A neurovascular unit-on-a-chip: culture and differentiation of human neural stem cells in a three-dimensional microfluidic environment

Introduction

In vitro models are the most valuable tools for studying cell behavior in a controlled and replicable environment. However, most biological studies over the past century relied on a simple monolayer cell culture, which does not reflect the complex functional characteristics of human tissues and organs, or their real response to external stimuli. Microfluidic technology has advantages of high-throughput screening, accurate control of the fluid velocity, low cell consumption, long-term culture, and high integration. By combining the multipotential differentiation of neural stem cells with high throughput and the integrated characteristics of microfluidic technology, an in vitro model of a functionalized neurovascular unit was established using human neural stem cell-derived neurons, astrocytes, oligodendrocytes, and a functional microvascular barrier. The model comprises a multi-layer vertical neural module and vascular module, both of which were connected with a syringe pump. This provides controllable conditions for cell inoculation and nutrient supply, and simultaneously simulates the process of ischemic/hypoxic injury and the process of inflammatory factors in the circulatory system passing through the blood-brain barrier and then acting on the nerve tissue in the brain. The in vitro functionalized neurovascular unit model will be conducive to central nervous system disease research, drug screening, and new drug development.

Key Words: neural differentiation; astrocyte; blood-brain barrier; brain microvascular endothelial cells; central nervous system; microfluidics; neural stem cells; neuron; neurovascular unit; oligodendrocyte; organ-on-a-chip

Abstract

Biological studies typically rely on a simple monolayer cell culture, which does not reflect the complex functional characteristics of human tissues and organs, or their real response to external stimuli. Microfluidic technology has advantages of high-throughput screening, accurate control of the fluid velocity, low cell consumption, long-term culture, and high integration. By combining the multipotential differentiation of neural stem cells with high throughput and the integrated characteristics of microfluidic technology, an in vitro model of a functionalized neurovascular unit was established using human neural stem cell-derived neurons, astrocytes, oligodendrocytes, and a functional microvascular barrier. The model comprises a multi-layer vertical neural module and vascular module, both of which were connected with a syringe pump. This provides controllable conditions for cell inoculation and nutrient supply, and simultaneously simulates the process of ischemic/hypoxic injury and the process of inflammatory factors in the circulatory system passing through the blood-brain barrier and then acting on the nerve tissue in the brain. The in vitro functionalized neurovascular unit model will be conducive to central nervous system disease research, drug screening, and new drug development.

Researchers first reported a microfluidic device to construct cell modules and simulate the microenvironment in 2004 (Andersson and van den Berg, 2004; Sin et al., 2004). The microfluidic device, as a bioreactor, was called “organ-on-a-chip”. After more than 10 years of development, these organ-on-a-chips have successfully been used to reproduce many tissues and organs (Oleaga et al., 2016; Wu et al., 2020; Rothbauer et al., 2021) such as lung (Stucki et al., 2015; Shrestha et al., 2020), liver (Banaeyyan et al., 2017; Moradi et al., 2020), kidney (Zhou et al., 2016; Lee and Kim, 2018), and skin (Wufuer et al., 2016; Lee et al., 2017). These precisely constructed models are used to clarify physiological phenomena that are difficult to dynamically observe, systematically evaluate, and quantitatively in vivo, and can serve as effective tools for high-throughput screening of therapeutic drugs. Microfluidic technology has advantages of high-throughput screening, accurate control of the fluid velocity, low cell consumption, long-term culture, and high integration (Sackmann et al., 2014; van Duinen et al., 2015). Through microfluidic or tissue engineering technology, different cells of the same organ are integrated into a limited culture space according to a specified organization to form a living cell structure unit with a certain tissue structure and physiological function. Reardon (2015) reported that the organ-on-a-chip is a revolutionary technology that may replace animal experiments.

