Inhibition of Glycogen Synthase Kinase 3ß Enhances Functions of Induced Pluripotent Stem Cell-Derived Brain Microvascular Endothelial Cells in the Blood–Brain Barrier

Tomoko Yamaguchi, Misae Nishijima, and Kenji Kawabata

INTRODUCTION

The blood–brain barrier (BBB) is mainly composed of brain microvascular endothelial cells (BMECs), which working together with other central nervous system (CNS) cells such as astrocytes and pericytes. BMECs possess barrier properties such as a high-resistance paracellular barrier, low rates of transcytosis, and expression of selective transporters and receptors. In vitro BBB models have been widely reported with primary BMECs isolated from animals, although it is known that the expression patterns and levels of transporters and receptors in BMECs differ between humans and animals. Recently, several methods to differentiate BMECs from human induced pluripotent stem (hiPS) cell have been developed. However, the expression of P-glycoprotein (P-gp), which is a key efflux transporter, in hiPS cell-derived BMECs was detected at a relatively low level compared with primary human BMECs. In this study, we examined the involvement of the canonical Wnt signaling pathway, which contributes to the development of BBB formation, in the regulation of P-gp expression in hiPS cell-derived BMECs. We found that the barrier integrity was significantly enhanced in hiPS cell-derived BMECs treated with glycogen synthase kinase-3ß (GSK-3ß) inhibitors, which are known to positively regulate the canonical Wnt signaling pathway. In addition, our data also showed P-gp expression level was increased by treatment with GSK-3ß inhibitors. In conclusion, physiological barrier function and P-gp expression in BMECs can be enhanced by the canonical Wnt signaling pathway. Our results may be useful for promoting the development of drugs for central nervous system diseases in vitro BBB model.

Key words: blood–brain barrier; Wnt signaling; P-glycoprotein; induced pluripotent stem (iPS) cell

Brain microvascular endothelial cells (BMECs) are essential component of the blood–brain barrier (BBB). BMECs strictly regulate the entry of various molecules into the central nervous system from the peripheral circulation by forming tight junctions and expressing various influx/efflux transporters and receptors. In vitro BBB models have been widely reported with primary BMECs isolated from animals, although it is known that the expression patterns and levels of transporters and receptors in BMECs differ between humans and animals. Recently, several methods to differentiate BMECs from human induced pluripotent stem (hiPS) cell have been developed. However, the expression of P-glycoprotein (P-gp), which is a key efflux transporter, in hiPS cell-derived BMECs was detected at a relatively low level compared with primary human BMECs. In this study, we examined the involvement of the canonical Wnt signaling pathway, which contributes to the development of BBB formation, in the regulation of P-gp expression in hiPS cell-derived BMECs. We found that the barrier integrity was significantly enhanced in hiPS cell-derived BMECs treated with glycogen synthase kinase-3ß (GSK-3ß) inhibitors, which are known to positively regulate the canonical Wnt signaling pathway. In addition, our data also showed P-gp expression level was increased by treatment with GSK-3ß inhibitors. In conclusion, physiological barrier function and P-gp expression in BMECs can be enhanced by the canonical Wnt signaling pathway. Our results may be useful for promoting the development of drugs for central nervous system diseases using in vitro BBB model.

Key words: blood–brain barrier; Wnt signaling; P-glycoprotein; induced pluripotent stem (iPS) cell

INTRODUCTION

The blood–brain barrier (BBB) is mainly composed of brain microvascular endothelial cells (BMECs), which working together with other central nervous system (CNS) cells such as astrocytes and pericytes. BMECs possess barrier properties such as a high-resistance paracellular barrier, low rates of transcytosis, and expression of selective transporters and receptors. In vitro BBB models have been widely reported with primary BMECs isolated from animals, although it is known that the expression patterns and levels of transporters and receptors in BMECs differ between humans and animals. Recently, several methods to differentiate BMECs from human induced pluripotent stem (hiPS) cell have been developed. However, the expression of P-glycoprotein (P-gp), which is a key efflux transporter, in hiPS cell-derived BMECs was detected at a relatively low level compared with primary human BMECs. To overcome these problems, hCMEC/D3 cells have been used as a culture model for human BMECs because they have several characteristics of BMECs, such as BMEC-specific transporter expression. However, hCMEC/D3 cells lack barrier integrity, which makes it difficult to study BBB permeability. Accordingly, we and other researchers have developed methods to differentiate BMECs from human induced pluripotent stem (hiPS) cells. These cells also possess several characteristics of tissue-derived BMECs, such as physiological barrier function. However, we and other group previously reported that the expression of P-glycoprotein (P-gp), which plays the most important role as an efflux transporter in BMECs, is significantly lower in hiPS cell-derived BMECs than in hCMEC/D3 cells and primary human BMECs. Therefore, it is necessary to upregulate the P-gp expression in hiPS cell-derived BMECs.

