

A Thesis for the Degree of Ph.D. in Engineering

Development of an in vitro neurovascular unit model
by on-chip tissue engineering

February 2019

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Thesis Abstract

Neurovascular unit (NVU) is a conceptual unit in the brain, composed of neurons, glial cells such as astrocytes, microvasculatures covered by pericytes and extracellular matrix. This warns against focusing on only neurons and indicates the importance of investigating interactions between neurons and other brain cells for therapeutic approaches for neurodegenerative diseases. Microvasculatures in NVU have brain-specific functions called blood-brain barrier (BBB) and limit the transport of drugs to neurons. Although many studies have been performed for investigating BBB functions, an *in vitro* three-dimensional (3D) NVU model has not been established because of the gap between *in vitro* cell culture microenvironments and *in vivo* cellular conditions. Therefore, the aim of this study is to establish an *in vitro* 3D NVU model using a microfluidic device.

Chapter 1 summarizes the background, previous studies and objectives of this study.

Chapter 2 summarizes materials and methods of this study.

Chapter 3 describes the establishment of an *in vitro* brain angiogenesis model focusing on microvasculatures in NVU. In particular, organ-specificity of endothelial cells (ECs) is focused on, and brain ECs and human umbilical vein endothelial cells (HUVECs) are cultured to construct microvasculatures. These organ-specific microvasculatures are compared in terms of microvascular formation processes, endothelial barrier functions and endothelial junction protein expressions. Consequently, an *in vitro* brain angiogenesis model is successfully established. In addition, it is found that endothelial barrier functions are greater in brain microvasculatures compared to microvasculatures formed by HUVECs.

Chapter 4 describes the integration of brain angiogenesis and neurogenesis models toward the construction of an *in vitro* NVU structure. First, brain ECs and mesenchymal stem cells are cocultured to investigate cell culture conditions for inducing angiogenesis. Next, the optimized culture condition for angiogenesis is applied to the culture of neural stem cells (NSCs), which succeeds in inducing 3D neurite extension. Finally, these angiogenesis and neurogenesis models are combined, which results in the construction of neurovascular tissues.

Chapter 5 describes investigation of long-term culture to construct NVU tissues including astrocytes. NSCs differentiate into astrocytes in long-term culture, which allows to construct a tissue composed of neurons, astrocytes and microvasculatures. However, further investigation is needed to construct more physiological NVU structures.

Chapter 6 summarizes the results of this study and future perspectives.

論 文 要 旨

【訳題】

オンチップ組織工学による生体外 Neurovascular Unit モデルの開発

Neurovascular Unit (NVU) とは、主にニューロン、アストロサイトなどのグリア細胞、ペリサイトを伴う微小血管、細胞外マトリクスから成る概念的な構成単位である。このことは、神経変性疾患の治療に対する治療戦略として、ニューロンのみに着目するのではなく、周辺の細胞との相互作用の調査を行うことの重要性を示唆している。NVU における微小血管には血液脳関門という脳血管特有のバリア機能があり、ニューロンに対する薬剤の供給を制限する。これまで血液脳関門の機能について多くの研究が行われてきたが、生体外の脳血管モデルは生体内の環境との乖離があり、いまだに NVU の 3 次元生体外モデルは実現していない。そこで本研究では、マイクロ流体デバイスという細胞の 3 次元培養が可能なチップを用いて、神経幹細胞と脳の血管内皮細胞を培養し、生体外 NVU モデルを構築することを目的とした。

第 1 章に、本研究に関する基礎事項、従来の研究、本研究の目的を概説した。

第 2 章に、本研究で用いた試薬類や実験手法などについてまとめて述べた。

第 3 章では、まず NVU の中の微小血管のみに着目し、生体外血管新生モデルの確立のために行った実験について述べた。特に血管内皮細胞の臓器特異性について着目し、脳血管内皮細胞およびヒト臍帯静脈内皮細胞を用いてそれぞれ微小血管網の構築を行い、その構築プロセス、血管バリア機能の評価、血管内皮細胞のジャンクションタンパク群の発現評価などを行った。その結果、生体外脳血管新生モデルを確立するとともに、脳血管において血管バリア機能が優れていることを見出した。

第 4 章では、生体外 NVU 構造の構築を目指し、血管新生モデルと神経新生モデルの統合を試みた。まず、脳血管内皮細胞と間葉系幹細胞の共培養によって血管新生を誘導できるような細胞培養条件を検討した。次に、その培養条件下で神経幹細胞を培養し、3 次元的な神経網形成を誘導することに成功した。さらに、血管新生と組み合わせることで、神経網および微小血管からなる神経・血管組織の構築に成功した。

第 5 章では、第 4 章で述べた培養系を長期培養にすることにより、神経網と微小血管に加えアストロサイトを含んだ NVU 様組織の構築に必要な培養条件を検討した。長期培養によって神経幹細胞からアストロサイトへの分化誘導が確認され、神経・血管・アストロサイトを含む組織の構築に成功したが、生体内の模擬のため、さらなる検討が必要であることが示唆された。

第 6 章に、結論として各章の内容をまとめて研究成果を要約し、今後の展望について述べた。

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List of abbreviations and symbols

2D: two-dimensional

3D: three-dimensional

AD: Alzheimer's disease

Ang: angiopoietin

AQP: aquaporin

A β : amyloid- β

BBB: blood-brain barrier

BDNF: brain derived neurotrophic factor

bFGF: basic fibroblast growth factor

BM: basement membrane

BMEC: brain microvascular endothelial cell

CNTF: ciliary neurotrophic factor

DAPI: 4',6-diamidino-2-phenylindole

Dll4: delta-like ligand 4

EC: endothelial cell

ECM: extracellular matrix

FBS: fetal bovine serum

FITC-dextran: fluorescein isothiocyanate-conjugated dextran

GFAP: glial fibrillary acidic protein

Glut: glucose transporter

HIF-1 α : hypoxia inducible factor-1 α

HUVEC: human umbilical vein endothelial cell

IGF-1: insulin-like growth factor-1

MAP2: microtubule associated protein 2

MMP: matrix metalloproteinases

MSC: mesenchymal stem cell

NG-2: neuron-glia antigen-2

NSC: neural stem cell

NVU: neurovascular unit

PBS: phosphate buffered saline

PDGF: platelet-derived growth factor

PDMS: polydimethylsiloxane

PECAM-1: platelet endothelial cell adhesion molecule

SMC: smooth muscle cell

TEER: transendothelial electrical resistance

VE-cadherin: vascular endothelial cadherin

VEGF: vascular endothelial growth factor

α -SMA: α -smooth muscle actin

Symbols used in chapters 2 and 3

c_m : solute concentration inside cell membrane [mol/cm³]

c_0 : solute concentration difference across vessel wall [mol/cm³]

D : diffusion coefficient [m²/s]

I : fluorescence intensity

I_0 : initial intravascular fluorescence intensity

J : flux [mol/cm²/s]

l : vessel length [cm]

N : amount of dextran molecules [mol]

P : permeability coefficient [cm/s]

r : vessel radius [cm]

S : area of vessel wall [cm²]

V : volume of vessel [cm³]

β : partition coefficient

Chapter 1 General Introduction

1-1 Neurovascular unit (NVU)

The brain consumes almost 20% of all nutrients present in the blood (Zlokovic, 2008). For the proper function of the brain, neurons always demand nutrients, mainly glucose, from closely-existing capillaries. Almost all neurons in human brain have capillaries in close proximity (Zlokovic, 2005). Astrocytes exist in close proximity between neurons and capillaries. This neuron-astrocyte-capillary organization is important for astrocytic transport of nutrients from blood to neurons, and this unique multicellular structure is called the neurovascular unit (NVU) defined as a structure composed of neurons, astrocytes, capillaries and extracellular matrix (ECM).

The concept of NVU was proposed in the Stroke Program Review Group held in 2001 by the National Institutes of Neurological Disorders and Stroke (<https://www.ninds.nih.gov/>) (Lo et al., 2003). The concept of NVU implies the importance of considering multicellular structure of NVU. The loss of neuronal functions causes neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Neuronal functions are supported by surrounding other cells. It is important to consider interactions between neurons and other cell types in NVU for the further understanding of pathological states in neurodegenerative diseases (Iadecola, 2017).

1-2 Blood vessels and capillaries

1-2-1 Fundamental features of blood vessels and capillaries

Blood vessels are composed of endothelial cells (ECs), basement membrane (BM), mural cells and ECM. ECs form monolayer wall of blood vessels. BMs are various secreted proteins from ECs and mural cells, including type IV collagen, laminin, fibronectin, heparan sulfate proteoglycan and so on. Mural cells are smooth muscle cells

(SMCs) and pericytes. Mural cells exist closely around blood vessel wall and interact with endothelial monolayer. Pericytes exist around capillaries (Fig. 1-1) while SMCs exist around larger vessels. The average diameter of capillaries is 5–10 μm . ECM is composed of collagens, fibronectin, laminin, proteoglycans and so on (Frantz et al., 2010). ECM forms net fiber structure and almost all cells attach the fiber structure to live and form organs. Blood vessels are distributed throughout the body and mainly work as a conduit of blood. Blood vessels supply oxygen and nutrients to parenchymal cells. ECs are connected with each other through various junctions. Leakage of macromolecules and cells floating in blood into parenchyma is normally restricted by tight junctions and adherens junctions while oxygen and nutrients are properly transported to organ parenchyma through vascular walls.

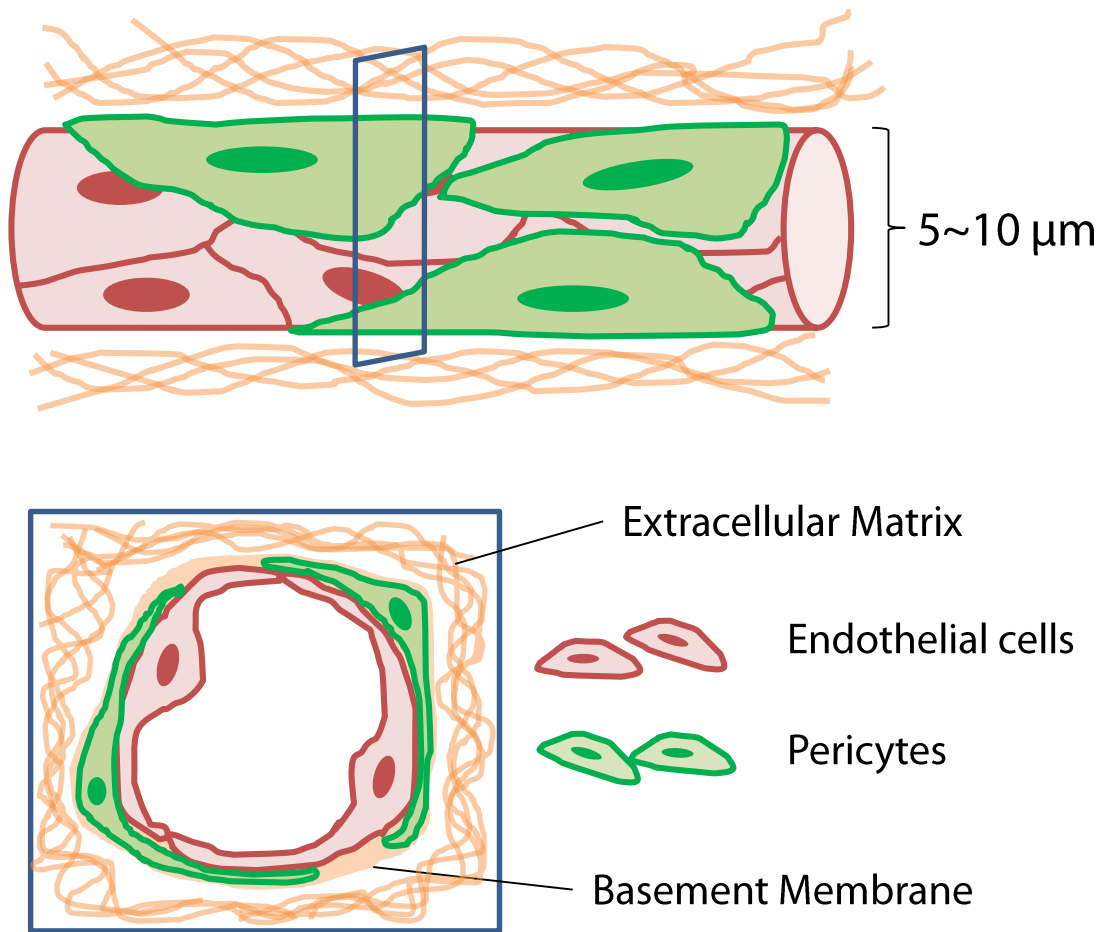


Fig. 1-1 Schematic illustrations of capillary microenvironment.

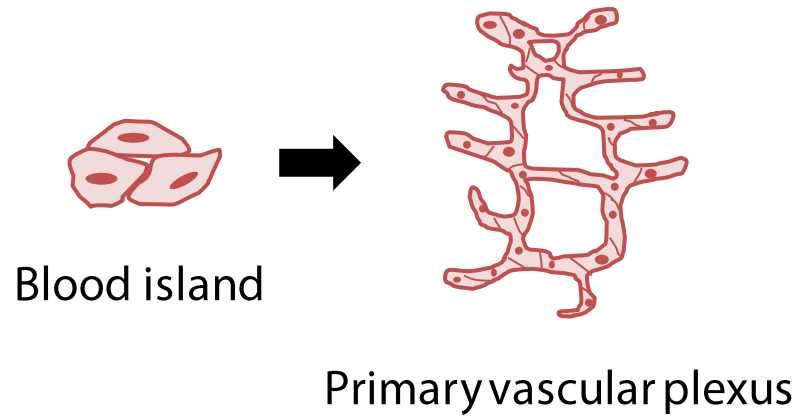
Capillaries are small blood vessels whose diameter ranges from 5 to 10 μm . Capillaries are sparsely wrapped by pericytes. BM proteins localize at outer capillary surface.

1-2-2 Vasculogenesis and angiogenesis

Blood vessels constitute a circulatory system throughout human body. They transport oxygen and nutrients throughout our body to maintain functions of each organ and sustain our lives. In the generation of our body through embryogenesis, there are two main mechanisms of creating blood vessel networks.

Vasculogenesis is the first step to build up vascular networks (Fig. 1-2). In the embryonic stage, hemangioblasts, which are stem cells of ECs, proliferate to create a group of cells called blood island. Hemangioblasts existing in the periphery of blood islands differentiate into ECs and create primary vascular plexus. The series of processes of creating primary vascular plexus is called vasculogenesis. As a next step, ECs sprout from the pre-existing vascular plexus and form more complex vascular networks through branching and pruning processes. These growing vascular networks are then wrapped by other cell types such as SMCs and pericytes through interactions among these cell types. Pericytes wrap around capillaries to stabilize their structures. Capillaries wrapped by pericytes become mature and stable. This morphogenesis from vascular plexus to mature vascular networks is called angiogenesis.

Vasculogenesis



Angiogenesis

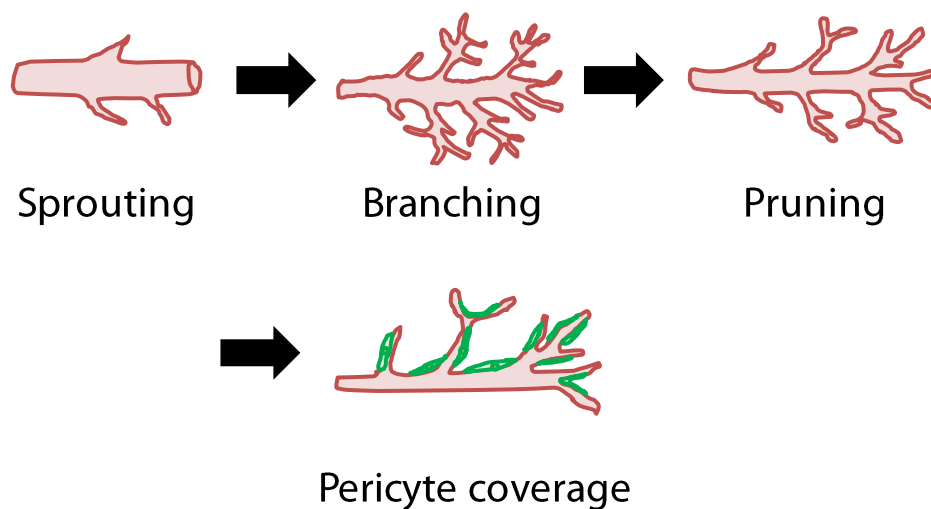


Fig. 1-2 Schematic illustrations of vasculogenesis and angiogenesis.

Vasculogenesis is the first step of vascular formation processes in which endothelial stem/progenitor cells expand and form primary vascular plexus. Angiogenesis is the following step for forming more complex vascular networks. Angiogenic processes are classified into sprouting, branching, pruning and pericyte coverage.

Angiogenesis is a multi-step morphogenesis of creating new blood vessels. In angiogenic processes, ECs show different morphologies and functions depending on the parts of growing vessels (Ribatti and Crivellato, 2012) (Fig. 1-3). As a first step, vascular sprouts are formed by leading ECs. these ECs are called tip cells (Gerhardt et al., 2003). Tip cells exist at the front edge of vascular sprouts and guide other ECs to elongate vascular sprouts. Tip cells extend filopodial structures, which sense guidance cues such as vascular endothelial growth factor (VEGF) (Ruhrberg et al., 2002). ECs following tip cells are called stalk cells, which proliferate and form vascular lumen structures. The selection of tip cells out of pre-existing vascular ECs is performed by signaling processes between VEGF and VEGF receptor 2 (VEGFR2), one of the receptors for VEGF (Bentley et al., 2014). ECs constituting vascular monolayer have VEGFR2. When VEGF bind VEGFR2 in ECs, these ECs activate Notch receptor in adjacent ECs by supplying delta-like ligand 4 (Dll4). ECs whose Notch receptors are activated by Dll4 behave like stalk cells while ECs which have high VEGF/VEGFR2 signaling level stay located at the leading edge of vascular sprouts as tip cells. Position changes between tip cells and stalk cells can happen depending on the balance between VEGF/VEGFR2 signaling and Dll4/Notch signaling. ECs residing in the pre-existing capillary with a stable morphology are called phalanx cells. Phalanx cells have VEGFR1, which acts as a suppressor of VEGF binding to VEGFR2. Phalanx cells contribute to stop VEGF/VEGFR2 signaling and finish angiogenic processes (Bautch, 2009).

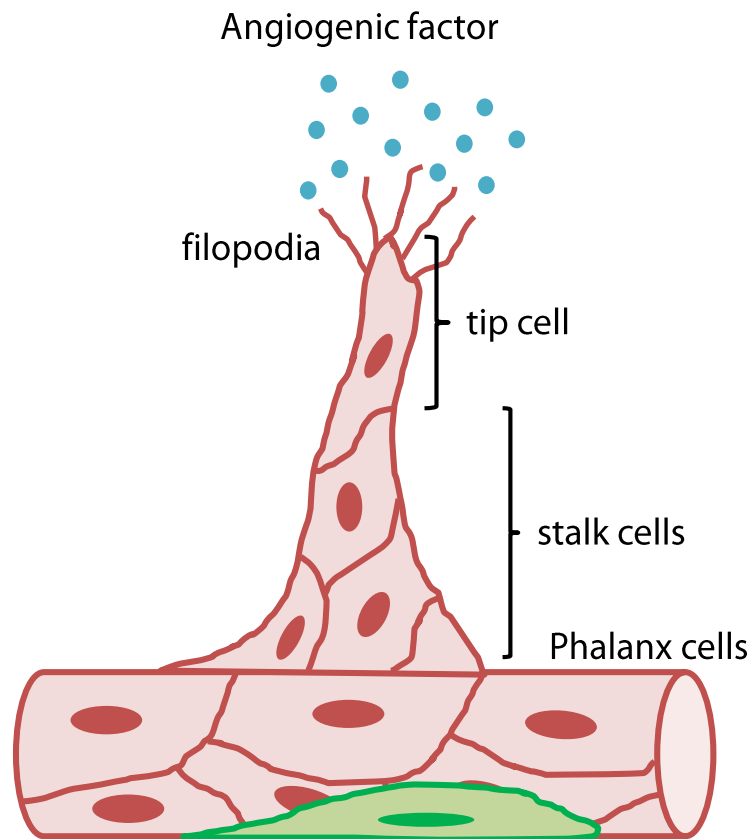


Fig. 1-3 Types of ECs in processes of angiogenesis.

Tip cells are leading cells of growing vasculatures and extend filopodial structures sensing angiogenic factors. Stalk cells are proliferating cells in growing vasculatures. Stalk cells form vascular lumen structures. Phalanx cells exist in the root of growing vessels and contribute to finish angiogenic processes.

VEGF promotes EC proliferation, inhibits EC apoptosis and increase endothelial permeability. VEGF regulates vascular development throughout embryogenesis. The expression of VEGF is induced by hypoxic conditions. Especially, hypoxia inducible factor-1 α (HIF-1 α) promotes the expression of VEGF (Zimna and Kurpisz, 2015).

Growing capillaries become mature through interactions between ECs and pericytes. ECs secrete platelet-derived growth factor-B (PDGF-B) to recruit pericytes. Pericytes have PDGF receptor- β (PDGFR- β). Proliferation and migration of pericyte progenitor cells are promoted through PDGF/PDGFR- β signaling, resulting in pericyte covering around growing endothelial tubes.

Angiopoietin-1 (Ang-1) is a major angiogenic factor to promote vascular maturation (Ribatti et al., 2011) (Fig. 1-4). Ang-1 is secreted by pericytes and binds to endothelial Tie-2, a receptor for Ang-1. Angiopoietin-2 (Ang-2) is expressed in ECs and acts as a de-stabilizing factor of vessels. VEGF increases the production of Ang-2 by ECs, and Ang-2 binds to pericyte Tie-2 receptor, resulting in the dissociation of pericytes from vessels. In this state, VEGF induces angiogenesis by stimulating ECs without pericytes. ECs without the coverage of pericytes and stimulus of VEGF undergo apoptosis.

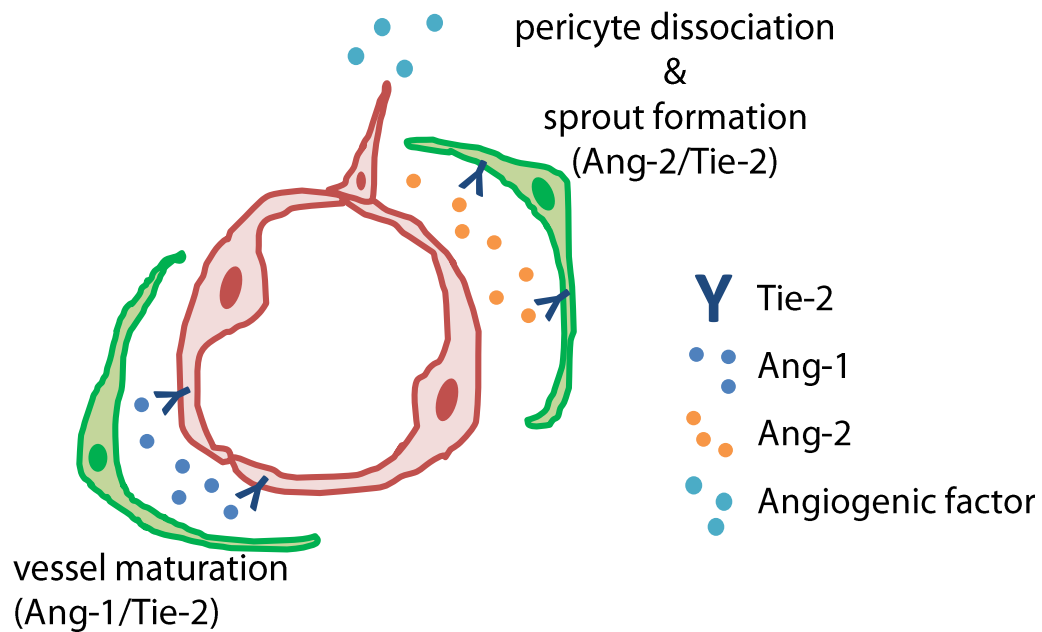


Fig. 1-4 Interactions between ECs and pericytes.

Ang-1 secreted by pericytes binds to endothelial Tie-2, which promotes pericyte association and capillary maturation. Endothelial Ang-2 binds to Tie-2 in pericytes, which generates pericyte dissociation. ECs without pericyte coverage can elongate vascular sprouts toward the region with abundant angiogenic factors such as VEGF.

1-3 Extracellular matrix (ECM)

ECM is a scaffold of organs and has fiber network structures. ECMs are essential for cells to live and function in every organ. Cells attach to ECM structure through integrins. Cell–ECM attachment through integrins is important for cell–cell or cell–ECM interactions. Main components of ECM are collagens, fibronectin, laminin, proteoglycans (Frantz et al., 2010). The components of ECM are different in every organ. In general, collagen I and fibronectin are abundant in many organs. However, brain ECM has much less collagen I and fibronectin (Bonneh-Barkay and Wiley, 2009; Zimmermann and Dours-Zimmermann, 2008). Hyaluronan is abundant in brain ECM and various proteoglycans bind to hyaluronan. Brain cells attach to these proteoglycans.

1-4 Mesenchymal stem cell (MSC)

Mesenchymal stem cells (MSCs) exist in many tissues, and are isolated from brain, spleen, liver, kidney, lung, thymus and pancreas (Lai et al., 2015). MSCs are originally defined as multipotent stem cells which have an ability to differentiate into adipocytes, chondrocytes and osteocytes (Lai et al., 2015). MSCs are now known to differentiate into stromal support cells and secrete factors to support other cells (Lai et al., 2015; Volarevic et al., 2011). In particular, MSCs are known to have some effects on angiogenesis (Bronckaers et al., 2014; Nassiri and Rahbarghazi, 2014; Volarevic et al., 2011). MSCs secrete VEGF, basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1) and PDGF to support EC survival and promote angiogenesis in a paracrine manner. It was reported that co-implantation of ECs and mesenchymal precursor cells led to the formation of long-lasting blood vessels in mice (Koike et al., 2004). The implanted mesenchymal precursor cells expressed mural cell marker, suggesting that MSCs can differentiate into pericytes. In addition to the proangiogenic effects of MSCs, MSCs are also known to promote endothelial junction protein expressions such as vascular endothelial cadherin (VE-cadherin) and Claudin-1 in a

juxtacrine manner (Menge et al., 2013; Nassiri and Rahbarghazi, 2014). MSCs can inhibit angiogenic processes by enhancing vascular integrity. Taken together, MSCs have both positive effects and negative effects on angiogenesis and play important roles throughout angiogenic processes. The relationship between MSCs and pericytes still remains to be elucidated. However, some previous studies reported that MSCs and pericytes have many common features (Caplan, 2017; de Souza et al., 2016).

1-5 Blood-brain barrier (BBB)

1-5-1 Fundamental features of BBB

The blood-brain barrier (BBB) is a brain-specific endothelial barrier. ECs constituting brain microvasculatures are connected with each other by tight junction proteins and strictly regulate the transport of oxygen and nutrients to brain parenchyma.

Brain microvasculatures are formed by ECs, pericytes and astrocytes (Fig. 1-5). Brain microvasculatures are wrapped by pericytes and both ECs and pericytes are surrounded by BM. Astrocytic endfeet ensheath ECs and pericytes. BMs are composed of different proteins including type IV collagen, laminin, nidogen and so on (Thomsen et al., 2017). These BM proteins are secreted by ECs and pericytes.

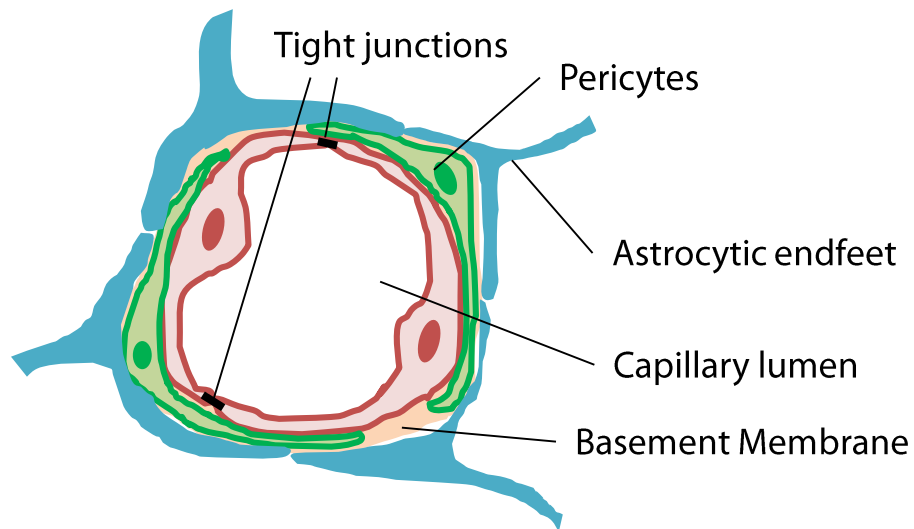


Fig. 1-5 BBB microenvironment.

ECs are sealed with each other by tight junction proteins and form capillary lumen. Pericytes wrap capillary lumen structures. Basement membranes ensheath both ECs and pericytes. Astrocytic endfeet wrap ECs and pericytes from further outside.

