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Differentiation and characterization of human pluripotent stem cell-derived brain microvascular endothelial cells

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ABSTRACT

The blood–brain barrier (BBB) is a critical component of the central nervous system (CNS) that regulates the flux of material between the blood and the brain. Because of its barrier properties, the BBB creates a bottleneck to CNS drug delivery. Human *in vitro* BBB models offer a potential tool to screen pharmaceutical libraries for CNS penetration as well as for BBB modulators in development and disease, yet primary and immortalized models respectively lack scalability and robust phenotypes. Recently, *in vitro* BBB models derived from human pluripotent stem cells (hPSCs) have helped overcome these challenges by providing a scalable and renewable source of human brain microvascular endothelial cells (BMECs). We have demonstrated that hPSC-derived BMECs exhibit robust structural and functional characteristics reminiscent of the *in vivo* BBB. Here, we provide a detailed description of the methods required to differentiate and functionally characterize hPSC-derived BMECs to facilitate their widespread use in downstream applications.

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1. Introduction

1.1. Scope

The brain vasculature is key to central nervous system (CNS) health through its regulation of molecular and cellular traffic across the blood–brain interface. In doing so, brain blood vessels also create a barrier to ions, drugs, pathogens, and CNS-damaging toxins. This so-called blood–brain barrier (BBB) restricts the delivery of CNS therapeutics, thereby hindering CNS drug development. An increasing number of studies have also indicated BBB dysfunction in many CNS-related pathologies, including Alzheimer's disease [1], stroke [2], and traumatic brain injury [3].

At the cellular level, the BBB is comprised of brain-specific endothelial cells that line the brain vasculature. These brain microvascular endothelial cells (BMECs) are distinct from other tissue-specific endothelium through expression of a unique subset of tight junction proteins, nutrient transporters, and efflux transporters [4,5] that play important roles in BBB physiology. For instance, tight junction proteins limit paracellular diffusion, nutrient transporters regulate the flow of energy and amino acids across the CNS, and efflux transporters remove a wide array of therapeutics from the CNS [4,6]. *In vitro* BBB tissue models, including primary cells isolated from brain capillaries [7–10] and immortalized primary BMEC lines [11–13], recapitulate many properties of the *in vivo* BBB. However, species-specific differences in BMEC efflux transporter expression reduce the utility of animal-derived *in vitro* BBB modeling for human health applications [14,15]. In addition, scarce availability of primary human BMECs [16] and reduced barrier properties of immortalized human BMEC lines limit application of these models [13].

Recent advancements in stem cell technology have overcome several of these material challenges leading to protocols whereby BMEC-like cells are derived from expandable and renewable human stem cell sources. These sources include cord blood-derived hematopoietic stem cells [17], circulating cord blood-derived endothelial progenitors [18], and human pluripotent stem cells (hPSCs) [19]. Human PSC-derived BMECs exhibit physically

Abbreviations: BBB, blood–brain barrier; CNS, central nervous system; hPSCs, human pluripotent stem cells; BMECs, brain microvascular endothelial cells; P-gp, P-glycoprotein; RA, retinoic acid; UM, unconditioned medium; EC, endothelial cell; EC + RA medium, EC medium supplemented with RA; TEER, transendothelial electrical resistance; NEAA, nonessential amino acids; KOSR, knockout serum replacement; hESFM, human endothelial serum-free medium; PDS, platelet poor plasma-derived serum; bFGF, basic fibroblast growth factor; ECM, extracellular matrix; iPSCs, induced pluripotent stem cells; hESCs, human embryonic stem cells; RFU, relative fluorescence units; CsA, cyclosporin A.

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tight barriers similar to the *in vivo* BBB and functionally active transporters, including the most well characterized transporter, P-glycoprotein (P-gp). In addition, hPSC-derived BMECs exhibit permeabilities to a range of small molecules that correlate well with those observed in rodents [19]. Human PSC-derived BMECs also offer the unique ability to examine cellular signaling events involved in human BBB development as undifferentiated hPSCs undergo cell fate decisions towards BMECs. Disease phenotypes with putative BMEC involvement may also be examined using induced pluripotent stem cell (iPSC) lines derived from patients.

The hPSC-derived BMECs are produced through simultaneous differentiation of endothelial and neural progenitors [19]. This mixed, co-differentiation environment recapitulates some aspects of early BBB development [19], including components of WNT signaling activation [20–22] and responsiveness to retinoic acid (RA) signaling [23], a pathway implicated in BBB regulation [24]. After 8 days of differentiation, the mixed neural progenitor/endothelial population is subcultured onto collagen/fibronectin. Endothelial cells selectively attach to the collagen/fibronectin matrix while neural progenitor cells do not and are thus removed following medium exchange, yielding virtually pure BMEC monolayers. Supplementing differentiating BMEC cultures with RA further enhances hPSC-derived BMEC tightness to levels comparable to the *in vivo* BBB [23]. BMECs also exhibit responsiveness to other cells from the neurovascular unit, such as pericytes and astrocytes and neurons, offering the ability to examine cellular crosstalk at the BBB [19,23]. In this article, we detail optimized differentiation strategies as well as the characterization required to validate hPSC-derived BMEC monocultures.

