Wnt signaling mediates acquisition of blood-brain barrier properties in naïve endothelium derived from human pluripotent stem cells

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ABSTRACT

Endothelial cells (ECs) in the central nervous system (CNS) acquire their specialized blood-brain barrier (BBB) properties in response to extrinsic signals, with Wnt/β-catenin signaling coordinating multiple aspects of this process. Our knowledge of CNS EC development has been advanced largely by animal models, and human pluripotent stem cells (hPSCs) offer the opportunity to examine BBB development in an in vitro human system. Here we show that activation of Wnt signaling in hPSC-derived naïve endothelial progenitors, but not in matured ECs, leads to robust acquisition of canonical BBB phenotypes including expression of GLUT-1, increased claudin-5, decreased PLVAP and decreased permeability. RNA-seq revealed a transcriptome profile resembling ECs with CNS-like characteristics, including Wnt-upregulated expression of LEF1, APCDD1, and ZIC3. Together, our work defines effects of Wnt activation in naïve ECs and establishes an improved hPSC-based model for interrogation of CNS barriergenesis.

KEYWORDS

Endothelial cells, blood-brain barrier, barriergenesis, Wnt signaling

IMPACT STATEMENT

Activation of Wnt/β-catenin signaling in endothelial progenitors derived from human pluripotent stem cells partially induces the specialized blood-brain barrier phenotype, while the same treatment in matured endothelial cells is less efficacious.
INTRODUCTION

In the central nervous system (CNS), vascular endothelial cells (ECs) are highly specialized, with complex tight junctions, expression of a spectrum of nutrient and efflux transporters, low rates of vesicle trafficking, no fenestrae, and low expression of immune cell adhesion molecules (Reese and Karnovsky, 1967; Obermeier et al., 2013). ECs bearing these attributes, often referred to as the blood-brain barrier (BBB), work in concert with the other brain barriers to facilitate the tight regulation of the CNS microenvironment required for proper neuronal function (Daneman and Engelhardt, 2017; Profaci et al., 2020). During development, the Wnt/β-catenin signaling pathway drives both CNS angiogenesis, during which vascular sprouts originating from the perineural vascular plexus invade the developing neural tube, and the coupled process of barriergenesis by which resulting ECs begin to acquire BBB properties (Liebner et al., 2008; Stenman et al., 2008; Daneman et al., 2009; Engelhardt and Liebner, 2014; Umans et al., 2017). Specifically, neural progenitor-derived Wnt7a and Wnt7b ligands signal through Frizzled receptors and the obligate co-receptors RECK and GPR124 (ADGRA2) on endothelial cells (Kuhnert et al., 2010; Cullen et al., 2011; Vanhollebeke et al., 2015; Cho et al., 2017; Eubelen et al., 2018; Vallon et al., 2018). Other ligands function analogously in other regions of the CNS, including Norrin in the retina and cerebellum (Ye et al., 2009; Wang et al., 2012) and potentially Wnt3a in the dorsal neural tube (Daneman et al., 2009). Furthermore, Wnt/β-catenin signaling is required for maintenance of CNS EC barrier properties in adulthood (Tran et al., 2016), with astrocytes as a major source of Wnt7 ligands (He et al., 2018; Vanlandewijck et al., 2018; Guérit et al., 2021).

Molecular hallmarks of Wnt-mediated CNS EC barriergenesis are (i) acquisition of glucose transporter GLUT-1 expression, (ii) loss of plasmalemma vesicle-associated protein
(PLVAP), and (iii) upregulation of claudin-5 (Daneman et al., 2009; Kuhnert et al., 2010; Cho et al., 2017; Umans et al., 2017; Wang et al., 2019). Notably, the Wnt-mediated switch between the “leaky” EC phenotype (GLUT-1\(^{-}\) PLVAP\(^{+}\) claudin-5\(^{\text{low}}\)) and the barrier EC phenotype (GLUT-1\(^{+}\) PLVAP\(^{-}\) claudin-5\(^{\text{high}}\)) correlates with reduced permeability to molecular tracers (Wang et al., 2012; Cho et al., 2017) and is conserved in multiple contexts. For instance, medulloblastomas that produce Wnt-inhibitory factors have leaky vessels (Phoenix et al., 2016). Moreover, vasculature perfusing circumventricular organs is leaky due to low levels of Wnt signaling (Benz et al., 2019; Wang et al., 2019). Notably, ectopic activation of Wnt in ECs of circumventricular organs induces GLUT-1 and suppresses PLVAP (Benz et al., 2019; Wang et al., 2019).

