition exhibited the highest fluorescent intensities for all basement membrane proteins studied. Scale bars are 100µm.
2.4 Discussion

The decrease in average branch length and increase in number of branches per vascularized area with the addition of PCs and ACs are both indicative of greater vascular branching and interconnectivity. Moreover, the average lateral diameter was significantly reduced with the addition of PCs and ACs to the culture, although the average transverse diameter remained constant in the three culture conditions. This suggests that the shape of the vessel cross-sections went from being elliptical, in the mono-culture system, to adopting a more circular morphology and reduced cross-section area with PCs and ACs, indicating that vessels are more stable and capillary-like. The BBB properties of the vasculatures formed in the tri-culture system were also confirmed via permeability studies, indicating significant barrier tightening with the addition of PCs and ACs, for both 10kDa and 40kDa dextran perfusion studies. Permeability values obtained were similar to measurements reported in rat brains for the same dextran sizes, further validating the physiological relevance of our model[87]. The increase in barrier tightness was also confirmed via immunostaining. All endothelial barriers expressed ZO-1 and Claudin5, however, it could be observed that the fluorescent intensity of both TJ proteins was increased in the tri-culture model. ZO-1 fluorescent intensity was also observed to be more localized at the adjacent EC borders in the tri-culture model when compared to the co- and mono-culture platforms. This confirms that the presence of PCs and ACs in the culture enhances TJ protein expression. Finally, basement membrane deposition, indicative of vascular stability and maturation, was assessed in the three models used. While laminin, collagen IV and HSPG2 were expressed in all three platforms, the tri-culture system exhibited the highest fluorescent intensity representative of these proteins, suggesting that PCs and ACs strongly contribute to basement membrane deposition.

In summary, the tri-culture platform developed with iPS-ECs, PCs and ACs is highly relevant and representative of a BBB model on-a-chip. Vessel stability over time, vascular morphology, permeability, TJ expression and basement membrane deposition were all employed as metrics to assess the platform. These parameters converged to indicate high physiological relevance of the tri-culture system as a 3D vascularized BBB model on-a-chip that can be further employed for several assays, including disease modeling and drug delivery studies.
Chapter 3: Tumor extravasation at the brain from *in vitro* blood-brain barrier microvascular networks

3.1 Introduction

The spread of metastasis is characterized by the invasion of surrounding tissues at the primary cancer site, followed by intravasation into adjacent vessels, circulation of CTCs in the bloodstream, extravasation from the vasculature at the secondary site and proliferation. Although extravasation has been studied in animal models and generic *in vitro* platforms (Transwell assays or HUVEC microvascular networks), the precise mechanisms and cellular interactions governing extravasation at the brain remain poorly understood. The elevated incidence of brain metastases in cancer patients (20-40%), coupled with low survival rates post-diagnosis (30% survival rate five years post-diagnosis), shed light on the tremendous need for high-resolution assays to elucidate the mechanisms and cellular interactions characteristic of tumor cell extravasation at the brain.

Currently, most brain metastasis studies are performed in low-resolution and low-throughput *in vivo* systems, focusing on endpoint data collection rather than cellular interactions and mechanisms employed by tumor cells to cross the barrier. State-of-the-art *in vitro* brain extravasation assays rely on the use of monolayer systems where ECs are either plated on a 2D cell culture insert or coat the inner membrane of a hollow 3D channel. While these models allow for high-throughput cell migration studies in platforms employing human cells, they fail to accurately replicate the morphology and features of brain vasculatures, as well as their effect on tumor cell extravasation. The use of precisely controlled and physiologically relevant *in vitro* human BBB self-assembled vasculatures for extravasation studies would provide tremendous insight into the effects of the different brain stromal cells on CTCs, as well as the time-course of extravasating tumor cells with different metastatic abilities at the brain.

