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Keywords: blood-brain barrier, organ-on-a-chip, *in vitro* modeling, neurological diseases

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ABSTRACT

The blood-brain barrier (BBB) is an interface between cerebral blood and the brain parenchyma. As a gate keeper, BBB regulates passage of nutrients and exogeneous compounds. Owing to this highly selective barrier, many drugs targeting brain diseases are not likely to pass through the BBB. Thus, a large amount of time and cost have been paid for the development of BBB targeted therapeutics. However, many drugs validated in *in vitro* models and animal models have failed in clinical trials primarily due to the lack of an appropriate BBB model. Human BBB has a unique cellular architecture. Different physiologies between human and animal BBB hinder the prediction of drug responses. Therefore, a more physiologically relevant alternative BBB model needs to be developed. In this review, we summarize major features of human BBB and current BBB models and describe organ-on-chip models for BBB modeling and their applications in neurological complications.

INTRODUCTION

The blood-brain barrier (BBB) is a highly selective barrier in the brain. It protects the brain from exogenous substances by strictly regulating the transport of molecules from the blood vasculature into the brain. The BBB has a multicellular structure. It mainly comprises endothelial cells (ECs), pericytes, and astrocytes (1). Brain ECs are core cellular components of the BBB. They line the inner surface of cerebral blood vessels. Pericytes wrap around brain ECs and astrocytes extend their endfeet to contact with blood vessels. ECs, pericytes, and astrocytes interplay with each other to maintain the structural and functional integrity of the BBB (2). Additionally, with surrounding neurons, microglia and extracellular matrix (ECM) cooperate with BBB, forming a more functional structure called a neurovascular unit (NVU) which plays a critical role in regulating cerebral blood flow and BBB functions to maintain brain homeostasis (3) (**Fig. 1A**).

As a gate-keeper, BBB protects the brain from toxic substances and pathogens (4). The barrier function results from the restriction of both paracellular and transcellular transport. The paracellular transport of ions and hydrophilic solutes is severely limited by tight junctions between adjacent ECs with proteins including zonula occludens 1 (ZO-1) and claudin (5). Transporters such as P-glycoprotein serve as efflux pumps to remove harmful agents from the brain (6) (**Fig. 1B**). In addition, a low level of transcytosis in cerebral ECs further increases the selectivity of the BBB (7). Owing to the presence of the BBB, the entry of many therapeutic drugs targeting human brain are prevented, as more than 98% and approximately 100% of small- and large-molecule drugs cannot cross the BBB, respectively (8).

MODELS OF THE BLOOD-BRAIN BARRIER

Many BBB research studies have been conducted to resolve neurological complications (9). Development of a physiologically relevant BBB model has been a great interest as current BBB

models could not represent the complexity of the human BBB. Animal models have been used in pharmaceutical development to predict drug efficacy and toxicity. They provide major human health benefits as good models to predict human physiology and pathology. The clear advantage of utilizing animals is that they provide basic biological knowledge of a living organism. Therefore, animal testing has been considered as the default and gold standard in preclinical research. It has been widely accepted for a long time. While mouse and rat are the most widely used animal models, various other animal species (*e.g.*, zebrafish) have also been exploited as alternatives (10). Animal models have elucidated fundamental cellular architecture and morphological features, barrier permeability, and transport mechanisms (11). However, the reliability and predictability of animal testing for drug responses and physiological alterations in human have been revealed to be very low. Over 80% drugs validated in animal models have failed in human clinical trials (12) mainly due to genetic, molecular, and immunologic differences between human and other animals. Moreover, in some disease, even pathological mechanisms are different between human and animal models (13). Additionally, ethical issues as well as high costs also hamper the use of animals (14).

