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Nanostructured Materials as Models for Cell Motility and Metastasis

Daniela Kalafatovic, PhD and Rein V Ulijn, PhD
Advanced Science Research Center (ASRC)
City University of New York, New York, NY 10031

Introduction

Metastasis, i.e. cancer cells migrating from the primary tumor to a distant site in the body, where secondary tumors develop, is a major contributor to mortality[1]. Despite progress, many questions remain unresolved regarding the mechanisms involved. It is now clear that it is not just the cells, but also their environment - and in particular the dynamic interplay between them - that dictates whether metastasis is likely to occur. Thus, there is a need for well-defined model systems that enable determinants of metastasis to be studied systematically. We summarize recent breakthroughs and future opportunities for nanostructured materials to contribute to this area.

Metastasis, adhesion and migration

Stages of the development of metastases (**Figure 1**) can be summarized as follows: (1) detachment of cancer cells form the primary tumor by reduced adhesion to neighboring cells; (2) invasion through surrounding tissues by clearing the path to allow cell migration; (3) intravasation of cells through the vasculature to enter the bloodstream and remaining in circulation under flow; (4) attachment to endothelial tissue and subsequent extravasation to the secondary site; (5) proliferation and establishment of secondary tumor[2]. Changes in interactions of cells with their environment, typically adhesion and migration, are critical at every step. Adhesion in this context can refer to cell-cell and/or cell-matrix (ECM) interactions. Migration for our purpose can be either adhesion-dependent or -independent, and may involve active matrix degradation by cell-secreted or cell-surface expressed enzymes- typically matrix metalloproteases (MMPs). Interestingly, there is a substantial body of literature focused on the use of model systems to show how biochemical, mechanical and topographical signals in the cell's environment (typically focusing on stem cells[3]) influence cell fate. The development of exactly such in vitro model systems is now gaining pace for cancer metastasis research.

Designed 3D matrices as model systems to study metastasis

Designed nanostructured materials with precisely tunable properties that mimic aspects of the extracellular environment have the potential to lead us to a better understanding of the role that the tumor microenvironment plays in triggering metastasis[4]. It is now well established that 3D models are more relevant to mimic the tumor/metastasis microenvironment *in vivo*[5]. Commonly used matrices are naturally derived, including commercially available 3D culture systems such as Matrigel™, collagen gels or fibroblast-derived matrices. These materials can be informative as model systems- for example, collagen scaffolds were used to study and identify MMP independent migration pathways relevant to metastatic invasion[6]. Recognizing that natural ECM possesses a highly complex 3D organization that dictates function (which is currently impossible to mimic), matrices have been prepared by decellularizing of various tissues in order to preserve the native integrity of ECM and explore its ability to influence metastasis[7]. While effective in certain contexts, these naturally derived materials are unlikely to reveal molecular level understanding of cell-matrix interactions, as natural systems are not fully defined, have variable compositions, cannot be easily tailored and often contain biologically active materials (e.g. growth factors).

A range of synthetic materials have therefore been developed that can serve as a 'blank canvas' upon which bioactive groups can be rationally introduced. Typically, 'base' materials are selected which have seen previous use in biomedical context, such as poly-ethylene glycol (PEG), poly(lactic-co-glycolic acid) (PLGA) and poly-ε-capronolactone. Synthetic peptide-based materials such as commercially available Puramatrix™ are simplistic mimics of the ECM, which allow for cell culture under well-defined conditions. A number of designs of such self-assembling systems have been developed over the years, typically involving building blocks of 8-20 amino acid residues that can be easily functionalized with bioactive peptides. More specifically for the three primary components necessary to study metastatic disease, we discuss the current state-of-the-art for each.

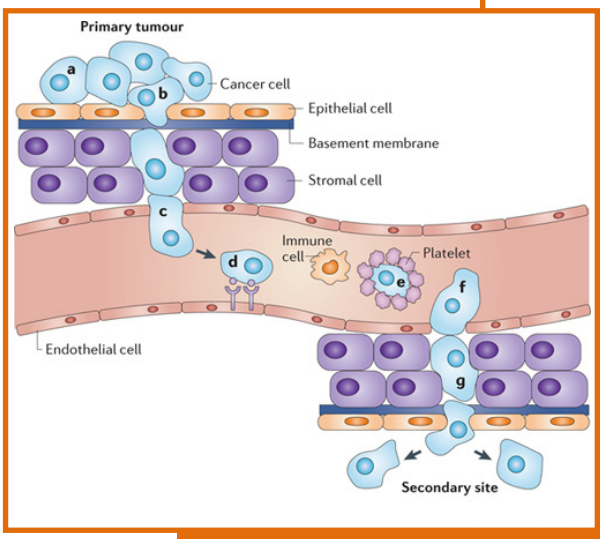

Figure 1. (*Reprinted with permission from Schroeder et al., 2012*)[2].