1-5-2 BBB molecules

Brain ECs are connected with each other by tight junction proteins. Tight junction proteins expressed in a brain are occludin, claudin 1, claudin 3, claudin 5 and claudin 12, and tight junction accessory proteins, ZO-1, ZO-2 and ZO-3, are also expressed in endothelial intercellular region (Reinhold and Rittner, 2017; Tietz and Engelhardt, 2015). In addition, adherens junction proteins such as platelet endothelial cell adhesion molecule (PECAM-1) and VE-cadherin are known to regulate BBB functions (Gavard and Gutkind, 2006).

1-5-3 BBB transport pathways

Previous studies investigated BBB-specific transport pathways (Abbott et al., 2010; Sweeney et al., 2018). ECs connected by tight junctions strictly prohibit the passage of substances through endothelial monolayer as a paracellular route. While ECs of brain microvasculatures have low rate of paracellular and transcellular transport, they have specific transporters and receptors for the entry of specific substances into brain parenchyma. BBB protects neurons from neurotoxins existing in blood circulation and nurture them by supplying nutrients. O₂ and CO₂ cross the BBB by simple diffusion. Water is transported via water channels called aquaporin (AQP) receptors. Glucose, which is the main energy source of a brain, is transported by glucose transporter isotype-1 (Glut1). Brain ECs highly express Glut-1 protein. Glucose transport depends on the concentration gradient between blood and brain parenchyma. Peptides and proteins are transported via receptor-mediated transcytosis. The transport of amino acids, hormones and vitamins is performed by carrier-mediated transport.

1-6 Neural stem cell (NSC)

The brain is composed of neurons, glial cells including astrocytes and oligodendrocytes, blood vessels and ECM. Neurons, astrocytes and oligodendrocytes are generated by the same stem cell source called neural stem cell (NSC). While NSCs can generate neurons, astrocytes and oligodendrocytes, NSCs can divide symmetrically to generate the same stem cell (Ahmed, 2009; Temple, 2001). Undifferentiated cells which can self-renew and generate neurons, astrocytes and oligodendrocytes are defined as NSCs. Cultured NSCs also have an ability to generate differentiated cells (Fig. 1-6 A, B, C) while they can proliferate symmetrically (Fig. 1-6 D, E).

In terms of cultured NSC differentiation, previous *in vitro* studies investigated effects of soluble factors on the NSC differentiation into neuronal or glial lineages (Bhattacharya et al., 2008; Chen et al., 2013). For example, it was reported that brain-derived neurotrophic factor (BDNF) promoted NSC proliferation in a dose-dependent manner, and BDNF effectively promoted neuronal and oligodendrocytic differentiation (Chen et al., 2013). In terms of retinal stem cell differentiation, ciliary neurotrophic factor (CNTF) have concentration-dependent effects on neuronal or glial differentiation (Bhattacharya et al., 2008). It was reported that low concentration of CNTF induced neuronal differentiation while high concentration of CNTF induced glial differentiation. Fetal bovine serum (FBS), which is a widely-used essential supplement containing many factors required for cell growth and survival (Brunner et al., 2010) was also reported to have different effects on NSC differentiation (Hu et al., 2013; Hung and Young, 2006). These studies reported that FBS promoted NSC differentiation into astrocytes, not neurons. Taken together, previous *in vitro* studies suggested that some additional factors in NSC culture medium can promote neuronal or glial differentiation, further suggesting that specially-designed growth factor cocktail can be used for the desired expansion of neuronal and glial lineages in NSC culture.

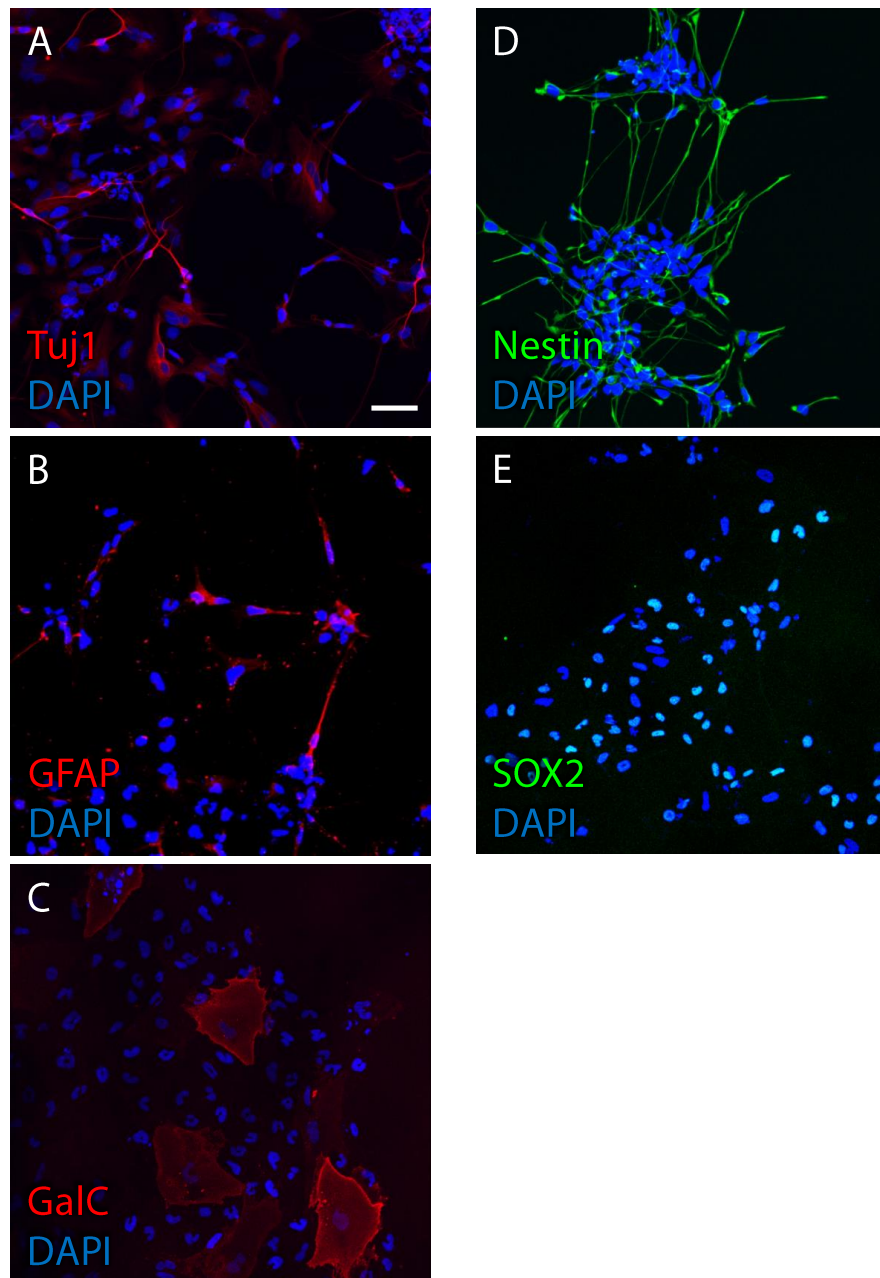


Fig. 1-6 Neural stem cell culture. (A: neurons, B: astrocytes, C: oligodendrocytes, D, E: neural stem cells) (Scale bar, 50 μ m)

NSCs were cultured using culture dishes for 14 days. Immunofluorescence staining was performed for differentiated cells (A, B, C) and undifferentiated stem cells (D, E). NSCs have an ability to generate differentiated cells in addition to the ability of self-renewal. Antibodies used here are described in chapter 2.

1-7 Organ-specific ECs

Every organ has its own vascular characteristics and patterning that are generated through interactions between ECs and parenchymal cells (Géraud et al., 2014). ECs in different organs are heterogeneous depending on organ-specific functions. Differences of ECs were previously reported (Géraud et al., 2014; Rafii et al., 2016). ECs are classified into three groups; continuous non-fenestrated ECs, continuous fenestrated ECs and discontinuous ECs. For example, brain ECs are continuous non-fenestrated ECs. ECs in choroid plexus are continuous and fenestrated. Liver sinusoidal ECs are discontinuous. Brain ECs strictly restrain the passage of molecules over 1 nm in size, while liver sinusoidal ECs allow permeation of molecules of 60 nm in size (Géraud et al., 2014).

ECs always interact with parenchymal cells. Organ-specific ECs are different not only in terms of fenestrae but also in terms of abilities to secrete angiocrine factors. Angiocrine factors are secreted by ECs and can stimulate organ-repair of damaged or diseased organs. A previous study showed that expression profiles of angiocrine factors in ECs were different among various organs (Nolan et al., 2013).

These differences of organ-specific ECs are not negligible when engineering multicellular structure such as BBB. To mimic microvascular environment by a tissue engineering approach, in which ECs interact with parenchymal cells, organ-specific ECs derived from the target organ need to be used for the recapitulation of the multicellular organization and its formation processes.

1-8 *In vitro* vasculogenesis/angiogenesis models

Previous studies reported *in vitro* vasculogenesis and angiogenesis models based on microfluidic cell culture systems (Kim et al., 2015, 2013; Lim et al., 2013; Moya et al., 2013). Microfluidic devices are made of polydimethylsiloxane (PDMS) and have designed microchannels. Microfluidic devices enabled us to culture cells

three-dimensionally within injected ECM scaffolds in the devices. By culturing ECs in ECM scaffolds such as collagen and fibrin and supplying growth factors, vascular formation can be induced through vasculogenesis or angiogenesis processes.

In previous studies, vascular formation was demonstrated by ECs, and most of these studies used human umbilical vein endothelial cells (HUVECs) (Kim et al., 2015, 2013; Lim et al., 2013). Vascular formation was induced within a week. Engineered vascular networks expressed not only endothelial junction markers such as VE cadherin, but also expressed basement membrane proteins, which were signs for the maturation of engineered vascular networks.

Functions of engineered vasculatures were also investigated. Especially, endothelial barrier function was analyzed. To analyze the barrier function of engineered vasculatures, it is needed to perfuse fluorescent dextran solution into the vasculatures. Previous studies successfully perfused the dextran solution into the engineered vasculature, and furthermore, the passage of the solution through the vascular wall was analyzed to measure the endothelial barrier function (Bang et al., 2017; Bichsel et al., 2015; Lee et al., 2014; Sobrino et al., 2016).

The construction of vascular networks and the perfusion of solution into the networks were of great importance for fundamental studies of vascular biology and drug screening studies. However, engineering of capillary-level microvascular networks with the coverage of pericytes has not been realized. In addition, as far as I know, *in vitro* BBB model of functional brain microvascular networks has not been reported while most previous studies were performed with HUVECs. Until now, previous *in vitro* BBB models have investigated endothelial barrier functions with/without pericytes and astrocytes in 2D-based cell culture systems.

1-9 *In vitro* BBB models

The main components of BBB are ECs, astrocytes, pericytes and BMs. ECs in BBB express tight junction proteins, and pericytes and astrocytes closely exist around ECs. Previous studies focused on this characteristic morphology of brain microvasculatures and demonstrated BBB microenvironment by culturing these cell types.

Transwell cell culture system has been mostly used as an *in vitro* BBB model (Banerjee et al., 2016; Bicker et al., 2014) (Fig. 1-7). Transwell system is a combination of well plates and semipermeable membrane inserts. Cells can be cultured on the top surface of the insert, the bottom surface of the insert and the bottom of wells. To mimic the *in vivo* microenvironment, ECs are basically cultured on the top surface of the insert. When ECs are cocultured with pericyte or astrocyte, pericytes or astrocytes are cultured on the bottom surface of the insert or the bottom of the well. Tri-culture system is realized by culturing ECs on the top surface of the insert and culturing other cell types on the bottom surface of the insert or the bottom of the well, which is a mimicry of *in vivo* BBB microvascular environment. BM proteins such as laminin or collagen IV can be coated on the top surface of the insert before seeding ECs on the insert. Transwell cell culture system not only mimics the *in vivo* brain microvascular environment, but also is useful for analyzing interactions among these cell types in paracrine manner.

Using Transwell cell culture system, endothelial barrier functions have been investigated. Transendothelial electrical resistance (TEER), which represents the resistance towards ion diffusion through endothelial monolayer, has been analyzed in the Transwell cell culture system to estimate endothelial barrier functions. This analysis is realized by employing two electrodes in apical side and basal side of EC monolayer respectively. Previous studies demonstrated that astrocytes or pericytes augmented the endothelial barrier functions estimated by TEER analysis in Transwell coculture and tri-culture systems.

Although previous *in vitro* BBB models have established a platform to investigate

interactions among ECs, astrocytes and pericytes in terms of endothelial barrier functions, there is still a gap between *in vitro* BBB models and native EC conditions *in vivo* (Bicker et al., 2014). Native *in vivo* capillaries are three-dimensional, while ECs in previous *in vitro* BBB models formed 2D monolayer on a culture substrate. In addition, ECs are covered by pericytes and astrocytic endfeet and the percentage of pericyte coverage around capillaries are the highest in the brain compared to the other organs (Daneman et al., 2010; Keaney and Campbell, 2015; Thomsen et al., 2017). These fundamental features of brain capillaries were not included in previous *in vitro* studies, highlighting the disparity between *in vitro* BBB models and native EC conditions *in vivo*.

Instead of Transwell cell culture system, a three-dimensional *in vitro* BBB model has been reported using a microfluidic cell culture system (Adriani et al., 2017). This study focused on the organization of the *in vivo* NVU structure in which astrocytes lie between neurons and microvasculatures. To mimic this organization, neurons and astrocytes were seeded within two adjacent collagen gel channels and ECs were seeded in medium channel to form EC monolayer. This study demonstrated that cultured neurons showed neurite elongation and exhibited neuronal activity. Endothelial barrier functions were analyzed by introducing fluorescent dextran solution into the EC-coated microchannel. However, this study implied some difficulties in engineering functional NVU structures. One was the difficulty in the mimicry of the NVU organization. This study indeed mimicked the cell arrangement composed of neurons, astrocytes and microvasculatures by seeding these cells in adjacent microchannels. However, these cell types were cultured within compartmentalized gel scaffolds respectively while astrocytes closely exist between neurons and microvasculatures *in vivo*. Another difficulty was the analysis of endothelial barrier functions. Permeability coefficients were calculated based on the fluorescence intensity of introduced fluorescent dextran into the EC-coated microchannel. However, cultured ECs formed monolayer, not lumen structures, and consequently had relatively large permeability coefficients compared to

previous studies. In addition, permeability coefficients were smaller in EC monoculture condition than coculture condition with neurons and astrocytes. This result contradicted previous reports that astrocytes augmented endothelial barrier functions performed by Transwell cell culture system and TEER approach. It is still challenging to construct a functional 3D NVU structure *in vitro*.

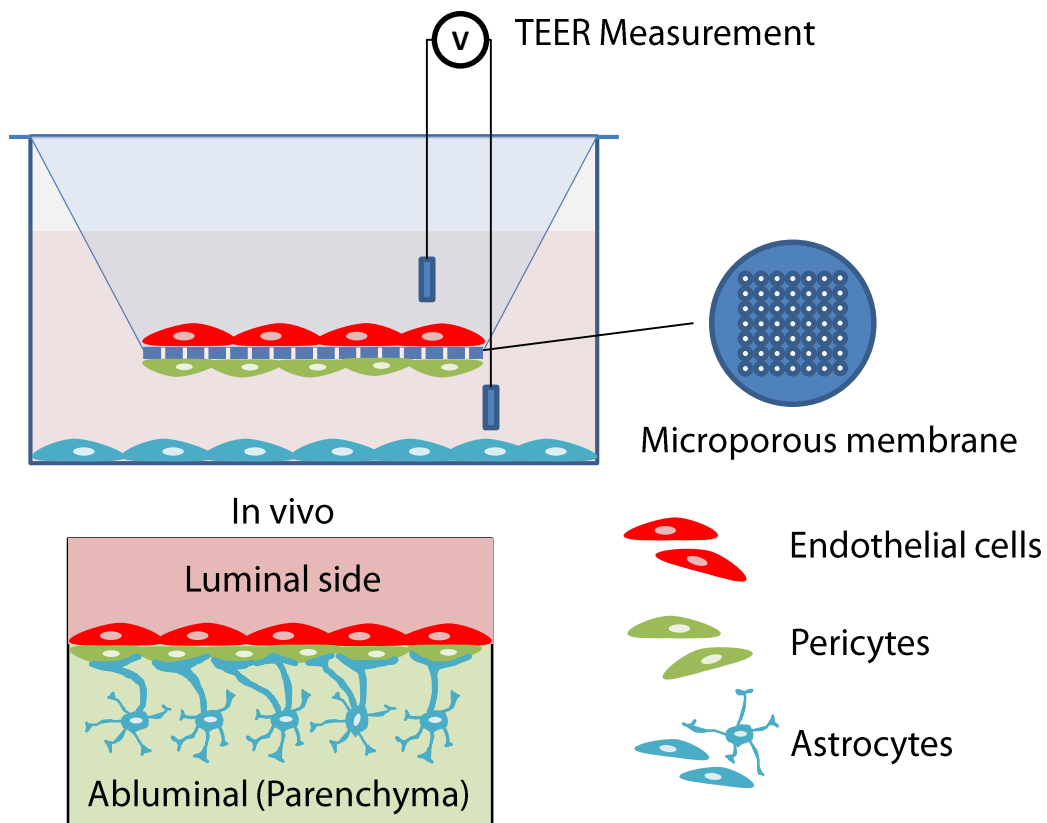


Fig. 1-7 Previous *in vitro* BBB model using Transwell cell culture system.

ECs were cultured in Transwell system and endothelial barrier functions were estimated by TEER analysis, which is the measurement of electrical resistance across an endothelial monolayer. Endothelial barrier function is augmented by culturing ECs with astrocytes and pericytes.

1-10 Application of *in vitro* BBB models to drug screening studies

One of the main objectives of establishing an *in vitro* BBB model is the technical development to investigate the drug delivery to brain parenchyma. BBB excludes almost 100% of large molecules (>500 Da) from the brain parenchyma and more than 98% of all small molecules (Pardridge W., 2005). Only 7% of drugs for brain diseases in clinical development reach the marketplace (Booth and Kim, 2012). The difficulty in the prediction of BBB passage makes it quite difficult to develop a new drug for neurodegenerative diseases. To overcome the difficulty, an *in vitro* BBB model as a tool of testing drug efficacy is needed.

In vitro disease models were reported previously. For example, pathological states of Alzheimer's disease (AD) were mimicked in an *in vitro* manner (Park et al., 2015). Neural progenitor cells were cultured in a microfluidic device which has a series of hemisphere wells on the bottom of a microfluidic channel. These cells were cultured in hemisphere wells as spheroid culture. A shear flow was applied in the channel. As a result, the shear flow enhanced differentiation of neural progenitor cells into neurons and the formation of neuronal networks among hemisphere wells. In pathological states of AD, the accumulation of amyloid- β ($A\beta$) is widely recognized. When $A\beta$ was added to the cell culture medium, viabilities and connectivity of neurons were decreased especially in flow condition compared to static condition. This previous study investigated the effects of fluid flow and $A\beta$ protein toxicity on neurospheroids and suggested the potential of the *in vitro* model as a drug screening model.

Another previous study established an *in vitro* BBB model focusing on the transport of solutes through endothelial monolayer cultured in a microfluidic channel, in which fluid flow can be applied (Booth and Kim, 2012). The fluid flow can be applied by the connection of a pump system to the microfluidic cell culture system. This previous study reported that fluid flow augmented the endothelial barrier function measured by TEER approach. In addition, ECs were exposed to histamine, known as an

inflammatory mediator for BBB disruption, resulting in the temporal disruption of the endothelial barrier function. This *in vitro* BBB model can be used as a tool for testing effects of barrier-enhancing or barrier-opening drugs.

Previous *in vitro* drug screening studies recapitulated some characteristics of pathological states or brain endothelial barrier functions. However, previous *in vitro* disease models and BBB models have some limitations, which prevents the mimicry of *in vivo* 3D characteristics of the BBB. Previous *in vitro* disease models have been performed using only neural cells, and mimicking the BBB has not been realized (Yi et al., 2015). In addition, there are many BBB studies performed using rodent cells or *in vivo* rodent models. However, these models reflect only some aspects of human neurodegenerative diseases because of species differences. Therefore, to fully understand the pathology of human diseases and the prediction of drug efficacy, human cell-based *in vitro* BBB model is needed, which will potentially be of great use for drug screening studies.

1-11 Objectives

The final goal of this thesis is the engineering of an NVU structure (Fig. 1-8). Main cellular components of NVU are ECs, pericytes, neurons and astrocytes. Morphological features of NVU include microvascular networks, coverage of pericytes and astrocytic endfeet around microvasculatures and neighboring neural networks, which generate neuron-astrocyte-microvascular system. If we can establish a cell culture system of NVU, we can possibly apply the system to studies for neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease by a drug screening approach or by a recapitulation of pathological processes and the investigation of the processes.

Previous *in vitro* NVU models have mainly focused on endothelial barrier functions specific for brain microvasculatures using Transwell cell culture system. In these studies, cellular microenvironment of BBB was mimicked, and endothelial barrier functions

were estimated by TEER approach. However, there is still a gap between the cellular microenvironment in the Transwell system and *in vivo* BBB microenvironment. *In vivo* complex microvascular structure has neither been considered in previous *in vitro* cell culture systems nor has been engineered by a tissue engineering approach.

One of the potential approaches of the engineering NVU structure *in vitro* is a microfluidic device. Microfluidic device has a set of microchannels designed to realize specific features of a target tissue or to investigate interactions among different cell types. Tissue engineering approaches can be performed using microfluidic devices because of the capability of culturing cells three-dimensionally within ECM scaffolds to investigate formation processes of a target tissue or to recapitulate morphological features of a target tissue.

To achieve the engineering of functional NVU organization, it is quite important to construct the characteristic structure of NVU including the features of BBB. In this organization, capillary networks can be engineered through the recapitulation of angiogenic processes. As well as capillary networks, neural and glial networks can be engineered by inducing the differentiation of NSCs and morphogenesis of differentiated cells. Therefore, *in vitro* NVU model can be considered as the combination of angiogenesis model and neurogenesis model. Hence, cell culture conditions for neural cells and ECs were investigated to induce neurogenesis and angiogenesis respectively.

In this thesis, effects of organ-specific ECs on angiogenic microvascular formation and its function were first investigated by culturing brain microvascular ECs (BMECs) or HUVECs with MSCs in chapter 3, which was an essential milestone in the construction of an *in vitro* BBB model. Next, an *in vitro* neurogenesis-angiogenesis model was established in chapter 4. In chapter 5, culture conditions for a long-term triculture of NSCs, BMECs and MSCs were investigated to construct a tissue composed of neurons, astrocytes and microvasculatures. Finally, summary and future perspectives were described in chapter 6.

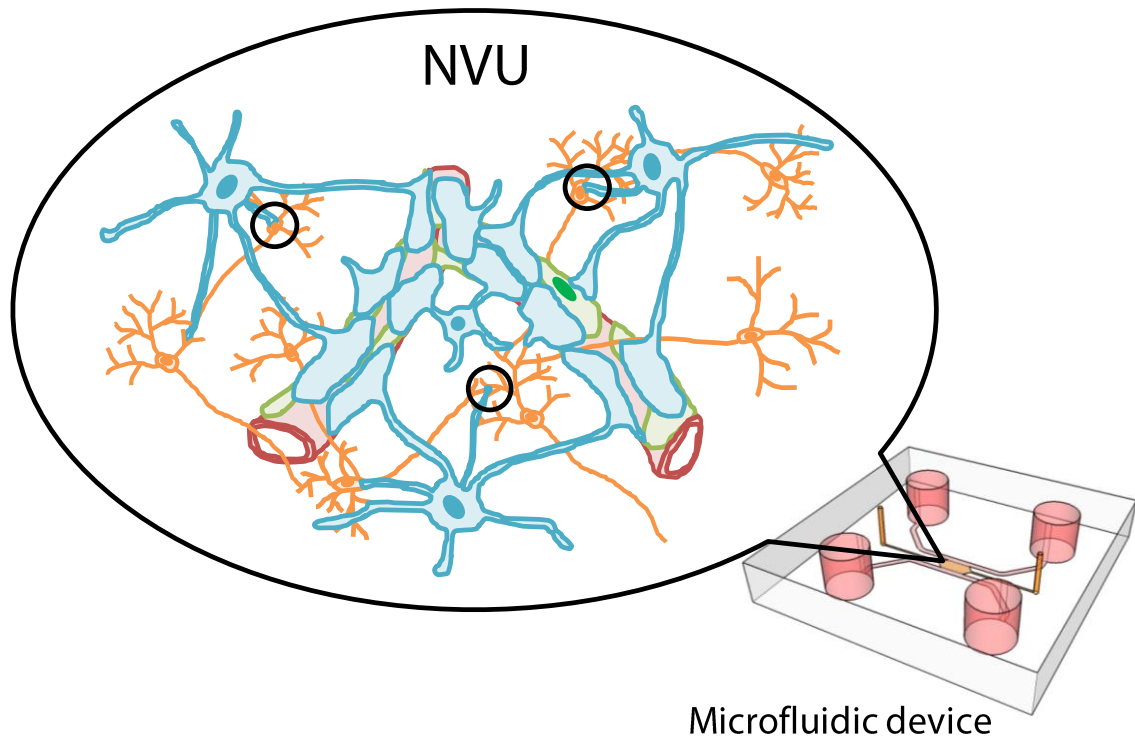


Fig. 1-8 Schematic illustration of an *in vitro* NVU model in a microfluidic device.

The purpose of this study is the construction of an *in vitro* NVU model which is composed of neurons (orange), astrocytes (blue) and microvasculatures (red) covered by pericytes (green). Especially, two characteristics of NVU are focused on; coverage of astrocytic endfeet around microvasculatures and close contact between astrocytes and neurons (circles).

Chapter 2 Materials and methods

2-1 Cell culture^(chapter 3, 4, 5)

Cells used in this thesis were human brain microvascular endothelial cells (BMECs), human umbilical vein endothelial cells (HUVECs), human H9-ESC derived neural stem cells (NSCs) and human mesenchymal stem cells (MSCs).

BMECs were commercially obtained (Cell Systems Corporation, Kirkland, WA, USA). BMECs were cultured in Endothelial Cell Growth Medium-2 BulletKit (EGM-2, Lonza, Basel, Switzerland) or CSC complete medium (Cell Systems Corporation), depending on experiments. BMECs were expanded in fibronectin-coated culture dishes for no more than 7 passages.

HUVECs were commercially obtained (Lonza). HUVECs were cultured in EGM-2. HUVECs were expanded in fibronectin-coated culture dishes for no more than 7 passages.

NSCs were commercially obtained (GIBCO, Gaithersburg, MD, USA) and expanded in fibronectin-coated culture dishes. NSCs were cultured in NSC medium kit (StemPro[®] NSC SFM, GIBCO): Knockout DMEM/F12 with 2% StemPro[®] Neural Supplement, 1% GlutaMAX, 20 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor and 1% Antibiotic-Antimycotic (GIBCO). NSCs were expanded for no more than 5 passages.

Isolation of MSCs was described in a previous study (Yamamoto et al., 2013). Briefly, MSCs were isolated from human bone marrow using LNGFR (CD271) and Thy-1 (CD90) surface markers. First, bone marrow mononuclear cells (Poietics[™]; Lonza) were suspended at 1 to 5×10^7 cells/mL in ice-cold Hank's balanced salt solution supplemented with 2% fetal bovine serum (FBS), 10 mM HEPES and 1% penicillin/streptomycin. Next, the cells were stained for 30 minutes on ice with the monoclonal antibodies. To eliminate dead cells from the flow cytometric cell sorting

(FACS) system, propidium iodide (2 $\mu\text{g/mL}$) was used. A triple-laser MoFlo (Beckman Coulter, CA, USA) or FACSVantage SE (Becton Dickinson, Heidelberg, Germany) were used for FACS analysis.

MSCs were used to promote vascular formation and stabilize vascular structures (Yamamoto et al., 2018, 2013). MSCs were cultured in the MSC growth medium: Dulbecco's Modified Eagle Medium with low glucose (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 20% FBS and 1% Antibiotic-Antimycotic. MSCs were expanded for no more than 8 passages.

2-2 Microfluidic device preparation^(chapter 3, 4, 5)

Fabrication processes of the microfluidic device used in this thesis were described in detail in a previous study (Shin et al., 2012). Briefly, the microfluidic device was made of poly-dimethylsiloxane (PDMS; Silgard184, Dow Corning, Midland, MI, USA) and was produced by soft lithography with SU-8 patterned wafers. After peeling the cured PDMS from the SU-8 mold, it was cut into pieces and punched out to make media inlets and gel inlets. To form microchannels, each device was plasma-bonded with a coverglass. Microchannels were filled with 1 mg/mL poly-d-lysine solution (Sigma-Aldrich, St. Louis, MO, USA), and the devices were placed in a humidified 5% CO₂ incubator at 37°C for overnight. The devices were then rinsed twice with sterile deionized water and dried at 60°C for 24 hours.

This microfluidic device has three channels. The central channel, which is filled with hydrogel, is sandwiched by two parallel microfluidic channels. The central channel has two gel-inlets which are 1.2 mm in diameter, and two microfluidic channels have two media-inlets which are 4.0 mm in diameter. The height of each channel is 135 μm . The width of the central channel is 750 μm or 1300 μm , which were used depending on experiments, and the width of microfluidic channels is 500 μm .