The Wnt signaling pathway controls various developmental processes, including differentiation, proliferation, and migration. When the canonical signaling pathway is activated, inactivation of glycogen synthase kinase-3ß (GSK-3ß) via phosphorylation at Ser9 increases the cytoplasmic ß-catenin protein, leading to stabilization of ß-catenin and its translocation to the nucleus, where it regulates the expression of canonical Wnt target genes. Therefore, GSK-3ß inhibitors, such as Chir99021 and 6-bromoindirubin-3'-oxime (BIO), are widely used for activating the canonical Wnt signaling pathway. Stenman et al. have demonstrated that the canonical Wnt signaling pathway is an important signaling for brain vascularization and the BBB formation in vivo. This pathway is also known to be involved in the formation of tight junction proteins in developing brain blood vessels. Accordingly, the canonical Wnt signaling pathway contributes to the differentiation of BMECs. However, as far as I know, the effect of the inhibition of GSK-3ß and the resultant activation of the canonical Wnt signaling pathway on functions of hiPS cell-derived BMECs has not been reported yet. In this study, we aimed to elucidate whether the inhibition of GSK-3ß could regulate both the physiological barrier function and P-gp expression in hiPS cell-derived BMECs.

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MATERIALS AND METHODS

**Cell Cultures** The hiPS cell line iMR90-4 (WiCell) was cultured in mTeSR1 medium (Stem Cell Technologies, Vancouver, Canada), and dissociated into clumps using 0.5 mM ethylenediamine tetraacetic acid (EDTA)/phosphate-buffered saline (PBS) and plated onto growth factor-reduced basement membrane matrix (Corning, Corning, NY, U.S.A.). The hiPS cell line 201B7 was maintained in E8-Flex (Thermo Fisher Scientific, Waltham, MA, U.S.A.), which was changed daily, and dissociated into clumps using 0.5 mM EDTA/PBS and plated onto Laminin-521 (Thermo Fisher Scientific). The differentiation protocol for the induction of BMECs from hiPS cells was described previously.\(^{11}\) Chir99021 (Lot: 5B/198191) and BIO (Lot: 3A/178335) were purchased from Tocris. Chir99021 was reconstituted in dimethyl sulfoxide (DMSO) and included at concentrations of 3–6 µM depending on the experiments. BIO was reconstituted in DMSO and included at concentrations of 2–3 µM depending on the experiments. They were added into the culture medium from day 6 to day 8.

**Trans-Endothelial Electrical Resistance (TEER) Measurement and Permeability Experiments** To measure TEER, hiPS cell-derived BMECs were incubated on the Transwell inserts. The TEER values were measured using Millicell ERS-2 (Merk Millipore, Burlington, MA, U.S.A.). The calculation of the TEER values was described previously.\(^{20,21}\) We confirmed the value of TEER was peaked on day 10. Therefore, all experiments were performed on day 10.

Before the permeability tests, hiPS cell-derived BMECs were washed with Dulbecco’s PBS (DPBS, Sigma, St. Louis, MO, U.S.A.) supplemented with d-glucose and N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) (DPBS-H). DPBS-H containing 10 μg/mL sodium fluorescein (NaF, Sigma) as a paracellular marker was then added into the upper chamber for 30 min at 37°C. After incubation, the medium in the lower chamber was collected and then the concentration of NaF in the medium was measured using a fluorescence multi-well plate reader (Genios, TECAN, Männedorf, Switzerland). The permeability coefficient was calculated as previously reported.\(^{20}\)

**Cell Viability Assay** hiPS cell-derived BMECs were incubated with 10% alamarBlue reagent (Thermo Fisher Scientific) for 2 h at 37°C. After incubation, the medium was collected. The absorbance was measured using Sunrise (TECAN).

**Quantitative PCR (qPCR)** Total RNA from hiPS cell-derived BMECs was prepared using RNAiso Plus reagent (TaKaRa). SuperScript VILO cDNA synthesis kit (Thermo Fisher Scientific) was used to generate cDNA. The mRNA quantification was performed using StepOnePlus real-time PCR system with FAST SYBR Green Master Mix (Thermo Fisher Scientific). Results were normalized against the input determined for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. Gene expression levels in the DMSO-treated cells were taken as 1.0. The sequences of the primers used are summarized in Table 1.

