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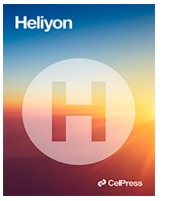

Research article

GDNF and cAMP significantly enhance *in vitro* blood-brain barrier integrity in a humanized tricellular transwell model

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ABSTRACT

Blood-brain barrier (BBB) is a crucial membrane safeguarding neural tissue by controlling the molecular exchange between blood and the brain. However, assessing BBB permeability presents challenges for central nervous system (CNS) drug development. *In vitro* studies of BBB-permeable agents before animal testing are essential to mitigate failures. Improved *in vitro* models are needed to mimic physiologically relevant BBB integrity. Here, we established an *in vitro* human-derived triculture BBB model, coculturing hCMEC/D3 with primary astrocytes and pericytes in a transwell format. This study found that the triculture BBB model exhibited significantly higher paracellular tightness (TEER 147.6 ± 6.5 Ω × cm^2) than its monoculture counterpart (106.3 ± 1.0 Ω × cm^2). Additionally, BBB permeability in the triculture model was significantly lower. While GDNF and cAMP have been shown to promote BBB integrity in monoculture models, their effect in our model was previously unreported. Our study demonstrates that both GDNF and cAMP increased TEER values (around 200 Ω × cm^2 for each; 237.6 ± 17.7 Ω × cm^2 for co-treatment) compared to untreated control, and decreased BBB permeability, mediated by increased claudin-5 expression.

Abbreviations: Akt, protein kinase B; BBB, blood-brain barrier; BMECs, brain microvascular endothelial cells; cAMP, cyclic adenosine monophosphate; CNS, central nervous system; CREB, cAMP-response element binding protein; FITC, fluorescein isothiocyanate; FoxO1, forkhead box protein O1; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GFR-α1, GDNF receptor α1; HASTR/ci35, human brain astrocyte ci35; HBMEC/ci18, human brain microvascular endothelial cell ci18; HBPC/ci37, human brain pericyte ci37; hCMEC/D3, human cerebral microvascular endothelial cell D3; IL-1β, interleukin-1β; iPSCs, inducible pluripotent stem cells; NVU, neurovascular unit; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PnMEC, peripheral nerve microvascular endothelial cell; RET, REarranged during transfection; RTK, receptor tyrosine kinase; α-SMA, α-smooth muscle actin; TEER, transepithelial electrical resistance; TJ, tight junction; ZO-1, zonula occludens-1.

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In summary, this humanized triculture BBB model, enhanced by GDNF and cAMP, offers an alternative for exploring *in vitro* drug penetration into the human brain.

1. Introduction

The ability of molecules, especially harmful molecules, to infiltrate from the bloodstream into the brain parenchyma is primarily restricted to brain endothelial cells rather than peripheral endothelial cells [1]. Brain vascular endothelial cells form a continuous layer interconnected by transmembrane tight junction proteins, effectively preventing passive transfer of cells and molecules between the brain and blood. Factors such as size, surface electrical charge, and lipid solubility play roles in this membrane permeability [2–4]. This special mechanism is in contrast to the endothelial cells lining the peripheral vasculature [2]. BBB serves as a selective physiological barrier that controls the passage of ions and molecules between the circulating blood and the brain, and, therefore, protecting neural tissue from toxins and pathogens [5–8]. One of the challenges in CNS drug development is assessing whether the drug candidates can effectively penetrate the BBB [9,10]. Currently, numerous candidate compounds fail in clinical trials due to their inability to cross the BBB especially in the areas of brain tumor drug discovery and development [11–13]. The discovery and development of the candidate compounds typically commence at the *in vitro* level [14]. The promising hit compounds from the *in vitro* study then progress into the systemic investigation using animal models [15]. Unfortunately, several candidate compounds face failure in clinical trials often due to variations in protein expression and activity levels across different animal species utilized in the studies [16,17]. In the pre-clinical phase, the development of candidate compounds relies heavily on an animal model [18,19]. Therefore, the drug discovery for BBB-permeable agents in the *in vitro* study before progressing into animal trials is a crucial step to minimize pre-clinical and clinical failures as well as resource utilization.

With advanced technologies, primary cells such as brain microvascular endothelial cells or inducible pluripotent stem cells (iPSCs)-derived brain endothelial cells have been used as a representative of BBB in establishing *in vitro* models [20,21]. However, working with those cells is challenging and time-consuming [22,23]. Moreover, the cultivation of both primary cells and iPSC-derived cells requires complicated techniques and is costly [23,24]. Thus, immortalized brain endothelial cell emerges as another viable option in constructing *in vitro* BBB models [25]. Meanwhile, multiple studies have indicated a decrease in the expression of efflux transporters in immortalized brain microvascular endothelial cells (BMECs) compared to primary cells [25–27]. In contrast, the downregulation of these efflux transporters was reversed in co-cultures involving immortalized human BMECs (HBMEC/ci18) and their supporting cells, immortalized human brain pericytes (HBPC/ci37) and astrocytes (HASTR/ci35). This co-culture model is commonly referred to as the human immortalized BBB (hiBBB) model [26,28–30]. However, the TEER value, a reliable indicator of the integrity of the cellular barriers [31], in the hiBBB model did not reach the relevant value indicative of a fully intact BBB [25,26]. It has been proposed that a nominal TEER value equal to or exceeding 500 $\Omega \times cm^2$ denotes an intact BBB, with values surpassing this threshold indicating physiologically significant BBB barriers [25,32]. Therefore, the primary objective of this study is to establish and characterize an *in vitro* co-culture BBB model using immortalized BMECs (hCMEC/D3 cells) in conjunction with primary pericytes and primary astrocytes in a transwell format. The transwell *in vitro* BBB model is widely used in research due to its ease to manipulate and setup allowing continuous visualization of cells throughout the experimental duration. It demonstrates reproducibility and scalability and facilitates the straightforward assessment of BBB integrity and permeability [25,28–30,33–35].