2-3 Hydrogel filling and cell seeding in microfluidic devices^(chapter 3, 4, 5)

Fibrinogen, CorningTM MatrigelTM GFR (Growth Factor Reduced) Membrane Matrix and hyaluronan (HyStem[®] Cell Culture Scaffold Kit, Sigma-Aldrich) were used as gel scaffolds for cell culture in microfluidic devices.

Fibrinogen was dissolved in phosphate buffered saline (PBS) to prepare pre-gel solution. The final concentration of fibrinogen solution was 4 mg/mL in chapter 3, and 2 mg/mL in chapter 4 and chapter 5. In chapter 4 and chapter 5, Matrigel and hyaluronan were additionally used with fibrinogen. The final concentration of Matrigel was 2, 4 or 8 mg/mL, depending on experiments. The final concentration of hyaluronan was 2 mg/mL. Pre-gel solutions of Matrigel and hyaluronan were mixed with fibrin pre-gel solution to make fibrin-Matrigel solution and fibrin-hyaluronan solution before injection into microfluidic devices. Pre-gel solution was injected through the two gel inlets immediately after thrombin (10 units/mL, Sigma-Aldrich) was added to the solution to start gelation. After the gel injection, devices were placed in a humidified 5% CO₂ incubator at 37 °C for 30 minutes to complete polymerization of the pre-gel solution. When injecting pre-gel solution, it was easily confined within the central channel because of surface tension between PDMS posts (Shin et al., 2012). After gelation, microfluidic channels were filled with culture medium from media inlets. The microfluidic devices were kept in an incubator until use.

BMECs, HUVECs, NSCs or MSCs were suspended in their own culture medium after these cells were dissociated from culture dishes using TrypLE (GIBCO). The cell concentration was 1×10^6 cells/mL for all cell types. After aspirating culture medium from reservoirs of a microfluidic device, 1×10^4 cells were seeded in the microfluidic device through media reservoirs. When seeding cells, devices were tilted by 90° to allow cells to adhere to the gel surface between trapezoid posts. After cell seeding, devices were incubated for 30 minutes in a humidified 5% CO₂ incubator at 37 °C to complete the cell attachment on the gel surface.

Culture medium was exchanged every day. Phase-contrast images were taken every day to monitor the process of cell migration and morphogenesis.

2-4 Immunocytochemistry

2-4-1 Immunofluorescence staining procedures^(chapter 3, 4, 5)

For the imaging of constructed neurites and microvasculatures, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 5 minutes. After permeabilization, the cells were treated with BlockAce (Dainippon Pharmaceutical, Japan) for 1 hour to block non-specific staining. The cells were then incubated at 4°C overnight with primary antibodies. Thereafter, the cells were incubated with secondary antibodies at room temperature for 2 hours. The cells were finally incubated with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) for staining cell nuclei. The cells were rinsed with PBS between each step.

Z-stack fluorescence images were taken by a confocal laser-scanning microscope (LSM 700, Carl Zeiss, Germany). Two-dimensional projection immunofluorescence images were generated with the z-stack images using ImageJ (National Institutes of Health, Bethesda, MD, USA).

2-4-2 Antibodies^(chapter 3, 4, 5)

Primary antibodies used in this thesis are as follows:

(product name; source; catalog number; company; dilution)

BMECs and HUVECs

Human CD31/PECAM-1 Antibody; sheep; AF806; R&D Systems; 1:100.

VE-Cadherin (D87F2) XP® Rabbit mAb; rabbit; #2500; Cell Signaling Technology; 1:100.

ZO-1 Monoclonal Antibody (ZO-1-1A12); mouse; 33-9100; Thermo Fisher Scientific; 1:50.

Occludin Polyclonal Antibody; rabbit; 71-1500; Thermo Fisher Scientific; 1:100.

Pericytes differentiated from MSCs

Monoclonal Anti-Actin, α -Smooth Muscle antibody produced in mouse; mouse; A2547; Sigma-Aldrich; 1:200.

Anti-PDGFR beta antibody [Y92] – C-terminal; rabbit; ab32570; abcam; 1:100.

Human NG2/MCSP PE-conjugated Antibody; mouse; FAB2585P; R&D Systems; 1:50.

Basement membranes

Anti (mouse) Laminin; mouse; LB-1013; LSL; 1:200.

Anti Collagen IV α 5(IV) Chain, Human (Mono); rat; C-452-EX; Chigei Medical Research Institute; 1:50.

NSCs

Purified anti-Tubulin β 3 (TUBB3) [Tuj1]; mouse; 801202; BioLegend; 1:500.

Microtubule associated protein 2 (MAP2) Antibody (M13); mouse; 13-1500; Thermo Fisher Scientific; 1:100.

GFAP Polyclonal Antibody; rabbit; PA5-16291; Thermo Fisher Scientific; 1:100.

Anti-Galactocerebroside Antibody, clone mGalC; mouse; MAB342; Merck Millipore; 1:100.

Anti-Human/Mouse SOX2 Monoclonal Antibody; mouse; MAB2018; R&D Systems; 1:100.

Anti-Nestin antibody – Neural Stem Cell Marker; rabbit; ab92391; abcam; 1:200.

Secondary antibodies (1:100 dilution) and the antibody for staining cell nuclei (DAPI, 1:250 dilution) used in this thesis were purchased from Invitrogen.

2-5 Quantitative analysis

2-5-1 Microvasculatures^(chapter 3, 4)

Quantitative analyses were performed for sprout and microvascular length, sprout number, branch points, vessel outer diameter and pericyte number. Microvascular length was quantified using z-stack immunofluorescence images of engineered microvasculatures. After 2D projection images were generated with z-stack images of microvasculatures, sprout length and microvascular length in the 2D projection images were traced using the freehand line tool in ImageJ. The length of the traced lines was measured to quantify sprout length and microvascular length. Sprout number and branch point were quantified by manually counting the sprout number and branch points in each image. Vessel diameter was measured at 1/3 and 2/3 of the width of the central channel along the Y-axis. Pericyte number was quantified by counting α -SMA-expressing cell nuclei on the outer surface of microvascular configurations.

Quantitative analyses of junction proteins were also performed using ImageJ. After thresholding each immunofluorescence image, image calculator, a plugin of ImageJ, was used to calculate fluorescence intensity of junction proteins in ECs constituting microvasculatures.

2-5-2 Neurites^(chapter 4)

Neurite length was measured by the same method of measuring microvascular length. To quantify the number of three-dimensionally migrating cells, cell nuclei which located at least 15- μ m far from the top or bottom surfaces of a 3D hydrogel scaffold were counted using ImageJ. This counted cell number was divided by the total cell number in the 3D hydrogel scaffold to calculate the percentage of cells in 3D region.

2-5-3 Gel collapse in triculture of NSCs, BMECs and MSCs^(chapter 4)

Gel collapse occurred in triculture experiments in chapter 4. The quantification of

gel collapse was performed using ImageJ by surrounding the devoid area in each z-projection image.

2-5-4 Dextran perfusion and measurement of microvascular permeability coefficient^(chapter 3)

To analyze microvascular permeability, fluorescein isothiocyanate-conjugated dextran (FITC-dextran, 2000 kDa) or Texas Red-conjugated dextran (70 kDa) were introduced into engineered microvasculatures. After aspirating culture medium from reservoirs of a microfluidic device, 10 μ L of 50 μ g/mL FITC-dextran or Texas Red-dextran solution was added to a microchannel containing seeded ECs and MSCs. Fluorescence images were obtained every 10 seconds using the 20 $\times$ objective lens of a confocal laser-scanning microscope (LSM 700).

Permeability coefficients (P) of BMEC/HUVEC microvasculatures were calculated using Fick's First Law. In general, Fick's First Law is represented by the following equation:

$$J = -D \frac{dc_m}{dx} \quad (1)$$

where J is the flux of solute across microvascular wall, D is the diffusion coefficient of solutes in endothelial monolayer constituting microvascular wall and c_m is the concentration of the solute inside EC membrane. When the width of the cell membrane is given by d , concentrations of solutes at the intravascular border ($c_m(0)$) and extravascular border ($c_m(d)$) are represented using a partition coefficient β as:

$$c_m(0) = \beta c_1 \quad (2)$$

$$c_m(d) = \beta c_2 \quad (3)$$

where c_1 is the concentration of solutes in microvascular lumen and c_2 is the concentration of solutes in extravascular region. Substitution of equations (2) and (3) into equation (1) yields:

$$J = -D \frac{dc_m}{dx} = -D \frac{c_m(d) - c_m(0)}{d} = \frac{D\beta(c_1 - c_2)}{d} \quad (4)$$

Equation (4) shows that the flux is in proportion to the concentration difference between intravascular region and extravascular region. It is difficult to determine each proportionality constant of $D\beta/d$ individually. Therefore, these constants are expressed in a single constant P as a permeability coefficient, yielding the following equation.

$$J = P(c_1 - c_2) \quad (5)$$

Permeability coefficient is calculated from the flux of solute for a concentration difference (c_0) across an area of microvascular wall (S) using Fick's First Law (Adamson et al., 1994; Bichsel et al., 2015; Kim et al., 2013).

$$P = J \times \frac{1}{c_0} \quad (6)$$

The flux J is calculated from the rate of the transport of dextran molecules (N) across the microvascular wall after the microvasculature was filled with the dextran solution.

$$J = \frac{dN}{dt} \times \frac{1}{S} \quad (7)$$

The concentration c_0 is represented using the amount of dextran molecules that initially fill the microvasculature.

$$c_0 = \frac{N_0}{V} \quad (8)$$

where V is the volume of the microvasculature. It is assumed that the number of solute molecules was proportional to the fluorescence intensity (I), substitution of equations (7) and (8) into equation (6) yields

$$P = \frac{V}{S} \times \frac{dI/dt}{I_0} \quad (9)$$

where I_0 is the initial intravascular fluorescence intensity, and dI/dt is the change in fluorescence intensity per unit time in the extravascular region. It was assumed that the microvascular lumens were circular.

$$S = 2\pi rl \quad (10)$$

$$V = \pi r^2 l \quad (11)$$

where r is the microvascular radius and l is microvascular length. Substitution of equations (10) and (11) into equation (9) yields

$$P = \frac{r}{2} \times \frac{dl/dt}{I_0} \quad (12)$$

Permeability coefficient P was calculated using equation (12).

2-5-5 Statistical analysis

Experiments were repeated three times to confirm the reproducibility of results. Data were represented as means $\pm$ SEM. Welch's t-test was used to test for differences between the two groups, which were considered statistically significant at $p < 0.05$.

Chapter 3 Comparison of organ-specific endothelial cells in terms of microvascular formation and endothelial barrier functions

3-1 Introduction

Microvascular networks are essential for correct organ functioning. Each organ has its own vascular characteristics and functions that are maintained through interactions between ECs and parenchymal cells within vascular niches (Géraud et al., 2014). Angiocrine and paracrine factors secreted by ECs regulate organ homeostasis and function (Rafii et al., 2016), and the fundamental function of microvascular networks is to deliver oxygen and nutrients throughout organs. ECs that coat microvascular walls adhere to each other through junction proteins such as VE-cadherin, occludin, and ZO-1 (Abbott et al., 2010; Greene and Campbell, 2016). While the transport of macromolecules and undesired substances such as neurotoxins out of the microvasculature is strongly inhibited by endothelial barrier functions, ECs regulate the active transport of nutrients and oxygen into the organ parenchyma by paracellular and transcellular pathways (Abbott et al., 2010; Barar et al., 2016; Greene and Campbell, 2016). In addition to this universal function, ECs display slightly different features depending on the organ in which they reside. Notably, brain ECs cooperate with astrocytes and pericytes to form a characteristic vascular microenvironment called BBB (Zhao et al., 2015). Brain microvascular networks strictly prohibit the transport of toxic substances to the brain parenchyma and effectively transfer compounds essential for brain homeostasis. On the other hand, ECs of the kidney glomeruli and liver effectively maintain active transport of blood-borne molecules to the parenchyma (Ben-Zvi et al., 2014; Géraud et al., 2014). In addition to these morphological features of organ-specific

ECs, ECs from different organs exhibit tissue-specific expression profiles of transcription factors, angiocrine growth factors, adhesion molecules, and chemokines (Nolan et al., 2013). Such differences in both the structural organization and function of organ-specific ECs suggest that organ specificity should be considered when investigating interactions between ECs and other cell types constituting a target organ.

The brain has organ-specific ECs that play important roles in the functioning of the BBB. Many studies have investigated BBB development and functions because the regulation of the BBB is important for both drug delivery and drug screening (Banerjee et al., 2016; Bicker et al., 2014). Transport properties of the brain capillary endothelium and interactions between ECs and perivascular cells, such as pericytes and astrocytes, have also been the focus of previous studies (Banerjee et al., 2016; Helm et al., 2016; Keaney and Campbell, 2015; Sweeney et al., 2018). By culturing ECs with pericytes and/or astrocytes, cell culture systems have been observed to mimic *in vivo* microvascular environments. In some studies, a Transwell cell culture system was used to obtain TEER measurements that were further analyzed to estimate endothelial barrier functions. These studies established that endothelial barrier function is augmented when ECs are cultured with astrocytes and pericytes. However, the ECs in these studies formed a two-dimensional (2D) monolayer rather than a native *in vivo* three-dimensional (3D) capillary structure. It has also been reported that ECs isolated from brain capillaries tend to lose their barrier properties (Banerjee et al., 2016). In addition, ECs are covered by pericytes and astrocytic endfeet and the percentage of pericyte coverage around capillaries are the highest in the brain compared to the other organs (Armulik et al., 2010; Daneman et al., 2010; Keaney and Campbell, 2015; Thomsen et al., 2017; Trost et al., 2016; Zlokovic, 2008). These fundamental features of brain capillaries were not included in previous *in vitro* studies, highlighting the disparity between *in vitro* BBB models and native EC conditions *in vivo*.

To bridge this gap, it is imperative that a successful 3D brain microvascular network be constructed. An *in vitro* BBB model requires two key features: the construction of

functional capillary-level microvascular networks and the coverage of pericytes and astrocytic endfeet around the microvasculatures. The construction of capillary-level microvasculatures is particularly challenging, especially in terms of capillary diameter: indeed, most previously reported microvasculatures have been between 20 and 100 μm in diameter (Kim et al., 2015, 2013; Nguyen et al., 2013), much larger than that of real capillaries. Toward the establishment of an *in vitro* BBB model, construction of capillary-level microvascular networks with pericyte coverage was first focused on. Previous studies that have constructed *in vitro* microvascular networks have used microfluidic cell culture platforms in which cells were cultured in 3D gel scaffolds mimicking an *in vivo* ECM (Kim et al., 2015; Kim et al., 2013). We created an angiogenesis model using ECs and MSCs (Yamamoto et al., 2018). HUVECs were used in these studies.

In this chapter, a microfluidic cell culture platform was developed by coculturing BMECs and MSCs, an essential milestone in the construction of an *in vitro* BBB model. To compare the characteristics of organ-specific ECs, HUVECs were also cultured with MSCs as opposed to BMECs. MSCs were used in the coculture because of their ability to differentiate into pericytes both *in vivo* (Koike et al., 2004) and *in vitro* (Yamamoto et al., 2018, 2013). Whether organ-specific ECs would affect vascular formation processes and whether microvascular networks constructed using such ECs would exhibit their own characteristics in terms of endothelial barrier functions and fundamental morphology were assessed in this chapter.

3-2 Experimental design

Detailed methods for the experiments described in this chapter are summarized in chapter 2. Here, experimental settings are described below.

Microfluidic device:

Schematic illustrations of the microfluidic device used in this chapter are shown in Fig. 3-1. This microfluidic device has three microchannels. The central channel is used as cell culture channel by injecting gel scaffolds. Two parallel microfluidic channels are used as media channels.

The width of the central channel is 750 μm . The width was determined by preliminary experiments. When ECs and MSCs are seeded in one microfluidic channel in the 750- μm device, they elongate vascular structures and these vasculatures reach the opposite side of the channel about 5-7 days. The penetration of microvasculatures across the central channel is needed to perfuse dextran solutions into the microvasculatures to investigate endothelial barrier functions. Therefore, we used the 750- μm device for EC-MSC cocultures.

The width between trapezoidal posts is 50 μm , which is determined by following reasons. First one is to prevent the leakage of gel solutions out of the central channel when injected. Gel solutions are easily fixed in the central channel because of the surface tension between these posts (Huang et al., 2009; Shin et al., 2012). Second is to minimize the undesired leakage of fluorescent dextran solution from microfluidic channel to the central channel. When constructing microvasculatures, some spaces exist between microvasculatures and trapezoidal posts. Some of the solutes of fluorescent dextran solution can pass through the space, which may diffuse throughout the central channel. To avoid this, the width between each post was set to be 50 μm . Microfluidic devices whose width between posts was 30 μm was tried to make, only to find it difficult to produce these devices stably.

Cells:

BMECs, HUVECs and MSCs were used in this chapter. The amount of all cell types seeded in each device was 1.0×10^4 cells. The ratio of ECs and MSCs was 1:1, which was optimized in a previous study (Yamamoto et al., 2013).

Angiogenesis is the growth of new blood vessels from pre-existing ones. Endothelial vascular sprouts are formed by degrading surrounding ECM around blood vessels through production of matrix metalloproteinases (MMPs). ECs and MSCs were seeded on the gel surface between each post in order to mimic angiogenic processes in a microfluidic device. Cultured ECs proliferate and form vascular-like structure inside the microfluidic channel where cells are seeded. Some ECs migrate into the gel scaffold and form vascular sprouts. These sprouts are elongated and become mature by the coverage of pericytes which are differentiated from MSCs. It was reported that the differentiation of MSCs into pericytes was induced by culturing MSCs with ECs (Koike et al., 2004; Yamamoto et al., 2018).

Medium:

For all experiments in this chapter, 1:1 mixture of EGM-2 and MSC growth medium was used.

Additional supplement to medium:

The medium was supplemented with 10 ng/mL VEGF and 10 ng/mL bFGF to promote cell proliferation and microvascular formation.

Gel scaffold:

Fibrin (4 mg/mL) was used for all experiments in this chapter because fibrin has been widely used as a gel scaffold for EC culture to induce angiogenic processes *in vitro* (Dietrich and Lelkes, 2006; Kim et al., 2013; Nakatsu and Hughes, 2008).

Dextran perfusion experiments:

To analyze endothelial barrier function of constructed microvasculatures, fluorescent dextran perfusion was performed. Molecular weights of the dextran were 2,000 kDa or 70 kDa. The 2,000 kDa dextran was used to test if the dextran solution would be perfused without instant leakage out of vessels. The measurement of microvascular permeability was performed using 70 kDa dextran solution. Some *in vitro* or *in vivo* studies demonstrated fluorescent dextran perfusion into constructed vascular structures or cerebral microvessels of rats (Alimperti et al., 2017; Bang et al., 2017; Kim et al., 2013; Sobrino et al., 2016; Yuan et al., 2009). There are many kinds of fluorescent dextran in terms of molecular weight, and 70 kDa dextran has been mostly used in previous studies because the molecular weight is closest to that of human albumin (66 kDa), which was reported to be neurotoxic (Hassel et al., 1994). Therefore, 70 kDa dextran was used not only to test the endothelial barrier function but also to compare the result to previous studies.

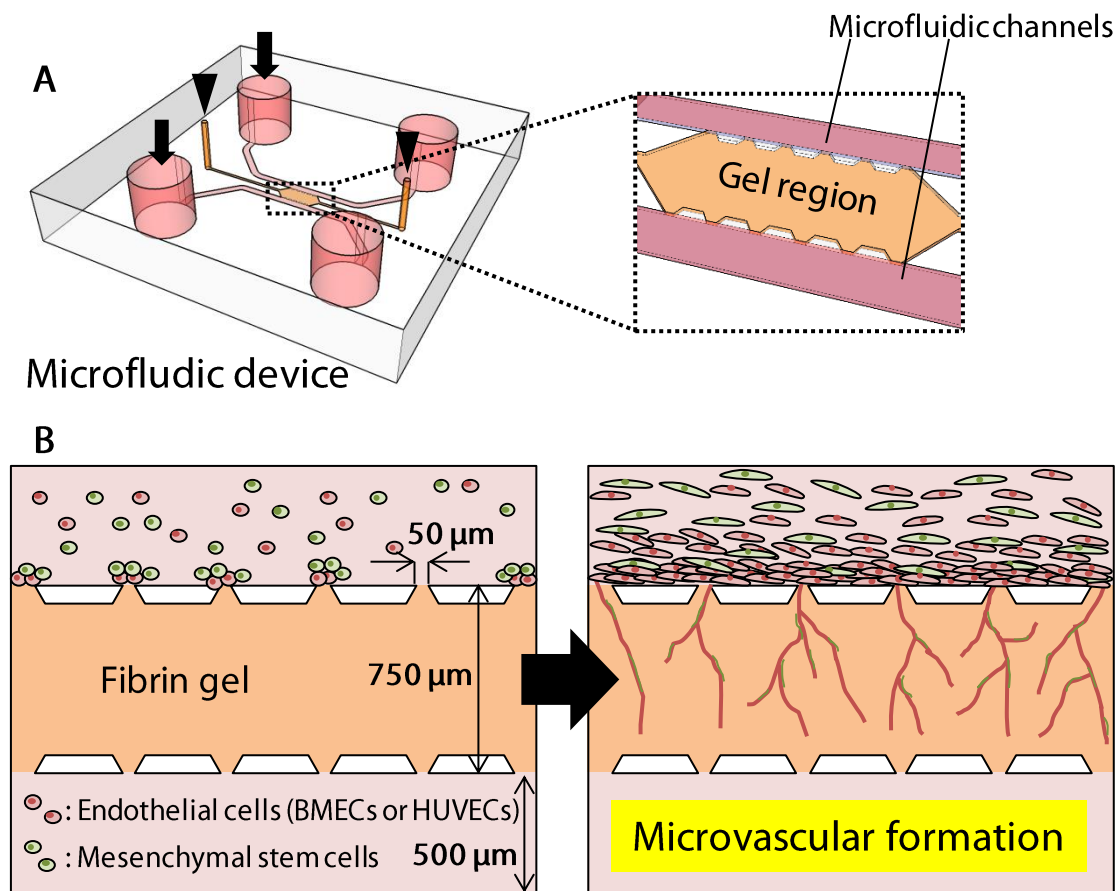


Fig. 3-1 Schematic illustration of an EC-MSc coculture in a microfluidic device.

A: A microfluidic device and an enlarged image of its central channel filled with fibrin gel (gel region). The central channel is sandwiched between two microchannels filled with cell culture media (microfluidic channels). Fibrin gel pre-polymer solution was injected into the central channel from two gel inlets (arrowheads) immediately after thrombin was introduced to the pre-polymer solution. Culture medium was added using the inlets of the microfluidic channels (arrows) following gel polymerization. B: Schematic illustrations of cell seeding and microvascular formation. A cell suspension of ECs and MSCs was added to one microfluidic channel and cells were allowed to attach to the side wall of the fibrin gel (left). ECs migrated into the fibrin gel to form microvascular structures (red) covered with pericytes differentiated from MSCs (green) (right).

3-3 Results

3-3-1 Initial vascular sprout formation in BMEC/HUVEC-MSC cocultures

First, an early stage of microvascular formation recapitulated by BMEC-MSC coculture and HUVEC-MSC coculture was focused on. ECs and MSCs were cultured together in a microfluidic device (Fig. 3-1). Both ECs and MSCs were seeded in the same microchannel and attached to the surface of the gel region (Fig. 3-2A, Day 0). These cells then actively migrated into the gel region from the start of coculture regardless of EC origin (Fig. 3-2A, Day 2, arrowheads). Corresponding phase-contrast images demonstrated that these cells migrated across the gel region within 3 days of seeding (Fig. 3-2A, Day 3). Immunofluorescence images showed that vascular sprouts stained with PECAM-1 were formed by day 3 (Fig. 3-2B, arrowheads). Quantitative analyses of the immunofluorescence images revealed that both sprout number and microvascular length were significantly greater in HUVEC-MSC cocultures than in BMEC-MSC cocultures (Fig. 3-2B–D). These results suggest that vascular formation in the early stage is different depending on EC origin.

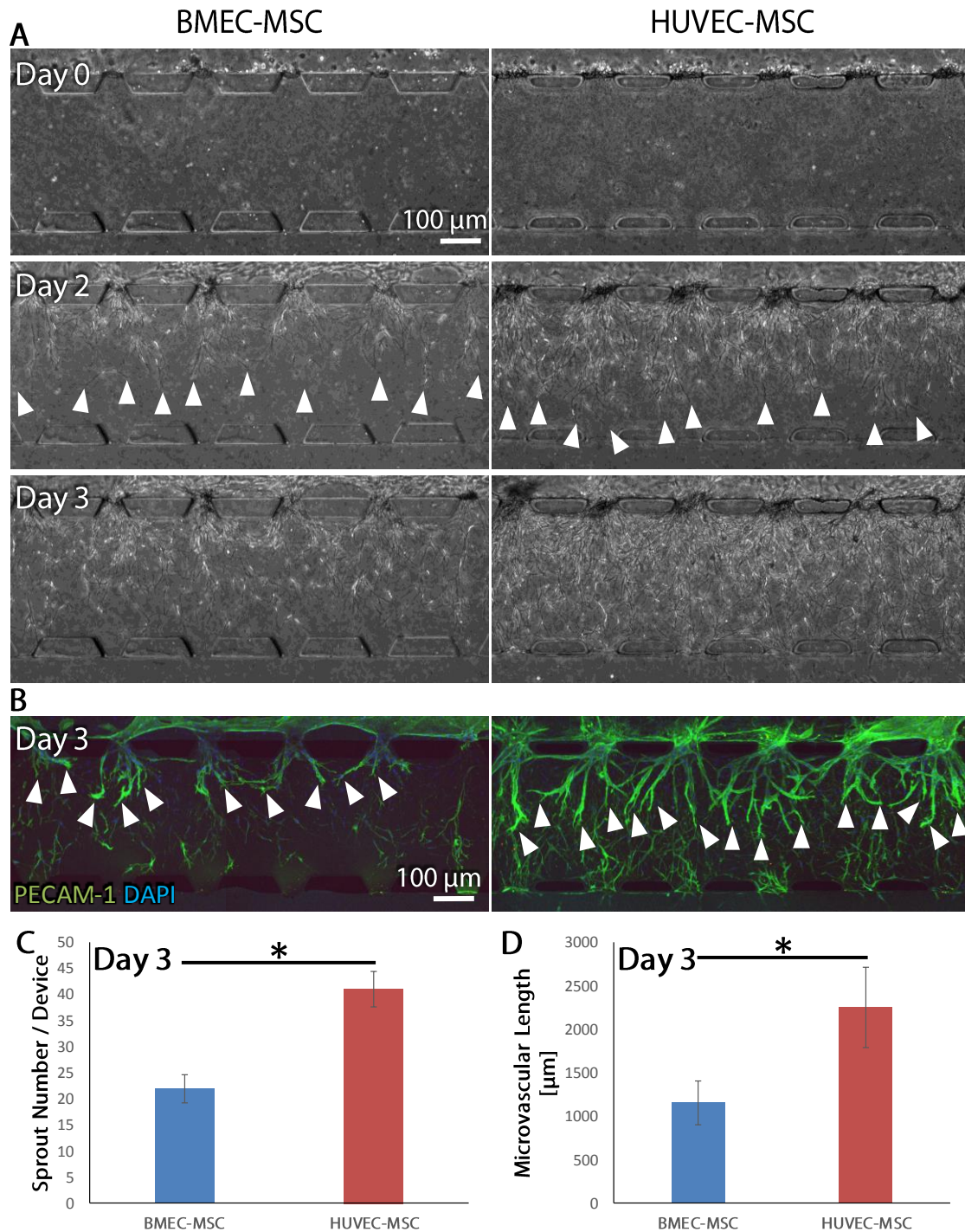


Fig. 3-2 An early stage of EC-MSC coculture. A: Phase-contrast images of BMEC-MSC (left) and HUVEC-MSC (right) cocultures on days 0–3. Arrowheads indicate migrating cells at the front edge. B: Representative immunofluorescence images of ECs and MSCs on day 3. Arrowheads indicate vascular sprouts stained with

PECAM-1 (green). Cell nuclei were stained with DAPI (blue). C: Quantitative analyses of the number of EC sprouts. D: Quantitative analyses of microvascular length. Data are shown as the mean $\pm$ SEM (N=3, n $\geq$ 9). * p <0.05.

3-3-2 Microvascular network formation in BMEC/HUVEC-MSK cocultures

Next, EC-MSK cocultures until day 7 were performed to observe the extension and the maturation of constructed microvasculatures. According to phase-contrast microscopy, the number of migrating cells into the gel region was increased on day 7 compared to that on day 3 (Fig. 3-3A versus Fig. 3-2A). Microvascular networks were constructed by day 7 regardless of EC origin, which was observed by immunofluorescence staining of microvascular structures with PECAM-1, α -SMA and DAPI (Fig. 3-3B). Enlarged images showed that some perivascular cells expressing α -SMA, which were derived from MSCs, existed closely around microvascular configurations (Fig. 3-3C).