However, similar ectopic activation of Wnt in liver and lung ECs produces only very minor barriergenic effects (Munji et al., 2019), and Wnt activation in cultured primary mouse brain ECs does not prevent culture-induced loss of barrier-associated gene expression (Sabbagh and Nathans, 2020). The reasons for the apparent context-dependent impacts of Wnt activation in ECs remain unclear and motivate systematic examination of this process in a simplified model system. Further, given species differences in brain EC transporter expression (Uchida et al., 2011), drug permeability (Syvänen et al., 2009), and gene expression (Song et al., 2020), this process warrants investigation in human cells to complement mouse \textit{in vivo} studies.

Prior studies have evaluated the impact of Wnt activation in immortalized human brain ECs and observed only modest effects on barrier phenotype (Paolinelli et al., 2013; Laksitorini et al., 2019). Combined with the aforementioned deficits observed in primary adult mouse brain endothelial cells that are not rescued by ectopic Wnt activation (Sabbagh and Nathans, 2020), one possibility is that mature, adult endothelium is largely refractory to Wnt activation, and that Wnt responsiveness is a property of immature endothelial cells analogous to those in the
perineural vascular plexus. Human pluripotent stem cells (hPSCs) offer an \textit{in vitro} human model system for systematic investigation of molecular mechanisms of BBB phenotype acquisition, especially given their ability to model early stages of endothelial specification and differentiation. However, currently available hPSC-based models of CNS endothelial-like cells are not well suited for modeling the BBB developmental progression as they do not follow a developmentally-relevant differentiation trajectory, lack definitive endothelial identity, or have been incompletely characterized with respect to the role of developmental signaling pathways (Lippmann et al., 2020; Workman and Svendsen, 2020; Lu et al., 2021). As a potential alternative, hPSCs can also be used to generate immature, naïve endothelial progenitors (Lian et al., 2014) that could be used to better explore the induction of BBB phenotypes. For example, we recently reported that extended culture of such hPSC-derived endothelial progenitors in a minimal medium yielded ECs with improved BBB tight junction protein expression and localization which led to improved paracellular barrier properties (Nishihara et al., 2020). However, as shown below, these cells exhibit high expression of PLVAP and little expression of GLUT-1, indicating the need for additional cues to drive CNS EC specification.

In this work, we aimed to define the effects of activating Wnt/β-catenin signaling in hPSC-derived, naïve endothelial progenitors and assess the extent to which this strategy would drive development of a CNS EC-like phenotype. We found that many aspects of the CNS EC phenotype, including the canonical GLUT-1, claudin-5, and PLVAP expression effects, were regulated by CHIR 99021, a small molecule agonist of Wnt/β-catenin signaling. CHIR treatment in matured ECs produced a more limited response. Whole-transcriptome analysis revealed definitive endothelial identity of the resulting cells and CHIR-upregulated expression of known CNS EC transcripts, including \textit{LEF1, APCDD1, AXIN2, SLC2A1, CLDN5, LSR, ABCG2, SOX7,}
and ZIC3. We also observed an unexpected CHIR-mediated upregulation of caveolin-1, which did not, however, correlate with increased uptake of a dextran tracer. Thus, we provide evidence that Wnt activation in hPSC-derived naïve endothelial progenitors is sufficient to induce many aspects of the CNS barrier EC phenotype, and we establish a model system for further systematic investigation of putative barriergenic cues.
RESULTS