In this chapter, we present a microfluidic platform used to model the entire process of tumor cell extravasation at the brain from within *in vitro* microvascular networks assembled via vasculogenesis. Our platform offers several advantages over existing *in vitro* extravasation assays:

a. Increased physiological relevance compared to planar *in vitro* BBB extravasation assays and generic 3D vascular networks formed with HUVECs
b. Ability to perform parametric studies with high-resolution imaging in a controlled and high-throughput microenvironment
We employ our model to determine the effect of both PCs and ACs on tumor cell extravasation efficiency of different tumor cell lines. We demonstrate the effect of tumor cell extravasation on barrier permeability in our platform, and the extravasation capabilities of metastasized tumor cells collected from different organs. Observations from our model result in a deeper understanding of tumor cell extravasation patterns in the presence or absence of brain pericytes and astrocytes, suggesting that our assay could be employed to discover elements that play a key role in promoting extravasation at the brain.

3.2 Methods

3.2.1 Blood-brain barrier microvascular network formation and maintenance

iPS-ECs alone, iPS-ECs with PCs, and iPS-ECs with PCs and ACs were suspended in a fibrin gel, seeded and cured in the central gel region of the device to generate the three in vitro vascular platform discussed in Chapter 2. The mono-culture model was employed as a control platform to perform studies without the addition of any brain stromal cells. The co-culture and tri-culture systems were employed to study the effect of the successive addition of brain PCs and brain ACs on microvascular network formation and tumor cell extravasation.

3.2.2 Tumor cell perfusion in the microvascular networks

MDA-MB-231, A549 (ATCC, VA), MDA-MB-231-BrM2 and MDA-MB-231-LM2 (Massague lab), all cultured in DMEM (Sigma, MA) supplemented with 10% FBS and 1% Pen-Strep (Invitrogen), were trypsinized and resuspended at a final concentration of 17,000 cells per mL in Vasculife (Lifeline Cell Technology). Media from the two reservoir channels lining the central gel channel were aspirated and 60μL of the tumor cell suspension was introduced into one of the reservoirs, leaving the reservoir on the opposite side of the gel channel empty to create a 5.0 mmH₂O pressure drop across the central gel channel. This pressure drop draws the tumor cells into the vasculature. Following perfusion, devices were incubated at 37°C and 5% CO₂ for 10 minutes to equilibrate and for tumor cells to adhere to the vessel walls or be entrapped. Fresh media was then added via a pressure drop in the same direction in which the tumor cells were perfused to wash off any non-adherent cells.
3.2.3 Tumor cell extravasation quantification

MDA-MB-231, A549, MDA-MB-231-BrM2 and MDA-MB-231-LM2 were transduced to stably express cytoplasmic fluorescence (GFP for all MDA-MB-231 cells and their metastatic variants, and mCherry for A549). Following perfusion into the three different platforms (mono-, co- and tri-culture), devices were subsequently fixed and stained at three different time points (t=6h, t=12h and t=24h post-perfusion, n=6 devices per time point per platform) to observe and quantify extravasation, using the immunostaining protocol described in Chapter 2. iPS-ECs were stained with anti-CD31 (Abcam) to visualize the position of the tumor cell with respect to the endothelial barrier. Devices were then imaged at 20X using confocal microscopy (Olympus) to obtain at least 6 z-stack ROIs.

Using the IMARIS software, z-stack images were analyzed and only fully transmigrated cells with cell body found on the abluminal side of the vessels were counted as an extravasation event. All ROIs were selected in the central region of the gel channel to avoid edge effects at the boundary between gel and media channels. Care was taken to analyze single cells entrapped or adhered to the microvessel walls, rather than tumor cell clusters. Extravasation efficiency was computed as the ratio of extravasated cells to total number of cells found in each ROI considered. A minimum of 6 ROIs per device were analyzed to compute extravasation efficiencies. The dimensions of each ROI were 800µm by 800µm by 150µm.