To evaluate microvascular formation by BMECs and HUVECs, characteristics of microvasculatures formed by these ECs were compared. Specifically, quantitative analyses were performed for microvascular length, branch points, outer diameter, and pericyte number. As expected from the results of vascular formation in the early stages as shown in Figure 3-2, HUVECs formed more complex microvascular networks compared to BMECs. The length of constructed microvascular networks was greater in HUVEC-MSK cocultures than in BMEC-MSK cocultures (Fig. 3-3E). Branch points were also greater in HUVEC-MSK cocultures (Fig. 3-3F), but microvascular outer diameter did not differ between cell types (Fig. 3-3G). Finally, pericyte numbers were comparable between cocultures (Fig. 3-3H).

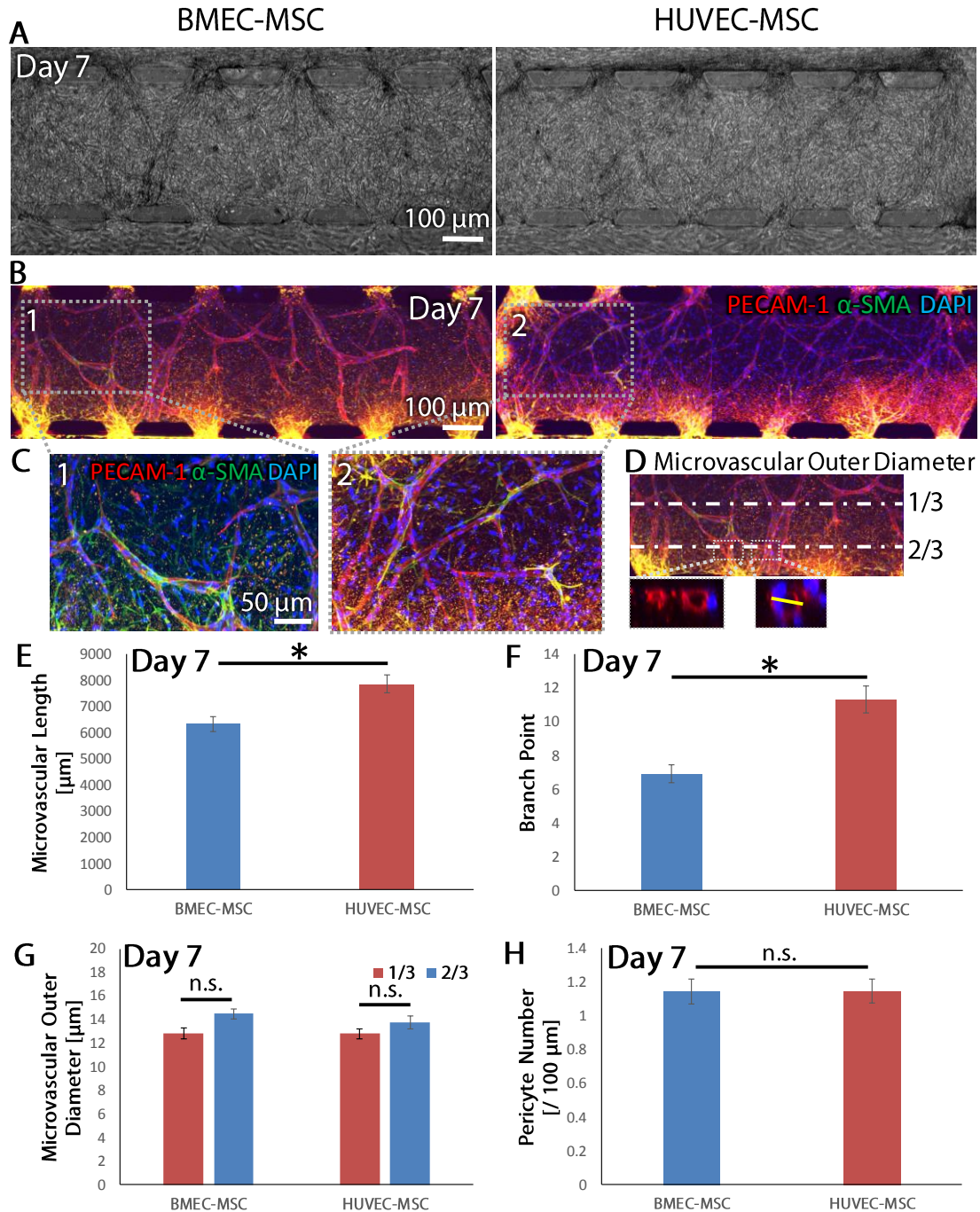


Fig. 3-3 A late stage of EC-MSC coculture. A: Representative phase-contrast images of ECs and MSCs on day 7 (left: BMEC-MSC, right: HUVEC-MSC). B: Corresponding immunofluorescence images of ECs (PECAM-1, red) and perivascular cells (α -SMA, green) on day 7. Cell nuclei were stained with DAPI (blue). C: Enlarged images of constructed microvasculatures, corresponding to boxes 1 and 2 in panel B, respectively.

D: Orthogonal views of constructed microvasculatures showing microvascular diameters. E-H: Quantitative analyses of microvascular length (E), branch point (F), microvascular outer diameter (G), and pericyte number (H) in both BMEC-MSC and HUVEC-MSC cocultures. Data are shown as the mean $\pm$ SEM (N=3, n $\geq$ 9). * p <0.05.

3-3-3 Basement membrane formation and pericyte coverage around microvasculatures

Next, the formation of basement membranes and the coverage of pericytes around microvasculatures, which are important stages in vascular maturation, were investigated. Immunofluorescence staining revealed that basement membrane proteins such as laminin and type IV collagen were expressed along with microvasculatures regardless of EC origin (Fig. 3-4A, 3-4B). Laminin was localized in the basal domain of the microvascular configurations (arrowheads, Fig. 3-4A), suggesting that ECs constituting microvasculatures recognized apical-basal polarity. Type IV collagen was localized in the outer space of microvasculatures. Some type IV collagen was co-expressed with α -SMA (arrows, Fig. 3-4A), suggesting potential cooperation between ECs and MSCs in type IV collagen production. In terms of pericyte coverage, we performed immunofluorescence staining of microvasculatures for pericyte markers, NG2 and PDGFR β , in addition to α -SMA. These markers were also expressed in perivascular cells in both BMEC-MSC and HUVEC-MSC cocultures (Fig. 3-4C, 3-4D). Cross-sectional images showed that these pericytes wrapped PECAM-1-positive microvasculatures. These results indicate that the constructed microvasculatures became mature and stabilized within 7 days of EC-MSC coculture.

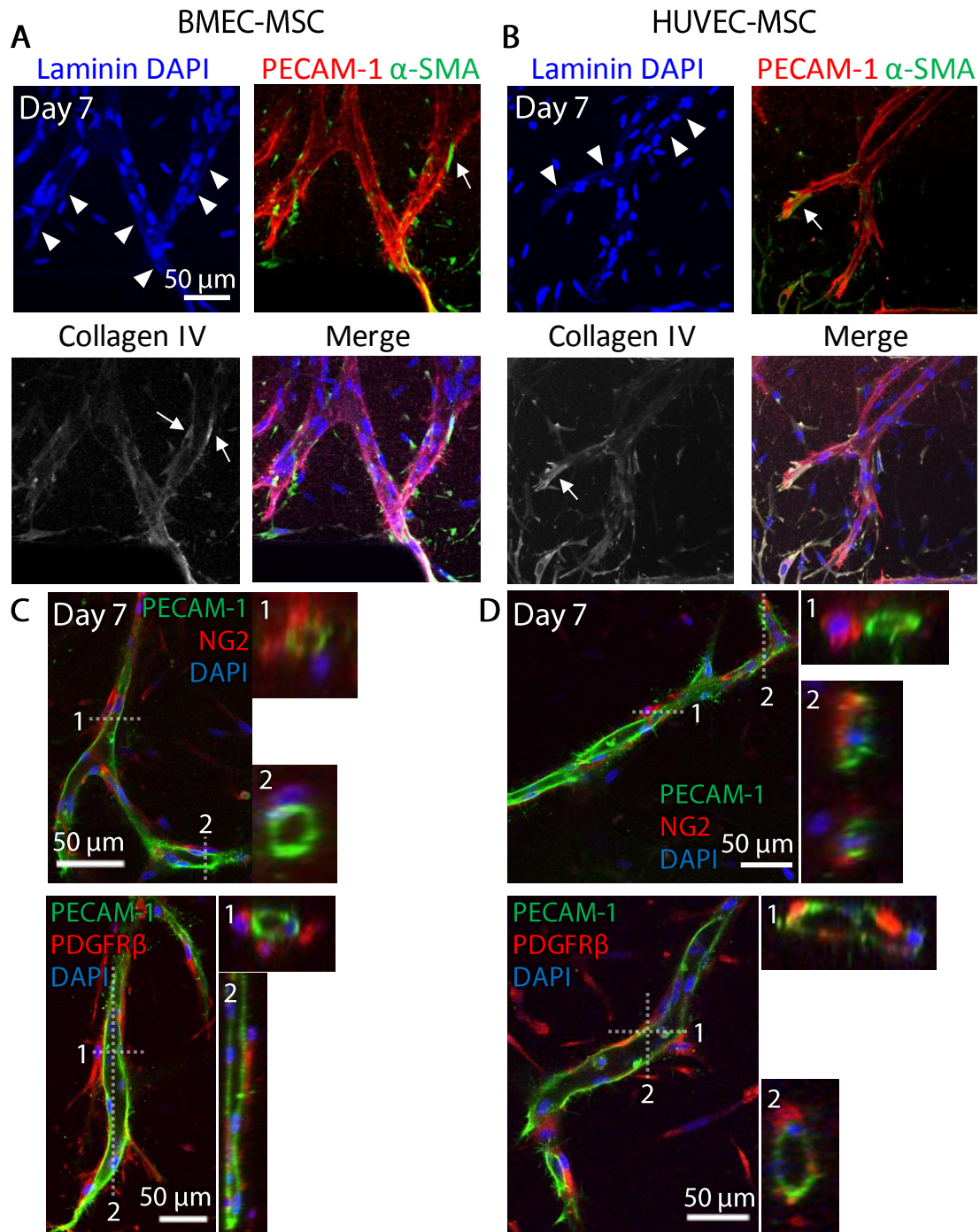


Fig. 3-4 Immunofluorescence staining for basement membrane proteins and pericytes. Representative immunofluorescence images of constructed microvasculatures (PECAM-1), pericytes (α -SMA, NG2 and PDGFR β), basement membrane proteins (laminin and type IV collagen), and cell nuclei (DAPI) on day 7 in BMEC-MSC coculture (A, C) and HUVEC-MSC coculture (B, D). Arrowheads indicate

laminin localization around microvasculatures. Arrows indicate the colocalization of α -SMA and type IV collagen. Perivascular cells, which were positive for NG2 and PDGFR β , were observed around microvasculatures (C, D). Cross-sectional images corresponding to the dotted lines in C and D showed the coverage of pericytes around microvasculatures.

3-3-4 Permeability assay of microvascular walls using fluorescent dextran solution

Next, endothelial barrier function of 7-day-old microvasculatures constructed from either BMECs or HUVECs was tested because the BBB is a specific feature of neurological tissue. Fluorescent dextran solution was perfused into microvascular networks, and time-lapse imaging was performed to investigate the time course of the dextran distribution. First, introducing fluorescent dextran solution into constructed capillary-level microvasculatures with diameter $< 10\ \mu\text{m}$ (Fig. 3-5A–C) was successfully performed. Cross-sectional images of microvasculatures clearly demonstrated that the luminal space was filled with the dextran solution without leakage (Fig. 3-5B). Next, the diffusion of the dextran solution from the intravascular region to the extravascular region was analyzed. Quantitative analyses of the fluorescence intensity showed that the intensity of the dextran solution significantly decreased across the capillary wall (Fig. 3-5D). To analyze endothelial barrier function, the time transition of fluorescence intensity of dextran in the extravascular region within $3\ \mu\text{m}$ from the capillary wall was quantified. The extravascular fluorescence intensity increased gradually with increasing time in both BMEC and HUVEC microvasculatures (Fig. 3-5E). The permeability coefficients of 70 kDa dextran solution across capillary walls were $0.9 \pm 0.4 \times 10^{-7}\ \text{cm/s}$ for BMEC microvasculatures and $2.7 \pm 0.6 \times 10^{-7}\ \text{cm/s}$ for HUVEC microvasculatures, respectively (Fig. 3-5F). These results indicate that the endothelial barrier function of BMEC microvasculatures was significantly more robust than that of HUVEC microvasculatures.

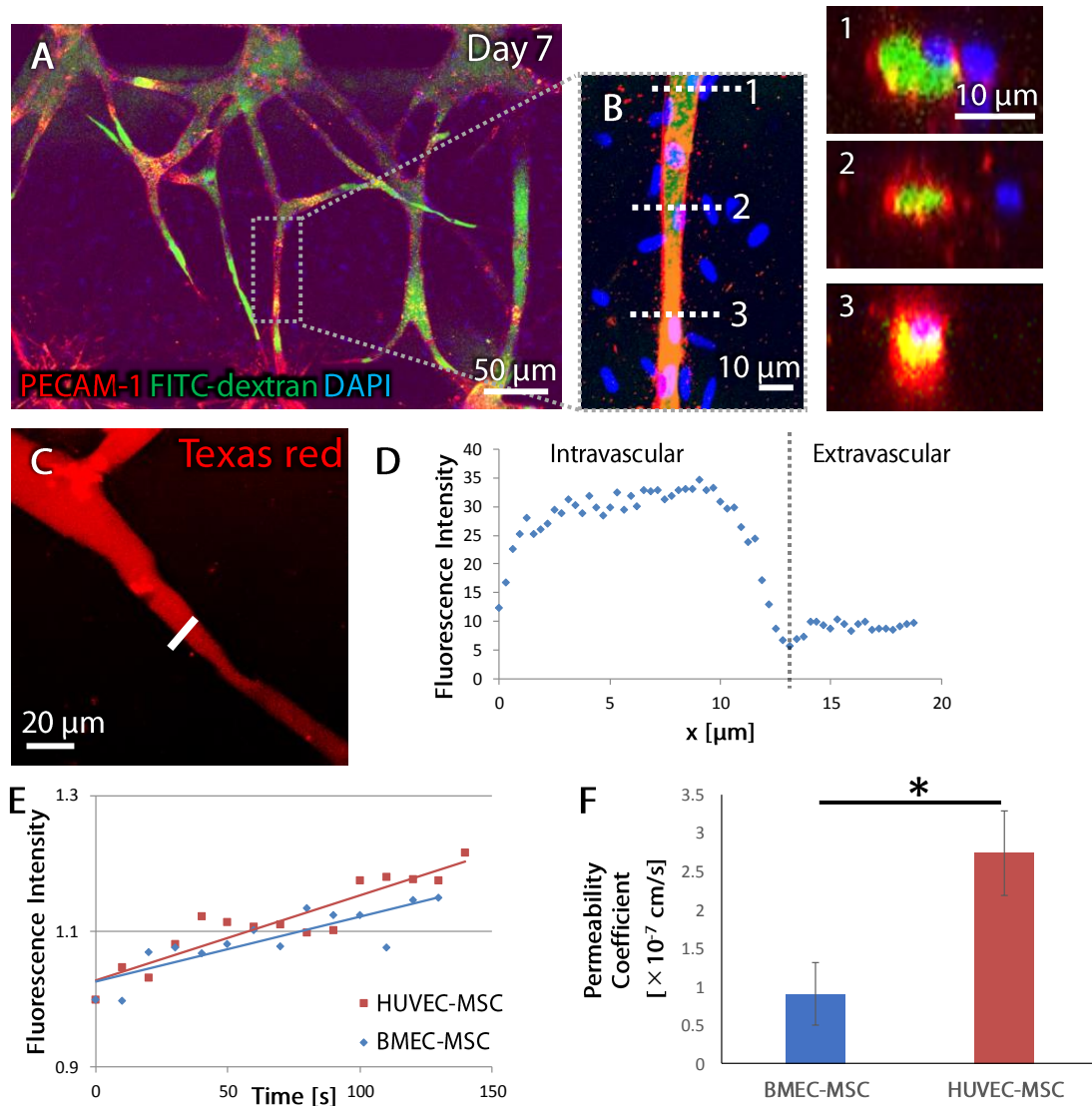


Fig. 3-5 Fluorescent dextran perfusion into constructed microvasculatures.

A: Immunofluorescence image of microvascular networks filled with FITC-dextran solution (2000 kDa). Dextran solution was introduced into microvascular networks on day 7. B: An enlarged image corresponding to the box in panel A and cross-sectional images of a dextran-perfused capillary (diameter $< 10 \mu\text{m}$). C: Immunofluorescence image of a dextran-perfused capillary on day 7. Fluorescent dextran solution (Texas red; 70 kDa) was introduced into microvascular networks. D: Representative fluorescence intensity distribution in intravascular and extravascular regions quantified along the axis of the white line in panel C. E: Representative time transition of fluorescent dextran intensity in extravascular regions. F: Quantitative analyses of permeability coefficients

for perfused microvasculatures in BMEC-MSC and HUVEC-MSC cocultures. Data are shown as the mean $\pm$ SEM (N=3, n $\geq$ 3). * p <0.05.

3-3-5 Junction protein expression in microvascular networks

It was hypothesized that the different permeabilities of the different microvasculatures were related to differences in endothelial junctional properties, in particular the greater tightness of BMEC microvascular walls. Therefore, immunofluorescence staining of endothelial junction proteins such as VE-cadherin, ZO-1, and occludin was performed to investigate the junctional properties of 7-day-old microvasculatures. VE-cadherin was expressed in intercellular regions and the cytoplasm of both BMECs and HUVECs (Fig. 3-6A, VE-cadherin). ZO-1 was localized in intercellular regions and the cytoplasm of BMEC microvasculatures while that in HUVEC microvasculatures was found only in cytoplasmic regions (Fig. 3-6A, ZO-1). Occludin was expressed in the cytoplasm of both BMECs and HUVECs contributing towards microvasculature structures (Fig. 3-6A, Occludin).

Quantitative analyses of VE-cadherin expression showed no significant differences between BMEC and HUVEC microvasculatures (Fig. 3-6B). Conversely, expression levels of ZO-1 and occludin were significantly higher in BMEC microvasculatures (Fig. 3-6C, 3-6D). These results demonstrated that the endothelial junctional properties differed by EC origin.

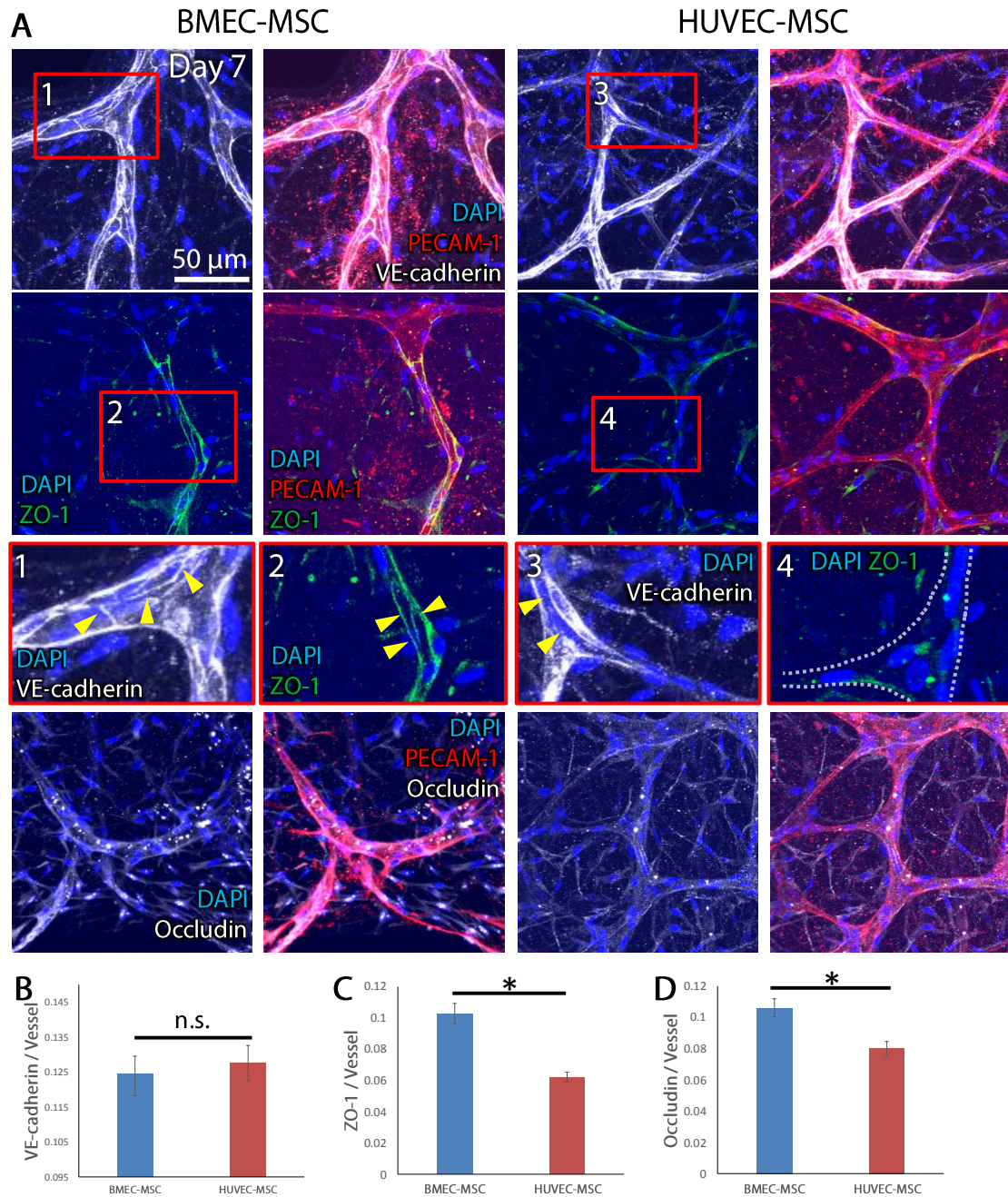


Fig. 3-6 Junctional properties of constructed microvasculatures in EC-MSC cocultures. A: Immunofluorescence images of constructed microvascular networks stained with VE-cadherin, ZO-1, occludin, PECAM-1 and DAPI. VE-cadherin, ZO-1, and occludin were expressed in the constructed microvasculatures. Arrowheads indicate intercellular expressions of junction proteins. Dotted lines in the enlarged image of HUVEC-MSC ZO-1 represent the microvascular configuration. B, C, D: Quantitative

analyses of VE-cadherin (B), ZO-1 (C), and occludin (D) expressions in the microvasculatures. Data are shown as the mean $\pm$ SEM (N=3, n $\geq$ 12). * p <0.05.

3-3-6 Inflammatory response of BMEC microvasculature induced by thrombin

Finally, the inflammatory response of microvasculatures was tested focusing on BMECs because it is important for the study of BBB in pathological states. BMEC microvasculatures were incubated with culture medium supplemented with 1 unit/mL thrombin to induce an inflammatory response. Before thrombin stimulation, the fluorescent dextran solution introduced in a microchannel passed through the microvascular networks across the gel region (Fig. 3-7A, 3-7C). The dextran solution came out of open ends of microvasculatures toward the other microchannel. The fluorescent dextran solution was detected in the other microchannel, the intensity of which increased with time (Fig. 3-7A, asterisks). However, the BMEC microvasculatures became leaky following thrombin stimulation (Fig. 3-7B). Although the solution was detected in the other microchannel, the increase in intensity was attenuated (Fig. 3-7B, asterisks). Moreover, it was detected throughout extravascular regions of the gel scaffold (Fig. 3-7B), suggesting that it passed across the walls of microvasculatures. To quantify these observations, fluorescence intensity of the dextran-perfused microvasculatures was measured along the line across microvasculatures (Fig. 3-7C, enlarged image). The time-series of fluorescence intensity profiles clearly showed that fluorescence intensity corresponding to extravascular regions increased with increasing time after thrombin stimulation (Fig. 3-7C–E, open circles).

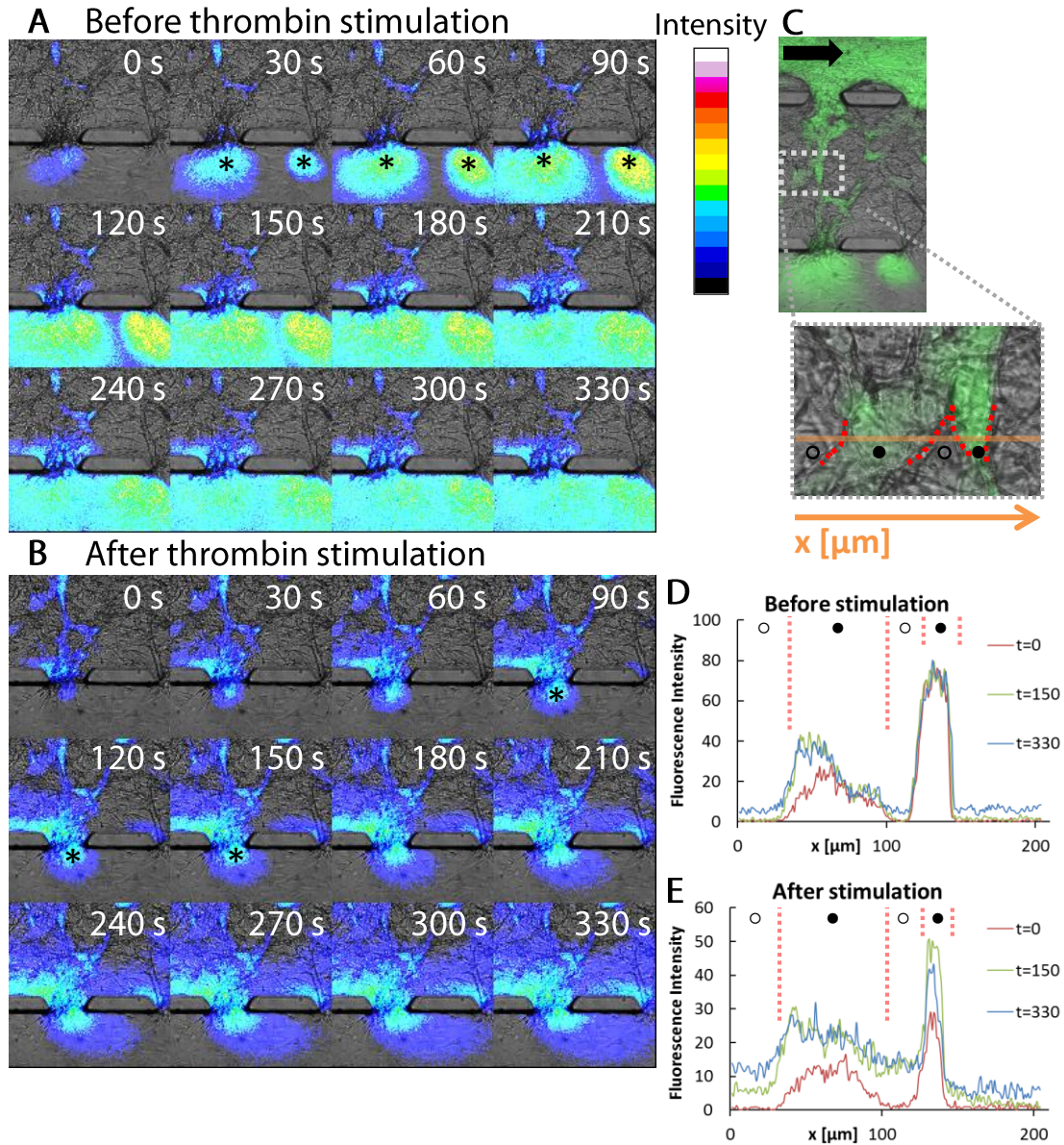


Fig. 3-7 Inflammatory response of BMEC microvasculatures. A: Time-lapse images of microvasculatures perfused with the dextran solution on day 5. Images were obtained every 30 s for 330 s using a confocal microscope. Asterisks represent FITC-dextran solution detected in the other microchannel, which passed through the microvasculatures. B: Time-lapse images of the thrombin-treated microvasculatures perfused with dextran solution on day 5. Asterisks represent the dextran solution detected in the other microchannel. The fluorescence intensity in extravascular spaces within the gel region was significantly increased after thrombin stimulation. C: A

merged image of phase-contrast and fluorescence images of microvasculatures perfused with FITC-dextran solution (2000 kDa) on day 5. Dotted lines in enlarged image represented microvascular configurations. D, E: Quantitative analysis of fluorescence intensity before thrombin stimulation (D) and after stimulation (E) along the straight line in the enlarged image in panel C. Fluorescence intensity of three different time points ($t=0s$, 150s, 330s) were shown together. Open circles inside dotted straight lines represent extravascular region and closed circles inside dotted straight lines represent intravascular region.

3-4 Discussion

An *in vitro* angiogenesis model using BMECs/HUVECs and MSCs was demonstrated in this chapter. Both microvasculatures had continuous lumens with average diameters close to 10 μm . In addition, the microvasculatures were sparsely covered by pericytes and expressed basement membrane proteins such as laminin and type IV collagen. These results indicate that the engineered microvasculatures were stable. Furthermore, introducing fluorescent dextran solution into the microvascular networks was successfully performed and their endothelial barrier functions were analyzed. The calculated permeability coefficients of BMEC and HUVEC microvasculatures were $0.9 \pm 0.4 \times 10^{-7} \text{ cm/s}$ and $2.7 \pm 0.6 \times 10^{-7} \text{ cm/s}$, respectively. These permeability coefficients are close to that of *in vivo* microvessels (Shi et al., 2014; Yuan et al., 2009). Permeability coefficients of *in vitro* microvasculatures perfused with 70 kDa dextran were also measured, which ranged from 4.5×10^{-7} to $2.0 \times 10^{-6} \text{ cm/s}$ (Bichsel et al., 2015; Lee et al., 2014; Sobrino et al., 2016). Taken together, these data indicate that the constructed microvasculatures had robust barrier functions. A previous study demonstrated that microvessels constructed by HUVEC-pericyte coculture showed more robust endothelial barrier function than those constructed by only HUVECs (Bichsel et al., 2015). Constructed microvasculatures in this chapter were covered by α -SMA-expressing pericytes, suggesting that endothelial barrier function was supported by these pericytes and through interactions between ECs and pericytes.