In addition to the evolution of the transwell *in vitro* models, other signaling molecules are also reported to promote BBB formation and integrity. Previous research has demonstrated that glial cell line-derived neurotrophic factor (GDNF) enhances BBB integrity by up-regulating tight junction (TJ) proteins, such as claudin-5, leading to an increase in the TEER value in various models including the porcine BBB, the human brain microvascular endothelial cell (BMEC) line, and peripheral nerve microvascular endothelial cell (PnMEC) line models [36,37]. In addition to GDNF, cyclic AMP (cAMP) has been shown to play a role in BBB formation through the cAMP-dependent protein kinase A (PKA) signaling pathway [38,39]. An increase in intracellular cAMP concentration activates the PKA, which, in turn, regulates the expression of TJ proteins such as occludin and claudin-5 in endothelial cells [40]. Therefore, both GDNF and cAMP are important players in the regulation of BBB formation and maintenance. However, the impact of GDNF or cAMP on BBB integrity in the transwell *in vitro* BBB triculture model remains unexplored. Thus, this study aims to assess whether both GDNF and cAMP enhance the physiologically relevant BBB integrity in this model.

2. Materials and methods

2.1. Cells and reagents

The human blood-brain barrier cell line (hCMEC/D3 cells) (Cat. #SCC066) was purchased from Merck (Schuchardt, Darmstadt, Germany). hCMEC/D3 cells were cultured with EndoGRO™-MV complete medium (Cat. #SCME004, Merck), which was supplemented with 1 ng/mL FGF-2 (Cat. #GF003, Merck). The primary human astrocytes (HAs) (Cat. #1800) were purchased from ScienCell™ Research Laboratories (Carlsbad, CA, USA). Astrocytes were cultured with astrocyte medium (AM) (Cat. #1801), which was supplemented with astrocyte growth supplement (AGS) and 2 % FBS. The primary human brain vascular pericytes (HBVPs) (Cat. #1200) were purchased from ScienCell™ Research Laboratories (Carlsbad, CA, USA). HBVPs were cultured with pericyte medium (PM) (Cat. #1201), which was supplemented with pericyte growth supplement (PGS) and 2 % FBS. The primary human umbilical vein endothelial cells (HUVECs) (Cat. #C-12203) were purchased from PromoCell (Heidelberg, Germany). HUVEC cells were cultured with

endothelial cell growth medium (Ready-to-use) (Cat. #C22010, PromoCell, Heidelberg, Germany).

2.2. Generation and characterization of in vitro BBB monoculture model

The insert transwell (apical side) was coated with collagen type I at 150 μg/mL in D.W. for 2 h in an incubator. After the incubation, the collagen-coated plate was washed with DPBS twice and dried. The hCMEC/D3 cells were seeded onto the collagen-coated 12-insert well plate with a cell density of 3×10^5 cells/well. The fresh medium was added into the bottom well (basolateral side) at 1,600 μL/well and the plate was incubated for 24 h. After 24 h of incubation, the measurement of transendothelial electrical resistance (TEER) was performed by using an EVOM2™ epithelial voltohmmeter (World Precision Instruments, Hitchin, Hertfordshire, UK). The culture insert's area was used to calculate TEER values ($\Omega \times cm^2$) (Fig. 1A) [33,41].

2.3. Generation and characterization of in vitro BBB triculture model

The insert transwell (apical side) was coated with collagen type I (Merck, Germany) at 150 μg/mL in D.W. for 2 h in an incubator. In the meantime, the insert transwell (basolateral side) was coated with 15 μL poly-L-lysine (ScienCell™ Research Laboratories, USA) in 10 mL D.W. for 2 h in an incubator. After 2 h of incubation, the coated plate was washed twice with DPBS (Cytiva, USA) and dried. Primary human astrocytes (HA) were seeded onto poly-L-lysine-coated insert transwell (basolateral side) at a cell density of 3.13×10^5 cells/well and incubated with astrocyte medium (AM), which was supplemented with astrocyte growth supplement (AGS) and 2 % FBS, for 2–3 h in an incubator (37°C and 5 % CO_2). After 2–3 h of incubation, the transwell inserts were inverted and any excess medium was carefully removed by aspiration. Astrocyte medium was then added to both the apical and basolateral compartments with volumes of 800 μL and 1600 μL, respectively. The plates were then returned to the incubator for an additional 48 h. Following this incubation period, the plates were taken out of the incubator, and the astrocyte medium was cautiously removed to minimize disturbance to the cell layer on the basolateral side of the insert. The inserts were inverted once again, and a pericyte cell suspension of 6.25×10^4 cells per well was added to the astrocyte cell layer on the basolateral side of the transwell inserts, achieving an approximate ratio of 5:1 astrocytes to pericytes [33]. The plate lids were promptly replaced and returned to the incubator for 2–3 h. Following this incubation period, the transwell inserts were reverted, and any excess medium was aspirated. A mixture of astrocyte and pericyte medium in a 1:1 ratio was then added to both the apical and basolateral compartments [33]. When astrocytes and pericytes reached 90 % confluency (around day 4 from the initiation of the model), the mixture medium (consisting of astrocyte and pericyte medium) in the apical compartment was removed. Subsequently, blood-brain barrier hCMEC/D3 cells (3×10^5 cells/well) were added to each well of the apical compartment of the transwell inserts. The cells were allowed to adhere for a minimum of 5 h, following which the medium