Wnt activation in hPSC-derived endothelial progenitors

We adapted an existing protocol to produce endothelial progenitor cells (EPCs) from hPSCs (Lian et al., 2014; Bao et al., 2016) (Figure 1A). To achieve mesoderm specification, this method employs an initial activation of Wnt/β-catenin signaling with CHIR 99021 (CHIR), a small molecule inhibitor of glycogen synthase kinase-3 (GSK-3), which results in inhibition of GSK-3β-mediated β-catenin degradation. After 5 days of expansion, the resulting cultures contained a mixed population of CD34+CD31+EPCs and CD34−CD31−non-EPCs (Figure 1B-C). We used magnetic-activated cell sorting (MACS) to isolate CD31+ cells from this mixed culture and plated these cells on collagen IV-coated plates in a minimal endothelial cell medium termed hECSR (Nishihara et al., 2020). We first asked whether Wnt3a, a ligand widely used to activate canonical Wnt/β-catenin signaling (Kim et al., 2005, 2008; Liebner et al., 2008; Cecchelli et al., 2014; Praça et al., 2019), could induce GLUT-1 expression in the resulting ECs. After 6 days of treatment, we observed a significant increase in the fraction of GLUT-1+E Cs in Wnt3a-treated cultures compared to controls (Figure 1D-E). Consistent with previous observations (Nishihara et al., 2020), we also detected a population of calponin+ smooth muscle protein 22-α+ putative smooth muscle-like cells (SMLCs) outside the endothelial colonies (Figure 1–figure supplement 1) and these SMLCs expressed GLUT-1 in both control and Wnt3a-treated conditions (Figure 1D).

Based on these promising results with Wnt3a, we next tested a low concentration (4 µM) of the GSK-3 inhibitor CHIR because of its ability to activate Wnt signaling in a receptor/coreceptor-independent manner. In addition to GLUT-1, we evaluated expression of two other key proteins: claudin-5, which is known to be upregulated in CNS ECs in response to Wnt (Benz et
al., 2019), and caveolin-1, given the low rate of caveolin-mediated transcytosis in CNS compared to non-CNS ECs (Reese and Karnovsky, 1967; Andreone et al., 2017) (Figure 2A). 4 
µM CHIR robustly induced GLUT-1 expression in approximately 90% of ECs while increasing 
EC number and increasing EC purity to nearly 100% (Figure 2B). Furthermore, CHIR led to an 
approximately 1.5-fold increase in average claudin-5 abundance and a 10- to 30-fold increase in 
GLUT-1 abundance, but also a 2- to 4-fold increase in caveolin-1 (Figure 2B). We therefore 
titrated CHIR to determine an optimal concentration for EC expansion, purity, GLUT-1 
induction, and claudin-5 upregulation while limiting the undesirable non-CNS-like increase in 
caveolin-1 abundance. Although 2 µM CHIR did not lead to increased caveolin-1 expression 
compared to vehicle control (DMSO), it also did not elevate claudin-5 or GLUT-1 expression 
compared to control and was less effective in increasing EC number and EC purity than 4 µM 
CHIR (Figure 2–figure supplement 1). On the other hand, 6 µM CHIR further increased GLUT-1 
abundance but also further increased caveolin-1 abundance and did not improve EC number, EC 
purity, or claudin-5 expression (Figure 2–figure supplement 1). Therefore, we conducted further 
experiments using 4 µM CHIR. We confirmed that the CHIR-mediated increases in EC purity, 
EC number, and caveolin-1 and GLUT-1 expression were conserved in an additional hPSC line, 
although claudin-5 upregulation was not apparent (Figure 2–figure supplement 2). We also used 
two hPSC lines with doxycycline-inducible expression of short hairpin RNAs targeting CTNNB1 
(β-catenin) to confirm that CHIR-mediated upregulation of GLUT-1 in ECs was β-catenin-
dependent. Indeed, doxycycline treatment in combination with CHIR significantly reduced 
GLUT-1 abundance in ECs derived from these hPSC lines (Figure 2–figure supplement 3). 
Finally, we confirmed that increased EC number was the result of increased EC proliferation in 
CHIR-treated cultures (Figure 2–figure supplement 4). Together, these results suggest that
activation of the Wnt/β-catenin pathway is capable of inducing CNS-like phenotypes in hPSC-derived endothelial progenitors.

**Effects of CHIR-mediated Wnt activation in endothelial progenitors**

Since CHIR elicited a robust Wnt-mediated response, we next asked whether other aspects of the CNS EC barrier phenotype were CHIR-regulated. PLVAP, a protein that forms bridges across both caveolae and fenestrae (Herrnberger et al., 2012), is one such canonically Wnt-downregulated protein. We therefore first evaluated PLVAP expression in Passage 1 control (DMSO) or CHIR-treated ECs using confocal microscopy (Figure 3A). We observed numerous PLVAP⁺ punctate vesicle-like structures in both conditions, with CHIR treatment reducing PLVAP abundance by approximately 20% (Figure 3A-B). This effect was not apparent in Western blots of Passage 1 ECs, likely due to the relatively modest effect (Figure 4A-B).