The brain, as part of the central nervous system (CNS) of the human body, is an important target organ for drug screening and toxicity testing. The concept of the neurovascular unit (NVU) was first proposed by Harder et al. (2002). NVU comprises endothelial cells, extracellular matrix, astrocytes, pericytes, neurons and their axons, and other supporting cells (microglia, oligodendrocytes). The components are linked reciprocally and intimately, generating an anatomically and functionally efficient system that regulates the cerebral blood flow (McConnell et al., 2017; De Luca et al., 2020; Caffrey et al., 2021; Liu et al., 2021; Ye et al., 2022). NVU is the basic unit of the structure and function of the CNS. The NVU system is different from traditional blood-brain barrier (BBB) models that do not include glia or neurons with the other CNS elements. In the last 20 years, physiologic researchers have verified that cells in the vasculature intercellularly communicated with adjoining glia and neurons strongly. Thus, instead of working independently, BBB functions as...
Human BMECs (hBMECs; RRID: CVCL_4U95) were purchased from ScienCell medium, and then adding 10% fetal bovine serum. fibroblast growth factor and epidermal growth factor from the complete ng/mL basic fibroblast growth factor (Gibco BRL). The cells were cultured at 37°C under constant humidity in 5% CO2 to generate neurospheres (Wang et al., 2017). The growth medium of the NSCs was refreshed every 48 hours. The spontaneous differentiation of NSCs was induced by eliminating basic fibroblast growth factor and epidermal growth factor from the complete medium, and the NSCs were cultured at 37°C under constant humidity in 5% CO2 to generate neurospheres (Wang et al., 2017).

Flow cytometry identification
Flow cytometry was conducted for immunophenotyping of antigens on the NSCs (2nd to 4th passage) and BMECs (3rd to 5th passage). The cells were collected, adjusted to 1 x 10^6/ml, incubated with mouse anti-human nestin-fluorescein isothiocyanate (FITC) monoclonal antibody (1:100, BioLegend, San Diego, CA, USA) and rabbit anti-human CD31-FITC antibody (1:100; MilliporeSigma, St. Louis, MO, USA), then stained with 1% penicillin/streptomycin, 10% fetal bovine serum, and 2 ml L-glutamine. The cells were incubated in 5% CO2 at 37°C and 95% humidity until confluence.

Non-invasive neural vascular unit (NVU) construction
Neural stem cells (NSCs), as pluripotent cells in the CNS, have high self-renewal capacity. NSCs are of great significance to embryonic development, nervous system formation, and pathological homeostasis. In addition, they are suitable cellular systems for regenerative medicine and developmental biology in vitro research because of the potential to differentiate into almost all neuron lineages (Obernier and Alvarez-Buylla, 2019).

Materials and Methods

Cell isolation, culture, and differentiation
The aborted embryos were collected at 6–12 weeks of pregnancy, and hNSCs were cultured in vitro to identify their stemness features, specificity, and growth characteristics. This study was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Dalian Medical University. The protocols of the experimental animals were conducted using the guidelines for the care and use of laboratory animals (National Institutes of Health, 2011). All antibody and isotype controls were provided by Pharmigen (Becton Dickinson). Flow cytometry was conducted for immunophenotyping of antigens on the NSCs (2nd to 4th passage) and BMECs (3rd to 5th passage). The cells were collected, adjusted to 1 x 10^6/ml, incubated with mouse anti-human nestin-fluorescein isothiocyanate (FITC) monoclonal antibody (1:100, BioLegend, San Diego, CA, USA) and rabbit anti-human CD31-FITC antibody (1:100; MilliporeSigma, St. Louis, MO, USA), then stained with 1% penicillin/streptomycin, 10% fetal bovine serum, and 2 ml L-glutamine. The cells were incubated in 5% CO2 at 37°C and 95% humidity until confluence.

Quantitative reverse transcription polymerase chain reaction
Quantitative polymerase chain reaction (qRT-PCR) was used to detect marker genes in the samples from the disassembled microfluidic device after being induced by different perfusion times (5, 7, 9, 11 days). Briefly, total RNA was extracted from differentiated and control groups by TRIzol (Takara, Dalian, China) and reverse transcribed into complementary DNA with a PrimerScript™ reverse transcription kit (Takara, Tokyo, Japan). The qRT-PCR was then performed using a Roche LightCycler480 Real-time System (Roche, Basel, Switzerland) after 2 μl of complementary DNA was mixed with 10 μl of 2x SYBR Green PCR master mix (Takara, Dalian, China). The sequences of the primers are: nestin (NC marker) forward: 5’-GAG AGG GAG GAC AAA GTC CC-3’, reverse: 5’-TCC TTC AGA GAC TAG CAG TGC-3’; microtubule-associated protein 2 (MAP2), forward: 5’-ATG TCT CCA GGA CCC TCA AC-3’, reverse: 5’-TCA GCC CCA TGG TCC ACA CG-3’; glial fibrillary acidic protein (GFAP; astrocyte marker) forward: 5’-CTG TGG CAG AGT GAG GTT-3’, reverse: 5’-TCA TCG TCT AGG TCC TT-3’; myelin basic protein (MBP; oligodendrocyte marker) forward: 5’-CTG GCT GGG CTT-3’, reverse: 5’-GAG GGA GGT AAG CAG CCG GTT A-3’. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization. The sequence was designed as GAPDH forward: 5’-CTG CAG GCC ACA GCT TCA T-3’, reverse: 5’-CCA ATA CGA CCA ART CCG TGG-3’.