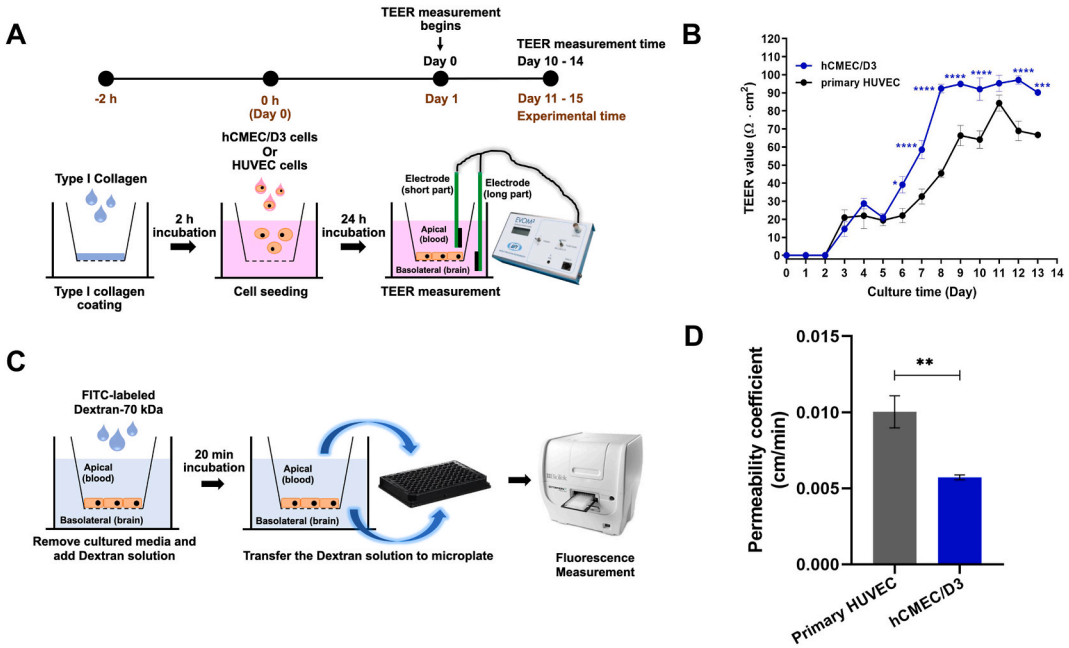

Fig. 1. *In vitro* endothelial barrier model in a transwell format. (A) Schematic illustration of endothelial barrier establishment procedure and barrier tightness measurement by TEER assay on a 12-well transwell culture plate. (B) Measured TEER values of brain endothelial cells (hCMEC/D3: blue) and peripheral primary endothelial cells (primary HUVEC: black) were increased over the course of culture time until the saturation of TEER were observed. (C) Schematic illustration of FITC-labelled dextran permeability assay. (D) The permeability coefficient of FITC-labelled dextran in hCMEC/D3 cells was compared with primary HUVEC cells. *, $p < 0.1$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$. All the experiments were performed in triplicate.

was replenished to 800 μL with hCMEC/D3 medium, and the plates were returned to the incubator [33]. The measurement of TEER was performed by using an EVOM2™ epithelial voltohmmeter (World Precision Instruments, Hitchin, Hertfordshire, UK). The culture insert's area was used to calculate TEER values ($\Omega \times cm^2$) (Fig. 2A) [33,41].

2.4. Immunocytochemistry

For biomarker-specific cell detection, hCMEC/D cells, or astrocytes or pericytes were seeded into the 96-well clear plate (Corning (Acton, MA, USA) at a cell density of 2×10^4 cells per well and incubated in 37°C under a humidified 5 % CO_2 incubator. After 24 h, cells were washed with cold PBS and fixed with 4 % paraformaldehyde at room temperature for 20 min, and permeabilized with permeabilizing buffers (0.3 % TritonX-100 and 0.3 % BSA in PBS) for 20 min. After that, cells were washed and incubated with a blocking solution (3 % BSA in PBS) at room temperature for 1 h. Then, cells were incubated with primary antibodies (ZO-1 (Thermo Fisher Scientific, USA) for hCMEC/D3; GFAP (Thermo Fisher Scientific, USA) for astrocytes; α-SMA (Thermo Fisher Scientific, USA) for pericytes), at 4°C for overnight. Cells were washed with PBS 3 times and then incubated with Alexa Fluor® 488 goat anti-rabbit IgG (H + L) (Invitrogen, USA) or Alexa Fluor® 568 goat anti-mouse IgG (H + L) (Invitrogen, USA) and Hoechst (stain nuclear) (Invitrogen, USA) at room temperature for 1 h. After that cells were washed with PBS 3 times. The samples were visualized at room temperature with an Operetta microscope (PerkinElmer, USA).