To overcome these limitations of animal models, human cell-based *in vitro* testing methods have been adopted. As the simplest way to conduct an *in vitro* experiment, single cell type of ECs cultured on plastic culture dishes in two dimensions has been used to recapitulate endothelial barriers (15). Although it is simple and reproducible, this model lacks brain compartment which plays an indispensable supporting role in barrier properties (1). Associated brain components, especially astrocytes, pericytes, and neurons, need to be cultured in one culture platform to facilitate cellular interplays (16). The most widely used co-culture platform is a Transwell system. An *in vitro* BBB model has also been developed using the Transwell system (17). Usually, ECs are seeded on the top of the porous membrane while astrocytes, pericytes, and neurons are seeded on the opposite side of the membrane or seeded at the bottom

4

of the well. The Transwell system offers many advantages. For example, the system enables the measurement of transendothelial resistance (TEER) and permeability assays (18). Still, it is too simple to represent the complexity and functionality of human BBB due to its two-dimensional geometry and the lack of an ECM. Thus, an innovative co-culture system needs to be developed.

Over the last decade, organoid technology which harnesses the developmental process of organogenesis has been developed to generate *in vitro* 3D tissue constructs to recapitulate human organs (19). BBB-like structures have been reconstituted in 3D brain organoids. For example, generated brain organoids are further added with ECs to create vascularized brain organoids (20-23). Cakir *et al*. developed vascular networks in cortical organoids with human embryonic stem cells overexpressing an angiogenic transcription factor human ETS variant 2 (*ETV2*) (23). Vascularization resulted in enhanced maturation of cortical organoid with higher growth rate and lower apoptosis. The developed vasculature in cortical organoid exhibited BBB characteristics including increased expression of tight junction markers and higher TEER value than in cortical organoid without vascularization. In another study, functional vascularization in brain organoids was induced *in vivo* by transplanting human brain organoids into the adult mouse brain (24). Organoid grafts integrated into the mouse brain underwent neurogenesis and vascularization, suggesting a strategy for *in situ* construction of BBB-like structures in brain organoids.

BLOOD-BRAIN BARRIER-ON-A-CHIP

Although animal models and recently advanced *in vitro* models have provided a lot of insights into BBB physiology and pathology, the complex BBB microenvironment limits current models to be effective platforms for studying neurological diseases and drug development. Fluid flow at the BBB plays an important role in maintaining barrier functions and homeostasis

of the central nervous system. Shear stress generated by circulatory blood flow in the brain capillaries ranges from 5 to 23 dyn/cm^2 (25). It can be applied to capillary endothelium to facilitate barrier functions and regulate transport (26-28). Fluid shear stress can also be used to regulate physiological functions in the brain parenchyma (11, 25). However, most of conventional *in vitro* cell culture models in static conditions cannot recapitulate such controlled fluid flow. BBB possesses a unique cellular architecture in which pericytes wrap around capillaries and astrocyte endfeet contact with capillary ECs (3). This complicated structure informing a distinctive BBB microenvironment and cell-cell interactions cannot be accurately reconstituted via conventional co-culture systems such as Transwell. Thus, a more advanced co-culture system needs to be developed.

Organ-on-a-chip technology, a microphysiological system on which cells and tissues can be cultured, has been applied to overcome limitations of conventional *in vitro* BBB models. Basically, it is composed of microfluidic devices that can simulate tissue- and organ- level physiological functions (29). In the 1990s, microfluidics was developed rapidly with the introduction of poly(dimethylsiloxane) (PDMS) (30, 31), the most frequently used material for microfluidics and organ-on-a-chip. The study that cultured cells on a microfluidic chip for simulating organ-level function of human physiology and pathology was conducted by Shuler group in 2004 (32, 33). In that study, lung, liver, fat, and other tissue compartments called cell culture analogue (CCA) systems were connected by channels and tissue culture medium as blood surrogate was recirculated through these tissue compartments. With this system, investigators modeled the absorption, distribution, metabolism, elimination, and potential toxicity of the chemical drugs that are mathematically simulated with a physiologically relevant pharmacokinetic model. Lung-on-a-chip introduced in 2010 by Ingber group (34) was a landmark study in the field of *in vitro* modeling. Since then, microfluidic devices for culturing cells have been rapidly developed, mimicking most parts of the body, especially the gut, kidney,

placenta, blood-retinal barrier, blood vessels, lymphatic vessels, and BBB (35). Using organ-on-a-chip, several cell types can be cultured simultaneously on one chip. They can be compartmentalized in plenty of shapes with channels or membranes. Besides, channels enable the application of fluid flow, facilitating the culture of cells, especially cells requiring fluid shear stress (30). Therefore, organ-on-a-chip can offer a more physiologically relevant culture environment for BBB modeling. Despite challenges remain in transitions into drug development pipelines, organ-on-a-chip systems are not limited to academic research in laboratories. Many types of chips have already been commercialized including BBB-on-a-chip systems (35-37). Meanwhile, organ-on-a-chip technologies have attracted interest from the pharmaceutical industry and regulatory agencies, and received increasing investments (37, 38).