**Western Blotting** The protein samples were prepared using radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific) supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland). They were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a 5–20% polyacrylamide gel (Wako Pure Chemical Corporation, Osaka, Japan), and subjected to Western blot analysis as described previously (ref). Claudin-5 was detected using 1/1000 diluted a mouse anti-claudin-5 antibody (Thermo Fisher Scientific) and HRP-conjugated anti-mouse immunoglobulin G (IgG) (Cell Signaling Technology, Danvers, MA, U.A.).

**Accumulation Assay** hiPS cell-derived BMECs were cultured with 10 µM rhodamine 123 (Sigma) in transport buffer (distilled water with 0.12 M NaCl, 25 mM NaHCO\(_3\), 3 mM KCl, 2 mM MgSO\(_4\), 2 mM CaCl\(_2\), 0.4 mM K\(_2\)HPO\(_4\), 1.0 mM HEPES, and 0.1% bovine serum albumin (BSA)). For inhibition experiments, cells were pre-incubated with P-gp specific inhibitor, 10 µM cyclosporin A (CsA; Sigma) in transporter buffer for 1 h at 37°C. After incubation for 2 h, cells were washed with PBS and lysed with RIPA buffer. The fluorescence of the cell lysate was assessed using Genios. Fluorescence was normalized on a per-cell basis by counting dissociated cells.

**Statistical Analysis** Results are presented as the mean +/- standard deviation (S.D.). Statistical analysis was performed using an unpaired two-tailed Student’s t-test. Statistical significance was set at \(p<0.05\).

**RESULTS**

**BBB Integrity in hiPS Cell-Derived BMECs Was Enhanced by GSK-3β Inhibitors** The canonical Wnt signaling pathway is known to contribute to the development of BMECs in mice.\(^{16,17}\) Therefore, we investigated the effect of the canonical Wnt signaling pathway, which could be activated by GSK-3β inhibitors, on BBB integrity and paracellular permeability.

**Table 1. Primer List Used in Quantitative Real-Time PCR**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>(5’) Forward primers (3’)</th>
<th>(5’) Reverse primers (3’)</th>
</tr>
</thead>
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<tr>
<td>GAPDH</td>
<td>GGTGGTCTCTCTGCAGCTCAACGT</td>
<td>GGTGGTCTGGAGGGAAGAAGT</td>
</tr>
<tr>
<td>Axin2</td>
<td>GAGGGCACTTGTGGCAGCTTC</td>
<td>GGTGGCTGTGCAAAGACATAG</td>
</tr>
<tr>
<td>CLDN5</td>
<td>GGTTGTGCTCTTCTCTGTA</td>
<td>GCTCGTACTTCTCGACACG</td>
</tr>
<tr>
<td>PECAM1</td>
<td>GGATTACCTGCAGCGCTCC</td>
<td>AAACATGAATAGGCTCTTTC</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>TACAGTAAACAGGCCACAAC</td>
<td>TGCCATCCCCATTGCTCGAG</td>
</tr>
<tr>
<td>Glut1</td>
<td>GCTATGGGGAGAGCATCCTG</td>
<td>AAGGCCAGGCTGATTCATCAT</td>
</tr>
<tr>
<td>MRP1</td>
<td>CAAGTTGAGTGGAAATGAGG</td>
<td>TTTCCTCACTTTGCTGGGCC</td>
</tr>
<tr>
<td>TR</td>
<td>AGGCCACTTGTGTATACGCT</td>
<td>GCAGCCACGTTATTCTCAGAG</td>
</tr>
<tr>
<td>LRP1</td>
<td>CAACAACACCTCGACTGCC</td>
<td>TTTTCCTCACAGCTGCC</td>
</tr>
<tr>
<td>BCRP</td>
<td>CTTTCCTGGTGGCAACACT</td>
<td>GGTGCACTTGGACTGAAAGA</td>
</tr>
<tr>
<td>P-gp</td>
<td>CACCCGACTTACAGATG</td>
<td>GGTGCACTTGGACTGAAAGA</td>
</tr>
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in hiPS cell-derived BMECs. First, we compared the expression level of axis inhibition protein 2 (Axin2) mRNA, which is one of the target genes of the canonical Wnt signaling pathway, in hiPS cell-derived BMECs treated with GSK-3β inhibitors, Chir99021 and BIO. We confirmed that the expression of Axin2 mRNA was higher in GSK-3β inhibitors-treated cells than in DMSO-treated cells (control cells) (Fig. 1), suggesting that GSK-3β inhibitors could activate the canonical Wnt signaling pathway in hiPS cell-derived BMECs.