Immunofluorescent staining
Immunofluorescent staining was performed to characterize the maintenance, proliferation, and differentiation of hNSCs as well as the maintenance and proliferation of BMECs. The cell samples were treated by 0.2% Triton-X100 (MilliporeSigma, St. Louis, MO, USA), 4% paraformaldehyde (MilliporeSigma), and 5% bovine serum albumin in PBS, incubated with primary rabbit anti-nestin antibody, rabbit anti-SOX2 (1:200; Abcam, Cat# ab75627, RRID: AB_1310697), rabbit anti-MAP2 (1:200; Abcam, Cat# ab212454, RRID: AB_1209900), mouse anti-GFAP (1:200; Abcam, Cat# ab10611, RRID: AB_831977), rabbit anti-MAP2 (1:200; Abcam, Cat# ab75627, RRID: AB_1310697), sheep anti-Von Willebrand factor (vWF) (BMEC marker; 1:200; Abcam, Cat# ab8822, RRID: AB_946122), and rabbit anti-zonula occludens-1-z-200; Abcam, Cat# ab2271824) antibodies overnight at 4°C following the manufacturer’s instructions, rinsed by PBS, and thereafter incubated with goat anti-rabbit IgG-FITC antibody (1:100; MilliporeSigma, Cat# F3828, RRID: AB_631744), and goat anti-mouse IgG-tetramethylrhodamine isothiocyanate (TRITC) antibody (1:100; MilliporeSigma, Cat# F0382, RRID: AB_631744), and goat anti-mouse IgG-tetramethylrhodamine isothiocyanate (TRITC) antibody (1:100; MilliporeSigma, Cat# F0382, RRID: AB_631744) overnight at room temperature for 90 minutes. Cell nuclei were stained and their positions were identified with 4’,6-diamidino-2-phenylindole staining solution (1:500, Invitrogen, Carlsbad, CA, USA) overnight at 4°C. The imaging settings and quantitative data were identical to those for cell viability evaluation. Fluorescent images were taken by fluorescence microscopy (Leica DMi 4000B, Solms, Hesse-Darmstadt, Germany) equipped with a digital camera (Leica DFC 500).

Assessment of cell viability
To characterize the maintenance and death of BMECs and neuro-glial cells in the microfluidic device, the PDMS layers were detached from each other for sample collection after 7 days of perfusion. The recycled samples were then incubated with calcein (1:1000, LIVE/DEAD viability/cytotoxicity kit, Molecular Probes, Eugene, OR, USA) and propidium iodide (1:1000) for 20 minutes at room temperature in the dark. Before and after incubation in the staining solution, the samples were washed by PBS twice for 5 minutes each time. The neural culture was observed with a Leica DMi 4000B fluorescence microscope.
microscope equipped with a Leica DFC 500 digital camera. BMEC samples cultured on the PC membrane were observed under a Leica SP8 laser scanning confocal microscope after being sectioned into 20 layers. Each image was acquired at identical light source intensity and pixel. Quantification was performed with Image-Pro Plus software of the Media Cybernetics (IPP 5.0 system (Rockville, MD, USA) by obtaining five individual stacks before projection along the z-axis. To measure the ratio of live cells to dead cells, the fluorescence images were recorded at 480 nm and 590 nm, respectively. The viability was assessed by calculating the percentage of live cells to total cells. The cells in five randomly selected visual fields (100×) were counted using Image Pro-Plus Software (Image-Pro-Plus 6.0, Media Cybernetics, Silver Spring, MD, USA).