3.2.4 Barrier permeability measurements following tumor cell extravasation

To assess the permeability of the vasculature following tumor cell perfusion, a similar protocol as the one described in section 2.2.4 was employed. MDA-MB-231 were perfused in the vasculatures formed in the three platforms used, as described in the section above, and allowed to extravasate for 24 hours. In parallel, separate devices of each culture condition were left without tumor cells. At t=24 hours, permeability was measured in the control platforms without any tumor cells and in the platforms with tumor cells, using two sizes of FITC-dextran (Sigma) in PBS (10kDa and 40kDa Dextran). The measurements and analyses were performed similarly as described in section 2.2.4, with one ROI imaged per device and n=6 devices per vasculature condition (mono-culture, co-culture or tri-culture) selected for analysis.
3.3 Results

3.3.1 Tumor cell perfusion and extravasation in the BBB microvascular networks

MDA-MB-231 and A549 tumor cells from the breast and lung, respectively, were chosen in this assay given their established propensity to form metastases at the brain in vivo (Fig. 3)\textsuperscript{37,88,89}. After varying tumor cell densities (from 17,000 cells per mL to 500,000 cells per mL), we selected a perfusion cell density of 17,000 tumor cells per mL, to prevent tumor cells from aggregating into clumps and clogging the microvascular networks, and to obtain comparable densities as CTC densities found in the brain in vivo\textsuperscript{42}. Tumor cells were seeded in the microvasculature by applying a 5.0 mmH\textsubscript{2}O pressure drop across the central gel channel. Fixed samples at different time points (t=6 hours, t=12 hours and t=24 hours) revealed the location and morphology of the perfused tumor cells and were used to quantify extravasation efficiencies in the different platforms perfused (Fig. 16).

![Image A](image1.png)
![Image B](image2.png)
![Image C](image3.png)

Figure 16. Visualization of MDA-MB-231 and A549 extravasation events. Different positions of tumor cells relative to the endothelium in the three different culture conditions. (A) Circular A549 cell inside the lumen extending its protrusions to cross the barrier and reach the extracellular matrix in the mono-culture system. ECs are stained with anti-CD31 (green) and A549 were stably transduced to express cytoplasmic fluorescence (white) (B) MDA-MB-231 transmigrated and spread outside of the lumens in the co-culture platform. ECs are stained with anti-CD31 (blue), PCs and MDA-MB-231 were stably transduced to express cytoplasmic fluorescence (red for PCs and green for MDA-MB-231). (C) Circular MDA-MB-231 intravascularly trapped in a vessel branch in the tri-culture system. ECs are stained with anti-CD31 (blue), and MDA-MB-231 were stably transduced to express cytoplasmic fluorescence (green). PCs and ACs are present on the abluminal side of the vessels but are not fluorescently labeled here. Scale bars are 50\textmu m.

The extravasation efficiencies of MDA-MB-231 and A549 were quantified in the three different platforms employed (mono-, co- and tri-culture) and at three different time points (t=6 hours, t=12 hours, and t=24 hours). 6 hours following tumor cell perfusion, the extravasation efficiency of MDA-MB-231 was found to be significantly higher in the tri-culture system (31.85 ± 1.62 %), when compared to the co- and mono-culture systems (28.97 ± 0.90 % and 24.06 ± 2.31 %, respectively). At the 12 hour time point, the differences in extravasation efficiencies of MDA-MB-231 were not statistically significantly different
across the three platforms employed. However, at t=24 hours post-perfusion, both the tri-culture and co-culture assays exhibited increased extravasation efficiencies when compared to the mono-culture assay with 43.82 ± 1.51 % for the tri-culture system, 43.02 ± 3.83 % for the co-culture system and 33.82 ± 1.08 % for the mono-culture system (Fig. 17A-C). The number of extravasated cells was observed to plateau at around t=12 hours post-perfusion for the mono-culture assay alone, with no significant difference in the number of extravasated cells at t=24 hours (p-value=0.276). This number increased significantly from t=12 hours to t=24 hours for both the co- and tri-culture assays, with a higher fold change in extravasation from 12 to 24 hours in the co-culture model (1.23 for co-culture and 1.18 for tri-culture, Fig. 17D). These results suggest that most extravasation events happen in the first 12 hours in the vasculature for the mono-culture system without any stromal cells. The addition of PCs and ACs has a significant impact on the extravasation time-course of tumor cells from within the vasculature.