Adhesion

Adhesion typically involves integrins, the trans-membrane portion of focal adhesions that connect the cytoskeleton inside the cell to the extracellular matrix on the exterior. They bind to bioactive ligands in the surrounding matrix, such as the tri-peptide

RGD (arginine-glycine-aspartic acid). Introduction of RGD ligands into synthetic polymers is now straightforward using well-established polymerization techniques. There is much scope here for the inclusion of different ligands beyond RGD. For example, when using PEG-based hydrogels functionalized with adhesion peptides RGD and YIGSR (the integrin-adhesive regions of fibronectin and laminin, respectively) it was found that cancerous and non-cancerous mammary epithelial cells responded differentially to the adhesion cues[8]. Methods are now also available to introduce bioactive ligands and even entire proteins in precisely defined rations in self-assembled peptide materials[9].

In addition to the concentration of bioactive ligands, their presentation (spatial orientation, clustering) is critical. Questions about spatial organization can be addressed using precisely patterned ligands on surfaces, which may be achieved utilizing block copolymer micellar nanolithography. This approach has been used to demonstrate adhesion dependence with varying distance between RGD ligands, which in turn influenced melanoma cell fate[10]. While this is a 2D approach, the information that is obtained may be used to inform spacing of ligands in 3D constructs. In addition to static presentation of RGD ligands, a number of approaches are now available to dynamically regulate adhesion using switchable RGD ligands (by photolytic uncapping of protected precursors)[11]. These approaches have not yet been used in the context of metastasis and hold great promise in controlling temporal presentation of bioligands.

Migration

Cancer cell migration makes use of a combination of adhesion and enzymatic degradation, involving MMPs and hyaluronases (although non-enzymatic migration is also known[6]). The first designed PEG based gels crosslinked by MMP cleavable peptides were described over a decade ago[12]. Introduction of MMP cleavable linkers in PEG gels was recently used in a metastasis model. A PEG-heparin hydrogel was described that mimics the tumor angiogenesis microenvironment by incorporating RGD (adhesive), MMP-9 responsive (matrix degradation) and glycosaminoglycan (bioactive building block) motifs to take into account different metastasis characteristics[13].

Stiffness

Matrix stiffness is a known determinant of cell fate[3]. Methods are now available to tune this parameter precisely in PEG based materials as well as synthetic self-assembled peptide structures. An example is the use of collagen coated polyacrylamide hydrogel systems with tunable stiffness to study the metastatic potential through matrix stiffness induced epithelial to mesanchymal transition (indication of cancer cell invasiveness)[14]. The effects of

bio-adhesion and matrix mechanics could be investigated separately by varying either the cross-link density or ligand concentration in a gel that also included MMP degradable linkers. Results were shown to be similar to that observed in matrigel, demonstrating that key cell behaviors can be accurately mimicked in fully synthetic gels[15].

Future aspects and conclusions

We note that designed nanomaterials could be used in conjugation with microfluidics, providing access to confined environments while under flow[16]. This would enable (i) mimicry of extravasation[17]; (ii) development of structures for the efficient capture of circulating tumor cells (CTCs)[18] or (iii) study of the interactions of CTCs with endothelial barriers[19]. Tumors contain a variety of cell types (stromal, immune, in addition to tissue specific cells) so accurate mimicry of the microenvironment would require the presence of mixtures of cells. Key to fully understanding migration and invasion will be the development of microscopy techniques. This could include visualization of the invasive protrusions associated with metastasis e.g. using super-resolution (STED) microscopy. This could be combined with FRET approaches to monitor MMP activity and cell migration in real time.

Looking out 10 years, it is highly likely that researchers will be able to use this information in the clinical translation of nanomaterial based models to new materials based therapies.