DESIGNS OF BBB-ON-A-CHIP

To replicate the complex structures and functions of the BBB, advances have been made for the design of BBB-on-a-chip (**Fig. 2**). One of the widely used BBB-on-a-chip design is the use of a porous membrane to separate chambers (39). One chamber comprises ECs and the opposite side of the chamber consists of astrocytes and pericytes (**Fig. 2A**). ECs can be seeded on the porous membrane or cultured to form monolayer with vascular lumen inside the chamber. Blood vessels can also be generated inside hydrogels with or without perivascular cells embedded in the hydrogel (**Fig. 2B**). These platforms can further recapitulate the *in vivo* BBB microenvironment with an ECM (40). Harnessing the inherent ability of vasculogenesis and angiogenesis, ECs can be self-organized to form vasculature (**Fig. 2C**). This approach mimics the vascular development *in vivo*, resulting in improved functionalities of the BBB (41). Recently, 3D spheroid model of BBB has been developed (**Fig. 2D**). 3D spheroids can mimic brain tissues by including various cell types of BBB or NVU in the spheroids. BBB spheroids can be created by assembling brain ECs, astrocytes, and pericytes with low-adherence culture

techniques or microfluidic devices (42-44).

BBB-ON-A-CHIPS FOR VARIOUS APPLICATIONS

Various neurological diseases are known to be associated with BBB (45-47). Brain disease can affect the BBB by disrupting tight junctions. Chronic BBB malfunction is also related to the development of multiple neurological disorders. The following sections discuss applications of BBB-on-a-chip in Alzheimer's disease, ischemic stroke, infectious disease, brain cancer, and transport mechanism studies.

Alzheimer's disease models

BBB disruption and impairment of barrier functions are associated with various neurological disorders and neurodegenerative diseases (48). In Alzheimer's disease (AD), the decreased expression of tight junction proteins increases the BBB permeability and the efflux pathway through receptor-mediated transcytosis, the major mechanism of beta-amyloid (Aβ) clearance across the BBB, is disrupted (49). Animal models are not suitable for human AD modeling due to their different genetic profiles and pathophysiology. The accumulation of Aβ and hyper-phosphorylated tau is observed in animals, but they cannot be considered as AD models since other biological features of human AD, such as neurofibrillary tangle pathology and sporadic forms of AD, are not exhibited in animal models (50, 51). Thus, pre-clinical results from animal models rarely translate into humans (52). Shin *et al.* (53) have developed a microfluidic model which comprises BBB components, including a tube-shaped brain EC monolayer and AD microenvironment (**Fig. 3A**). A unique AD pathological microenvironment has been developed by culturing human-origin neural progenitors expressing familial AD (FAD) mutations in 3D. Major AD pathologies were recapitulated with extracellular deposition of amyloid plaques and tauopathy followed by BBB impairment as observed in AD patients, such as elevated BBB

8

permeability, decreased expression of tight junctions and adherens junctions, and increased levels of reactive oxygen species and matrix metalloproteinase (MMP) (*e.g.*, MMP-2). More importantly, Aβ deposition was observed at the surface of the abluminal side of brain ECs.

Ischemic stroke models

In ischemic stroke, inadequate supply of blood leads to neurological pathologies where BBB is disrupted, resulting in the breakdown of tight junctions and ionic shifts in the brain (54, 55). However, drugs validated in animal models have failed in clinical trials probably because of physiological and pathological differences between human and animals (56). Lyu *et al*. (57) have created a microfluidic ischemic stroke model. A functional NVU model consisting of human brain microvascular ECs, pericytes, astrocytes, microglia, and neurons was established in the microfluidic device. An ischemic condition was developed by supplying 2% O_2 with depletion of serum and glucose for 24 hours to recapitulate the peri-infarct zone of ischemic injury. The developed BBB chip was tested for evaluating neurorestorative efficacy of various types of stem cells to investigate the influence of stem cells during disease progression and recovery processes.