After evaluating BBB integrity through TEER assessment under GNDF or cAMP treatment, the expression of claudin-5 in hCMEC/D3 cells was determined. Cells were washed with cold PBS and fixed with 4 % paraformaldehyde at room temperature for 20 min, and permeabilized with permeabilizing buffers (0.3 % TritonX-100 and 0.3 % BSA in PBS) for 20 min. After that, cells were washed and incubated with a blocking solution (3 % BSA in PBS) at room temperature for 1 h. Then, cells were incubated with Claudin 5 monoclonal antibody (4C3C2), Alexa Fluor™ 488 (Invitrogen, USA) at 4°C overnight. The nucleus was stained with Hoechst for 1 h at room temperature. After that cells were washed with PBS 3 times. The samples were visualized at room temperature with BioTek Cytation 7 Multi-Mode Reader (Agilent, Santa Clara, CA, USA). The imaging analysis was performed using ImageJ software (NIH, USA).

2.5. Assessment of GDNF and cAMP on BBB integrity

Following the establishment of an *in vitro* BBB triculture model, it underwent continuous cultivation for 48 h, during which the integrity of the BBB was monitored. The 10 ng/mL GDNF (Cat. #450–10, Thermo Fisher Scientific, Wilmington, DE, USA) and 125 μM cAMP (Cat. #16980-89-5, Sigma-Aldrich, St. Louis, MO, USA) were added to the apical compartment. BBB transwell plates were returned to the incubator. The TEER measurement was performed every day for 3–5 days. The culture insert's area was used to

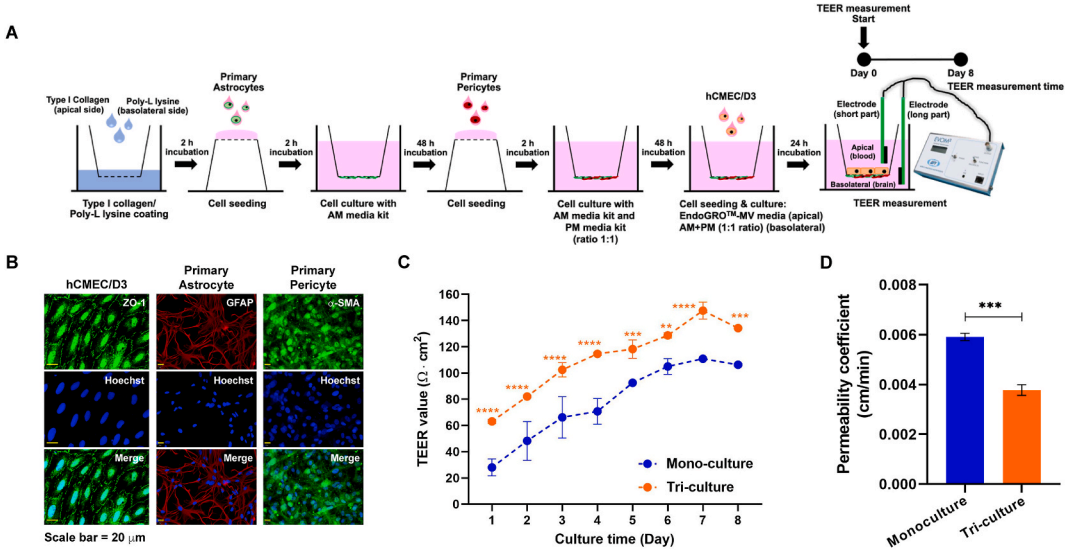

Fig. 2. Generation and characterization of the triculture BBB model. (A) Schematic illustration of the triculture BBB model establishment process on a 12-well transwell culture plate employing the co-cultivation of immortalized brain endothelial cells (hCMEC/D3) with two other primary supporting cells (primary astrocytes and primary pericytes). (B) Biomarkers of brain endothelial cells and two primary supporting cells. The tight junction proteins, specifically zonula occludens-1 (ZO-1), were observed in hCMEC/D3 cells. Glial fibrillary acidic protein (GFAP), an intermediate filament-III protein, is exclusively found in astrocytes within the central nervous system [64]. Brain pericytes were distinguished by the surface marker α-smooth muscle actin (α-SMA) [65]. The scale bar equals to 20 μm. (C) TEER values of triculture BBB model (orange) and monoculture (blue) in a vary culture time manner. (D) Permeability coefficient of FITC-labelled dextran in triculture BBB model (orange) compared with monoculture BBB model (blue) at day 8. **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$. All the experiments were performed in triplicate.

calculate TEER values ($\Omega \times cm^2$) (Fig. 3A) [33,41].