It was shown here that microvascular networks can indeed be constructed in BMEC-MSC as well as HUVEC-MSC coculture systems. Notably, the microvasculatures of different cell origin had different features. For example, both sprout formation and microvascular length were greater in HUVEC-MSCs than in BMEC-MSCs on day 3, the initiation stage of vascular formation. Previous studies have reported that MSCs produce angiogenic factors such as VEGF and bFGF (Bronckaers et al., 2014; Nassiri and Rahbarghazi, 2014; Volarevic et al., 2011). The results in this

chapter suggest that HUVECs were more sensitive to angiogenic factors than BMECs, or that HUVECs have greater potential than BMECs in terms of vascular formation, which might be due to their organ-specific characteristics. BMEC and HUVEC microvasculatures were also compared on day 7, the maturation stage of vascular formation. Similar to the initiation stage of vascular formation, HUVECs constructed more extensive networks in terms of both microvascular length and branch points. However, there were no significant differences between BMECs and HUVECs in terms of vessel diameter and the number of pericytes covering microvasculatures. A previous study reported that brain capillaries have higher pericyte coverage than any other capillaries (Daneman et al., 2010). Further investigation is needed to clarify whether reported differences in pericyte coverage are observed in EC-MSc cocultures 3D models in this chapter.

Pericytes stabilize capillary structures through the Ang1-Tie2 signaling pathway, resulting in inhibition of angiogenic processes (Armulik et al., 2005; Ribatti et al., 2011). The construction of microvascular networks was realized in two possible steps: EC sprout formation and elongation, and the stabilization of capillary networks by the coverage of pericytes differentiated from MSCs. In addition, the expression of basement membrane proteins such as laminin and type IV collagen were observed in both BMEC and HUVEC microvasculatures, suggesting that engineered microvasculatures were both stable and mature within 7 days of culture.

Fluorescent dextran perfusion into constructed microvascular networks was performed to compare endothelial barrier functions. Instant leakage was not observed. Furthermore, the permeability coefficient was significantly lower in BMEC microvasculatures. This indicates a more robust barrier function in BMEC microvasculatures. Tight junction proteins such as ZO-1 and occludin are key components that regulate endothelial paracellular barrier functions (Greene and Campbell, 2016; Reinhold and Rittner, 2017). VE-cadherin is also a key regulator of endothelial barrier functions (Dejana, 1996; Gavard, 2014; Koch and Claesson-Welsh,

2012). Therefore, the difference in barrier function observed in this chapter might be attributable to the junctional properties of each constructed microvasculature. To verify this, immunofluorescence staining for VE-cadherin, ZO-1, and occludin was performed. The expressions of ZO-1 and occludin were significantly higher in BMEC microvasculatures than in HUVEC microvasculatures, while VE-cadherin expression was confirmed in both microvasculatures at the same level. These data indicate that the lower permeability coefficients of BMEC microvasculatures are attributable in part to the higher expression of tight junction proteins. VE-cadherin and ZO-1 were localized in intercellular regions while occludin was not. Further investigation on occludin localization in intercellular region and its effect on endothelial barrier functions is required.

Finally, the potential of the engineered BMEC microvasculatures was demonstrated. A previous study presented an *in vitro* vascular model using HUVECs with bone marrow stromal cells to mimic barrier function and analyze the role of mural cells in vascular inflammation (Alimberti et al., 2017). Inflammatory factors such as lipopolysaccharides, thrombin, and tumor necrosis factor α have been used to induce an inflammatory response in vasculatures *in vitro*. Vasculatures treated with thrombin have shown the greatest increase in permeability among these inflammatory factors, and thrombin disrupts tight junctions (Hassanpour et al., 2017) and VE cadherin-dependent cell–cell contact (Konstantoulaki et al., 2003). Therefore, thrombin was selected as an inflammatory factor, and it was found that BMEC microvasculatures showed leaky characteristics after treatment. This suggests that *in vitro* BMEC-MSC coculture model in this chapter recapitulated an endothelial response to the proinflammatory stimulus, which may potentially be of use in the study of BBB pathologies.

In conclusion of this chapter, BMEC-MSC and HUVEC-MSC cocultures were performed to establish an *in vitro* brain angiogenesis model, and the effects of EC origin on the formation of microvasculatures were compared. The formation process differed depending on EC origin. Moreover, BMEC and HUVEC microvascular networks

exhibited different characteristics in terms of endothelial barrier functions as well as fundamental morphology. In addition, the inflammatory response of microvasculatures constructed with BMECs was also demonstrated. These results suggest that the organ source of ECs has to be considered when creating an organ-specific cell culture model because the organ-specificity can affect interactions between microvasculatures and parenchymal cells in the model.

3-5 Summary

Every organ demonstrates specific vascular characteristics and functions maintained by interactions of ECs and parenchymal cells. Particularly, brain ECs play a central role in the formation of a functional BBB. Organ-specific ECs have their own morphological features, and organ specificity must be considered when investigating interactions between ECs and other cell types constituting a target organ. Here, angiogenesis-based microvascular networks with perivascular cells were constructed in a microfluidic device setting by coculturing ECs and MSCs. Furthermore, endothelial barrier functions were analyzed as well as fundamental morphology, which was an essential step to build an *in vitro* BBB model. In particular, both BMECs and HUVECs were used to test if organ specificity of ECs affects the formation processes and endothelial barrier functions of an engineered microvascular network. It was found that microvascular formation processes differed by the source of ECs. HUVECs formed more extensive microvascular networks compared to BMECs while no differences were observed between BMECs and HUVECs in terms of both the microvascular diameter and the number of pericytes peripherally associated with the microvasculatures. To compare the endothelial barrier functions of each type of EC, fluorescent dextran perfusion on constructed microvasculatures was performed. The permeability coefficient of BMEC microvasculatures was significantly lower than that of HUVEC microvasculatures. In addition, there were significant differences in terms of tight junction protein expression.

These results suggest that the organ source of ECs influences the properties of engineered microvasculature and thus is a factor to be considered in the design of organ-specific cell culture models.

Chapter 4 Integration of neurogenesis and angiogenesis models for constructing a neurovascular tissue

4-1 Introduction

The brain consists of neural tissues including neurons and glial cells, blood vessels wrapped by smooth muscle cells or pericytes, and ECM. The brain cells interact with each other both structurally and functionally. For example, endothelial tight junctions form BBB to control the transport of substances to the brain parenchyma, and it is known that astrocytes and pericytes around blood vessels contribute to the regulation of BBB functions (Abbott et al., 2010; Wilhelm and Krizbai, 2014). This brain-specific structure is now well-known as the concept of NVU, which is a basic unit in the brain. In the field of brain tissue engineering, many studies have been conducted using single cell type, e.g. either neurons or glial cells, to simplify an experimental model. However, as the concept of NVU has been proposed, it becomes increasingly important to investigate interactions among brain cells for further understanding of brain functions and developing therapeutics of brain disorders. Therefore, it is necessary to construct a brain tissue with multiple cell types *in vitro*, which allows us to analyze the interactions of brain cells in NVU.

In vitro NVU culture models have been reported to recapitulate brain specific functions (Palmiotti et al., 2014). Especially, a Transwell assay has been used to analyze the function of brain ECs by coculturing ECs and astrocytes or pericytes (Wilhelm and Krizbai, 2014; Wong et al., 2013). These studies revealed that the expression of tight junction proteins increased in ECs when they were cocultured with astrocytes or pericytes. However, ECs were cultured two-dimensionally on a plastic dish or a cell culture insert, which are much stiffer than the ECM substrate exposed to cells *in vivo*. The difference in cell configuration and substrate stiffness may cause a different

morphogenesis of ECs in 2D culture compared to those in physiological conditions. To overcome the gap between 2D culture and *in vivo* conditions, it is required to develop a 3D culture model where neural and vascular networks can be constructed in an ECM scaffold.

Previous *in vitro* NVU culture models mimicked a specific structure of NVU in which an endothelial monolayer was in close proximity with astrocytes or pericytes (Booth and Kim, 2012; Wong et al., 2013). In these models, differentiated glial cells or pericytes were used to construct such a specific structure. However, ECs formed a monolayer in these models while ECs form vascular structures *in vivo*. To construct vascular structures, ECs are required to be cultured in 3D gel scaffolds to mimic angiogenesis, which is a multi-step morphogenesis of ECs and mural cells. Such a 3D gel scaffold also provides an environment for neural cells to mimic neurogenesis, which is also a multi-step process including neural differentiation from NSCs and neurite extension. Construction of a neurovascular tissue is realized through these processes, suggesting that the combination of *in vitro* neurogenesis and angiogenesis models is a critical step toward the NVU construction *in vitro*.

In this chapter, a 3D cell culture model in a microfluidic platform is presented, which allows us to construct neurovascular tissues by triculture of human NSCs, BMECs and human MSCs. As a first step, constructing neurovascular tissues including three-dimensionally extensive neurons and capillaries was focused on, because construction of neurovascular tissues is essential for the next step to construct a 3D NVU structure including neurons, capillaries and glial cells. In the present study, cells were cultured in ECM gel scaffolds composed of fibrin, Matrigel and hyaluronan. The effect of the gel components on both neurogenesis and angiogenesis was investigated to construct neurovascular tissues. The timing of cell seeding and cell distribution were also controlled to optimize cell–cell and cell–ECM interactions in the process of neurogenesis and angiogenesis. In terms of angiogenesis, our previous study demonstrated that MSCs promoted vascular formation of HUVECs and differentiated

into α -SMA-positive perivascular cells (Sudo, 2015). These MSC-derived pericyte-like cells sparsely wrapped constructed vasculatures, which had continuous luminal structures. Based on these results, it was hypothesized that BMECs could also construct vascular structures in BMEC-MSC coculture. The aim of this study was to investigate 3D microenvironments for culturing NSCs and BMECs with induction of neurogenesis and angiogenesis in a microfluidic platform, which is the critical step toward the development of an *in vitro* NVU model.

4-2 Experimental design

Detailed methods for the experiments described in this chapter are summarized in chapter 2. Here, experimental settings are described below.

Microfluidic device:

Schematic illustrations of the microfluidic device used in this chapter are shown in Fig. 4-1. The width of the central channel is 750 μm or 1300 μm . The 750- μm device was used for triculture experiments to enhance potential interactions by the diffusion of growth factors secreted by both neural and vascular cells. It was demonstrated that fluorescent dextran (molecular weight: 40 kDa) injected in one microfluidic channel can be transported across a gel region, whose width was 750 μm , by diffusion within 30 minutes (Sudo et al., 2009).

Cells:

BMECs, MSCs and NSCs were used in this chapter. The amount of all cell types seeded in each device was 1.0×10^4 cells. In triculture experiments, NSCs were seeded in one microfluidic channel and BMECs and MSCs were seeded in the other microfluidic channel. Neural cells and vascular cells were seeded separately to induce neurogenesis from one side and angiogenesis from the other side of the gel region. In

NSC pre-seeding condition, NSCs were first cultured alone for 7 days to allow them to migrate into the gel region and increase the neuronal population. Triculture was performed for additional 3 days to induce neurovascular formation. On the other hand, in BMEC-MSc pre-seeding condition, BMECs and MSCs were first cocultured for 7 days to induce microvascular formation in the gel region. NSCs were then seeded to start triculture. Triculture experiments were performed for 10 days for all conditions.

Medium:

NSC medium was used for NSC monoculture (4-3-1, 4-3-3 and 4-3-4) and triculture of NSCs, BMECs and MSCs. BMEC monoculture (4-3-5) and BMEC-MSc coculture (4-3-2) were performed using 1:1 mixture of CSC complete medium and MSC growth medium.

Additional supplement to medium: The mixture of CSC complete medium and MSC growth medium was supplemented with 10 ng/mL VEGF and 10 ng/mL bFGF to promote microvascular formation and cell proliferation.

Gel scaffold: Fibrin, Matrigel and hyaluronan were used in this chapter. The reason for using fibrin was mentioned in chapter 3 (3-2). Matrigel was commonly used for neural cell culture (Bang et al., 2016; Sun et al., 2016; Wevers et al., 2016). In addition, main components of Matrigel are laminin and collagen IV, which are components of brain ECM (Carbonetto, 1984). Hyaluronan is also a component of brain ECM (Margolis et al., 1975; Ripellino et al., 1988). Therefore, Matrigel and hyaluronan were used to test if these ECM components would be suitable for the culture of NSCs in terms of neurite extension.

The concentration of fibrin was 2 mg/mL for all experiments in this chapter. The concentration of Matrigel in fibrin-Matrigel mixed gel was 2 mg/mL in the first experiment (4-3-1), and 2, 4 and 8 mg/mL in the later experiment (4-3-3). The

maximum concentration of Matrigel was set to be 8 mg/mL because it becomes hard to seed cells onto the gel wall when the concentration of Matrigel is high. After optimization of the condition of gel concentration in 4-3-3, the concentration of Matrigel used in later experiments was 8 mg/mL. NSC monoculture (4-3-4) and triculture of NSCs, BMECs and MSCs (4-3-5) was performed in fibrin-Matrigel mixed gel. The concentration was 2 mg/mL and 8 mg/mL for fibrin and Matrigel, respectively.

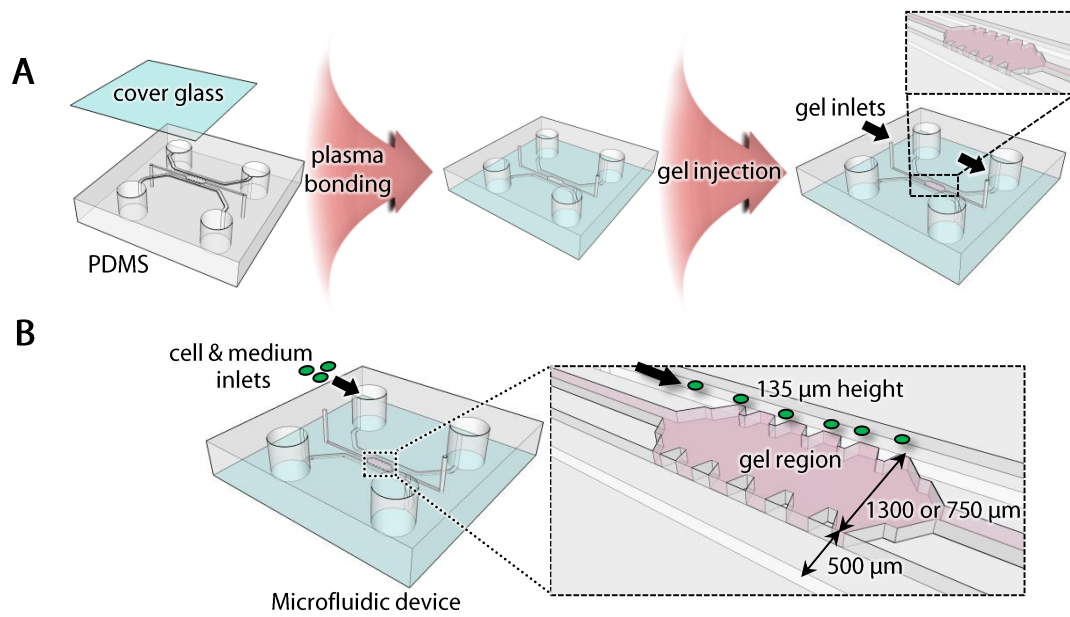


Fig. 4-1 Schematic illustrations of a microfluidic device. A: A PDMS device copied by an SU-8 mold was plasma-bonded with a coverglass to form microfluidic channels. Gel pre-polymer solution was then injected into the central channel from two gel inlets (arrows). B: There are two parallel microchannels separated by the gel region. BMECs and NSCs were injected into one of the microchannels (arrows).

4-3 Results

4-3-1 Neurite formation of neurons differentiated from NSCs in different gel scaffolds

First, NSCs were cultured in a microfluidic device, which includes a 3D gel region (Fig. 4-1). In the NSC monoculture, different gel scaffolds were used to optimize the gel condition in terms of neurite extension (Fig. 4-2A). NSCs were cultured in different gel compositions including fibrin, Matrigel and hyaluronan. Fibrin gel has been commonly used for 3D cell culture because of its structural stability. Matrigel and hyaluronan have often been used for the culture of neural cells because brain ECM is composed of laminin, hyaluronan and other proteins such as chondroitin sulfate proteoglycans (Zimmermann and Dours-Zimmermann, 2008). Laminin is the main component of Matrigel. Although Matrigel and hyaluronan were tested to use without fibrin, it was difficult to form these gels (data not shown). Therefore, fibrin was used as a basal gel scaffold and added Matrigel or hyaluronan, resulting in three gel conditions; fibrin gel, fibrin-Matrigel mixed gel and fibrin-hyaluronan mixed gel. NSCs were cultured in these gel conditions and cell morphology and migration were observed everyday by phase-contrast microscopy until day 9. The result showed that, in fibrin gel condition, some cells migrated into the gel on day 1 (Fig. 4-2B, Fibrin). These migrating cells exhibited a round-shape without neurite extension (e.g., arrowheads in Fig. 4-2B, Fibrin, day 1, inset) and the number of migrating cells did not increase until day 9. Similarly, NSCs exhibited a round-shape when cultured in fibrin-hyaluronan mixed gel (arrowheads, Fig. 4-2B, Fibrin-Hyaluronan, day 1, inset). The number of migrating cells gradually increased until day 9. On the other hand, the morphology of NSCs was different in fibrin-Matrigel mixed gel; cells extended within the gel (arrows, Fig. 4-2B, Fibrin-Matrigel, day 1, inset). In addition, migration of NSCs was the greatest in the gel.

To further investigate cellular morphology and differentiation of NSCs into neurons, immunofluorescence staining of cells on day 9 was performed for a neuronal marker,

MAP2. In fibrin gel condition, a few round-shape cells were positive for MAP2 but neurite extension was not observed (Fig. 4-2C, Fibrin). In fibrin-hyaluronan mixed gel, some NSCs were positive for MAP2 and exhibited short neurite-like structures (Fig. 4-2C, Fibrin-Hyaluronan). On the other hand, many NSCs in fibrin-Matrigel mixed gel were positive for MAP2 and extended neurites (Fig. 4-2C, Fibrin-Matrigel). Quantitative analysis of neurite length on day 9 revealed that neurite extension was significantly promoted in fibrin-Matrigel mixed gel (Fig. 4-2D).

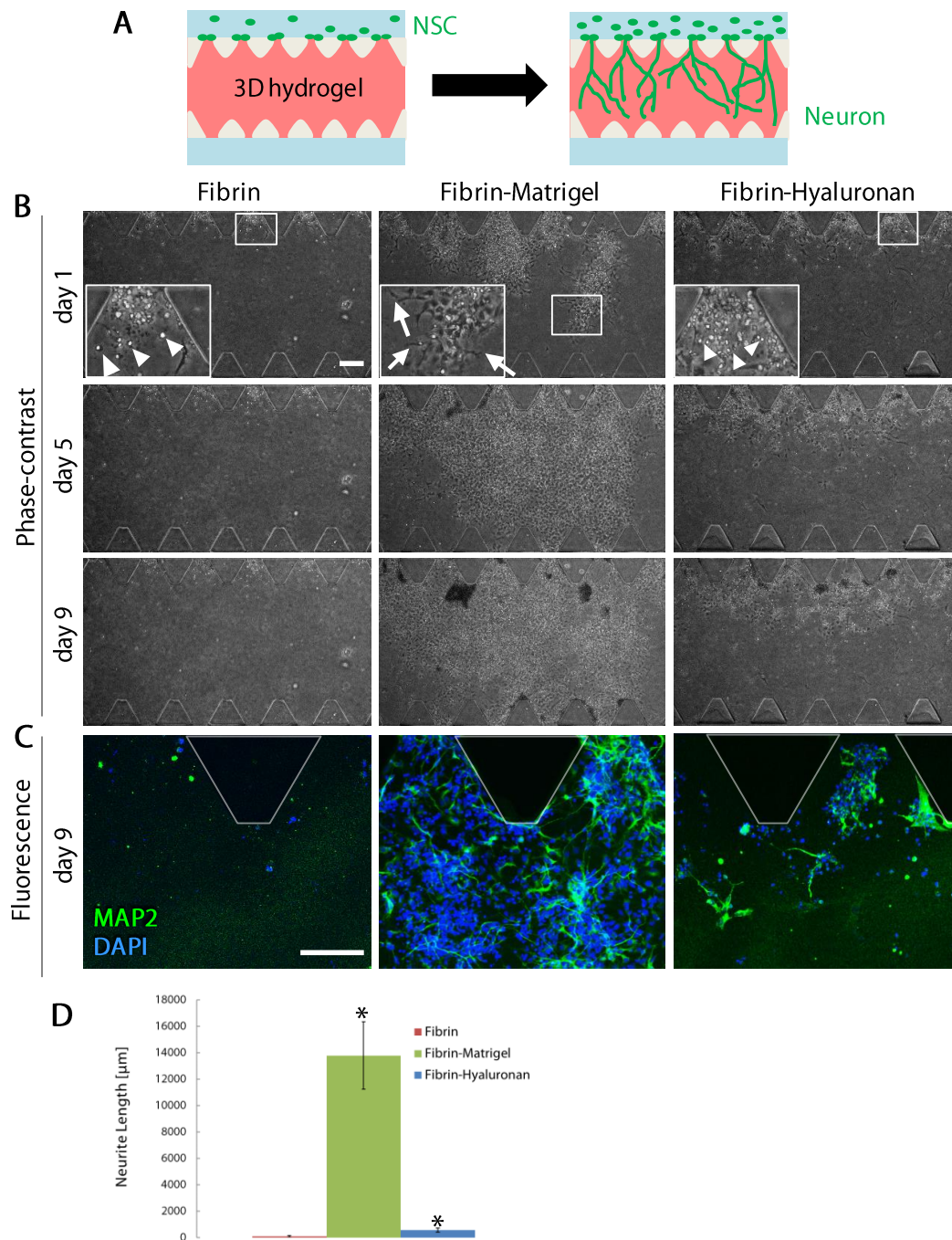


Fig. 4-2 Neurite formation of NSC-derived neurons in different gel scaffolds.

A: Schematic illustrations of NSC culture for constructing a neural network. B: Representative phase-contrast images of NSCs cultured in three different gel scaffolds; fibrin (left), fibrin-Matrigel mixed gel (middle) and fibrin-hyaluronan mixed gel (right). Arrowheads in insets indicate a round shape of NSCs (Fibrin, Fibrin-Hyaluronan), while

arrows in an inset indicate an extended NSCs (Fibrin-Matrigel). C: Representative immunofluorescence images of neurons (MAP2, green) and nuclei (DAPI, blue) on day 9. D: Quantitative analysis of neurite length. Data are shown as the mean $\pm$ SEM. (N=3, n $\geq$ 16). * p <0.05 vs. Fibrin. Scale bars, 100 μ m.

4-3-2 Formation of microvasculature in BMEC-MSc coculture in different gel scaffolds

Next, the same gel conditions, which were fibrin gel, fibrin-Matrigel mixed gel and fibrin-hyaluronan mixed gel as tested for NSCs, were tested in terms of capillary formation. Coculture of BMECs and MSCs was performed in these gel conditions. First, BMECs and MSCs were cultured in fibrin-Matrigel mixed gel to investigate whether capillary networks can be formed in the gel condition. BMECs were seeded into one channel while MSCs were seeded into the other channel (Fig. 4-3A). On day 1, MSCs dominantly migrated into the 3D gel scaffold toward BMECs, while some BMECs formed vascular sprouts (arrowheads, Fig. 4-3B, Fibrin-Matrigel, day 1, inset). Both cell types increasingly migrated into the gel region until day 9. Because it was difficult to distinguish between BMECs and MSCs by phase-contrast microscopy, immunofluorescence staining was performed for PECAM-1 and α -SMA, which are markers for BMECs and perivascular cells, respectively. The immunofluorescence image revealed that BMECs formed vascular sprouts and constructed branching microvasculatures while MSCs differentiated into α -SMA-positive perivascular cells on day 9. Similar microvasculatures were also formed in fibrin gel, while sheet-like structure was observed in fibrin-hyaluronan mixed gel (Fig. 4-3C, Fibrin, Fibrin-Hyaluronan, Fig. 4-3D). Quantitative analysis of network length on day 9 revealed that construction of microvasculatures was the greatest in fibrin-Matrigel mixed gel (Fig. 4-3E). In addition, microvasculatures in fibrin-Matrigel mixed gel was successfully constructed by culturing BMECs and MSCs in the same microchannel (Fig. 4-7).

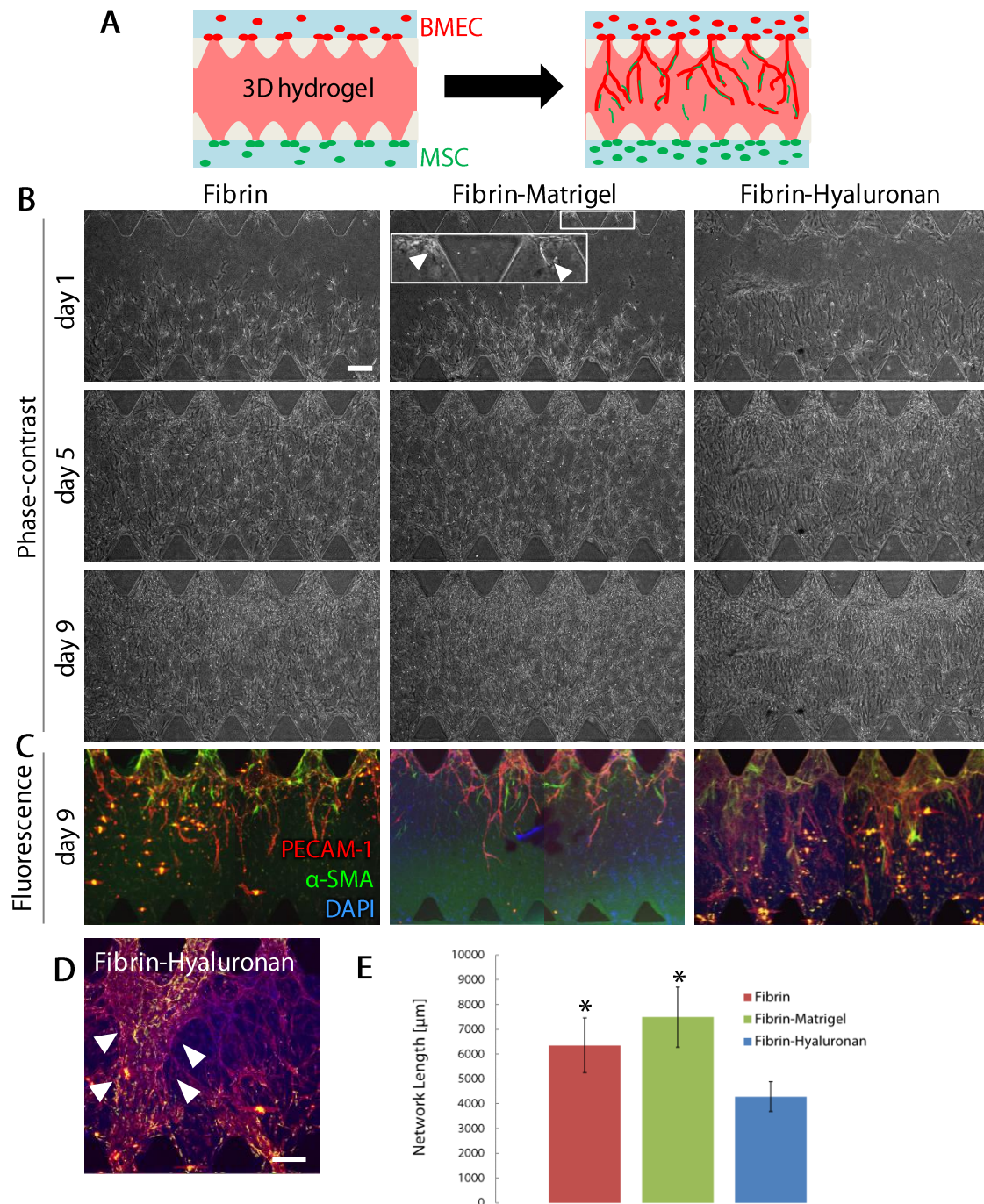


Fig. 4-3 Formation of microvasculatures in BMEC-MSC coculture in fibrin, fibrin-Matrigel mixed gel, and fibrin-hyaluronan mixed gel. A: Schematic illustrations of BMEC-MSC coculture. B, C: Representative phase-contrast images on days 1, 5 and 9, and corresponding immunofluorescence images of BMECs (PECAM-1, red), pericytes (α -SMA, green), and nuclei (DAPI, blue) on day 9. Arrowheads in an

inset indicates vascular sprouts (Fibrin-Matrigel, day 1). Scale bar, 200 μm . D: An enlarged image of cells in the fibrin-hyaluronan mixed gel. Arrowheads indicate a sheet-like structure of BMECs. Scale bar, 200 μm . E: Quantitative analysis of network length. Data are shown as the mean $\pm$ SEM. (N=3, n $\geq$ 9). * p <0.05 vs. Fibrin-Hyaluronan.