Clearly, a wide range of synthetic and natural materials, processing and functionalization methods is currently available to create *ex vivo* models to study aspects of metastasis. What is missing, are fully designed model systems, that could mimic all critical aspects of the tumor microenvironment in a more controlled way, opening up opportunities to rationally and systematically vary environmental factors and discover which ones dominate. Not only are designed nanomaterials likely to provide new insights, they can also inform new therapies. There are tremendous opportunities for nanoscience to design artificial (synthetic) cell-compatible hydrogels as models to study metastatic cancer.

Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers will be able to develop tunable scaffolds (stiffness, ligand incorporation, degradability) based on self-assembled structures as models to study each step of metastasis; biological findings

will inform materials design and, by close collaboration between cancer experts, chemists, materials scientists and engineers, new models should be developed to investigate specific aspects of metastatic disease; and superresolution fluorescence microscopy to visualize invasion. Looking further ahead over the next 5 years, researchers will be able to deliver specific, optimized matrices for establishment of secondary tumors; and a quantitative comparison of new *in vitro* models with current animal models. Looking out 10 years, it is highly likely that researchers will be able to use this information in the clinical translation of nanomaterial based models to new materials based therapies.

Microfluidic Models to Study Cell Extravasation and Metastasis

Roger Kamm, PhD
Biological and Mechanical Engineering
Massachusetts Institute of Technology, Cambridge, MA 02139

Introduction

Metastatic cancer remains the leading cause of mortality. While there have been considerable advances in the development of new approaches to the treatment of cancer, the control of metastasis is still one of the major challenges[20,21]. Despite its tremendous importance, a fundamental understanding of the processes that constitute the metastatic cascade remains elusive. As a result, there are few therapeutic approaches available to block the various steps of metastasis. Two factors contribute significantly to this glaring deficiency. First, modern animal models of metastatic disease[22,23,24], although responsible for much of what we have learned, provide inadequate insight into the disease process for lack of the ability to image the details of cancer progression, and because of the limited ability to control and monitor the local chemical and mechanical environments. In addition, there the inevitable questions regarding differences in behavior between cells from humans and those from test animals still exist. Second, the existing *in vitro* models using traditional cell culture methods such as well-plate systems and transwell assays[25], are unable to capture many of the key features that regulate the various stages of metastasis. The gap between *in vitro* and *in vivo* models is considerable, and both have severe limitations.

Further contributing to this knowledge gap is the enormous complexity of the metastatic cascade, which consists of multiple steps: local invasion of cells from the primary tumor into the surrounding tissue, entry into the circulation by intravasation, survival and transport via circulation to a remote site, extravasation into the metastatic site, and finally, recolonization (**Figure 2**)[26]. The challenges to producing a realistic *in vitro* model of any of these steps are enormous, yet recent progress in the development of microfluidic assays capable of 3D culture of multiple cell types, some with an intact endothelial monolayer, has given rise to optimism.

In the past several years, considerable progress has been made. This is largely due to projects funded through the new emphasis by the NCI on assay development and the physical aspects of cancer growth and invasion. And, although we are still at the early stages, advances have been impressive.

Current capabilities

Recent progress has resulted from new capabilities in several strategic areas, and advances in microfluidic technologies have enabled many of these. New approaches and models have appeared within the past decade, both in the context of primary tumor and metastasis[25], although for this chapter, we focus attention exclusively on the latter, with an emphasis on extravasation. Microfluidic assays typically consist of multiple channels or regions containing hydrogels with spatial arrangement and dimensions that facilitate chemical and mechanical signaling among various cell types seeded within the interconnected compartments. The goal of these devices is in creating a local microenvironment among the cellular compoents that replicates many aspects of *in vivo* interaction[25]. For some time, it has been possible to culture cells in 3D microenvironments, simulating the extracellular matrix of tissues[27]. Progress in 3D culture subsequently led to numerous studies in cell migration[28] and the culture of tumor spheroids with microvessels[29]. Studies have examined the role of various cytokines, including spatial concentration gradients, on the initiation of dispersion from a tumor, in some cases documenting the cells' transition from an epithelial to mesenchymal state (EMT)[30]. The capability to suspend cells in 3D and to generate gradients of either chemoattractants or hydrostatic pressure across matrix-containing regions has facilitated new studies on 3D migration[31], and the effects of matrix properties[32], other interacting cell types within the matrix[33], and interstitial flows such as exist at the tumor margin or in the vicinity of blood or lymphatic vessels[34].