Evaluation of the neurovascular interface integrity and permeability
To evaluate the integrity of the endothelial barriers between two chambers, a cover of cells in the NVU device was perfused by 25 μM FITC-conjugated dextran (4000 Da, Sigma) at 1 μl/min on the upper side. A device that did not contain an hBMECs layer was used as a negative control. Subsequently, the upper chambers were perfused with serum-free medium containing 10 ng/mL tumor necrosis factor-α (TNF-α; Sigma) at 1 μl/min. The perfusion fluid was collected from the lower outlet hourly to assess the permeation amount of FITC-dextran. A calibration curve of the concentration of FITC-dextran versus absorbance at 490 nm was plotted by an EL808 fluorescence microplate reader (BioTek, Highland Park, VT, USA). The concentration of the dye permeating the endothelial barrier was obtained through normalization to the calibration curve.

Stimulation of the inflammation via tumor necrosis factor-α
To evaluate the response of the NVU model to inflammatory stimulus, the neurovascular communication functions were biochemically modulated by 2 hours of exposure of the upper chambers (hBMECs) to serum-free medium containing 10 ng/mL tumor necrosis factor-α (TNF-α; Sigma) at 1 μl/min. The upper chambers were then perfused with serum-free medium containing FITC-dextran for 5 hours. The perfusion liquid was collected hourly from the lower outlet to assess the permeation amount of FITC-dextran.

Stimulation of oxygen and glucose deprivation/reperfusion
The upper and lower chambers were rinsed with sterile PBS, and the perfusion fluid was replaced with glucose-free and serum-free Dulbecco’s modified Eagle’s medium. The model was placed in a three-gas incubator at 37°C for 3 hours of anoxic culture, with an anaerobic mixture of 1% O2, 5% CO2, and 94% N2 (volume fraction). After the oxygen and glucose deprivation (OGD) treatment, the perfusion fluid of both chambers was replaced with conventional complete medium, and the model maintained oxygen and glucose reperfusion in an incubator with saturated humidity and 5% CO2 at 37°C. For assessing the level of cell death after OGD/R, the release of intracellular lactate dehydrogenase (LDH) into the perfusate was measured with a LDH Assay Kit (Solarbio, Beijing, China) according to the manufacturer’s instruction (Ma et al., 2019), where LDH (U/mL) = y/T × 10^{-3} (y was the concentration of standards, μmol/mL; T was the reaction time, 15 minutes). The LDH leaking rate and transmission rate of the FITC-labeled dextran in the NVU model were detected at 0, 6, 12, 24, and 48 hours. The control group was also detected at the same time points.

Statistical analysis
Quantitative data were expressed as mean ± standard deviation (SD). Quantitative analysis was performed according to Student’s t-test using SPSS 18.0 (SPSS, Chicago, IL, USA). Each experiment was performed at least in triplicate. P < 0.05 was considered statistically significant.

Results
Characterizations of neural stem cells and brain microvascular endothelial cells
Primary NSCs and BMECs were characterized before seeding into the microfluidic device. In the in vitro culture of hBMECs in the fusiform or polygon, cells were closely arranged without overlap, with a single pebble sample structure (Figure 1A), and they stably expressed endothelial cell-specific proteins vWF (Figure 1B), showed the top view of the chip. Figure 3C was the combined diagram of the chip model. The real object is shown in Figure 3D.

Composition of the microfluidic neurovascular unit model
The NVU model was divided into various functional areas of the integrated composite structure. As shown in Figure 3A, the microchip comprises upper PDMS layer 1, PC membrane in form of lower PDMS layer 3, and bottom glass sublate substrate 4. The upper PDMS layer 1 included liquid inlet 11 penetrating the upper PDMS layer 1 and communicating with 31, liquid outlet 12 penetrating the upper PDMS layer 1 and communicating with 32, blood vessel unit liquid inlet 13, blood vessel unit liquid outlet 14, and an upper PDMS layer culture chamber 15 for BMEC culture. Lower PDMS layer 3 included neuro-glial unit inlet 31, neuro-glial unit outlet 32, and lower PDMS layer culture chamber 33 for NSC inoculation. Upper chamber 15 and lower chamber 33 were aligned in the vertical direction, which seamlessly clamped to polycarbonate film 2. The culture medium filled the inlets (13, 11–31), microfluid channels, cell culture rooms (15, 33), and waste liquid ports (14, 12–32). The inlets were connected to the injection pump. Through a hose, and the microfluidic channels were closed inside the microfluidic chip. The cell culture rooms, liquid inlets, and waste liquid ports were connected through microfluidic channels. Figure 3B showed the top view of the chip. Figure 3C was the combined diagram of the chip model. The real object is shown in Figure 3D.