4-3-3 Optimization of the concentration of fibrin-Matrigel mixed gel

Following the experimental results above, fibrin-Matrigel mixed gel was used for subsequent experiments. Since neurons extend three-dimensionally in the brain tissue, 3D distribution of NSCs in the gel was investigated. Confocal microscope images revealed that Tuj1-positive neurons derived from NSCs migrated into the gel region in a 2D manner along with the top and bottom surface of the gel region (Fig. 4-4A, C). As shown in Fig. 4-2, fibrin-Matrigel mixed gel promoted neurite extension more than fibrin gel, which suggested that Matrigel promoted neurite extension. Therefore, the fibrin concentration was fixed as 2 mg/mL and Matrigel concentration was increased. Specifically, Matrigel concentration was increased to be 4 or 8 mg/mL to form more stable gel structure. Consequently, 3D neurite extension was induced when NSCs were cultured in the modified gel with higher Matrigel concentrations (Fig. 4-4B, D). To quantify this 3D distribution of NSCs, the percentage of the number of cell nuclei in a 3D region against the entire gel region was calculated (Fig. 4-4E). The number of cell nuclei in the 3D region significantly increased as Matrigel concentration increased (Fig. 4-4F). In particular, the greatest 3D distribution was obtained in 2-8 mg/mL fibrin-Matrigel mixed gel, and the percentage was $38 \pm 3.7\%$.

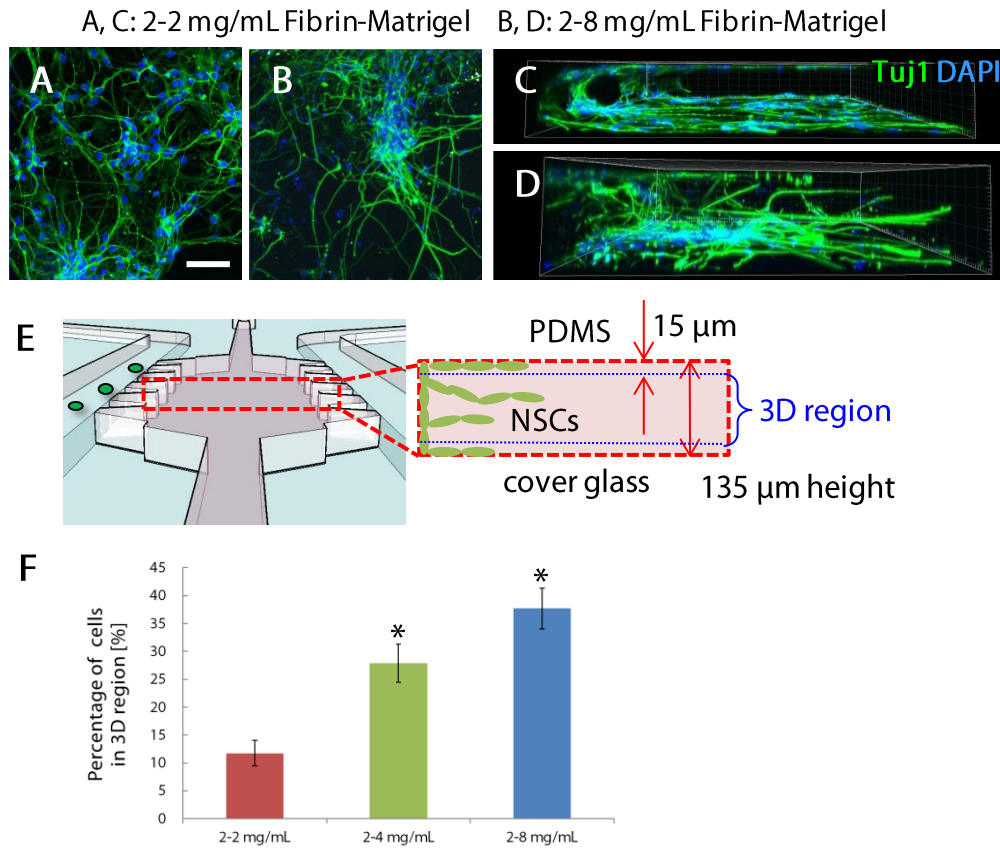


Fig. 4-4 Optimization of the concentration of fibrin-Matrigel mixed gel for 3D migration of neurons. A–D: Immunofluorescence projection images and corresponding 3D views of neurons in 2-2 mg/ml and 2-8 mg/ml fibrin-Matrigel mixed gel, respectively. Cells were fixed on day 21 and stained for neurons (Tuj1, green) and nuclei (DAPI, blue). Scale bar, 100 µm. E–F: Quantitative analysis of the percentage of migrating cells in a 3D manner. The number of NSCs migrating into the 3D gel region. Data are shown as the mean ± SEM. (N=3, n≥12). * $p < 0.05$ vs. 2-2 mg/ml.

4-3-4 The process of neurogenesis and neurite extension in NSC culture

To further investigate differentiation of NSCs into neurons and the process of neurite extension, NSCs cultured in 2-8 mg/mL fibrin-Matrigel mixed gel were fixed on days 7, 14 and 21, and immunofluorescence staining was performed for neurons (Tuj1). Representative projection images showed that cells on day 7 were positive for Tuj1 but exhibited only short processes (Fig. 4-5A, day 7). However, cells on day 14 extended neurites, which further extended to form long neurites on day 21 (Fig. 4-5A, day 14, 21). According to the histogram of neurite length, the distribution of the neurite length of neurons shifted towards longer area as they were cultured for 7, 14 and 21 days, suggesting that neurons continuously elongated their neurites until day 21 (Fig. 4-5B). The longest neurite length was 139 μm , 300 μm and 445 μm on day 7, 14 and 21, respectively. The average neurite length increased in proportion to the culture days (Fig. 4-5C). The average neurite length was $31 \pm 0.6 \mu\text{m}$, $51 \pm 2.0 \mu\text{m}$ and $86 \pm 2.3 \mu\text{m}$ on day 7, 14 and 21, respectively.

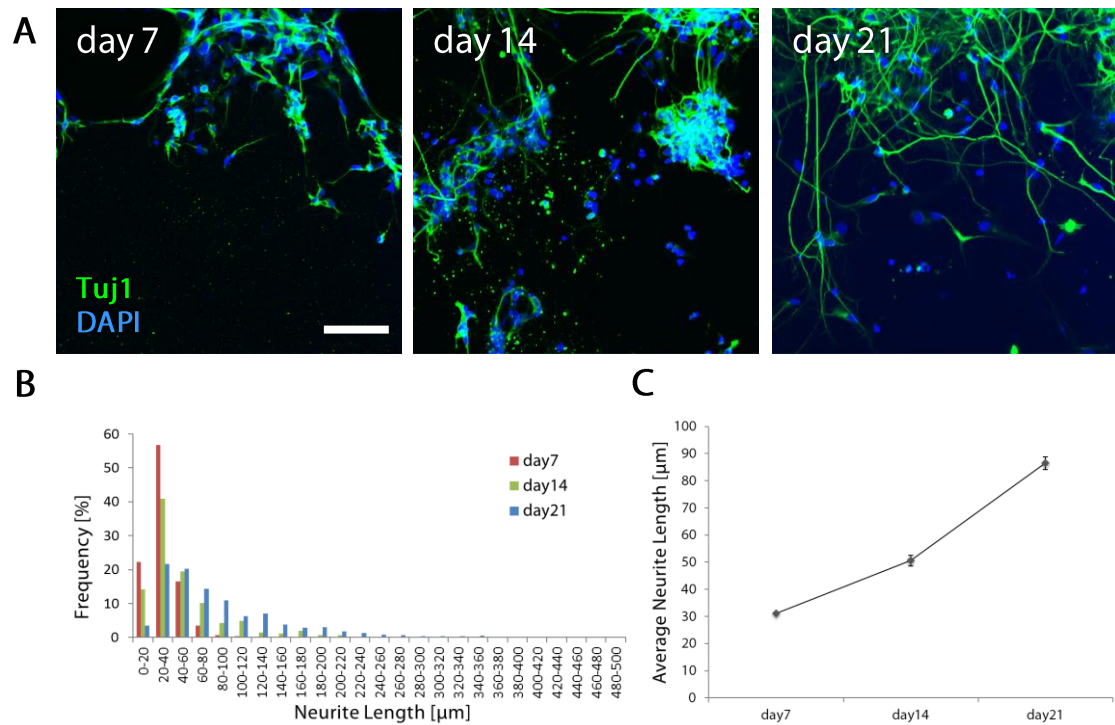


Fig. 4-5 The process of neurogenesis in 21-day culture. A: Immunofluorescence projection images of NSC-derived neurons in 2-8 mg/mL fibrin-Matrigel mixed gel. Cells were fixed on day 21 and stained for neurons (Tuj1, green) and nuclei (DAPI, blue). Scale bar, 100 μ m. B: A histogram of neurite length on days 7, 14, and 21. C: Time course of average neurite length in 21-day culture. Data are shown as the mean $\pm$ SEM. (N=3, n $\geq$ 12).

4-3-5 Triculture of NSCs, BMECs and MSCs for constructing neurovascular tissues

Finally, the NSC culture model, which recapitulates neurogenesis, and BMEC-MSC coculture model, which recapitulates angiogenesis, were combined for constructing neurovascular tissues. In the triculture, BMECs and MSCs were seeded in one microchannel and NSCs were seeded in the other microchannel to separate angiogenesis and neurogenesis. When culturing BMECs and MSCs, there are two ways of cell seeding; seeding both cell types into one microchannel or seeding BMECs into one microchannel and seeding MSCs into the other channel. It was confirmed that microvasculatures were constructed in both conditions (Fig. 4-7). Three conditions were performed in terms of the timing of cell seeding; “same-day seeding”, “BMEC-MSC pre-seeding”, and “NSC pre-seeding”. In the same-day seeding condition, BMECs-MSCs and NSCs were seeded on the same day. In the BMEC-MSC pre-seeding condition, BMECs and MSCs were seeded in the beginning of the culture, and NSCs were added on day 7 after vascular structures were constructed by BMECs and MSCs. In the NSC pre-seeding condition, NSCs were seeded in the beginning of the culture, and BMECs and MSCs were added on day 7 after NSCs migrated into the gel region and extended neurites.

In the same-day seeding condition, NSCs migrated into the gel region from the beginning of the triculture while BMECs started sprouting into the gel region. In the BMEC-MSC pre-seeding condition, BMECs also started sprouting into the gel region from the beginning of the culture, and some of microvasculatures reached the end of the gel region, which is 750 μm far from the seeding point. However, in these seeding conditions, the gel structure shrank as vascular sprouts elongated into the gel region with increasing culture time (Fig. 4-6A, Same-day seeding, BMEC-MSC pre-seeding). Therefore, enough space was not maintained for NSCs to migrate and expand in the shrunk gel structure. On the other hand, in the NSC pre-seeding condition, NSCs migrated into the gel region before seeding BMECs and MSCs. NSCs differentiated into

neurons and elongated neurite structures while microvasculatures were constructed inside the same gel region after seeding BMECs and MSCs on day 7 (Fig. 4-6A, NSC pre-seeding). The area of the gel collapse was quantified on day 10 of the triculture (Fig. 4-6B). Gel collapse was significantly reduced in the NSC pre-seeding condition compared to the other conditions. In addition, enlarged view of neurons and BMECs in the NSC pre-seeding condition showed that microvasculatures and neurites existed in close proximity in the gel region (Fig. 4-6C). The constructed neurovascular tissues maintained at least for 10 days.

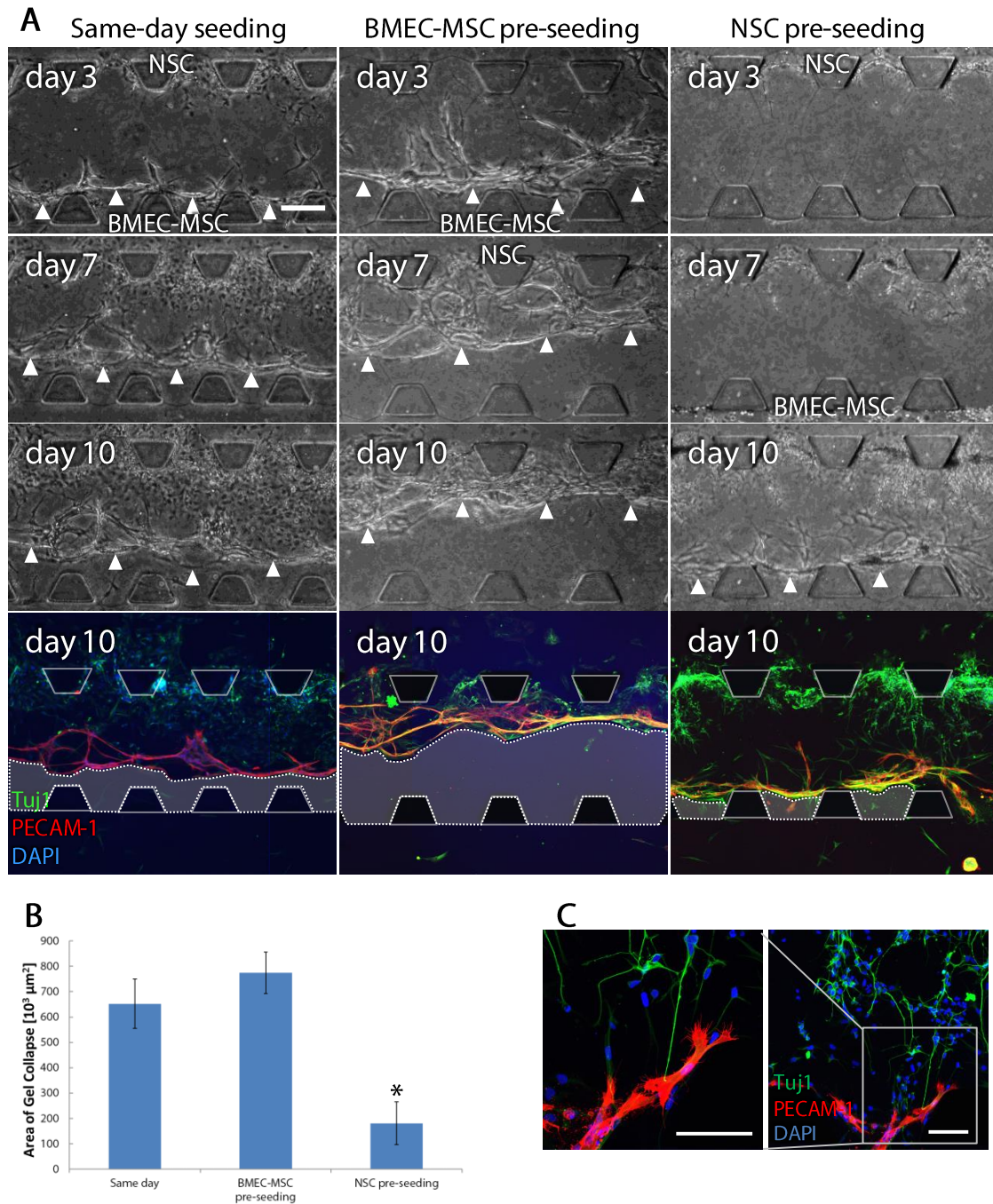


Fig. 4-6 Triculture of NSCs, BMECs and MSCs for constructing neurovascular tissues. A: Representative phase-contrast images on days 3, 7 and 10, and corresponding immunofluorescence images of neurons (TuJ1, green), BMECs (PECAM-1, red) and cell nuclei (DAPI, blue) on day 10. Arrowheads and areas surrounded by dotted lines in immunofluorescence images represent the gel collapse.

Scale bar, 200 μm . B: Quantitative analysis of the gel collapse in different seeding conditions on day 10. Data are the means $\pm$ SEM. (N=3, n $\geq$ 9). * p <0.05 vs. “Same day” and “BMEC-MSD pre-seeding”. C: Enlarged views of neurons and BMECs in NSC pre-seeding condition. Scale bars, 100 μm .

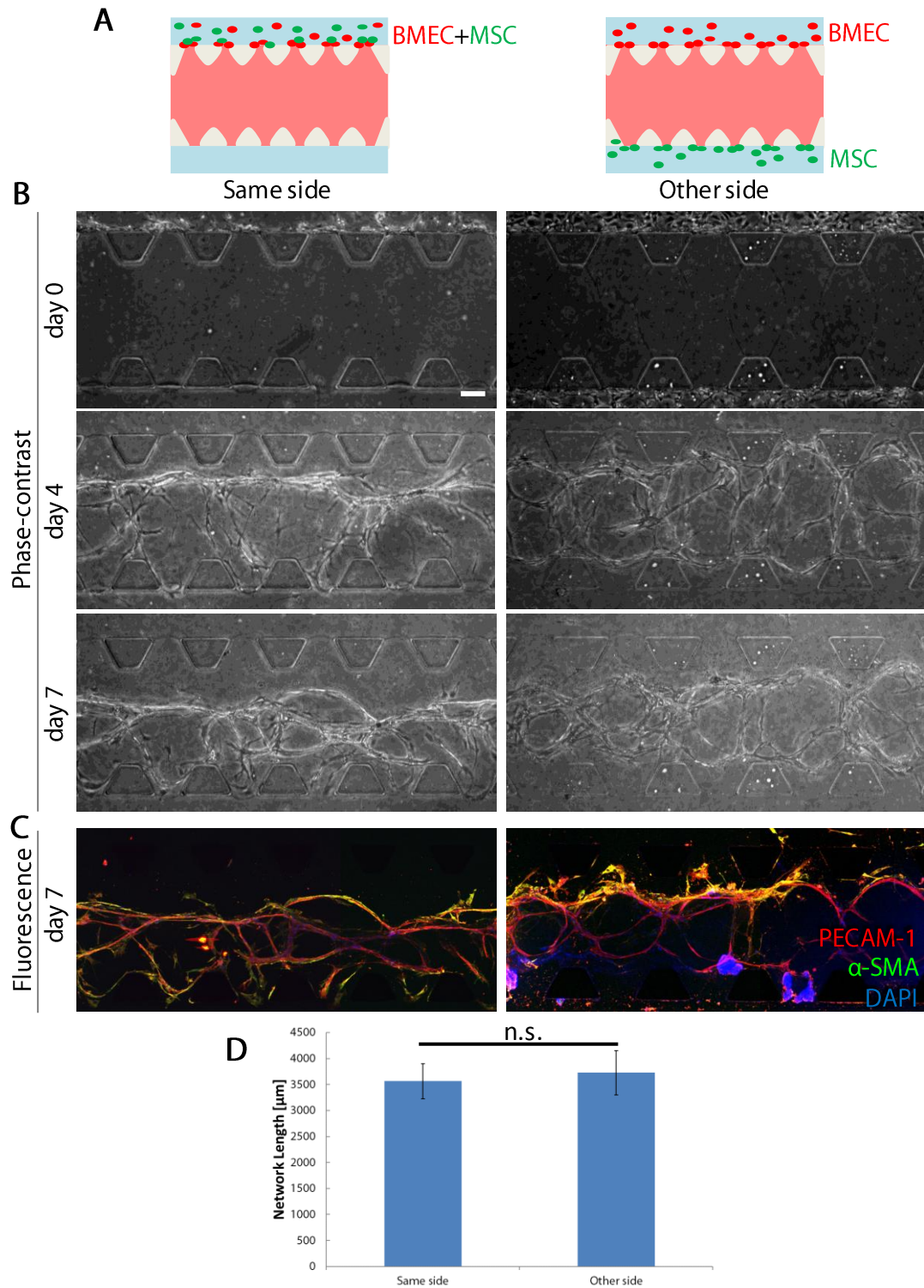


Fig. 4-7 Formation of microvasculatures in BMEC-MSC coculture in “same side” and “other side” seeding conditions. A: Schematic illustrations of BMEC-MSC coculture. B, C: Representative phase-contrast images on days 0, 4 and 7, and

corresponding immunofluorescence images of BMECs (PECAM-1, red), perivascular cells (α -SMA, green), and nuclei (DAPI, blue) on day 7. Scale bar, 100 μ m. D: Quantitative analysis of network length. Data are shown as the mean $\pm$ SEM. (N=3, n $\geq$ 9). n.s.: no significance.

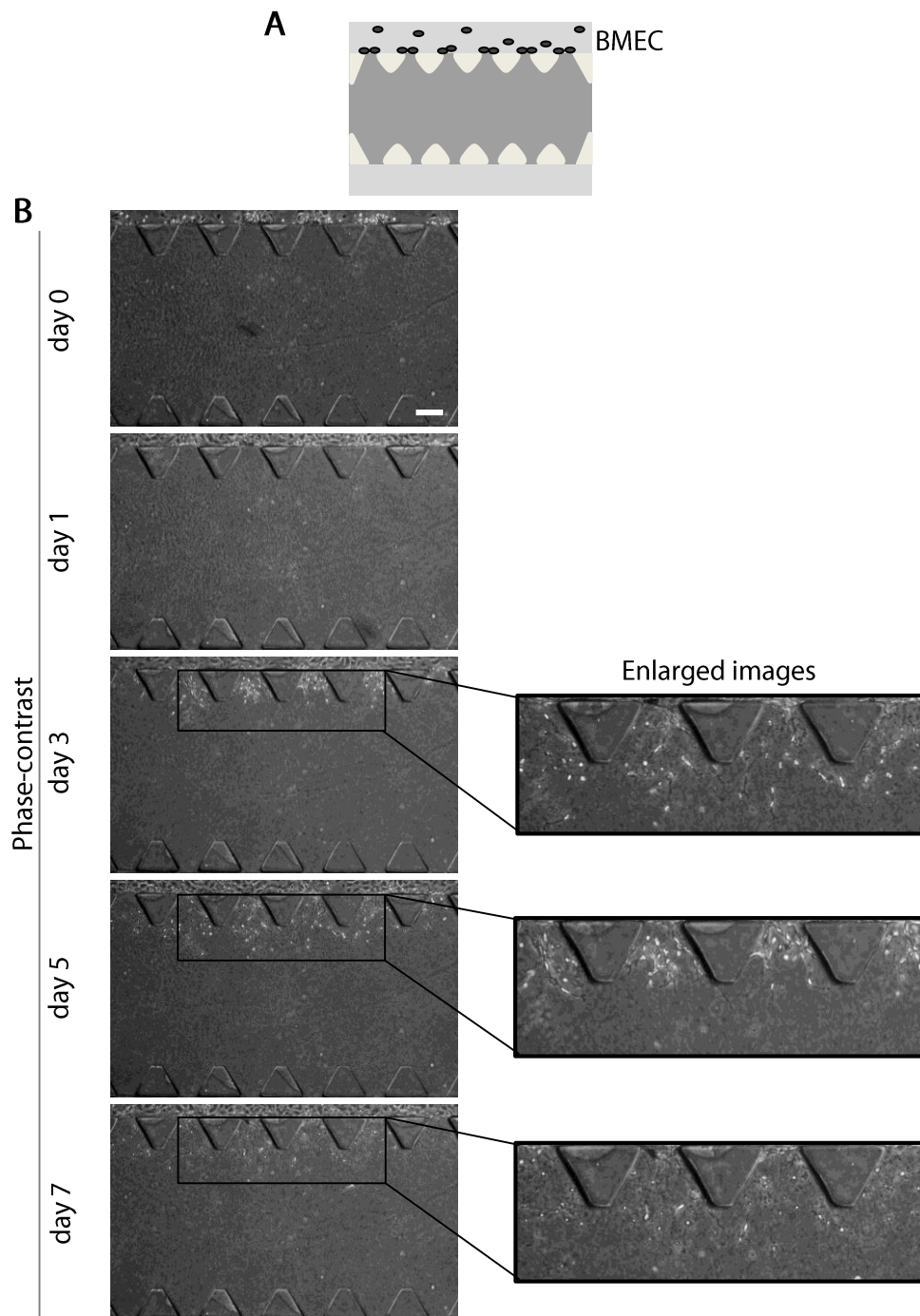


Fig. 4-8 BMEC monoculture in fibrin-Matrigel mixed gel. A: A schematic illustration of BMEC culture. B: Phase-contrast images on days 0, 1, 3, 5 and 7, and corresponding enlarged images on days 3, 5 and 7. Scale bar, 100 μm .

4-4 Discussion

In the present study, 3D neurovascular tissues in ECM gel scaffolds were successfully constructed by combining 3D angiogenesis and neurogenesis models. In the field of tissue engineering, the construction of a 3D NVU structure including neurons, glial cells, and blood vessels has not been demonstrated (Osipova et al., 2018). Previous studies reported *in vitro* NVU models using Transwell or microfluidic devices that were composed of ECs with/without astrocytes and pericytes to investigate BBB functions of ECs (Bicker et al., 2014; Cho et al., 2015; Nakagawa et al., 2009; Wilhelm and Krizbai, 2014; Xue et al., 2013). These culture models enabled us to investigate the functional characteristics of ECs such as endothelial permeability. However, cells in these models were cultured as endothelial monolayers, which is different from *in vivo* conditions.

Culturing brain cells in an ECM gel scaffold, which is closer to physiological conditions than a plastic substrate such as well plates, will enable us to recapitulate more physiologically relevant morphogenesis. Brain cells in the Transwell model cannot interact with each other in the juxtacrine manner because of the physical compartment, while astrocytes and pericytes wrap around blood vessels through the basement membrane in a physiological condition (Hawkins and Davis, 2005). This limitation of signaling pathway can cause a different morphology of cultured cells compared to those in physiological conditions. The stiffness of a plastic surface or coated ECM proteins on which cells adhere can also affect the morphology of these cells and differentiation of stem cells (Engler et al., 2006; Kothapalli and Kamm, 2013). To overcome such limitations in previous 2D NVU models, this cell culture model provided a 3D neurovascular tissue that is constructed by inducing neurogenesis and angiogenesis in a microfluidic device. The success in recapitulating neurogenesis and angiogenesis suggests that this microfluidic culture model provided a proper microenvironment for both neural and vascular cells to survive and function.

In this chapter, NSCs were first cultured in fibrin gel, fibrin-Matrigel mixed gel and fibrin-hyaluronan mixed gel. The proliferation of NSCs and the neurite extension in fibrin-Matrigel were significantly promoted compared to the other conditions. It is known that various factors can promote or inhibit neurite extension. A composition of ECM proteins is one of the important factors in regulating neurite extension. Previous studies showed that laminin can guide neurite extension and promote migration of growth cone of neurons (Kuhn et al., 1995; Turney and Bridgman, 2005). Neurons bind to laminin, which is the main component of Matrigel, through $\beta 1$ integrin (Hall et al., 2008; Lei et al., 2012). A previous report showed that axons of neurons which bound to laminin expressed a $\beta 1$ integrin, and laminin/ $\beta 1$ integrin signaling contributed to axon development and promoted axon elongation (Lei et al., 2012). Similarly, in the present experiments, it is suggested that NSC-derived neurons, which were cultured in fibrin-Matrigel mixed gel, bound to laminin and constructed 3D neural networks through the laminin/ $\beta 1$ integrin signaling.

In this neurovascular tissue culture model, NSCs differentiated into neurons and they continued elongating neurites at least for 21 days. Relatively short neurites which are within 60 μm were observed even on day 21, suggesting that cultured NSCs continued generating neurons for 21 days or some neurons stopped elongating their neurites in the beginning of the culture but still alive at the end of the culture. It is known that neural differentiation of NSCs is followed by glial differentiation (Temple, 2001). Therefore, it is expected that glial differentiation will be induced by continuing the NSC culture in this cell culture model, which will lead to the construction of the 3D NVU structure in long-term culture.

BMECs and MSCs were cocultured in three different gel conditions, fibrin gel, fibrin-Matrigel mixed gel and fibrin-hyaluronan mixed gel. It was found that microvasculatures with α -SMA-positive perivascular cells were constructed in these gel conditions. It was hypothesized that hyaluronan would be suitable to construct brain vasculature because it is the main component of brain ECM (Zimmermann and

Dours-Zimmermann, 2008). However, interestingly, most cells migrated two-dimensionally along the bottom glass surface when they were cultured in fibrin-hyaluronan mixed gel. Some microvasculatures were constructed in this condition, but sheet-like structures were also constructed resulting from the two-dimensional cell migration, suggesting that hyaluronan is not suitable for constructing capillaries. Given that hyaluronan is one of the main components of brain ECM and angiogenesis is suppressed in mature normal brain, these data suggest that hyaluronan has some anti-angiogenic effects which lead to the stabilization of pre-existing vasculature. Addition of hyaluronan to hydrogel changed not only biochemical properties of the gel but also its mechanical properties. The stiffness of ECM may also affect angiogenic processes (Burdick and Prestwich, 2011). For example, it was reported previously that the different stiffness of collagen gel led to the different vessel diameter and length in an angiogenic process (Chung et al., 2009; Yamamura et al., 2007). Therefore, the further investigation will be needed in terms of the effect of biochemical and biomechanical cues on angiogenesis to establish a stable *in vitro* experimental model of NVU tissues.