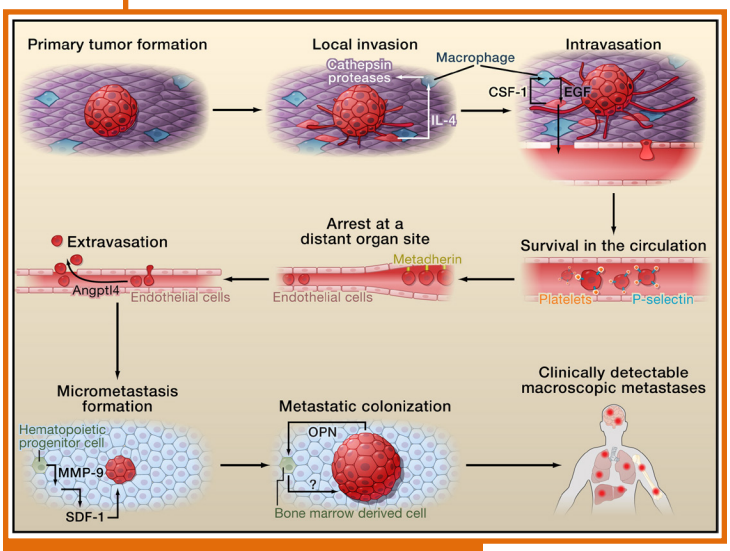

Figure 2. The metastatic cascade. From primary tumor to clinically observable metastases (*Reprinted with permission from Valastyan and Weinberg, 2011*)[26].

When one or more of the channels is lined with an endothelial monolayer, a model for intravasation can be produced by inducing cells seeded into the adjacent matrix to transmigrate into the channel[33]. Similarly, tumor cells introduced into the channel can adhere to the endothelium and transmigrate into the adjacent gel region, mimicking the process of extravasation into the remote host tissue[35]. In some cases, a microvascular network has been established within the gel region that can be perfused with a tumor cell-containing medium, leading to even greater realism in that the tumor cells can then either adhere to or become lodged in the smaller vessels, as they

would in the capillaries of the target organ[36]. Recent studies have also begun to introduce certain organ-specific cells into the matrix, demonstrating that the different rates of extravasation of a particular type of cancer can be replicated within relatively simple *in vitro* systems[37,38].

Future challenges

The use of microfluidics to model metastasis has been rapidly accelerating, but many barriers remain. One of the greatest challenges is to progressively improve the realism of the model while at the same time, keeping it sufficiently simple to use so that these methods remain accessible to the broader cancer research community. In the case of the primary tumor microenvironment, the introduction of cancer associated fibroblasts and tumor associated macrophages, along with the cells of the local microvessels will further enhance the realism of the models. Similarly, the addition of organ-specific stromal cells to models of the remote, metastatic organ will be an important step. Aside from the cellular environment, the matrix properties also need to be carefully considered, since the current choice of type 1 collagen, fibrin or even Matrigel has a significant influence on behavior. Most researchers currently use cell lines, but these should eventually give way to patient-derived tumor cells, and even to the potential for patient-derived induced pluripotent stem (iPS) cells for the creation of more realistic models.

Development of patient-specific models holds the potential for direct clinical application of microfluidics.

One of the greatest current limitations of microfluidics is that the cell numbers and volumes are small, thus making it difficult to employ many of the traditional biochemical or genetic analyses to probe cell function. Methods need to be developed for improved interrogation of the systems (e.g., protein analysis, RNA-seq) including the capability of real-time monitoring of signaling factors or cell function, beyond what can currently be accomplished by imaging.

As researchers expand to model other tissue types, new challenges will emerge. The difficulties in generating a realistic model of the blood-brain barrier are well recognized. Creating models of other organs such as those with high cell densities and intricate internal structural organization – liver, kidney, pancreas – will remain one of the most difficult problems to overcome.

Clinical potential

Development of patient-specific models holds the potential for direct clinical application of microfluidics. Use of iPS cell based systems, patient-derived explants, circulating tumor cells extracted from patient blood, or other similar models will eventually lead to the ability to screen for a therapeutic protocol that is optimized for each patient. In the context of metastasis, this implies an approach that would reduce the tendencies for the primary cancer to spread and recolonize. In addition, improvements in usability and increases in throughput will ultimately facilitate the transition into the clinic, and enable moderate to high throughput screening for combination therapies.

Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers will have been able to develop many more organ-specific models of metastasis; and patient-specific assays for drug selection based on surgical or biopsy specimens. Looking further ahead over the next 5 years, researchers will be able to deliver multiple organ models on a single chip; high-throughput drug screening platforms; and potentially metastatic cancer-on-a-chip. Looking out 10 years, it is highly likely that researchers will be able to deliver iPS cell based models for patient specific drug screening in the clinic as well as, the really important milestone of, point-of-care assays for diagnosis and treatment planning.

In Vitro Models of the Blood-Brain Barrier

Peter Searson, PhD
Department of Materials Science and Engineering, School of Medicine, and Institute for NanoBioTechnology
Johns Hopkins University, Baltimore, MD 21218

Introduction

The blood-brain barrier (BBB), or neurovascular unit, is a complex dynamic system responsible for providing nutrients and essential molecules to power the brain while at the same time ensuring that signaling in the brain is not disrupted by fluctuations in chemistry, inflammation, or the entry of toxins or pathogens[39,40]. The blood-brain barrier maintains homeostasis by transducing signals from the vascular system and the brain, and comprises the brain microvascular endothelial cells (BMECs) that form the 600 km of capillaries, the basement membrane, and surrounding pericytes, astrocytes, and neurons. For example, the brain regulates oxygen supply by signaling via astrocytes, which have end-feet that completely surround the capillaries.

The highly specialized endothelial cells that form the lumen of microvessels and capillaries in the brain are characterized by high transendothelial electrical resistance (TEER > 1000 Ω cm^2), low permeability, expression of tight junction proteins (e.g. claudin-5 and occludin), transporters (e.g. LAT-1), and broad spectrum efflux pumps (e.g. P-gp). The two main components of the blood-brain barrier security system are the tight junctions and the efflux pumps. The formation of tight junctions at the boundaries between endothelial cells almost completely prevents paracellular transport into the brain. The array of broad-spectrum efflux pumps, primarily on the luminal surface, returns almost all non-essential small molecules back into circulation. Notable exceptions are caffeine, alcohol, and anesthetics. A consequence of this security system is that it is extremely difficult to deliver drugs to the brain following oral or intravenous administration. More than 98% of small molecule drugs and 100% of large molecule drugs do not cross the blood-brain barrier[41]. As a result, there are many diseases of the brain for which there are no drug treatments. Treatable brain disorders are limited to depression, schizophrenia, chronic pain, and epilepsy.

Recently it has become recognized that many diseases of the brain are associated with disruption of the blood-brain barrier[40]. While the details of these disruptions are not well understood, they most likely result in local increases in permeability that can lead to the disruption of signaling.

Current state of In Vitro BBB Models for Translational Development.

In the pharmaceutical industry and in academic research, the initial screening of drugs for treatment of central nervous system (CNS) diseases is performed using the transwell assay where the permeability of a drug is determined from the amount that crosses a monolayer of type II Madin-Darby Canine Kidney cells (MDCK.II)[42]. These are dog kidney epithelial cells and not human brain endothelial cells although this represents state-of-the-art in the field of pharmaceutical development for CNS drug therapies. MDCK cells transfected to express different efflux pumps can be used to assess whether molecules are substrates for these pumps. In many cases permeability coefficients obtained from the transwell assay are in reasonable agreement with brain perfusion studies in animal models, although the correlation to humans is not well understood. The transendothelial resistance and hence paracellular transport can be decreased by seeding astrocytes and pericytes, or astrocyte extract, in the basolateral compartment of the transwell chamber, highlighting the importance of these cells in the neurovascular unit[43].

A fundamental problem in BBB research is that animal-derived cell lines and immortalized human BMECs do not fully recapitulate the characteristics of human BMECs. For example,