Infectious disease models

Bacteria and viruses can enter the BBB via receptor-mediated active transport or because of an increase of barrier permeability (58). The infection of pathogens can also be recapitulated in a functional BBB model. Kim *et al*. (59) have developed a microfluidic NVU model using human neural stem cells, brain microvascular ECs, and brain vascular pericytes in a brain-relevant 3D matrix which comprises collagen and hyaluronic acid. The microfluidic NVU chip with BBB structure was applied to model the BBB penetration of *Cryptococcus neoformans*, a fungal pathogen that causes fatal meningoencephalitis (**Fig. 3B**). The microfluidic chip was cultured

in a unidirectional flow driven by stepwise gravity without syringes or tubing, thus enabling high throughput culture of chips. *Cryptococcus neoformans* can penetrate the BBB without changes in tight junctions, proposing transcytosis-mediated transport of *Cryptococcus neoformans*. Furthermore, neurotropism of *Cryptococcus neoformans* was recapitulated in a multi-organ chip design. More recently, coronavirus disease 2019 (COVID-19) infection has been modeled in a microfluidic BBB device. Buzhdygan *et al*. (60) have used a 3D microfluidic model of BBB consisting of brain ECs injected into a channel created by mixing multiple hydrogels on a microfabricated device. SARS-CoV-2 spike proteins are then introduced to the model, showing significant influences on barrier functions. The spike proteins can trigger immune responses and elevate the expression of pro-inflammatory cytokines, chemokines, and adhesion molecules to disrupt the BBB integrity and increase barrier permeability.

Brain cancer models

Pharmaceutical treatments for brain cancer are limited because of the poor BBB penetration of drugs and the aggressiveness of primary brain tumors as well as the occurrence of metastatic brain tumors from various primary sites (61). Additionally, tumors can interact with the surrounding microenvironment including cellular and noncellular components to resist therapeutics. Thus, more effective *in vitro* brain tumor platforms including blood vessels are required (62). Seo *et al*. (40) have engineered a BBB-on-a-chip to replicate the brain tissue-specific microenvironment using human astrocytes and brain vascular pericytes embedded in a 3D hydrogel matrix (**Fig. 3C**). A monolayer of human brain ECs formed blood vessel inside the matrix and recapitulated the glioblastoma multiforme (GBM) microenvironment by adding GBM spheroids to the matured BBB model to predict drug responses.

Transport mechanism study

Poor BBB penetration of pharmaceutical drugs is one of the major issues in neurological disease treatments (63). Thus, BBB targeting studies have been conducted to deliver drugs across the BBB (9). Park *et al*. (39) have established a BBB-on-a-chip with human induced pluripotent stem cell-derived brain microvascular endothelium forming a vascular lumen in the lower channel separated by a porous membrane with primary human brain astrocytes and primary human pericytes seeded on the top of the porous membrane. During differentiation, cells were exposed to hypoxia, resulting in increased endothelial barrier functionality. Ahn *et al*. (64) have developed a BBB-on-a-chip with human brain microvascular ECs seeded on the top of the porous membrane and human brain vascular pericytes seeded on the opposite side of the membrane (**Fig. 3D**). Importantly, human astrocytes were seeded in 3D in the opposite side of the membrane to highlight the contribution of astrocytes to BBB pathology with decreased reactive gliosis as well as astrocytic network due to their extending endfeet contacting blood vessels and polarized expression of aquaporin-4 (AQP4) near the vasculature. High-density lipoprotein-mimetic nanoparticles were then introduced to demonstrate the distinct BBB penetration through lipoprotein receptor-mediated transcytosis. More recently, Eilenberger *et al*. (44) have demonstrated a microfluidic multi-size spheroid array to generate BBB spheroids composed of human brain ECs, human primary pericytes, and astrocytes with various spheroid sizes for studying size-dependent compound uptake. With consequential altered cell responses according to spheroid size, anticancer drug toxicity assessment and better prediction of penetration across the BBB were accomplished.