2.6. FITC-labelled dextran permeability assay

After evaluating BBB integrity through TEER assessment under GDNF or cAMP treatment, fresh phenol red-free medium was added to the bottom part of a new 12-well plate at 1,000 μL per well. The treatment solution was then removed from the BBB triculture and the insert transwells were transferred to the new 12-well plate containing fresh phenol red-free medium. FITC-labelled dextran 70 kDa (Sigma-Aldrich, USA), mixed with phenol red-free medium at a final concentration of 100 μg/mL, was added to the upper

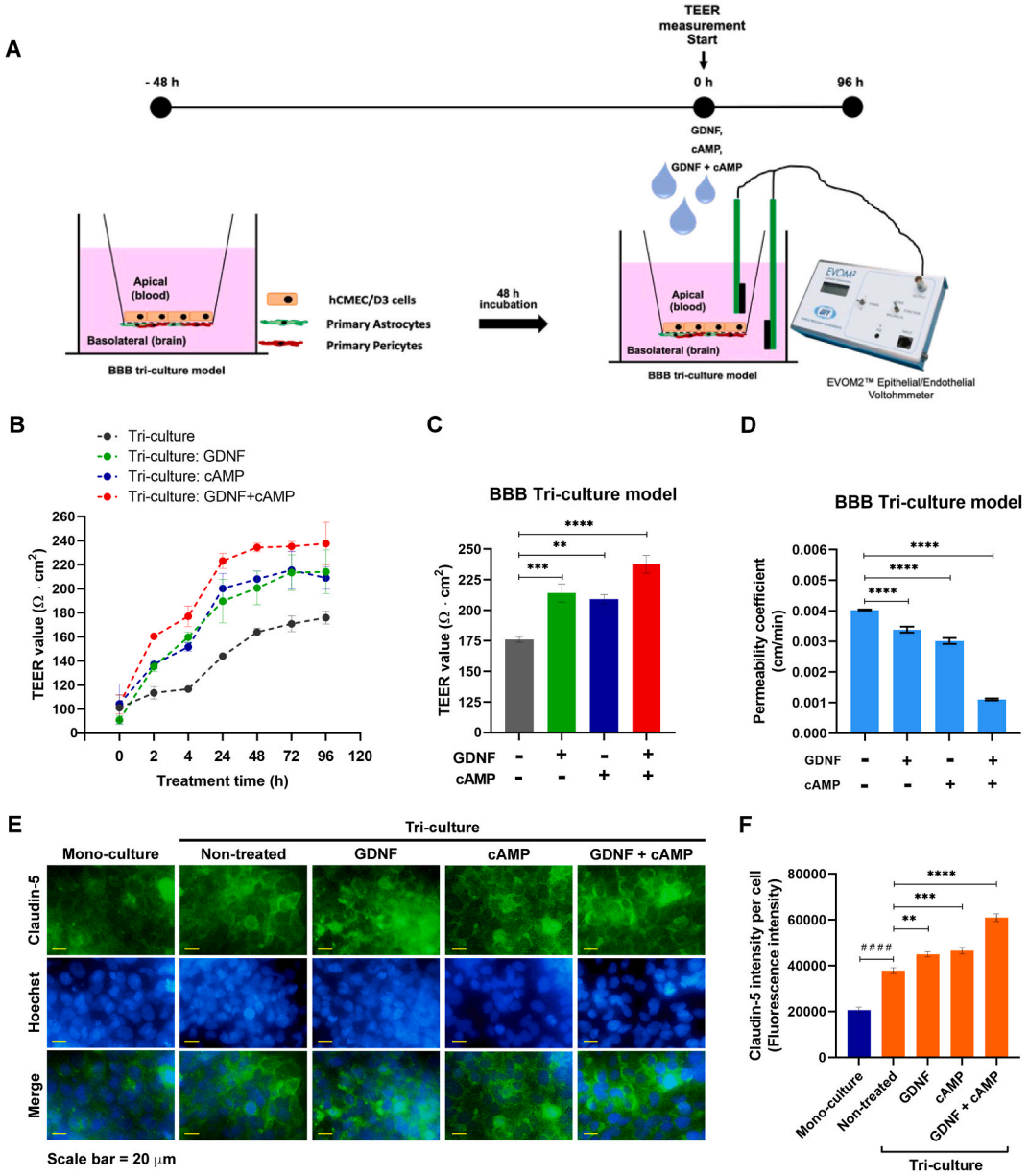

Fig. 3. The effect of GDNF and cAMP on triculture BBB integrity. (A) Schematic illustration of GDNF and cAMP treatment in triculture BBB model. The BBB integrity after treatment with GDNF and cAMP was evaluated by TEER assay. (B) TEER values over the course of the experiment of non-treatment (black), GDNF treatment (green), cAMP treatment (blue), and combined treatments (red). (C) Comparison of TEER values at 96 h after treatment. (D) The permeability coefficient of FITC-labelled dextran in non-treated and treated conditions. (E) The immunocytochemistry fluorescence of claudin-5 expression in monoculture BBB model and triculture BBB model with or without treatment. (F) Comparison of claudin-5 intensity in non-treated and treated conditions analyzed by ImageJ software. Scale bar equal to 20 μm **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$. All the experiments were performed in triplicate.

compartment of the triculture insert transwell and the plate was incubated in incubator for 20 min. Subsequently, the FITC-labelled dextran suspension basolateral media (lower compartment) was collected into 1.5 mL Eppendorf tube and vortex. 100 μL aliquots from each Eppendorf tube containing FITC-labelled dextran basolateral media were transferred to Corning™ 96-well clear bottom black polystyrene microplate in triplicate. Additionally, 100 μL of phenol red-free medium was transferred to an additional three wells to serve as the blank control. Fluorescence intensity measurements were conducted using a BioTek Cytation 7 Multi-Mode Reader (Agilent, Santa Clara, CA, USA) at 490/520 nm for excitation/emission (Fig. 1C). The permeability analysis of FITC-labelled dextran was calculated as permeability coefficient (Pe) [42].

2.7. Statistical analysis

All data were analyzed by using GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA). The multiple value comparisons were made using one-way analysis of variance (ANOVA). The unpaired Student t-test was used to compare the two values.