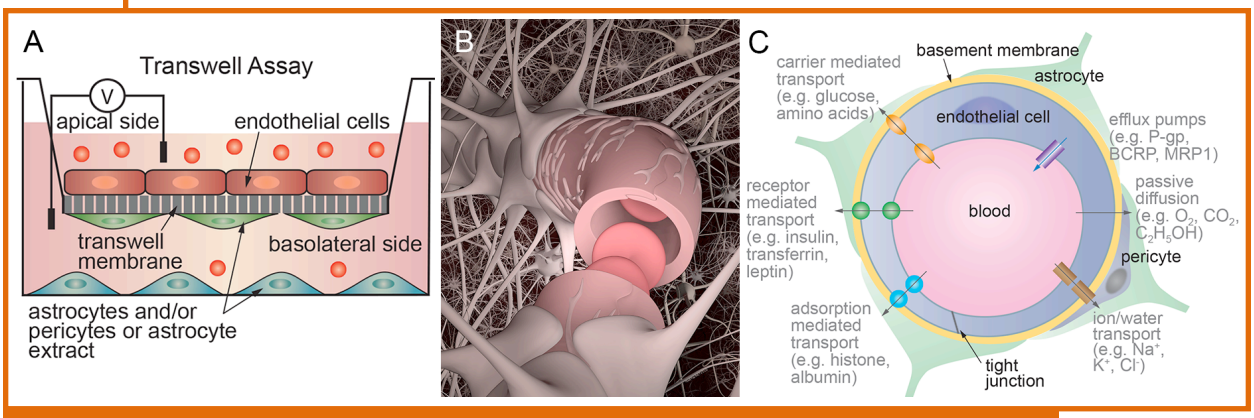

Figure 3. (A) The transwell assay is the standard in vitro tool for determining the permeability of a solute across the blood brain barrier. MDCK cells are widely used since they express tight junction proteins. Paracellular transport can be minimized by seeding astrocytes and/or pericytes, or astrocyte extract in the basolateral chamber. (B) The blood-brain barrier is modulated by functional interactions between brain microvascular endothelial cells, astrocytes, pericytes, and neurons, mediated by the 3D extracellular matrix and basement membrane. Shear flow in the microvessels and the high curvature also play a role in upregulating the blood-brain barrier phenotype. (C) The highly specialized endothelial cells in the brain are characterized by tight junctions that effectively limit paracellular transport, transporters that supply nutrients and other essential molecules, and an array of efflux pumps that return most solutes that cross the luminal membrane back into circulation.

the TEER values of MDCK monolayers are typically around 200 Ω cm^2, almost an order of magnitude lower than physiological values for the brain microvasculature (≈ 2,000 Ω cm^2). The disadvantages of primary hBMECs are that they are not readily available and lose some of their characteristics when cultured *in vitro*. Similarly, the distribution of efflux pump expression varies across species resulting in very different concentrations in the brain. Therefore the lack of physiologically relevant cell lines is a major limitation to advancing the field[44].

The traditional *in vitro* approach to screening drugs for cancer therapy is to assess efficacy by incubating the drug with the relevant cancer cells in culture, and then to assess permeability and brain penetration using the transwell assay (**Figure 3**). In recent work, the transwell assay has been modified to screen drugs for cancer therapy by seeding patient-derived glioma cells in the basolateral compartment and using a live/dead assay to assess efficacy. This approach mimics the pharmacokinetics by exposing the glioma cells to a concentration of the drug that is modulated by blood-brain barrier transport[45].

Recent developments suggest that stem cell engineering may be a solution to the lack of physiological endothelial cells for blood-brain barrier research.

Recent developments suggest that stem cell engineering may be a solution to the lack of physiological endothelial cells for blood-brain barrier research. Human brain microvascular endothelial cells have been derived from induced pluripotent stem cells[46,47]. The derived cells express relevant tight junction proteins, transporters, and efflux pumps, and treatment with retinoic acid results in TEER values in excess of 2,000 Ω cm^2. While more extensive characterization of these derived cells remains to be accomplished, these results could revolutionize the field.

Future of In Vitro BBB Models in Research and Development

The transwell assay provides a relatively high throughput assessment of blood-brain barrier transport, but does not capture the 3D cylindrical geometry of microvessels, the shear stress on the endothelium resulting from blood flow, or the local microenvironment. Engineered microvessel platforms using human cell lines that recapitulate the physiological blood-brain barrier have the potential to rapidly accelerate scientific discovery and the development of new therapies for diseases such as malignant brain cancer[48].

Further advances in stem cell engineering are likely to provide readily available human cell lines for blood-brain barrier research. Methods to harvest patient-derived cells will also be key in developing patient-specific therapies.

The blood-brain barrier remains a major roadblock in delivering drugs to the brain. New strategies for delivering drugs to the brain may include cell penetrating peptides, highjacking transporters (so-called Trojan horse approaches), or transiently increasing the permeability of the blood-brain barrier (e.g. vasomodulators, focused ultrasound, etc.).

The nature of disease-associated disruptions in modulating the local permeability of the blood-brain barrier and their role in disease remain important challenges that will be crucial to developing therapies for many diseases of the central nervous system.

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