1.2. Overview of BMEC differentiation and characterization procedures

The hPSC-derived BMEC differentiation occurs in a four-step process to generate pure BMEC populations. (1) Human PSCs are seeded as singularized cells onto Matrigel-coated plates. (2) Culture density is quantified to ensure it falls within the optimal range, and hPSCs are then differentiated to a mixed endothelial cell/neural progenitor cell culture by switching cells to unconditioned medium (UM) for 6 days (D6) (Fig. 1a). (3) Endothelial cells are next selectively expanded by switching to endothelial cell (EC) medium supplemented with RA (EC + RA medium) from D6–8 (Fig. 1a). By D8 of the differentiation, endothelial cells express BBB-specific tight junction proteins—including Claudin-5, Occludin, and ZO-1 – the nutrient transporter GLUT-1, and the efflux transporter P-gp [19]. (4) To purify BMECs, mixed cultures are subcultured at D8 onto collagen/fibronectin-coated filters or plates; BMECs selectively adhere to the collagen/fibronectin substrate while the neural cells do not, resulting in a virtually pure BMEC monolayer by D9. BMECs subcultured on filters can be used to measure transendothelial electrical resistance (TEER) starting at D9 of the differentiation, while other functional assays such efflux transporter activity are usually performed on D10 when TEER reaches a maximum (Fig. 1a). Cells cultured on plates are typically used for verifying BMEC quality by immunocytochemistry at D10, including expression of endothelial cell markers – VE-cadherin and PECAM-1 – tight junction proteins, the nutrient transporter Glut-1, and efflux transporters – P-gp, BCRP, and MRP1 (Fig. 2).

2. Materials

2.1. Human PSC lines validated for BBB differentiation

- IMR90-C4 (WiCell WB008)
- WA09 (WiCell RB-001)
- iPSC DF19-9-11T.H. (WiCell WB0219)

2.2. Materials

- Costar™ cell culture plates (96, 48, 24, 12, or 6 well plates) (Corning 3596, 3548, 3524, 3513, 3516)
- 15 mL conical tubes (Fisher 05-539-5)
- Microfuge tubes (Fisher 02-681-272)
- 500 mL filter-top bottles (Thermo 566-0020)
- Corning Transwell polyester filters 12 or 24 well (Sigma CLS3460, CLS3450)
- 5 mL and 10 mL glass pipettes

2.3. Equipment

- Hemacytometer
- Pipette aid
- Fluorescent plate reader
- EVOM with STX electrodes
- Biosafety cabinet
- Incubator (37 °C and 5% carbon dioxide), containing a rotating platform

2.4. Reagents

- mTeSR™1 (Stem Cell Technologies 05850)
- Matrigel™, growth factor reduced (BD Biosciences 354230)
- Versene (Invitrogen 15040-066)
- ROCK inhibitor, Y27632 dihydrochloride (Tocris 1254)
- Accutase (Invitrogen A1110501)
- Trypan blue (Invitrogen 15250-061)
- DMEM/F12 (Invitrogen 11330-057)
- Nonessential amino acids (NEAA) (Invitrogen 11140-050)
- Glutamax (Invitrogen 35050-061)
- Knockout serum replacement (KOSR) (Invitrogen 10828-028)
- β-Mercaptoethanol (Sigma M3148)
- Human endothelial serum-free medium (hESFM) (Invitrogen 11111)
- Platelet poor plasma-derived serum, bovine (PDS) (Fisher 50-443-029)
- Human basic fibroblast growth factor (bFGF) (R&D Systems 233-FB)
- All-trans retinoic acid (Sigma R2625)
- Fibronectin (Sigma F1141)
- Collagen IV (Sigma C5533)
- Glacial acetic acid (Sigma 537020)
- DMSO (Sigma D2650)
- Methanol (Fisher A412-4)
- 32% paraformaldehyde in water (VWR 100496-496)
- PBS (Sigma D8537)
- Goat serum (Sigma G9023)
- Triton-X 100 (Sigma T8787)
- DAPI (Life Technologies D1306) diluted to 5 mg/mL in deionized water
- Sodium fluorescein (Sigma F6337)
- Rhodamine 123 (Sigma 83702)
- Cyclosporin A (CsA) (Sigma C1832)
- RIPA buffer (Sigma R0278)
- HBSS buffer (Life Technologies 14025092)
- Ethanol (Fisher BP2818-4)
- Tissue-grade sterile water (Sigma 59900C)

2.5. Material preparation

All the following preparations are made within a biosafety cabinet under sterile conditions unless otherwise indicated.

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