3. Results

3.1. Immortalized brain microvascular endothelial cell line (hCMEC/D3) is suitable for establishing an in vitro BBB model

In this initial study, hCMEC/D3 cells were employed to assess their suitability for generating an *in vitro* BBB model in a transwell format. The hCMEC/D3 cells and peripheral primary endothelial cells (primary HUVEC) were cultivated as monoculture barriers on the insert transwell plate, and barrier integrity was evaluated. The TEER value was measured after cell seeding and incubation for 24 h (Fig. 1A). The investigation revealed that the TEER value of hCMEC/D3 cells was increased and showed a faster rate of increase than that of the primary HUVEC cells after day 5 (Fig. 1B). After days 9–10, the TEER value of both cell types reached saturation. On day 12, the hCMEC/D3 cells exhibited a TEER value of $97.2 \pm 4.0\ \Omega \times cm^2$ which was significantly higher than that of the primary HUVEC cells ($68.9 \pm 9.4\ \Omega \times cm^2$) (Fig. 1B). These results indicate that brain endothelial cells possess a higher barrier tightness compared to peripheral endothelial cells.

Additionally, beyond assessing barrier tightness, barrier permeability was investigated using the FITC-labelled dextran. After day 13, when the TEER value approached saturation, the FITC-labelled dextran in size 70 kDa was added to BBB monoculture models to evaluate barrier permeability by fluorescence signal measurement (Fig. 1C). The permeability of the hCMEC/D3 monoculture barrier was significantly lower than that of the primary HUVEC monoculture barrier (Fig. 1D). This result suggests that the higher tightness of the brain endothelial cell barrier renders a limitation on substrate permeability in comparison to the peripheral endothelial cell barrier. Therefore, the hCMEC/D3 cells are suitable for the development of an *in vitro* BBB model.

3.2. The triculture BBB model shows enhanced membrane integrity than the monoculture model

Even though the monoculture BBB model employing immortalized brain endothelial cells (hCMEC/D3) exhibits a higher TEER value than peripheral endothelial cells (primary HUVEC), its TEER value remains unsatisfied. To address this, hCMEC/D3 cells were co-cultured with other two supporting primary cells, i.e. primary astrocytes and primary pericytes, on a 12-well transwell culture plate (Fig. 2A–B). The apical compartment of the insert transwell was coated with collagen type I. The basolateral compartment of the insert transwell was coated with poly-L lysine. Incubation of coated insert transwell for 2 h before seeding of cells. First, the primary astrocytes were seeded on the insert transwell at the basolateral compartment and incubated for 48 h. Secondly, the primary pericytes were then seeded on the insert transwell at the same site as primary astrocyte seeding, and incubated for 48 h. The medium for the culture of both cell types was a mixed medium of 1:1 ratio of Astrocyte Medium and Pericyte Medium. The hCMEC/D3 cells were subsequently seeded on the apical compartment of the insert transwell and incubated in the incubator for 24 h before TEER measurement commences (Fig. 2A). Furthermore, the three cell types employed to establish the triculture BBB model were assessed for verification of identity utilizing biomarkers specific to each cell (Fig. 2B). The tight junction proteins, zonula occludens-1 (ZO-1), were expressed in hCMEC/D3. The Glial fibrillary acidic protein (GFAP) is an intermediate filament-III protein identified only in astrocytes in the CNS. Brain pericytes can be identified using the surface marker α-smooth muscle actin (α-SMA).

The result revealed that the TEER value of the triculture BBB model was increased and showed a faster rate of increase than the monoculture BBB model (Fig. 2C). After day 7, the TEER value of both triculture and monoculture BBB models approached saturation. On day 8, the triculture BBB model exhibited a TEER value of $147.6 \pm 6.5\ \Omega \times cm^2$which was significantly higher than that of the monoculture BBB model ($106.3 \pm 1.0\ \Omega \times cm^2$) (Fig. 2C). These results indicate that the triculture BBB model possesses a higher barrier tightness compared to the monoculture BBB model. The permeability of the triculture BBB model is notably reduced leading to a significant decrease in the infiltration of FITC-labelled dextran from the apical compartment to the basolateral compartment when compared to the monoculture BBB model (Fig. 2D). Therefore, the co-culture of brain endothelial cells (hCMEC/D3) with primary astrocytes and pericytes better mimics the anatomical and functional relationship known as the neurovascular unit (NVU), which represents the intact BBB structure in the human brain. Hence, these supporting cells were important cells for the regulation of BBB formation and maintenance.

3.3. Both GDNF and cAMP improve BBB integrity approaching the threshold indicative of an intact BBB

Previous studies have demonstrated that GDNF and cAMP enhance BBB integrity in several monoculture models [36–40]. Thus, this