In this microfluidic cell culture model, especially in “NSC pre-seeding” condition, neurovascular tissues were successfully constructed on day 10 by inducing neurogenesis and angiogenesis simultaneously in an optimized 3D ECM gel condition. However, in “same-day seeding” and “BMEC-MSc pre-seeding” conditions, the gel structure shrank within a few days from the BMEC-MSc side. This collapse of the gel can be explained by two factors. First, the gel structure was not strong enough against the traction force of BMECs and MSCs when they migrated into the gel. The other possibility is that BMECs or MSCs secreted MMPs to degrade the gel. Although there is still a possibility of further modification of the gel, the success in constructing neurovascular tissues including neurons and microvasculatures is a critical step to achieve the construction of NVU structures *in vitro*.

In conclusion of this chapter, a microfluidic platform for constructing a 3D neurovascular tissue was established. This cell culture model enables us to culture NSCs

and ECs three-dimensionally in a close proximity, which is mimicry of brain microenvironment composed of neural cells, microvasculatures and ECM. In addition, processes of neurogenesis, angiogenesis and the formation of neurovascular tissues can be monitored by phase-contrast microscopy. This microfluidic cell culture system has such advantages which cannot be achieved by conventional culture systems such as Transwell systems. In the future study, the differentiation of NSCs into astrocytes will be focused on, which is an additional key element constituting NVU microenvironments. A new *in vitro* NVU model which contains neurons, astrocytes, microvasculatures wrapped by pericytes and ECM proteins will be of great use for studies of neurovascular interactions and studies of neurovascular development.

4-5 Summary

NVU is a conceptual basic unit in the brain, including neurons, glial cells, blood vessels and ECM. This concept implies the importance of a 3D culture model including these cell types for investigating brain functions. However, little is known about the construction of an *in vitro* 3D NVU model. The aim of this study was to construct 3D neurovascular tissues by combining *in vitro* neurogenesis and angiogenesis models using a microfluidic platform, which would be a critical step toward the NVU construction *in vitro*. Three gel conditions, which were fibrin gel, fibrin-Matrigel mixed gel and fibrin-hyaluronan mixed gel, were investigated to optimize the gel components in terms of neurogenesis and angiogenesis. First, fibrin-Matrigel mixed gel was found to induce NSC differentiation into neurons and neurite extension. In particular, 3D neurite extension was realized in 2-8 mg/mL fibrin-Matrigel mixed gel. Second, it was found that microvasculatures were also formed in the fibrin-Matrigel mixed gel by coculturing BMECs and MSCs. Finally, both neural and vascular culture models were combined, resulting in constructing 3D neurovascular tissues with an optimized seeding condition of NSCs, BMECs and MSCs.

Chapter 5 Investigation of triculture conditions of NSCs, BMECs and MSCs for the construction of NVU-like structure

5-1 Introduction

The brain is one of the most complex organs in the human body. Neurons, astrocytes and oligodendrocytes are the major brain parenchymal cells that perform brain-specific functions. Because of the limited capacity to store energy, neurons need constant supply of oxygen and nutrients from blood vessels for proper neuronal activities (Zlokovic, 2011). Astrocytes contribute to the transport of nutrients from blood vessels to neurons. Malfunction of the supply can cause pathological states. Therefore, brain activities and homeostasis are maintained by this multi-cellular system composed of neural cells and vasculature. This fundamental group of cells is called neurovascular unit (NVU).

NVU structure comprises a brain-specific microvascular interface called BBB. Brain microvasculatures are covered by pericytes and astrocytes. Brain ECs lining along microvascular walls express tight junction proteins such as claudins and occludin. The paracellular and transcellular transport of hydrophilic substances are strictly restricted by BBB. This brain-specific structure inhibits the active transport of drugs into brain parenchyma and makes it difficult for drugs to exert the effects. Therefore, understanding of BBB regulation and penetration is critical for drug screening studies targeting neurodegenerative diseases.

Many *in vitro* studies have been performed to investigate BBB functions (Banerjee et al., 2016; Bicker et al., 2014; Helm et al., 2016). In these studies, brain ECs were cultured in a 2D manner, and the barrier tightness of the endothelial monolayer was estimated by measuring the resistance of ionic diffusion through endothelial monolayer

using TEER approach. However, TEER is an estimate, not a direct analysis of BBB function because TEER is unmeasurable for *in vivo* microvasculatures (Banerjee et al., 2016). An ideal approach to investigate BBB function is constructing *in vivo*-like 3D brain microvasculatures and perfusing solutions into the constructed microvasculatures to measure the transport of solutes through the microvascular wall.

Microfluidic cell culture platform can potentially be a robust method to realize the construction of 3D NVU structures. Microfluidic devices are made through microfabrication processes such as photolithography and soft lithography. Microfluidic devices are made of PDMS and have designed microchannels. Cells can be cultured three-dimensionally within ECM-like gel scaffolds by injecting cells and gel solution into the devices. Previous studies demonstrated the engineering of 3D vasculatures by culturing HUVECs using microfluidic devices, which was a mimicry of neovascularization processes such as vasculogenesis and angiogenesis (Kim et al., 2015, 2013; Lim et al., 2013). *In vitro* 3D brain angiogenesis model and 3D neurogenesis model were also established previously (chapter 3 and chapter 4). These studies indicate the possibility of constructing 3D NVU structure by combining angiogenesis and neurogenesis models. However, the construction of the NVU structure composed of neurons, astrocytes and microvasculatures has not been achieved.

If we can construct 3D NVU structures in an *in vitro* manner, the cell culture system will be of great use for studies of BBB development and drug screening studies for neurodegenerative diseases. Therefore, the present study was performed to investigate cell culture conditions for constructing NVU structures including neurons, astrocytes and microvasculatures using microfluidic devices.

5-2 Experimental design

Detailed methods for the experiments described in this chapter are summarized in chapter 2. Here, experimental settings are described.

Neurovascular tissue formation was realized by the induction of neurogenesis and angiogenesis in fibrin-Matrigel mixed gel in chapter 4. This tissue was composed of neurons and microvasculatures, not including astrocytes. Toward the construction of NVU structure, the next step is the generation of astrocytes and the induction of the coverage of astrocytic endfeet to microvasculatures. In terms of NSC differentiation, astrocyte differentiation follows neuronal differentiation (Ramasamy et al., 2013). It suggests that long-term culture of NSCs is needed in our culture model. However, as a result of the triculture of NSCs, BMECs and MSCs in chapter 4, gel collapse was observed, suggesting the difficulty of long-term triculture experiments. Therefore, BMEC-MSC culture conditions were first investigated focusing on cell culture medium. Next, triculture experiments were performed for constructing NVU structures. In particular, toward the construction of the NVU structure, the generation of astrocytes in the triculture model was focused on.

Microfluidic device:

The 750- μm device was used for all experiments in this chapter.

Cells:

NSCs, BMECs and MSCs were used in this study. The amount of these cells was 1.0×10^4 cells for each device. In triculture experiments, NSCs were seeded in one microfluidic channel, and BMECs and MSCs were seeded together in the other microfluidic channel to induce neurogenesis from one side the gel region and angiogenesis from the other side of the gel region.

Time course of the triculture experiment was shown in Fig. 5-1. NSCs were first seeded in microfluidic devices (day 0) and cultured until day 21 using NSC medium in order to increase the number of neurons and induce the abundant neurite growth. On day 21, BMECs and MSCs were seeded in the microfluidic devices to start their triculture changing the medium from NSC medium to the mixture of NSC medium and

EGM-2. The triculture was performed until day 31. In this triculture, NSCs were cultured for 31 days and BMECs-MSCs were cultured for 10 days. The triculture was performed for 10 days, which aimed at the vascular development and astrocyte differentiation from NSCs following neuronal differentiation.

Medium:

NSC medium, EGM-2 and MSC growth medium were used in this chapter. Basically, NSCs were cultured in NSC medium, BMECs were cultured in EGM-2, and MSCs were cultured in DMEM (chapter 2). When coculturing BMECs and MSCs, the mixture of EGM-2 and DMEM were used (chapter 3). In the triculture in chapter 4, only NSC medium was used to culture NSCs, BMECs and MSCs. In addition, the gel collapse occurred in the condition, which was caused by BMECs and MSCs (chapter 4). Therefore, BMEC-MSC coculture medium conditions were first investigated for the induction of angiogenesis without gel collapse.

To test if combinations of these culture medium would be effective for inducing angiogenesis without obvious gel collapse, BMECs and MSCs were cultured using NSC medium alone, the mixture of EGM-2 and MSC growth medium or the mixture of NSC medium and EGM-2. Following triculture experiments were performed using the mixture of NSC medium and EGM-2.

Additional supplement to medium:

The mixture of NSC medium and EGM-2 was supplemented with 10 ng/mL VEGF and 10 ng/mL bFGF to promote vascular formation and cell proliferation.

Gel scaffold:

The optimized gel scaffold in chapter 4, the mixture of 2 mg/mL fibrin gel and 8 mg/mL Matrigel, was used in this chapter.

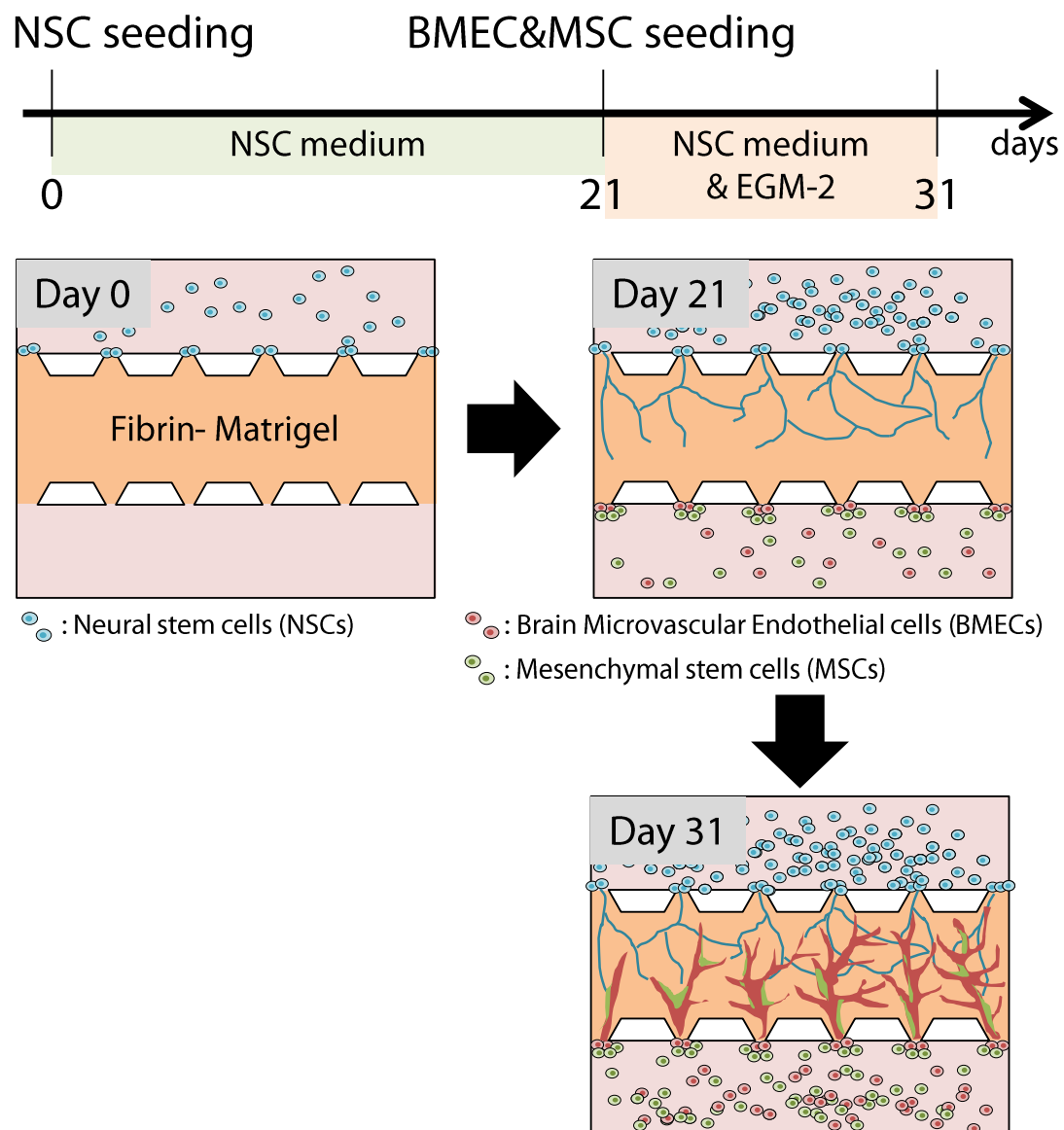


Fig. 5-1 Time course of triculture of NSCs, BMECs and MSCs, and schematic illustrations of cell seeding.

NSCs were first seeded in a microfluidic device (Day 0) until day 21. On day 21, BMECs and MSCs were seeded in the microfluidic device and started triculture. Triculture was performed until day 31. Culture medium was changed on day 21 from NSC medium to the mixture of NSC medium and EGM-2.

5-3 Results

5-3-1 BMEC-MSC coculture in combination medium of NSC medium, EGM-2 and DMEM

First, cell culture medium conditions for BMEC-MSC coculture were investigated to induce angiogenesis without gel collapse. BMECs and MSCs were cultured in a microfluidic device using NSC medium or mixture of EGM-2 and DMEM. NSC medium is the same culture medium condition for the triculture experiment in chapter 4. In NSC medium condition, microvasculatures were constructed on day 7. However, the gel structure shrank with increasing culture time (Fig. 5-2, NSC medium). In EGM-2-DMEM condition, the ideal cell culture medium condition for microvascular formation of BMECs and MSCs, microvasculatures were constructed as well as NSC medium condition. Furthermore, no obvious gel collapse was observed in this condition (Fig 5-2, EGM-2 + DMEM). These results indicate the possibility to culture BMECs and MSCs in fibrin-Matrigel mixed gel, the optimized gel condition in chapter 4, without the gel collapse.

In addition to the two culture medium conditions, BMEC-MSC coculture was performed using the mixture of NSC medium and EGM-2. As a result, microvasculatures were constructed as well as the other two conditions, and no obvious gel collapse was observed in this condition as well as EGM-2 + DMEM (Fig. 5-3).

Considering all results, the mixture of EGM-2 and DMEM or the mixture of NSC medium and EGM-2 was presumed to be suitable for the following triculture experiments. The mixture of NSC medium and EGM-2 was concluded to use for the next triculture of NSCs, BMECs and MSCs.

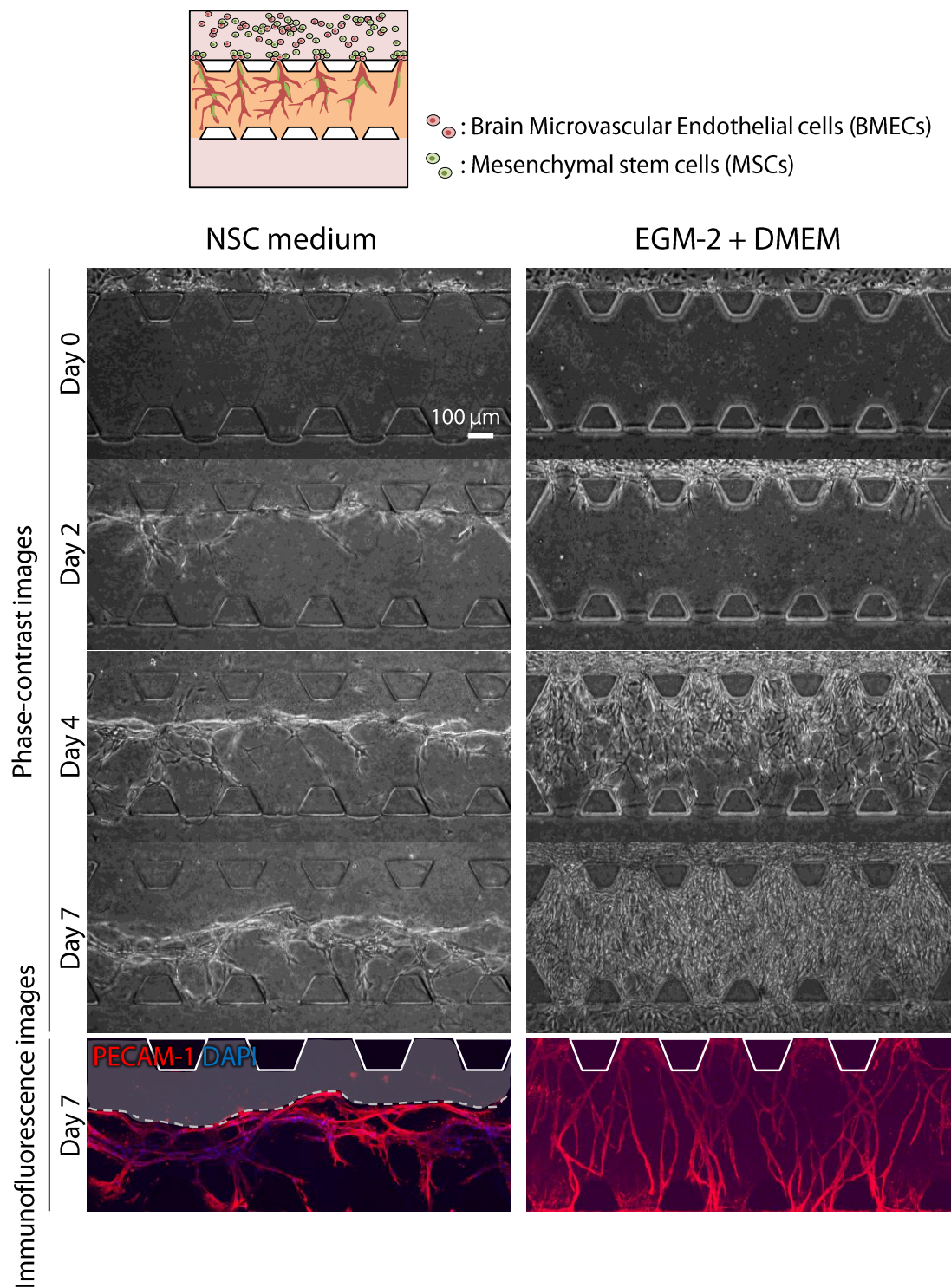


Fig. 5-2 BMEC-MSC coculture in NSC medium or EGM-2-DMEM mixture.

Microvascular formation was induced in both culture medium conditions. However, gel collapse was observed in the NSC medium condition (grey area with dotted line).

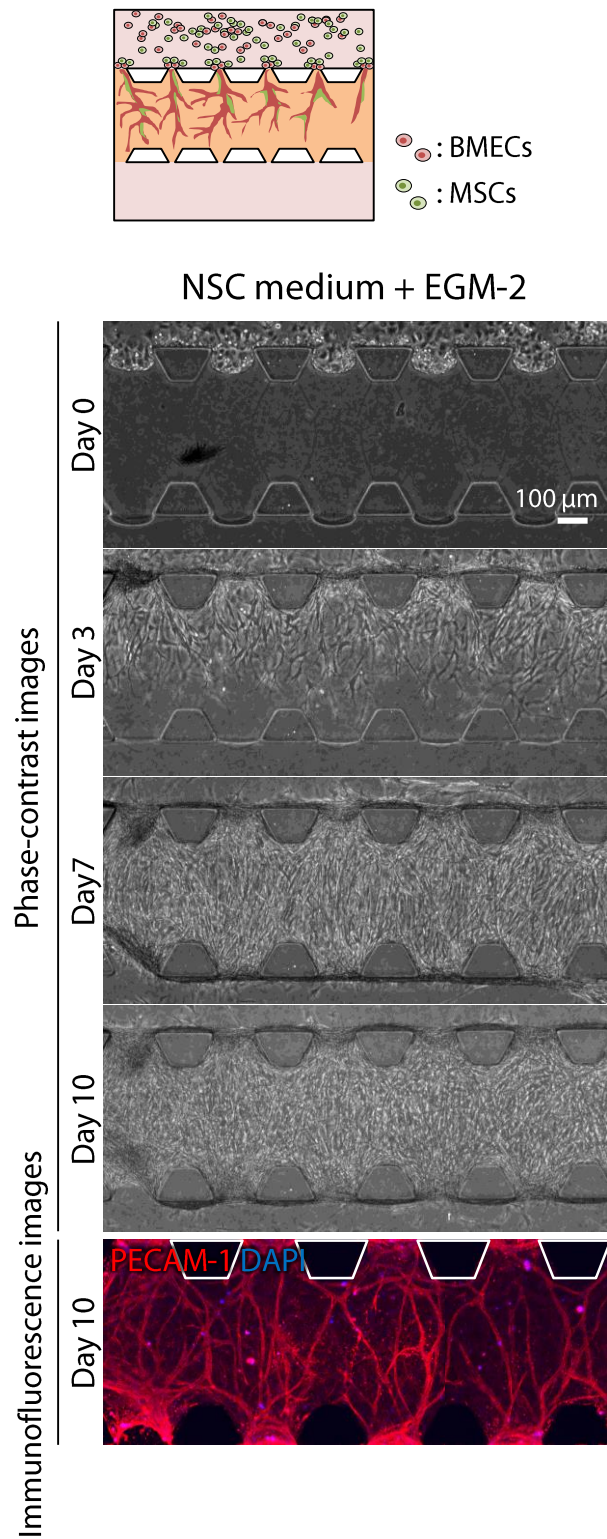


Fig. 5-3 BMEC-MSC coculture in the mixture of NSC medium and EGM-2.

Microvascular formation was induced in this culture medium condition. No obvious gel collapse was observed in this condition.

5-3-2 NSC monoculture in the mixture of NSC medium and EGM-2

Next, NSC monoculture was performed for 31 days. In prior study, 3D neurite extension was observed on day 21 (chapter 4, Fig. 4-5). Considering that astrocyte differentiation from NSCs follows neuronal expansion, NSCs were additionally cultured for 10 days, totally 31 days.

First, NSCs were first cultured in NSC medium until day 31 (Fig. 5-4A, B). This condition was the simple extension of prior NSC monoculture study (chapter 4, Fig. 4-5). After 31 days of NSC monoculture, samples were fixed and immunofluorescence staining was performed for Tuj1 and glial fibrillary acidic protein (GFAP), markers for neurons and astrocytes, respectively. As a result, NSCs proliferated with increasing culture time (Fig. 5-4C). Neurite extension was observed on day 31 (Fig. 5-4D). However, no clear GFAP expression was observed (Fig. 5-4E).

Next, NSCs were cultured in NSC medium until day 21, and the additional culture for 10 days was performed using the mixture of NSC medium and EGM-2 (Fig. 5-5A, B). Immunofluorescence staining was performed on day 31. As a result, NSCs expanded with increasing culture time until day 31 as well as the NSC medium condition (Fig. 5-5C). Immunofluorescence images showed abundant neurite extension in this condition (Fig. 5-5D, E), and the density of extended neurites was higher in the mixture of NSC medium and EGM-2 than NSC medium (Fig. 5-5E vs. Fig. 5-4E). In terms of GFAP expression, some GFAP-positive cells were observed on day 31 in this culture medium condition (Fig. 5-5F). Co-expression of Tuj1 and GFAP was not observed in this condition.

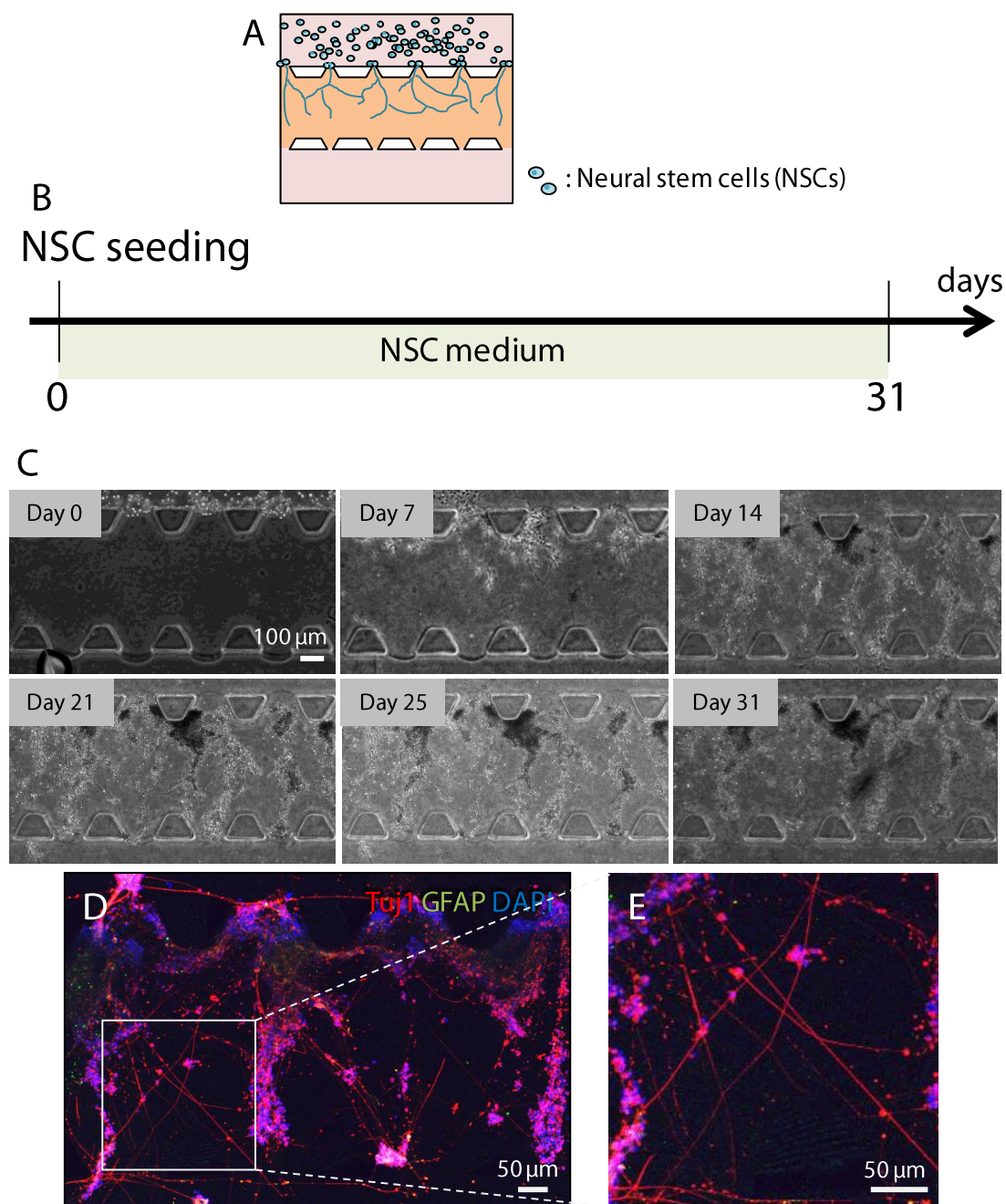


Fig. 5-4 NSC monoculture in NSC medium until day 31.

NSCs were cultured for 31 days in NSC medium (A, B). Neurite extension was observed as well as the result in chapter 4 (Fig. 4-5) (C, D). GFAP expression was not observed in this condition on day 31 (E).

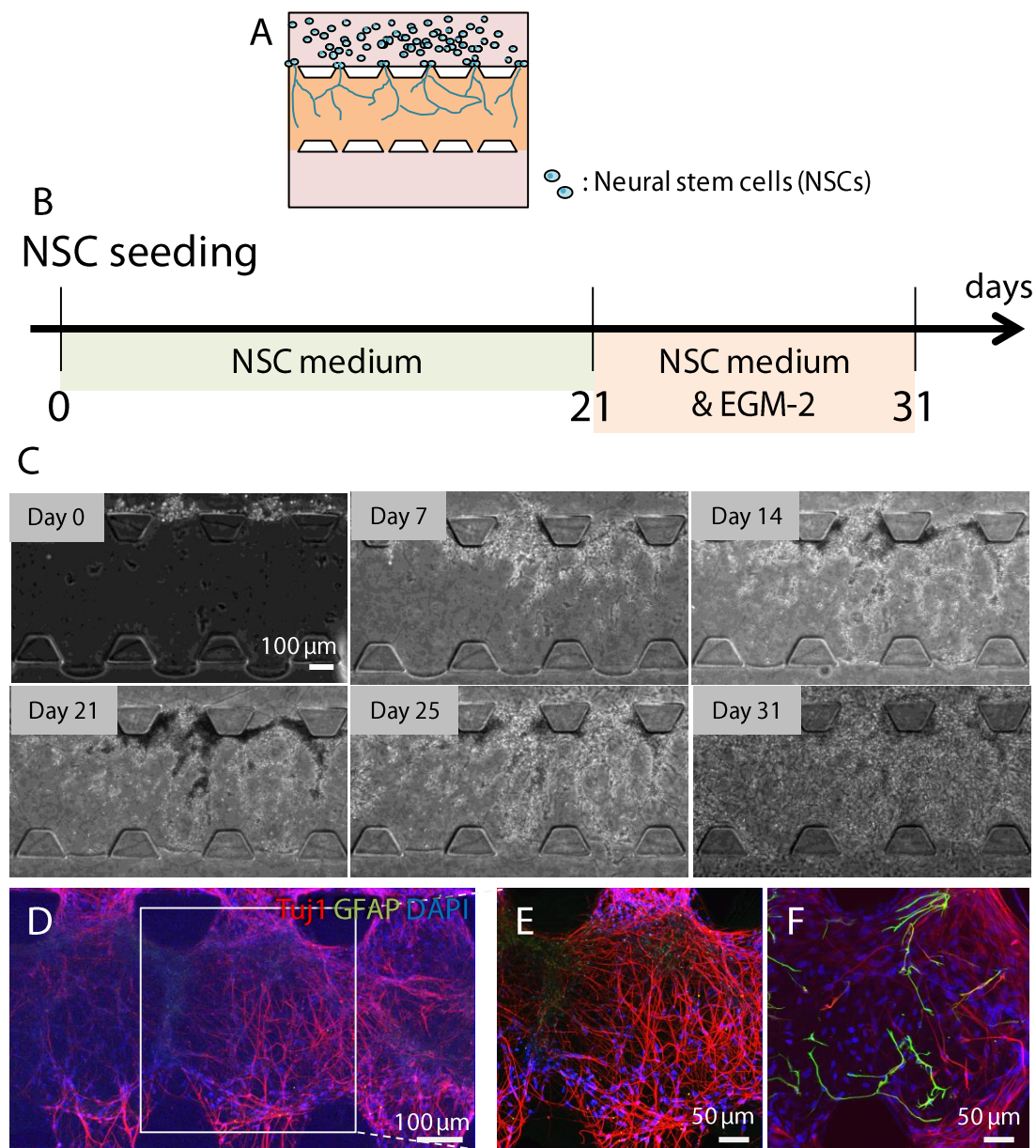


Fig. 5-5 NSC monoculture in NSC medium (until day 21) and the mixture of NSC medium and EGM-2 (from day 21 to 31).