Next, we investigated whether the addition of GSK-3β inhibitors could affect barrier tightness. Our results showed that treatment with GSK-3β inhibitors led to increased TEER values compared with DMSO treatment (Fig. 2A). In addition, the transcellular transport of sodium fluorescein (NaF) as a paracellular marker was significantly decreased by treatment with GSK-3β inhibitors (Fig. 2B). The same results were observed with both hiPS cell-derived BMECs (iMR90-4 and 201B7) (Supplementary Figs. S1A, B). Next, we examined the viability of GSK-3β inhibitor-treated hiPS cell-derived BMECs, and no reduction in the viability of GSK-3β inhibitor-treated cells was observed compared with DMSO-treated cells (Fig. 2C). Our results indicate that BBB integrity in hiPS cell-derived BMECs could be enhanced by GSK-3β inhibitors.

We investigated whether the effect on TEER value upon inhibition of GSK3β could be a result of increased expression levels of tight junction genes. Tight junction-related genes, such as claudin-5, are known to regulate barrier tightness in BMECs. Our data showed that the mRNA expression level of claudin-5 was higher in GSK-3β inhibitor-treated cells than in DMSO-treated cells (Fig. 3A, Supplementary Fig. S1C). We also found that the expression levels of claudin-5 protein were also upregulated by GSK-3β inhibitors (Fig. 3B). These results showed that GSK-3β inhibitors could upregulate barrier tightness by inducing the claudin-5 expression.

**The Endothelial Characteristics in hiPS Cell-Derived BMECs Could Not Be Influenced by GSK-3β Inhibitors**

![Fig. 1](image1.png)

**Fig. 1.** GSK-3β Inhibitors Could Activate Wnt Signaling in hiPS Cell-Derived BMECs

Axin2 expression level was assessed by measuring quantitative RT-PCR. Values are represented as the mean +/- S.D. from three independent experiments. **p < 0.01 vs. DMSO value.

![Fig. 2](image2.png)

**Fig. 2.** Barrier Tightness in hiPS Cell-Derived BMECs Is Enhanced by GSK-3β Inhibitors

(A) TEER values were measured in Chir99021-treated BMECs and BIO-treated BMECs. Values represent the mean +/- S.D. of four determinations. *p < 0.05 vs. DMSO value. (B) Sodium fluorescein permeability was measured. Values represent the mean +/- S.D. of four determinations. *p < 0.05 vs. DMSO value. (C) Cell viability was analyzed by alamarBlue. Values are represented as the mean +/- S.D. from three independent experiments.
Next, we performed the expression levels of endothelial cell marker genes, such as platelet endothelial cell adhesion molecule (PECAM1) and vascular endothelial (VE)-cadherin, in hiPS cell-derived BMECs treated with GSK-3ß inhibitors. No significant differences were observed in the mRNA expression levels of PECAM-1 and VE-cadherin in the absence or presence of GSK-3ß inhibitors (Fig. 4). These results showed that the expression of endothelial cell marker genes in hiPS cells-derived BMECs could not be influenced by GSK-3ß inhibitors.

**Functional P-gp Expression in hiPS Cell-Derived BMECs Was Increased by GSK-3ß Inhibitors** We examined the influence of GSK-3ß inhibitors on the expression of some influx/efflux transporters and receptors. No significant differences in the expression of glucose transporter 1 (Glut1/SLC2A1), multidrug resistance-associated protein 1 (MRP1/ABCC1), transferrin receptor (TfR), and low-density lipoprotein receptor-related protein 1 (LRP1) between DMSO-treated cells and GSK-3ß inhibitor-treated cells (Fig. 5A, Supplementary Fig. S2A). The expression of breast cancer resistance protein (BCRP/ABCG2) mRNA was increased by treatment with BIO, while the expression of BCRP mRNA was unchanged by treatment with Chir99021 (Fig. 5A, Supplementary Fig. S2A). The expression of P-gp/ABCB1 was higher in GSK-3ß inhibitors-treated cells than in DMSO-treated cells (Fig. 5A, Supplementary Fig. S2A). We found that P-gp expression could be upregulated by GSK-3ß inhibitor.