hNSCs can be induced into a variety of cell types, such as neurons, astrocytes, and oligodendrocytes, which are typically difficult to culture and proliferate in vitro (Figure 4A). As shown in Figure 4B, the entire microfluidic device comprised a neural chamber (for NSCs culture) and microfabricated vascular chamber (for endothelial cells culture). The two chambers were separated by a microporous PC membrane with a 10-μm thickness and 0.4-μm pore size, which was used to mimic BBB by loading hBMECs. The two chambers were fabricated with PDMS (weight ratio to curing agent, 10:1), which has been widely applied to develop microfluidic platforms for biological studies because of its high gas permeability and biocompatibility. There were independent inlets and outlets in each chamber (1-mm width). The microchannels were connected with a syringe pump to drive culture medium flow. The flow rate was adjusted to 1 μL/min, which approaches that of microvessels in vivo.
Neural and glial sub-populations from human NSCs

**Figure 3** | Model design of the neurovascular unit-on-a-chip.

(A) Model decomposition of the neurovascular unit-on-a-chip. The microfluidic chip comprises upper polydimethylsiloxane (PDMS) layer 1, microporous polycarbonate membrane 2, lower PDMS layer 3, and bottom glass substrate 4. The upper PDMS layer 1 included liquid inlet 11 penetrating the upper PDMS layer 1 and communicating with 31, liquid outlet 12 penetrating the upper PDMS layer 1 and communicating with 32, blood vessel unit liquid inlet 13, blood vessel unit liquid outlet 14, and upper PDMS layer culture chamber 15 for brain microvascular endothelial cell culture. The lower PDMS layer 3 included neuro-glial unit inlet 31, neuro-glial unit outlet 32, and lower PDMS layer culture chamber 33 for neural stem cells inoculation. (B) Top view of the neurovascular unit-on-a-chip. (C) Model combination diagram of the neurovascular unit-on-a-chip. (D) A photograph of the neurovascular unit-on-a-chip model.

**Figure 4** | Cell culture in the neurovascular unit model.

(A) The construction principle of the neural unit in the neurovascular unit model. (B) Side view of the cell culture chambers and cell inoculation of the neurovascular unit model.

Neural and glial sub-populations from human NSCs

qRT-PCR was used to detect the gene expression of hNSCs differentiated from the NVU model after being induced for different perfusion times. As shown in **Figure 5A**, the expression level of nestin in NSC-derived cells gradually decreased with the induction time until it reached the lowest level on the 11th day. The expression of neuron gene MAP2 (Figure 5B) and astrocyte gene GFAP (Figure 5C) reached a maximum on the 7th day of induction, and then decreased gradually. The expression level of the oligodendrocyte gene MBP (Figure 5D) gradually increased with the induction time, and reached the highest level on the 11th day. Because the neurons and astrocytes are important components of the human brain, 7 days was chosen as the optimal induction time of NSCs in the NVU models.

After staining with selective markers MAP2, GFAP, and MBP (**Additional Figure 1**), GFAP was significantly expressed in the NSC-derived cells under dynamic conditions, confirming the ubiquity of astrocytes (**Figure 5F and H**). NSC-derived neurons existed in patches, varying significantly in different samples (**Figure 5E and H**). There were small quantities of oligodendrocytes in these cultures (**Figure 5G and H**).