![Figure 17. Extravasation efficiencies of MDA-MB-231 over time.](image)

(A) Extravasation efficiency in the three different platforms at the t=6-hour time point. (B) Extravasation efficiency in the three different platforms t=12-hours post-perfusion. (C) Extravasation efficiency in the three different platforms at the t=24-hour time point. (D) Time course of extravasation efficiencies of MDA-MB-231 across all platforms and all time points. Data in the graphs is presented as mean ± SEM. Statistical significance was tested with student's t-test (n=6 per time point per culture condition, **p<0.01, ***p<0.001, ****p<0.0001).
In the case of the non-small-cell lung cancer cell line A549, the computed extravasation efficiency trends were similar to the ones observed for MDA-MB-231 at the t=6-hour and t=12-hour time points only (26.39 ± 1.47 % for the mono-culture model, 34.08 ± 3.76 % for the co-culture model, and 35.31 ± 0.89 % for the tri-culture at 6-hours, no significant differences in extravasation at t=12 hours). At t=24 hours, extravasation efficiencies were significantly higher for the tri-culture system (41.89 ± 0.22 %) when compared to the mono-culture system (38.49 ± 0.91 %). However, no significant differences were observed between the co-culture system and both the mono- and tri-culture systems at t=24 hours (Fig. 18). These results confirm the different extravasation ability of the breast MDA-MB-231 and lung A549, but also suggest differences related to their interactions with the different brain stromal cells incorporated in the platform.

Figure 18. Extravasation efficiencies of A549 over time. (A) Extravasation efficiency in the three different platforms at the t=6-hour time point. (B) Extravasation efficiency in the three different platforms t=12 hours post-perfusion. (C) Extravasation efficiency in the three different platforms at the t=24-hour time point. (D) Time course of extravasation efficiencies of A549 across all platforms and all time points. Data in the graphs is presented as mean ± SEM. Statistical significance was tested with student's t-test (n=6 per time point per culture condition, *p<0.05, ***p<0.001).
3.3.2 Extravasation potential of metastasized tumor cells collected from different organs

To further validate our BBB platform and study the extravasation potentials of metastasized tumor cells collected from different organs, we employed MDA-MB-231-BrM2 collected from the brain and MDA-MB-231-LM2 collected from the lung in our assay. Briefly, these cells were obtained from the parental MDA-MB-231 cell line by performing two rounds of *in vivo* selection in mice, to the brain (MDA-MB-231-BrM2) and to the lung (MDA-MB-231-LM2)\(^37\). The extravasation efficiency of the brain metastatic breast tumor derivative (MDA-BrM for brevity) was measured in the three culture conditions considered at three different time points, and directly compared to the extravasation efficiencies of the parental line and the lung derivative (MDA-Parental and MDA-LM for brevity). The lung metastatic breast tumor derivative was employed as a negative control to validate the organ-specificity of our BBB platform.