CONCLUSION

The high selectivity of the BBB has hampered the therapeutical treatment for neurological diseases. In addition, inappropriate *in vivo* and *in vitro* models have led to the requirement of a novel *in vitro* BBB model. Organ-on-a-chip has been employed to better recapitulate the human

in vivo microenvironment to facilitate the prediction of drug responses. BBB-on-a-chip has also been developed rapidly over the last decade. Various disease modeling and transport mechanism studies have been implemented using BBB-on-a-chip. In future studies, BBB-on-a-chip is expected to develop more clinically relevant models using patient-derived stem cells and integration of analyzing sensors.

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CONFLICTS OF INTEREST

The authors declare no competing financial interest.

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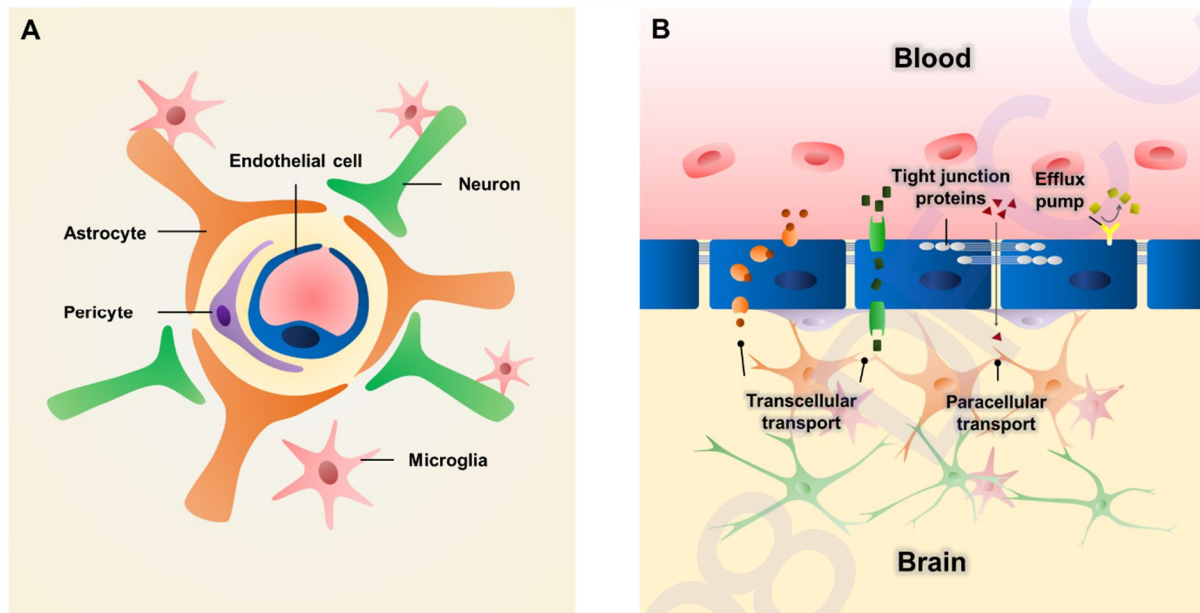
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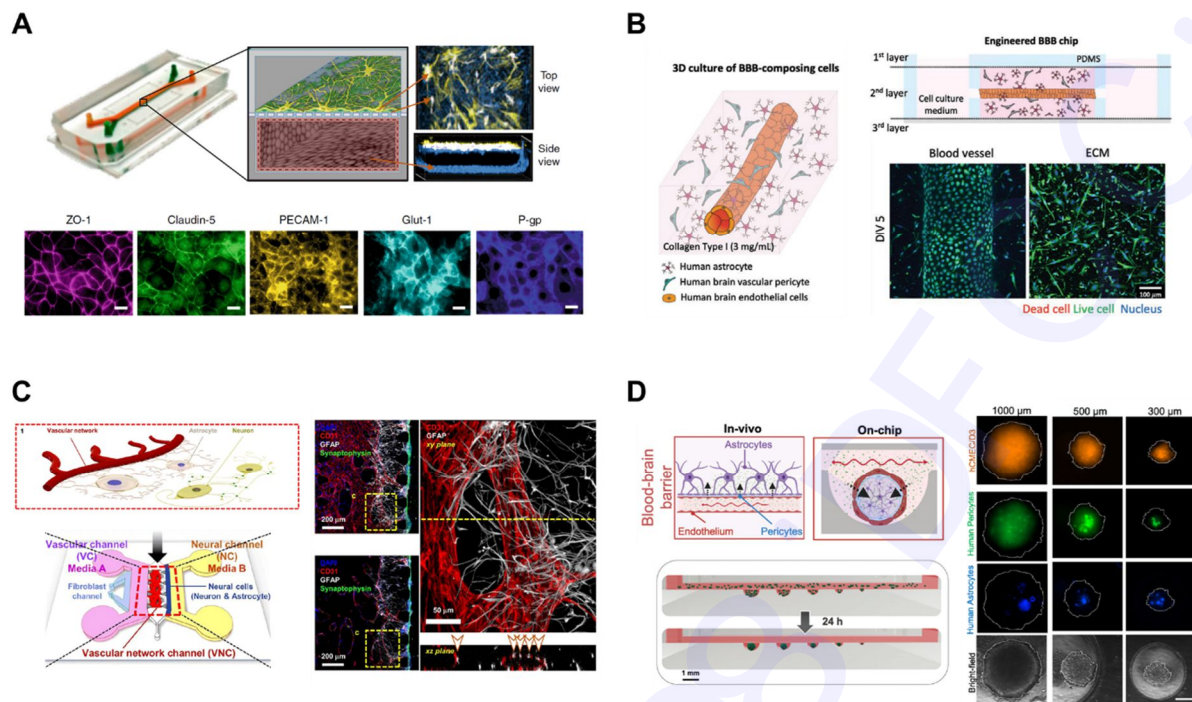
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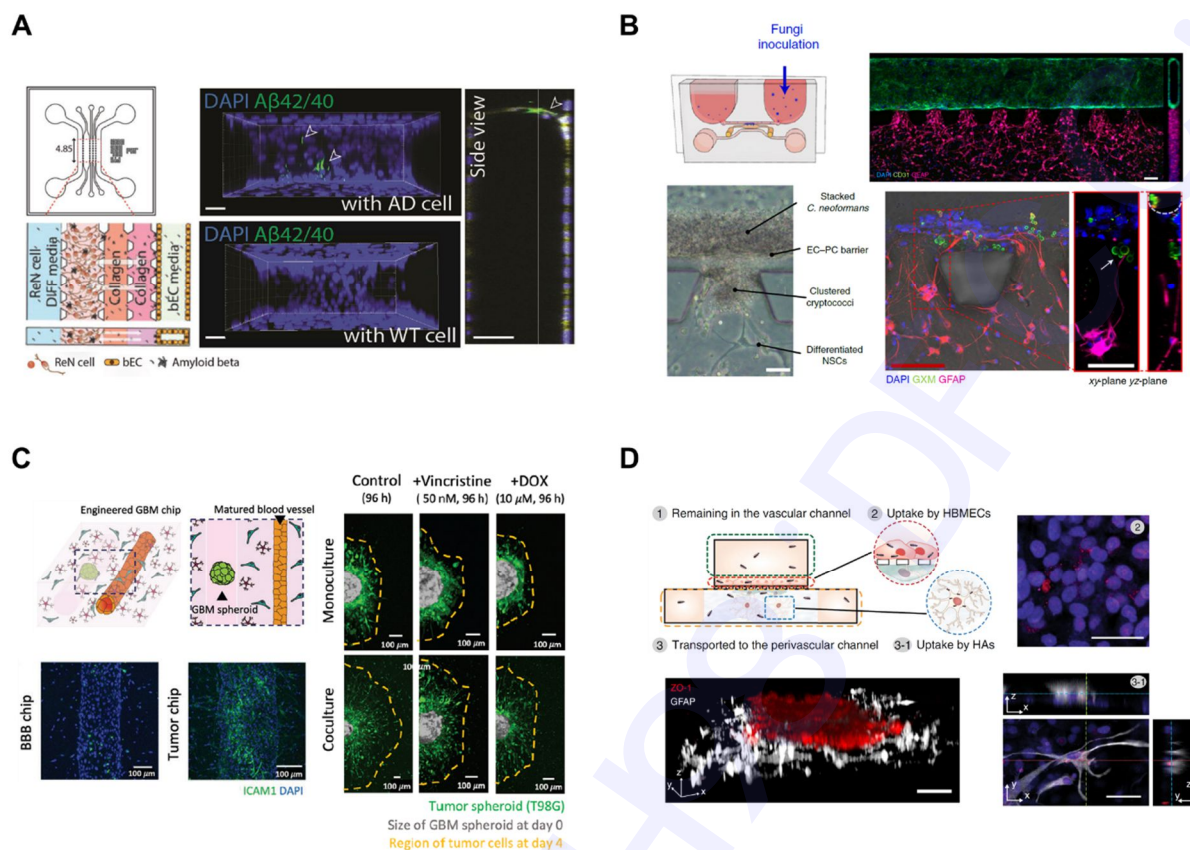
FIGURE LEGENDS