**Figure 5** | Comparison of the neuroglial differentiation of neural stem cells with different induction times using a quantitative real-time polymerase chain reaction. (A–D) Gene expression was detected by quantitative real-time polymerase chain reaction. (A) The expression of nestin in the differentiated cells derived from neural stem cells at different induction times. (B) The expression of microtubule-associated protein 2 (MAP2) in the differentiated cells derived from neural stem cells at different induction times. (C) The expression of glial fibrillary acidic protein (GFAP) in the differentiated cells derived from neural stem cells at different induction times. (D) The expression of myelin basic protein (MBP) in the differentiated cells derived from neural stem cells at different induction times. The target gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase. (E) The differentiation of neural stem cells into neurons in the neurovascular unit model after 7 days of induction. MAP2-positive cells showed green fluorescence after fluorescein isothiocyanate staining and nuclei staining showed blue fluorescence under 4′,6-diamidino-2-phenylindole. (F) The differentiation of neural stem cells into astrocytes in the neurovascular unit model after 7 days of induction. GFAP-positive cells showed red fluorescence after tetraethyl rhodamine isothiocyanate staining and nuclei staining showed red fluorescence after fluorescein isothiocyanate staining and nuclei staining showed blue fluorescence under 4′,6-diamidino-2-phenylindole. (G) The differentiation of neural stem cells into oligodendrocytes in the neurovascular unit model after 7 days of induction. MBP-positive cells showed red fluorescence after tetraethyl rhodamine isothiocyanate staining and nuclei staining showed blue fluorescence under 4′,6-diamidino-2-phenylindole. Scale bars: 200 μm. (H) Neuro-glial sub-population percentages of neural stem derived cells showing the relative distribution of neurons to glia after 7 days of induction culture. Data are expressed as mean ± SD and were analyzed by a Student’s t-test. The experiments were repeated three times.

Morphology and viability of the cells on the microfluidic device

After 7 days of perfusion culture, hBMECs were attached to the microporous PC membrane, forming a monolayer barrier mimicking BBB in vivo. The hBMECs were cobblestone-shaped and tightly in contact, producing a complete endothelium (**Figure 6A**). A single-cell suspension of NSCs was loaded into the lower chambers through independent inlets, and differentiated into neuro-glial cells stably after 7 days of induction. NSCs-derived neurons and glial cells were completely confluent and indistinguishable (**Figure 6B**). After culture in respective chambers, the viabilities of each cell component were determined by calcine-potassium iodide double staining (**Figure 6C and D**). More than 90% of the endothelial and neuro-glial cells were live, significantly exceeding the number of dead cells (P < 0.05; **Figure 6E and F**).
Effects of tumor necrosis factor-α (TNF-α) and oxygen and glucose deprivation (OGD) on tight junctions.

We screened OGD/reperfusion (OGD/R) injury conditions based on the changes of lactate dehydrogenase (LDH) activity in the perfusion fluid, which is sensitive to increase at 6 hours of reperfusion and reached a maximum at 24 hours and 48 hours of reperfusion (Figure 2A). After reperfusion, the permeability of the endothelial barriers in the NVU model increased after 12 hours of reperfusion, and reached a maximum at 24 hours and 48 hours of reperfusion (Figure 8B). The activity of LDH began to increase at 6 hours of reperfusion and reached a maximum at 24 hours (Figure 8C). The degree of damage of OGD/R in vitro can be quickly realized by the changes of LDH activity in the perfusion fluid, which is sensitive and convenient. The injury of OGD/R on the activity of the microvascular endothelial cells and neuro-glial cells in the NVU model is shown in Additional Figure 7. The survival rates of neuro-glial cells and hBMECs began to decline at 6 and 12 hours of reperfusion, respectively, and reached the lowest survival rate at 24 and 48 hours of reperfusion.

Integrity and permeability of the neurovascular unit

After 7 days of culture through perfusion, the integrity of the endothelial barrier in the vascular unit was detected by immunofluorescence staining. hBMECs in the chambers expressed vWF in the normal culture (Figure 7A), verifying that the microvascular endothelial activity was normal. Under the inverted fluorescence microscope, the positive expression of ZO-1 protein was detected among hBMECs (Figure 7B), indicating that tight junctions were formed between endothelial cells and the integrity of the barrier was established.

To determine permeability of the endothelial barrier, 4 kDa FITC-dextran was introduced to the prepared device. The upper channels were completely perfused with medium containing FITC-dextran. The perfusion liquid from the basolateral chamber outlet was detected hourly. According to the FITC-dextran calibration curve (only PC membrane, without cells), the absorbance increased rapidly, and the equilibrium state was reached in 1 hour. Moreover, the fluorescence intensity of the fully confluent endothelial barrier increased significantly less than that in the control group (P < 0.05). hBMECs remarkably increased the barrier permeability, as proven by the lower FITC-dextran intensity of the basolateral chamber.