In the mono-culture platform, the extravasation efficiencies of the parental line were significantly lower than the ones recorded for the lung and brain metastatic MDA-MB-231 derivatives for all time points considered (Fig. 19A, D). This result, in the generic mono-culture system, is in agreement with the fact that the lung and brain lines have a stronger metastatic potential due to the two rounds of *in vivo* selection performed to obtain these cell lines. In both the co-culture and tri-culture platforms, only the MDA-BrM extravasation efficiencies were significantly higher than the parental and lung lines for all time points considered (Fig. 19B, D). At t=24hours post-perfusion, the fold change of extravasation efficiency compared to the MDA-Parental cell line went from 1.39 ± 0.06 for MDA-BrM and 1.33 ± 0.06 for MDA-LM in the mono-culture system, to 1.201 ± 0.06 for MDA-BrM and 1.03 ± 0.04 for MDA-LM, suggesting that only the extravasation efficiency of the brain metastatic cells was increased by the addition of pericytes and astrocytes in the tri-culture system. The lung metastatic derivative showed no significant difference in extravasation efficiency when compared to the parental cell line in the co- and tri-culture platforms (p=0.786 and p=0.335 respectively, at t=24hours), suggesting that the addition of PCs and ACs in the culture did not have a significant effect on the lung metastatic derivative when compared to the parental line.
3.3.3 Effect of tumor cell extravasation on barrier permeability

It has been suggested that the endothelial barrier at the brain is altered following tumor cell extravasation\textsuperscript{27,90}. However, most of these alterations occur several days post-extravasation \textit{in vivo}, when tumor cells have proliferated and colonized the brain tissue to form a secondary tumor\textsuperscript{79,91}. In order to further investigate the role of tumor cells in altering barrier permeability at the brain, and to determine whether impaired barrier function plays a role in tumor cell extravasation, permeability measurements were obtained in our three culture systems with and without tumor cells, at t=24hours post-perfusion.

Barrier tightness was quantified 24 hours following the introduction of MDA-MB-231 breast tumor cells in the vasculature. Vessel permeability of separate devices without tumor cells was assessed in parallel at the same time point, for both 10kDa and 40kDa FITC-dextran. For all three culture conditions considered and for both solute sizes used in this assay, the permeability values recorded were not statistically significantly different with and without tumor cells (Fig. 20). For instance, when 40kDa dextran was perfused in the microvascular networks, the permeability value of $(3.56 \pm 1.80) \times 10^{-7}$ cm/s, recorded in the
tri-culture system with tumor cells, was not significantly different from $(2.74 \pm 2.00) \times 10^{-7}$ cm/s, recorded without tumor cells ($p=0.472$). Similarly, in the case of 10kDa dextran, permeability of the barrier in the BBB tri-culture model with tumor cells ($(3.88 \pm 1.57) \times 10^{-7}$ cm/s) was not statistically significantly different the permeability without tumor cells at the same time point ($(3.42 \pm 1.26) \times 10^{-7}$ cm/s) with a $p$-value of 0.573.

Figure 20. Effect of tumor cell extravasation on barrier permeability. (A) Average permeability values computed in each culture condition when vessels were perfused with 40kDa FITC-dextran. For the conditions with tumor cells, permeability was quantified at $t=24$ hours post-perfusion. Permeability was quantified at the same time point for the conditions without tumor cells. (B) Average permeability values computed in each culture condition when vessels were perfused with 10kDa FITC-dextran. For the conditions with tumor cells, permeability was quantified at $t=24$ hours post-perfusion. Permeability was quantified at the same time point for the conditions without tumor cells. Data is represented as mean ± SEM, with $n=6$ total devices over 3 experiments for each condition. Statistical significance was tested with student’s $t$-test (ns=non-significant).
The lack of significant differences in barrier permeability with and without tumor cells for the first 24 hours of perfusion, followed by tumor cell invasion, suggests that the initial step of extravasation does not rely on or promote barrier impairment. The initial extravasation of tumor cells at the brain must rely on different mechanisms of action, cell-cell interactions and signaling to take place.