study aims to evaluate whether both GDNF and cAMP collectively improve the physiologically relevant BBB integrity. Following the course of 48 h culture of the triculture BBB model, GDNF, cAMP, or a combination of both were introduced to the apical compartment of the triculture BBB model. TEER measurement commenced after 2 h post-treatment (Fig. 3A). The results at 96-h post treatment indicated a significant increase in TEER values with the administration of GDNF or cAMP alone ($214.1 \pm 18.1\ \Omega \times cm^2$ for GDNF, and $209.0 \pm 9.2\ \Omega \times cm^2$ for camp, respectively) compared to the untreated condition ($176.0 \pm 5.4\ \Omega \times cm^2$) (Fig. 3B–C). Notably, both substances expedited the increase of TEER values when compared to the non-treatment group over the course of the experiment. Interestingly, the TEER values of GNDF and cAMP treatments did not differ significantly. Remarkedly, the combined treatment of both GDNF and cAMP demonstrated a much higher TEER value ($237.6 \pm 17.7\ \Omega \times cm^2$) compared to the individual treatment (Fig. 3B–C) which, in turn, indicates a low permeability of the BBB. Further investigation on membrane permeability revealed a notable decrease in the permeability coefficient of FITC-labelled dextran in the treatment conditions, particularly with the combination treatment, compared to the non-treatment condition (Fig. 3D). Additionally, the expression of claudin-5, a tight junction protein, in the triculture BBB model was increased compared to the monoculture BBB model (Fig. 3E). Meanwhile, claudin-5 expression gradually increased in the treatment condition as compared to the non-treatment condition (Fig. 3F). Therefore, both GDNF and cAMP were found to enhance the physiologically relevant BBB integrity through the up-regulation of claudin-5 expression.

4. Discussion

The blood-brain barrier (BBB) acts as a special barrier, and its components have strong coordination in both anatomical and functional relationships. This special barrier function is challenging in the development of CNS drugs. The reliable platform of an *in vitro* BBB model that is relevant to the *in vivo* BBB is still being elucidated. This study used the human brain capillary microvascular endothelial cell line (hCMEC/D3 cells) as a representative brain endothelial cell to form the BBB monolayer on the insert transwell plate. The results showed that the hCMEC/D3 monolayer exhibited a stronger paracellular barrier than the peripheral endothelial cell (primary HUVEC) monolayer in terms of both TEER values and functional permeability, as illustrated in Fig. 1. This result is consistent with the previous research by L. Cucullo, which found that hCMEC/D3 cells are more capable than HUVEC cells of forming a tight barrier [43].

It is becoming widely recognized that coculturing hCMEC/D3 monoculture systems with astrocytes and/or pericytes is a useful approach in creating BBB models that closely replicate *in vivo* BBB physiology [44–46]. BBB cells sourced from various animal species have frequently been employed for this purpose. However, the difference in expression levels of BBB transporters have highlighted discrepancies in the functionality of the blood-brain barrier between animals and humans [47–49]. Therefore, a human-derived BBB model is required for CNS drug development. The present study investigated and established a humanized triculture BBB model that consists of hCMEC/D3 cells, primary human astrocytes, and primary human pericytes. This investigation found that the BBB integrity of the triculture BBB model had been raised up when compared with the monoculture BBB model (hCMEC/D3 monolayer), as shown in Fig. 2. Moreover, the BBB permeability had been decreased in triculture format when compared with monoculture (Fig. 2D). The results align with the previous work by Ito R., confirming the reliability of the human cell-derived triculture BBB model. This model closely resembles the neurovascular unit (NVU) in terms of both anatomical structure and functional relationships [26]. Their triculture BBB model used immortalized cell lines including brain endothelial cells: HBMEC/ci18, immortalized astrocytes: HASTR/ci35, and immortalized pericytes: HBPC/ci37 [26], which differs from our BBB model in that it uses primary supporting cells. The use of primary astrocytes and primary pericytes is beneficial cooperation in the triculture BBB model because these cells retain cell-to-cell contact properties [50,51]. This characteristic supports prolonged culture duration and prevents excessive cell growth or clumping within the model. However, the BBB integrity results of the triculture model in the present study are still not close to the relevant value that indicates an intact BBB [25,32].

The advanced development of the transwell *in vitro* BBB model does not only relies on utilizing supporting cells to enhance the physical properties of the BBB. Other factors are also being explored to facilitate the formation and maintenance of BBB integrity. Previous studies have reported that glial cell-derived neurotrophic factor (GDNF) and 3′,5′-cyclic adenosine monophosphate (cAMP) promoted BBB integrity in monoculture models [36–40]. As seen in Fig. 3, the current study discovered that GDNF and cAMP enhanced the physiologically significant BBB integrity by raising TEER value and lowering permeability through up-regulating claudin-5 expression. According to several findings, GDNF improves the integrity of the blood-brain barrier by upregulating the expression of claudin-5 and encouraging junction expression in brain microvascular endothelial cells [37,52]. However, the molecular mechanisms of GDNF-induced claudin-5 expression have not been clearly elucidated and need further investigation. Claudin-5 is widely regarded as the most crucial component in maintaining BBB function [53]. The transcription of the claudin-5 gene is regulated by Forkhead box protein O1 (FoxO1), which is the transcription factor [54]. The phosphorylation of FoxO1 is induced by the activation of the PI3K-Akt signaling pathway. This phosphorylation causes FoxO1 to move from its primary location in the nucleus to the cytoplasm, where it results in the derepression of the claudin-5 gene and promotes claudin-5 synthesis [55,56]. Therefore, the expression of claudin-5 relies on the PI3K-Akt signaling pathway activation. Several studies have reported that the PI3K-Akt signaling pathway is activated by the GDNF/GFR-α1/RET complex [57–60]. GDNF binds with GDNF receptor alpha 1 (GFR-α1) and then recruits RET (rearranged during transformation), which is a single transmembrane-spanning RTK (receptor tyrosine kinase) that acts as a GDNF family of ligand (GFL) co-receptor [61], to form a GDNF/GFR-α1/RET complex. This complex formation causes the autophosphorylation of specific tyrosine residues in the kinase domains of RET [57]. The phosphorylated RET subsequently activates a variety of signaling pathways, including PI3K-Akt, which enhance neuronal survival, differentiation, proliferation, and migration [59,60]. Thus, it can be hypothesized that GDNF up-regulates claudin-5 expression via RET-PI3K/Akt-FoxO1 cascade activation (Fig. 4). As demonstrated in the current work, GDNF was not the only factor that enhances BBB integrity; cAMP was also identified as a significant player. It is consistent with several