Figure 1. Schematic illustration of blood-brain barrier (BBB) structure. (A) Brain endothelial cells (ECs) are lined to form blood vessels. Pericytes wrap around the vessel and astrocytes extend endfeet to contact with ECs. Neurons and microglia cooperate with each other to form more functional neurovascular unit (NVU). (B) The barrier accounts for limited paracellular transport due to tight junction proteins (*e.g.*, zonula occludens 1 (ZO-1) and claudin) and transcellular transport due to efflux pumps (*e.g.*, P-glycoprotein).

Figure 2. Designs of BBB-on-a-chip. (A) Porous membrane separates blood components and brain components. As an example for this type, endothelial cells (ECs) forming vascular lumen are seeded in the lower chamber, and astrocytes and pericytes are seeded on the top of the porous membrane. Adapted with permission from Park *et al*. (39). Copyright (2019) Springer Nature. (B) Blood vessels form inside the matrix. As an example for this type, ECs can form blood vessels inside the matrix, with astrocytes and pericytes embedded around the blood vessel. Adapted with permission from Seo *et al*. (40). Copyright (2021) Wiley-VCH GmbH. (C) Self-assembled vasculatures of the BBB. As an example for this type, ECs are embedded in fibrin gel and vascular networks are formed in the matrix. Neurons and astrocytes are seeded in the adjacent channel by the side of the vascular networks. Adapted with permission from Bang *et al*. (41). Copyright (2017) Springer Nature. (D) 3D spheroid models of the BBB. As an example for this type, the BBB spheroids are formed with ECs, astrocytes, and pericytes in low adhesive microwells. Adapted with permission from Eilenberger *et al*. (44). Copyright (2021) Wiley-VCH GmbH.

Figure 3. Various applications of BBB-on-a-chip. (A) Alzheimer's disease (AD) can be modeled with BBB-on-a-chip. For example, AD-mimicking microenvironment can be reconstituted on the BBB-on-a-chip. Pathological features such as beta-amyloid (Aβ) deposition can be observed at the BBB. Adapted with permission from Shin *et al*. (53). Copyright (2019) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (B) Infectious disease model in an NVU chip. For example, infection of *Cryptococcus neoformans* has been modeled in a BBB-on-a-chip and neurotropism of the fungus has been elucidated with a multi-organ chip design. Adapted with permission from Kim *et al*. (59). Copyright (2021) Springer Nature. (C) Brain cancer can be recapitulated with co-culture of glioblastoma multiforme (GBM) in BBB-on-a-chip to observe different drug responses based on the interaction of the BBB with brain cancer. Adapted with permission from Seo *et al*. (40). Copyright (2021) Wiley-VCH GmbH. (D) Transport mechanisms are better studied using BBB-on-a-chip. For example, lipoprotein receptor-mediated transcytosis can be demonstrated with the introduction of high-density lipoprotein (HDL)-mimetic nanoparticles to BBB-on-a-chip. Adapted with permission from Ahn *et al*. (64). Copyright (2020) Springer Nature.