Biochemical modulation of the neurovascular unit

The influence of TNF-α, an inflammatory agent, on the microenvironment of the NVU model was evaluated. The vascular layer in which the endothelial barrier was not exposed to TNF-α was used as a negative control. After stimulation of TNF-α for 2 hours, FITC-dextran leakage across the neurovascular interface remarkably increased in the sample with TNF-α stimulation, resulting in an increase in fluorescence intensity of the perfusion liquid from the basolateral chamber. Therefore, TNF-α, which was circulated after perfusion, attenuated the endothelial barrier integrity that was increased rapidly, and the equilibrium state was reached in 1 hour. Moreover, the fluorescence intensity of the fully confluent endothelial barrier increased significantly less than that in the control group (P < 0.01). hBMECs remarkably decreased the barrier permeability, as proven by the lower FITC-dextran intensity of the basolateral chamber.

Discussion

Using microfabricated devices to mimic physiological structures, functions, and microenvironments requires the culture of diverse cells in devices with complex architectures and several compartments that are vertically or horizontally stacked (Phan et al., 2017; Ahadian et al., 2018). However, it is still challenging to culture more than two types of cells in these microdevices (Young and Beebe, 2010; Zervantonakis et al., 2011; Yan et al., 2021). First, it is rather difficult to seed cells in sealed chambers because of placement and viability. Second, continuous replenishment of fresh medium during cell growth in sealed systems is significantly hampered. Finally, if tissue vascularization is needed, the differentiation or growth rates of vascular and parenchymal cells should be optimized to expand the assay window. Particular attention should be paid to the process if there are more than three types of cells (e.g., our neuro-glial co-cultures) along the vascular endothelial barrier in a culture system.

Cell lines have been widely used to establish in vitro models because of their immortalization and easy culture characteristics (Cucullo et al., 2007; Ravi et al., 2015). However, the data obtained from cell lines to build functional in vitro models are quite different from the real situation of the human body. Because the limitations of nonhuman or immortalized cell lines are well recognized, researchers have increasingly isolated and cultured primary human cells for experimental research (Ghosh et al., 2011). Since Reynolds and Weiss (1992) isolated NSCs from the adult mammalian forebrain, new neurospheres have been confirmed to form in adulthood. As a result, lifelong neurogenesis has been
microfluidic chip technology. In addition to facile seeding and maintenance of the various cells together, with low sample requirements, the designed chip enabled building of complex models in a simple way (at least four types of cells were obtained using only two cell types). Furthermore, the endothelial barriers of the NVU model could mimic the role of BBB and have a good response to inflammatory stimulation. Additionally, the compartmentalized chip may be able to deliver hormones, cytokines, drugs, nutrients, viral/bacterial agents, and exosomes into the vascular channels, and NVU can be regarded as a whole system. We will use this microfluidic approach to study the pathogenesis of neurodegenerative diseases, CNS-related drug screening, biodefense, and individualized medication.

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Author contributions: Study conception and design, data analysis and manuscript writing: WJW; experiment implementation: WJW, YCW, WGC; manuscript revision: XG, JL. All authors read and approved the final version of the manuscript.

Conflicts of interest: None declared.

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Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Open peer reviewer: Kasum Azim, Universitätsklinikum Düsseldorf, Germany.

Additional files: Additional file 1: Open peer review report 1. Additional Figure 1: Differentiation of neural stem cells into neuro-glial cells in the model of neurovascular unit after 7 days of perfusion culture. Additional Figure 2: Effect of oxygen and glucose deprivation/reperfusion on the activity of brain microvascular endothelial cells and neuro-glial cells in neurovascular unit models.

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The primary purpose of the in vitro NVU microdevice is to model the basic structural and functional units, as well as the physiological microenvironment, of the human brain. The device is also applicable to predictive screening, assessment, and optimization of drug candidates for BBB permeation, as well as the study of neurovascular dysfunction. It can be used for in vitro modeling of the neurovascular unit after 7 days of perfusion culture. The device is also applicable to predictive screening, and individualized medication guidance.