previous studies that cAMP improves BBB integrity through up-regulation of claudin-5 expression [38–40]. When the porcine BBB endothelial cells were treated with the cAMP analogs, the protein kinase A (PKA) was activated by cAMP. The activated PKA then phosphorylated cAMP-response element binding protein (CREB), which is the transcription factor, to promote the transcription of the claudin-5 gene [62]. This molecular mechanism has been confirmed by the study on the effect of IL-1β on BBB integrity, which found that accumulated IL-1β disrupted cAMP/CREB activation and then attenuated transcription of claudin-5 [63]. Therefore, cAMP improves BBB integrity by increasing claudin-5 expression through the activation of the cAMP/PKA/CREB cascade (Fig. 4). Together, GDNF and cAMP play crucial roles in enhancing BBB integrity, approaching the threshold indicative of an intact BBB in the human-derived triculture BBB model. Moreover, this study emphasized the effectiveness of the transwell *in vitro* BBB model for assessing drug permeability.

5. Conclusion

In conclusion, BBB stands as a critical barrier of neural tissue regulating molecular exchange between the bloodstream and the brain and preventing harmful toxins from entering the brain. However, assessing a candidate compound's BBB permeability presents significant challenges in CNS drug development. *In vitro* permeability studies serve as essential precursors to animal testing helping to prevent potential clinical failures. Therefore, there is a need for improved *in vitro* models that closely mimic the physiological BBB integrity. In this study, an *in vitro* human-derived triculture BBB model in a transwell format employing a coculture of hCMEC/D3 cells with primary astrocytes and pericytes, is investigated. The results revealed that the triculture BBB model significantly exhibited higher paracellular tightness and decreased permeability compared to the monoculture model. This finding supports previous studies suggesting the importance of incorporating supporting cells for a more physiologically relevant BBB model. In addition, the present study also explored the role of GDNF and cAMP in enhancing BBB integrity through up-regulation of claudin-5 expression. The results suggest that both GDNF and cAMP contribute to strengthening the BBB, approaching the threshold indicative of an intact barrier. These findings highlight the potential of the transwell *in vitro* BBB model as an alternative tool for assessing drug permeability. In summary, this humanized triculture BBB model, enhanced by GDNF and cAMP, emerges as a valuable tool for CNS drug development research. Further investigation into the molecular mechanisms and continued development of this *in vitro* model will undoubtedly overcome the challenges of BBB permeability and facilitating a more effective CNS drug development strategy.

CRediT authorship contribution statement

Phongthon Kanjanasirirat: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Witchuda Saengsawang:** Resources, Conceptualization. **Pimonrat Ketsawatsomkron:** Resources. **Nithi Asavapanumas:** Resources. **Suparerk Borwornpinyo:** Resources. **Sunhapas Soodvilai:** Resources. **Suradej Hongeng:** Supervision, Resources, Funding acquisition, Conceptualization. **Sitthivut Charoensutthivarakul:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization.

Data availability

All the data presented here are included in this manuscript and available from the corresponding author upon request.

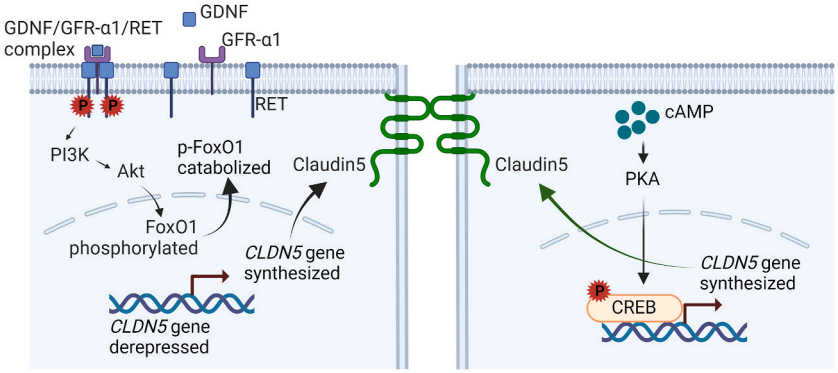

Fig. 4. The proposed mechanism involving GDNF, and cAMP enhances BBB integrity by up-regulating claudin-5. GDNF first binds with GFR-α1 recruiting RET to form complexes. Subsequently, the GDNF/GFR-α1/RET complex triggers RET autophosphorylation which then activates PI3K/Akt signaling cascades [57–60]. This activation induces FoxO1 phosphorylation causing its translocation from the nucleus to the cytoplasm, which leads to the derepression of claudin-5 gene transcription [54–56]. cAMP activates PKA, leading to CREB phosphorylation, which then promotes the transcription of the claudin-5 gene [62,63] (Created by BioRender.com/Mahidol University).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39343.

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