Finally, we examined the potential effects of GSK-3ß inhibitor on P-gp efflux transporter functionality via intracellular accumulation of the fluorescent P-gp substrate (rhodamine 123) in the presence and absence of the P-gp inhibitor (CsA). Both DMSO- and GSK-3ß inhibitor-treated BMECs exhibited an increase in rhodamine 123 accumulation in the presence of CsA (Fig. 5B, Supplementary Fig. S2B). In addition, the concentration of rhodamine 123 in the absence of CsA was lower in hiPS cell-derived BMECs in the presence of GSK-3ß inhibitors than that in the absence of GSK-3ß inhibitors (Fig. 5B, Supplementary Fig. S2B). These results showed that P-gp, which is induced by GSK-3ß inhibitors, could function in hiPS cell-derived BMECs.

**DISCUSSION** hiPS cell-derived BMECs possess several characteristics of tissue-derived BMECs, such as physiological barrier function. However, the major limitation of the use of hiPS cell-derived BMECs is the low level of P-gp expression compared with primary human BMECs and hCMEC/D3 cells. In this study, we found that GSK-3ß inhibitors could promote BBB integrity by increasing claudin-5 expression and could increase the P-gp expression in hiPS cell-derived BMECs. Thus, GSK-3ß inhibitors are useful for the development of in vitro BBB models using hiPS cell-derived BMECs.

It is well known that the Wnt signaling pathway has a hand in the barrier genesis in mice. Thus, activation of the canonical Wnt signaling pathway may promote the differentiation of BMECs from hiPS cells. To date, the effect of the inhibition of GSK-3ß on functions of hiPS cell-derived BMECs remains largely unknown. Thus, in this study, we demonstrated the
role of GSK-3ß inhibition in the differentiation of BMECs from hiPS cells. Our results showed that the inhibition of GSK-3ß led to the enhanced barrier integrity in hiPS cell-derived BMECs by increasing the expression of claudin-5, which plays a key role in barrier formation in BMECs. A previous study demonstrated that the inhibition of GSK-3ß in human primary BMECs could enhance barrier tightness by stabilizing claudin-5.22 These results are consistent with our results. GSK-3ß is known to control the activation of ß-catenin, which is involved in the regulation of expressions of tight junction-related genes, such as claudin-1, in patients with colorectal cancer.23 However, whether the expression of claudin-5 is directly regulated by ß-catenin in BMECs remains unclear. Further studies are clearly needed to obtain a detailed account of the mechanisms of the control of claudin-5 expression by GSK-3ß.

Our results showed that BCRP expression was influenced by BIO in hiPS cell-derived BMECs. Lim et al. have demonstrated that the expression of BCRP was upregulated by BIO in hCMEC/D3 cells.24 This result is consistent with our results. However, our data demonstrated that Chir99021 did not affect the expression of BCRP in hiPS cell-derived BMECs. BIO is also known to control the inhibition of cyclin-dependent kinases (CDKs). On the other hand, Chir99021 does not exhibit cross-reactivity against CDKs. Previous study has shown that inhibition of CDK could be enhanced the expression of BCRP.25 Therefore, BIO might regulate the expression of BCRP via the cyclin-CDKs pathway in BMECs.

Qin and Sato have demonstrated that Wnt signaling pathways are activated in the developing brain blood vessels at the stage of P-gp appearance.26 It is also known that T-cell factor 4 (TCF4)/ß-catenin could directly regulate the expression of P-gp in human colorectal carcinoma cell lines.27 In addition, ß-catenin signaling can significantly influence the expression of P-gp by activating the MDR1 promoter in primary rat BMECs and hCMEC/D3 cells,24 although these cells can express a high level of P-gp. Therefore, the Wnt signaling pathway might contribute to the induction of P-gp expression. Indeed, our data showed that functional P-gp expression in hiPS cell-derived BMECs could be induced by the inhibition of GSK-3ß that resulted in the activation of the canonical Wnt
signaling pathway.

Overall, we demonstrated that GSK-3ß inhibitors, such as Chir99021 and BIO, could promote BBB integrity by increasing the expression of claudin-5. We also observed that P-gp expression in hiPS cell-derived BMECs could be regulated by GSK-3ß inhibitors. Our data indicate that the regulation of both P-gp and claudin-5 in hiPS cell-derived BMECs can be influenced by Wnt signaling. Therefore, the present results would provide useful information for improving cell quality.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

REFERENCES