The neurovascular interface in our device was built using primary hBMMECs in which the tight junction protein ZO-1 was expressed. The permeation of FITC-dextran was also monitored in the NVU device in real time. We studied whether the NVU device was impossible using transwell chambers. As evidenced by the lower FITC-dextran intensity of the basolateral chamber, hBMMECs remarkably decreased the barrier permeability, indicating that tight junctions formed in the NVU model device. In addition, the endothelial barrier after TNF-a stimulation leaked more FITC-dextran than the unstimulated barrier. Accordingly, TNF-a may augment barrier leakage by triggering a pro-inflammatory reaction. Furthermore, we used the NVU model to investigate how the OGD/R to mimic ischemic encephalopathy, which accounts for approximately 85% of all cerebrovascular diseases. The results suggested that our NVU model showed good biocompatibility and neurovascular interface integrity, as well as good retention of the physiological chemical stimulus. However, the module design was not integrated with a trans-endothelial electrical resistance (TER) electrode as shown by Grieß et al. (2013), aiming to simplify the fabrication process.

The NVU model device in this study had the following advantages. (1) By combining an assembled, integrated, 3D dynamic perfusion microfluidic device with primary human-derived cells (rather than genetically engineered cell lines or animal cells), we developed a reliable, versatile, easily operated in vitro model to establish brain structural units that mimicked the in vivo cellular microenvironment. (2) Using the differentiation potential of NSCs to obtain three types of neural cells (neurons, astrocytes, and oligodendrocytes), which cannot be readily expanded and proliferated in vitro. This system is beneficial to the complex model of NVU using simple methods and reference cells. (3) The scarcity of human source cells from primary extractions can be solved by simply combining primary and less cell consuming cell lines. The cell survival rate can be increased by continuous perfusion and dynamic culture. (4) This system inspires further construction of pathological models of various neurological diseases, such as hypoxic-ischemic brain damage, and Alzheimer’s disease for drug safety and efficacy evaluation, dose screening, and individualized medication guidance.

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In summary, we successfully established an in vitro brain NVU model based on the differentiation potential of NSCs and integrated characteristics of the
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Additional Figure 1 Differentiation of neural stem cells into neuro-glial cells in the model of neurovascular unit after 7 days of perfusion culture.

(A) The differentiation of neural stem cells into neurons in the neurovascular unit model after 7 days of induction. Microtubule-associated protein 2 (MAP2)-positive cells showed green fluorescence after fluorescein isothiocyanate staining and nuclei staining showed blue fluorescence under 4′,6-diamidino-2-phenylindole. (B) The differentiation of neural stem cells into astrocytes in the neurovascular unit model after 7 days of induction. Glial fibrillary acidic protein (GFAP)-positive cells showed red fluorescence after tetraethyl rhodamine isothiocyanate staining, and nuclei staining showed blue fluorescence under 4′,6-diamidino-2-phenylindole. (C) The differentiation of neural stem cells into oligodendrocytes in the neurovascular unit model after 7 days of induction. Myelin basic protein (MBP)-positive cells showed red fluorescence after tetraethyl rhodamine isothiocyanate staining, and nuclei staining showed blue fluorescence under 4′,6-diamidino-2-phenylindole. Scale bars: 200 μm.
Additional Figure 2 Effect of oxygen and glucose deprivation/reperfusion on the activity of brain microvascular endothelial cells and neuro-glial cells in neurovascular unit models.

(A) The survival of brain microvascular endothelial cells in the neurovascular unit model. (B) The survival of brain microvascular endothelial cells in the neurovascular unit model after 3 hours of oxygen and glucose deprivation. The living cells showed green fluorescence after Calcein staining and the dead nuclei showed red fluorescence under propidium iodide. (C) The percentage of survival rate of brain microvascular endothelial cells in the neurovascular unit model at different reperfusion time points. (D) The survival of neural stem cells derived neuro-glial cells in the neurovascular unit model. (E) The survival of neural stem cells derived neuro-glial cells in the neurovascular unit model after 3 hours of oxygen and glucose deprivation. The living cells showed green fluorescence after Calcein staining and the dead nuclei showed red fluorescence under propidium iodide. Scale bars: 200 μm. (F) The percentage of survival rate of neural stem cells derived neuro-glial cells in the neurovascular unit model at different reperfusion time points. Data are expressed as mean ± SD. **P < 0.01, vs. control group; ###P < 0.01, vs. 0 h group (Student’s t-test). The experiments were repeated by three times.