3.4 Discussion

Our tri-culture BBB extravasation platform draws on the advantages of using a microfluidic system since it allows for greater control of the tumor microenvironment and the parameters of study, as well as greater throughput and high imaging resolution. In addition, the platform presented provides increased physiological relevance with the incorporation and spatial re-organization of PCs and ACs around a 3D self-assembled vascular network formed with iPS-ECs. The ability to progressively add brain stromal cells to the platform provided useful insight into the contributions of PCs and ACs to barrier function, vascular morphology, TJ and basement membrane protein expression, as well as extravasation potentials of different tumor cell lines. The increase in extravasation efficiencies of breast MDA-MB-231 and lung A549 suggested a role for PCs and ACs in promoting tumor cell invasion, despite their measured contributions in tightening the endothelial barrier in our model.

Although certain genes associated with brain metastatic breast tumor cells, namely ST6GALNAC5, have been identified to promote invasion across the BBB in mice, the role of these genes in relation to PCs and ACs in the specific step of extravasation across the human BBB remains unclear. It has been showed that the brain metastatic derivative of breast MDA-MB-231, as well as the lung metastatic MDA-LM transduced with ST6GALNAC5, exhibit increased trans-BBB migration relative to the respective parental lines across a monolayer of human brain primary endothelial cells\textsuperscript{37}. Here, we measured increased extravasation efficiencies of the brain metastatic derivative MDA-BrM with respect to the parental MDA-MB-231 lines across 3D vascularized networks with the addition of PCs and ACs, while the efficiencies of the lung derivative MDA-LM remained non-significant with respect to the parental breast line in the same tri-culture system. In the simple 3D vascular system with iPS-ECs alone, the extravasation measurements were not significantly different for any of the tumor cell lines considered. These findings validate the brain-specificity of our platform, but more importantly shed light on the significance of these genes, in relation with PCs and ACs, in promoting tumor cell extravasation across 3D BBB vasculatures in the metastatic cascade.

It has been reported that barrier function at the brain is impaired following extravasation and tumor cell colonization\textsuperscript{37,87,90,91}. While most of these changes in permeability occur several day following the onset of
brain metastatic lesion, we investigated the correlations between barrier tightness and tumor cell extravasation at the brain. At t=24hours post-tumor cell perfusion, barrier permeability was not significantly altered when compared to the control platforms for all three culture conditions. These results reveal that the initial step of extravasation is not dependent on changes in barrier function, with and without PCs and ACs in the platform, suggesting that alterations in BBB permeability are not a significant when it comes to promoting tumor cell extravasation.
Chapter 4: Conclusions

We have presented a BBB microfluidic extravasation assay consisting of self-assembled 3D vascular networks formed with iPS-ECs, PCs wrapped around the vasculature and ACs extending their end-feet to the abluminal side of the vessels. This model allows for the study of tumor cell extravasation across BBB vasculatures employing human cell lines. We demonstrate the physiological relevance of our system in terms of vascular stability and morphology, barrier permeability compared to values from *in vivo* rat BBB, as well as TJ and basement membrane protein expression and deposition. We determine, for the first time, the extravasation potentials of breast and lung tumor cell lines in a highly physiologically relevant *in vitro* platform employing only human cell lines self-organized in 3D BBB-like vasculatures. Our assay is further validated by the characterization of extravasation efficiencies of brain and lung metastatic breast tumor cells with respect to the parental line. Barrier function is also revealed to remain unaltered by tumor cell extravasation across our BBB model, providing useful insight into the relevant mechanistic questions to be addressed and answered regarding the brain as a secondary metastatic site. In the future, by transducing the relevant cell types in this model to express stable fluorescence, this platform could be employed to characterize the physical interactions between brain stromal cells and tumor cells in real-time. Moreover, by blocking the expression of certain proteins of interest, greater insight can be obtained on the molecular alterations or activated signaling pathways that play a defining role in tumor cell extravasation at the brain in the presence of pericytes and astrocytes. This model thus provides a robust platform to perform high-throughput and high-resolution parametric studies in a highly-controlled and physiologically relevant assay where the influence of different factors can be segregated and individually analyzed to obtain useful insight into the complex tumor cell-brain stromal cell interactions occurring during extravasation across the BBB.
References