NSCs were cultured in NSC medium until day 21 and in the mixture of NSC medium and EGM-2 from day 21 to day 31 (A, B). NSCs expanded and elongated abundant neurites in fibrin-Matrigel mixed gel (C, D, E). GFAP expression was also observed in this condition on day 31 (F). Co-expression of TuJ1 and GFAP was not observed.

5-3-3 Triculture of NSCs, BMECs and MSCs to construct a neuron-astrocyte-capillary tissue

Next, triculture of NSCs, BMECs and MSCs was performed for 31 days (Fig. 5-6A). NSCs were first monocultured in NSC medium for 21 days, and BMECs and MSCs were additionally seeded on day 21 (Fig. 5-1). Triculture was performed for 10 days using the mixture of NSC medium and EGM-2 (Fig. 5-6B). NSCs were cultured 31 days totally. Immunofluorescence staining was performed on day 31.

As a result, NSCs, BMECs and MSCs actively expanded in the gel region with increasing culture time (Fig. 5-6C). In addition to the construction of microvasculatures, GFAP-positive cells were also observed in the gel region (Fig. 5-6D, G). GFAP-positive astrocytes elongated some processes (Fig. 5-6E, H). No direct contacts of astrocytic endfeet and microvasculatures were observed. However, an orthogonal image of Fig. 5-6E showed that astrocyte existed closely around microvasculatures (Fig. 5-6F, orthogonal image along the white dotted line). Tuj1-positive neurons were also detected in the same gel region together with microvasculatures and astrocytes (Fig. 5-6G, I). These neurons located three-dimensionally with microvasculatures in a three-dimensional view of Fig. 5-6I (Fig. 5-6J).

In this microenvironment, it was confirmed that neurons and astrocytes existed closely around microvasculatures. However, the unique structure of NVU, the cell arrangement of astrocyte intervention between neurons and microvasculatures was not observed. In addition, the coverage of astrocytic endfeet around microvasculatures was not confirmed. Further investigation is needed to induce the coverage of astrocytic endfeet around microvasculatures and the recapitulation of the unique NVU structure.

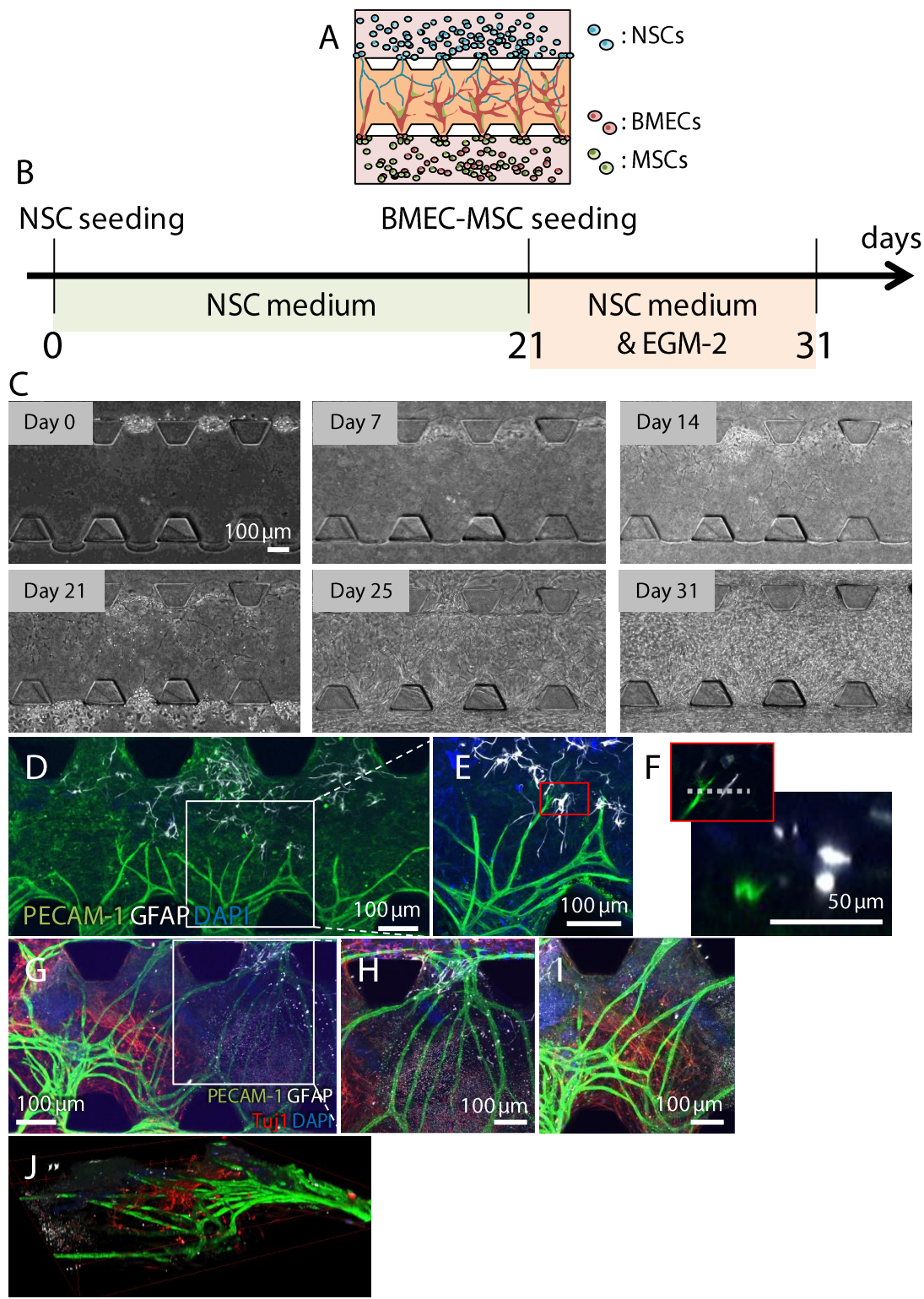


Fig. 5-6 Triculture of NSCs, BMECs and MSCs in a microfluidic device for 31 days.

NSCs, BMECs and MSCs were tricultured in a microfluidic device (A). NSCs were first cultured for 21 days, and BMECs and MSCs were additionally seeded on day 21.

Triculture was performed for 10 days, and the total culture days of NSCs were 31 (B).

Three cell types expanded in the gel region with increasing culture time (C). In addition to the construction of microvasculatures, astrocyte differentiation was also confirmed

(D, E, H). Astrocytes existed closely to microvasculatures (F). Neurons were also

observed in the same gel region together with microvasculatures and astrocytes (G, I).

Neurons located three-dimensionally in the gel region close to microvasculatures (J).

5-4 Discussion

In this chapter, cell culture medium conditions for the triculture of NSCs, BMECs and MSCs for 31 days were investigated. In chapter 4, neurovascular tissues composed of neurons and microvasculatures were successfully constructed. However, gel collapse was observed, suggesting the difficulty in extending the culture time to induce astrocyte differentiation in the triculture. Therefore, culture medium conditions were first investigated to construct microvasculatures without gel collapse. Consequently, culture medium including EGM-2 suppressed the gel collapse during angiogenesis. The results suggest that EGM-2-derived soluble factors have some effects on BMECs or MSCs in terms of the suppression of the gel collapse. Although the mechanisms of suppressing gel collapse remain to be elucidated, angiogenesis was successfully induced without the gel collapse, which was a critical step for a long-term triculture experiment aiming to induce astrocyte differentiation.

Three conditions were tested for culture medium: NSC medium, mixture of EGM-2 and DMEM, and mixture of NSC medium and EGM-2. EGM-2 is the medium for BMECs, and DMEM is the medium for MSCs (chapter 2). The major difference of these medium is in FBS. NSC medium does not contain FBS. EGM-2 contains 2% FBS. DMEM as an MSC medium contains 20% FBS. FBS is derived from fetal blood, and a commonly used supplement for cell culture medium. FBS includes many factors for cell adhesion, growth and survival (Brunner et al., 2010). Effects of FBS on cultured embryonic rat cerebral cortical neurospheres were investigated previously (Hung and Young, 2006). Neurospheres include NSCs and neural progenitor cells. According to this study, FBS promoted astrocyte differentiation from neurospheres, while the population of neurons was significantly lowered, suggesting that the supplement of FBS is unfavorable for neurons while favorable for astrocytes. The supplement of FBS may have some negative effect on neurons. This idea is supported by the fact that neurons in *in vivo* conditions are normally free of substances in blood because of BBB. However,

considering the previous study reporting that FBS promoted astrocyte differentiation from NSCs (Hung and Young, 2006), FBS may be needed for the triculture to induce astrocyte differentiation. The result of NSC monoculture in NSC medium, free of FBS, showed that there was no GFAP expression (Fig. 5-4). On the other hand, the 1:1 mixture of NSC medium and EGM-2, which contained totally 1% of FBS, induced astrocyte differentiation from NSCs (Fig. 5-5F). This coincided with the previous report (Hung and Young, 2006). It was confirmed that this culture medium condition was useful for the construction of microvasculatures without gel collapse (Fig. 5-3). Therefore, the mixture of NSC medium and EGM-2 was concluded to use as the triculture medium.

An NVU-like tissue in ECM gel scaffolds was constructed by combining brain angiogenesis and neurogenesis models. This NVU-like tissue was composed of neurons, astrocytes and microvasculatures. This result suggests the possibility to construct *in vivo* NVU structure, in which astrocytes exist closely to both neurons and microvasculatures and astrocytic endfeet wrap around microvasculatures. Toward the construction of NVU structure, the next step is the induction of the coverage of astrocytic endfeet around microvasculatures. In the triculture experiment, astrocytes existed closely to microvasculatures. However, direct contacts of GFAP-positive astrocytic endfeet to microvasculatures were not observed. *In vivo* astrocytes cover >99% of vascular surfaces by their endfeet (Iadecola and Nedergaard, 2007). In addition, it was reported that endfeet around capillaries were negative for GFAP and positive for connexin 43, a gap junction protein, and AQP 4, a water channel expressed in astrocytes (Iadecola and Nedergaard, 2007; Simard et al., 2003). Therefore, further investigation is needed to test the expression of these proteins in the engineered NVU-like tissue.

To induce astrocyte differentiation and the coverage of astrocytic endfeet around microvasculatures, additional days may be needed for the triculture. One of the aims of the extension of culture time is to increase the number of pericytes existing around microvasculatures. A previous study reported that pericyte-deficiency resulted in

changes in astrocytic endfeet distribution (Armulik et al., 2010). Pericytes are partly sandwiched by ECs and astrocytic endfeet. Therefore, there may potentially be some interactions between pericytes and astrocytes in terms of astrocytic endfeet coverage to capillaries. The mechanisms of interactions between astrocytes and microvasculatures in terms of endfeet coverage are not fully understood. However, the induction of astrocyte differentiation from cultured glial-progenitor cells were investigated in a previous study (Mabie et al., 1997). This study reported that the effect of bone morphogenetic proteins (BMPs) on astrocyte differentiation was similar to the effect of FBS. This suggests that FBS includes BMPs and promotes astrocyte differentiation by BMP-dependent manners. In addition, it was reported that retinoic acid and leukemia inhibitory factor promote astrocyte differentiation from NSCs (Asano et al., 2009). Therefore, addition of such factors in the cell culture medium may induce astrocyte differentiation more effectively toward the construction of microvasculatures covered by astrocytic endfeet.

5-5 Summary

In this chapter, cell culture medium conditions were investigated for the triculture of NSCs, BMECs and MSCs. In chapter 4, a neurovascular tissue composed of neurons and microvasculatures were successfully constructed. To construct NVU structure, it was aimed to induce astrocyte differentiation from NSCs.

To induce astrocyte differentiation, a long-term triculture was needed. However, gel collapse was occurred in the triculture in chapter 4. This gel collapse was caused by BMECs and MSCs. Therefore, cell culture medium conditions for BMEC-MSC coculture were first investigated to induce angiogenesis without the gel collapse. Consequently, it was found that the mixture of NSC medium and EGM-2 was suitable for inducing angiogenesis without the gel collapse in BMEC-MSC coculture.

In NSC monoculture experiments, astrocyte differentiation was not confirmed when NSCs were cultured in NSC medium, while astrocyte differentiation was induced by

changing medium from NSC medium to the mixture of NSC medium and EGM-2 on day 21 in 31 days-monoculture. Using this cell culture medium condition, an NVU-like tissue composed of neurons, astrocytes and microvasculatures was constructed on day 31. It was confirmed that astrocytes and neurons located closely to microvasculatures respectively. However, the direct contact between astrocytic endfeet and microvasculatures and the unique structure of NVU, astrocyte intervention between neurons and microvasculatures were not confirmed. Further investigation will be needed to construct NVU structures *in vitro* by an additional cell culture period or addition of soluble factors that are known to promote astrocyte differentiation.

Chapter 6 Concluding remarks

6-1 Summary

This thesis deals with neurovascular tissue engineering using NSCs, BMECs and MSCs toward the construction of NVU structures *in vitro*. NVU is a conceptual basic unit in the brain, which is composed of neurons, glial cells, microvasculatures and ECM. Toward the construction of NVU structure, there are some points to be considered. One of them is BBB. BBB is a brain-specific endothelial barrier and BBB microvasculatures are composed of ECs, pericytes and astrocytic endfeet. Another is the unique cell arrangement, in which astrocytes exist between neurons and microvasculatures through astrocytic endfeet. To realize the construction of NVU structure, inducing neurogenesis and angiogenesis in 3D ECM gel scaffolds was focused on.

In chapter 3, an *in vitro* brain angiogenesis model was established by coculturing BMECs and MSCs in a microfluidic device. The formation processes and endothelial barrier functions were compared with BMEC microvasculatures and HUVEC microvasculatures. This study was motivated by the brain-specific endothelial barrier, BBB. Every organ has its own vascular functions, and organ-specificity of ECs has to be considered to construct a targeted tissue such as BBB microvasculatures. The results in chapter 3 showed significant difference between BMEC microvasculatures and HUVEC microvasculatures in terms of endothelial barrier function and expressions of tight junction proteins. The study in chapter 3 suggests the importance of using brain-specific ECs toward the establishment of an *in vitro* BBB model.

Toward the construction of NVU structures *in vitro*, cell culture conditions were investigated to construct a neurovascular tissue composed of neurons and microvasculatures by culturing NSCs, BMECs and MSCs in chapter 4. The brain angiogenesis model in chapter 3 was performed using fibrin gel as a cell culture scaffold. However, it was found that fibrin-Matrigel mixed gel was more suitable for the

expansion and neurite extension of neurons differentiated from NSCs than fibrin gel alone. On the other hand, it was confirmed that brain angiogenesis was induced within the fibrin-Matrigel mixed gel as well as fibrin gel. In addition, 3D neurite extension was induced within the mixed gel. Finally, brain angiogenesis model and neurogenesis model were combined to construct neurovascular tissue. The results in chapter 4 suggest the possibility of constructing NVU structure in the fibrin-Matrigel mixed gel.

The constructed neurovascular tissue in chapter 4 was composed of neurons and microvasculatures. Considering that *in vivo* NVU structure is composed of neurons, astrocytes and microvasculatures, astrocyte differentiation from NSCs was focused on as a next step. In terms of the process of NSC expansion and differentiation, astrocyte generation follows the generation of neurons (Ramasamy et al., 2013; Temple, 2001). Therefore, it was hypothesized that astrocyte generation would be realized by extending the period of the triculture of NSCs, BMECs and MSCs. However, in the triculture study (chapter 4), gel collapse was observed, suggesting the difficulty in extending culture time. Following this result, conditions of cell culture medium in BMEC-MSC coculture were first investigated to induce angiogenesis without the gel collapse. As a result, microvasculatures were constructed without the obvious gel collapse in the 1:1 mixture of NSC medium and EGM-2. The condition of cell culture medium was concluded to use for the following triculture experiments in addition to the optimized condition of the gel scaffold in chapter 4.

Triculture of NSCs, BMECs and MSCs was performed in the optimized cell culture condition until 31 days after astrocyte differentiation was confirmed in the NSC monoculture for 31 days. As a result, an NVU-like tissue composed of neurons, astrocytes and microvasculatures was constructed. This *in vitro* microfluidic cell culture model has a potential to succeed in engineering *in vivo* NVU structures.

Although the *in vitro* microfluidic cell culture platform in this study still have some points to be solved, this study provided some important advantages toward the establishment of an *in vitro* NVU model. First, this microfluidic cell culture system

provided a stable model to realize the construction of capillary-level brain microvascular networks covered by pericytes. As far as I know, this is the first *in vitro* cell culture model of brain angiogenesis. Some previous studies reported *in vitro* angiogenesis models using HUVECs (Kim et al., 2015, 2013; Lim et al., 2013; Moya et al., 2013). These studies successfully induced vascular formation in 3D gel scaffold. However, the diameter of the constructed vasculatures is ranged from 20 μm to 150 μm , and most of these studies did not focus on the coverage of pericytes around microvasculatures, which implied the difficulty of engineering *in vivo*-like capillary structures. Second, this microfluidic cell culture system demonstrated the perfusion of fluorescent dextran solution into the constructed capillary-level microvasculatures. Brain capillaries have a specific barrier called BBB. The *in vitro* BBB model described in chapter 3 is a critical step to establish an *in vitro* NVU model, which will possibly be established by combining the BBB model with a neural cell culture model described in chapters 4 and 5.

In chapter 5, NVU-like tissue was constructed by the triculture of NSCs, BMECs and MSCs. A previous study reported microfluidic cell culture systems in which neural cells and ECs were cultured together in microfluidic devices (Adriani et al., 2017). This previous study investigated effects of neural cells on endothelial barrier functions and effects of ECs on neuronal growth and maturation, which is important for understanding interactions among neural cells and ECs. However, cultured ECs in this cell culture system form endothelial monolayer, not vascular lumen structures. It is important to construct *in vivo*-like microvascular structures to analyze barrier functions of microvasculatures in NVU structures. The *in vitro* NVU model in this thesis solved this problem by culturing BMECs with MSCs together in a microfluidic device.

So far, *in vitro* cell culture models aiming at construct NVU structures have not provided *in vivo*-like NVU structures in which microvasculatures are covered by pericytes and astrocytic endfeet and astrocytes exist between microvasculatures and neurons physically and functionally. The main contribution of the study in this thesis is

the construction of an NVU-like tissue composed of microvasculatures, neurons and astrocytes. The important point of the study in this thesis is that this *in vitro* cell culture model demonstrated the construction of the NVU-like tissue by recapitulating angiogenesis and neurogenesis simultaneously in one gel region. This *in vitro* cell culture system still have challenges to be solved. However, the fact that the construction of the NVU-like tissue was induced by self-organization processes such as angiogenesis and neurogenesis implies that this model is potentially of use for constructing *in vivo*-like NVU structures and investigating the development processes, which might not be recapitulated by the simply-mixed cell culture model using differentiated cells.

6-2 Future perspectives

An ideal *in vitro* NVU model is the mimicry of *in vivo* NVU structures. Key features of NVU were as follows: BBB microvascular networks including capillaries with the coverage of pericytes and astrocytic endfeet, neuronal network with synaptic activities, astrocyte regulation of synaptic activities, oligodendrocyte myelination of neurons and so on. In particular, in this thesis, BBB microvasculatures and neuron-astrocyte-microvascular tissue formation was focused on. By constructing NVU structures *in vitro*, the cell culture system can be applied to drug screening studies for neurodegenerative diseases and studies for the understanding of pathological states of NVU. Previous *in vitro* brain disease models have been performed using neural cells, not including vasculatures (Yi et al., 2015). Therefore, these models still have limitations related to the lack of vasculatures. The *in vitro* cell culture system in this thesis will potentially solve the pre-existing problems in drug screening studies; the difficulty of predicting BBB penetration of drugs and following drug efficacy on neural cells (Osaki et al., 2018a). These major problems in drug screening and drug development processes are difficult to solve by *in vitro* models composed of only neural cells. These problems are also difficult to solve by *in vivo* studies because of cost and

time for tests and ethical problems.

In addition to the application of the *in vitro* model to drug screening studies, the cell culture system can also be applied to studies of developmental morphogenesis such as BBB formation processes. Mechanisms of BBB development is still unclear, and monitoring the processes is quite difficult in *in vivo* studies because of present technical problems. This *in vitro* microfluidic cell culture system has a potential to become a robust tool for such studies by recapitulating development processes of BBB and monitoring these morphological changes.

The next step following this thesis will be the induction of astrocytic endfeet coverage around microvasculatures. A previous study reported that Ang-2, an angiogenic factor, induced astrocyte apoptosis via integrin signaling (Yun et al., 2016). The level of Ang-2/Tie-2 signaling is decreased in the angiogenic maturation stage in which pericytes cover around vascular lumen (Ribatti et al., 2011). This suggests that astrocytes start to cover around vascular lumen with pericytes from this time point. This idea is supported by the fact that astrocytes cover both ECs and pericytes (Abbott et al., 2010; Keaney and Campbell, 2015). To investigate this hypothesis, it is needed not only to extend culture time but also to regulate the supply of such angiogenic factors in the microfluidic cell culture system by adding these factors in the cell culture medium.

To investigate whether the direct coverage of astrocytic endfeet around microvasculatures are well-organized, it is needed to confirm the expression of proteins such as connexin 43, AQP 4, which are expressed in the interface between astrocytes and microvasculatures when they are well-organized (Simard et al., 2003). Endothelial expression of BBB-specific transporters is also needed to be confirmed. These investigations will support the integrity of the engineered NVU structures.

In addition to the assessment of morphological features of engineered NVU structures, the assessment of their function is also needed. In terms of microvascular function, the point is that ECs constituting microvasculatures are connected with each other by junctional proteins and these microvasculatures have an endothelial barrier

which restricts the undesired leakage of solutes flowing in the microvasculatures. It was confirmed that the *in vitro* brain angiogenesis model in chapter 3 met such characteristics. On the other hand, it is needed to make it clear that neurons in the NVU model in this thesis are functional. Previous studies performed Ca^{2+} imaging for cultured neurons using microfluidic devices to analyze neuronal activities (Adriani et al., 2017; Osaki et al., 2018b). In the NVU model in this thesis, although cultured neurons were positive for Tuj1 or MAP2, functions of these neurons were not assessed. However, neuronal activities can potentially be analyzed in this model by applying the techniques reported previously. As a next step of this study, the assessment will be important for investigating interactions between neurons and other cell types such as astrocytes and ECs.

In chapter 3, *in vitro* brain angiogenesis model was established in which fluorescent dextran solution was perfused into the engineered microvasculatures. If the construction of the NVU structure is realized, dextran perfusion into the astrocyte-covered microvasculatures will be performed to investigate the astrocytic regulation of endothelial barrier functions. Astrocytic endfeet coverage around microvasculatures can be monitored in a microfluidic device. By analyzing microvascular permeability before and after astrocyte-microvascular development, mechanisms of BBB development will potentially be investigated. The potential availability to use this cell culture system for studies of BBB development is one of the advantages of this cell culture system, which recapitulates self-organization processes. Furthermore, the study can be applied to drug screening studies by focusing on the transport of biomolecules from blood to astrocytes, or even to neurons. After the achievement of the construction of the NVU structure, the next step will be the investigation of functions of the engineered NVU structure, which is a multi-cellular system. This microfluidic cell culture system will become a robust tool not only for the fundamental study of brain functions but also for the drug screening study as a therapeutic strategy.

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<https://doi.org/10.1038/nrn3114>

Achievements

Publications

Main publications

1. Uwamori, H., Higuchi, T., Arai, K. and Sudo, R., “Integration of neurogenesis and angiogenesis models for constructing a neurovascular tissue”, Scientific Reports, Vol. 7, No. 1, 17349 (11 pages), (2017).
2. Uwamori, H., Ono, Y., Yamashita, T., Arai, K., and Sudo, R., “Comparison of organ-specific endothelial cells in terms of microvascular formation and endothelial barrier functions”, Microvascular Research, Vol. 122, pp. 60–70, (2019). [Accepted for publication].

Other publication

1. Yamamoto, K., Tanimura, K., Watanabe, M., Sano, H., Uwamori, H., Mabuchi, Y., Matsuzaki, Y., Chung, S., Kamm, R. D., Tanishita, K. and Sudo, R., “Construction of Continuous Capillary Networks Stabilized by Pericyte-like Perivascular Cells”, Tissue Engineering Part A, [Accepted for Publication].

International conferences

1. Uwamori, H., Tanimura, K., Sudo, R., “Effect of unidirectional VEGF supply on the formation of capillary networks with pericytes in a microfluidic device”, 7th World Congress of Biomechanics, Boston, USA, (2014).
2. Uwamori, H., Tanimura, K., Sudo, R., “HUVEC-MSC Interactions in the Initiation of Capillary Network Formation”, IEEE EMBS Micro and Nanotechnology in Medicine Conference, Hawaii, USA, (2014).
3. Uwamori, H., Higuchi, T., Sudo, R., “The development of neurovascular tissue culture model by coculturing NSCs and ECs in a microfluidic device”, BMES 2016

Annual Meeting, Minneapolis, USA, (2016).

4. Uwamori, H., Higuchi, T., Sudo, R., “Integration of neurogenesis and angiogenesis models for constructing a neurovascular tissue”, 5th Switzerland-Japan Workshop on Biomechanics, Zermatt, Switzerland, (2017).

Domestic conferences

1. 上森寛元, 谷村耕平, 須藤亮, “マイクロ流体デバイスを用いた細胞及び増殖因子位置制御による毛細血管網再構築の検討”, 日本機械学会 2014 年度年次大会, 東京, (2014).
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4. 上森寛元, 樋口拓哉, 須藤亮, “Neurovascular Unit の構築を目指した神経・血管新生モデルの検討”, 第 55 回日本生体医工学会大会, 富山, (2016).
5. 上森寛元, 樋口拓哉, 須藤亮, “マイクロ流体デバイスを用いた脳微小環境の生体外構築の検討”, 第 59 回日本脳循環代謝学会学術集会, 徳島, (2016).
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Acknowledgments

My research activities for this thesis were carried out at the School of Integrated Design Engineering, Keio University under the guidance of associate professor Ryo Sudo. First of all, I would like to thank him for his constant encouragement and guidance on my research. He has taught me interesting aspects of conducting research from a standpoint of tissue engineering. He helped me in many ways not only for conducting research but also for my showing a leadership to guide the members in the laboratory. I would also like to thank assistant professor Tadahiro Yamashita for his encouragement and invaluable scientific suggestions. He has taught me many things not only on research but also technical issues necessary for research.

In my first year in the graduate school, I visited associate professor Ken Arai at Harvard Medical School in Boston. I would like to thank him for giving me much advice on my research from a standpoint of neuroscience as the co-author of my papers and as one of sub-chief examiners for my thesis. I visited his laboratory when I was in the first year in the doctoral course. That experience was precious for me and I had a great time with the members in the laboratory.

I would like to thank my thesis examiners: professor Kotaro Oka at the School of Fundamental Science and Technology, associate professor Yoshihiro Taguchi at the School of Integrated Design Engineering and associate professor Hiroaki Onoe at the School of Integrated Design Engineering, for giving me insightful comments and questions from their academic standpoints. Furthermore, they encouraged me a lot and let me notice what is important as a researcher.

In my first and second year in the doctoral course, I conducted research with Mr. Yuuichi Ono, who belongs to Mitsubishi Tanabe Corporation, Japan. I would like to thank him. His much advice helped me a lot in my thinking about my career.

I also thank the members of Sudo laboratory for their supports. In particular, I am grateful to Mr. Masafumi Watanabe and Mr. Yuta Chonan, doctoral students in Sudo

Acknowledgments

laboratory. They set great examples as doctoral students to guide other members. I also thank the members of “Neurovascular Unit” group in Sudo laboratory. I had a great time with them having discussion on our research.

Finally, I would like to express my gratitude for my family to support me for many years.

February 2019

Hiroyuki Uwamori