However, after two more passages (Figure 1A), Passage 3 ECs demonstrated a robust downregulation of PLVAP in CHIR-treated cells compared to controls (Figure 4C-D). We also used Western blotting to confirm CHIR-mediated upregulation of GLUT-1 and claudin-5 both at Passage 1 and Passage 3 (Figure 4A-D). We next evaluated expression of the tricellular tight junction protein LSR (angulin-1) because of its enrichment in CNS versus non-CNS ECs, and the temporal similarity between LSR induction and the early stage of Wnt-mediated CNS barriergenesis (Sohet et al., 2015). We found that CHIR treatment led to a strong increase in LSR expression in both Passage 1 and Passage 3 ECs (Figure 4A-D), suggesting that Wnt signaling upregulates multiple necessary components of the CNS EC bicellular and tricellular junctions.

CHIR treatment produced two apparently competing changes in ECs related to vesicular transport: an expected downregulation of PLVAP and an unexpected upregulation of caveolin-1.
We therefore asked whether the rate of total fluid-phase endocytosis differed between CHIR-treated and control ECs, using a fluorescently-labeled 10 kDa dextran as a tracer. After incubating Passage 1 cultures with dextran for 2 h at 37°C, we used flow cytometry to gate CD31+ ECs and assess total dextran accumulation (Figure 5A-B). In ECs incubated at 37°C, CHIR treatment did not change the geometric mean dextran signal compared to DMSO (Figure 5B,C), but did cause a broadening of the distribution of dextran intensities as quantified by the coefficient of variation (CV), indicative of sub-populations of cells with decreased and increased dextran uptake (Figure 5B,D). We confirmed that the dextran signal measured by this assay was endocytosis-dependent by carrying out the assay at 4°C and with inhibitors of specific endocytic pathways (Figure 5–figure supplement 1A-C). Compared to vehicle control, chlorpromazine (inhibitor of clathrin-mediated endocytosis) and rottlerin (inhibitor of macropinocytosis) both decreased dextran uptake, while nystatin (inhibitor of caveolin-mediated endocytosis) did not significantly affect uptake (Figure 5–figure supplement 1B,C), consistent with the very small number of dextran+caveolin-1+ puncta observed by confocal imaging (Figure 5–figure supplement 1D). Thus, despite the generally uniform elevation of caveolin-1 and decrease of PLVAP observed by immunocytochemistry in CHIR-treated ECs, our functional assay suggests neither an overall increase nor decrease in total fluid-phase endocytosis. Instead, it indicates that CHIR increases the heterogeneity of the EC population with respect to the rate of endocytosis.

We also compared the paracellular barrier properties of DMSO- and CHIR-treated ECs. Because Passage 1 cultures contain SMLCs that preclude formation of a confluent endothelial monolayer, we evaluated paracellular barrier properties of Passage 3 ECs that had undergone selective dissociation and replating (see Materials and Methods), a strategy that effectively purifies the cultures (Nishihara et al., 2020). CHIR-treated Passage 3 ECs had elevated...
transendothelial electrical resistance (TEER) (Figure 5E) and decreased permeability to the small molecule tracer sodium fluorescein (Figure 5F). Together, these results are consistent with CHIR-mediated increases to tight junction protein expression (e.g., claudin-5 and LSR) and suggest Wnt activation leads to functional improvements to paracellular barrier in this system.

Given the relatively weak responses to Wnt activation in adult mouse liver ECs in vivo (Munji et al., 2019) and adult mouse brain ECs cultured in vitro (Sabbagh and Nathans, 2020), we sought to determine whether the immature, potentially more plastic state of hPSC-derived endothelial progenitors contributed to the relatively robust CHIR-mediated response we observed. To test this hypothesis, we matured hPSC-derived ECs in vitro for 4 passages (until approximately day 30) prior to initiating CHIR treatment for 6 days, and compared the resulting cells to differentiation-matched samples treated with CHIR immediately after MACS (Figure 6A). Both Passage 1 DMSO-treated ECs and Passage 5 DMSO-treated ECs, which are analogous to EECM-BMEC-like cells we previously reported (Nishihara et al., 2020), did not have detectable GLUT-1 expression (Figure 6B). Compared to DMSO controls, the CHIR-treated Passage 5 ECs exhibited no increase in GLUT-1 abundance (Figure 6B-D), which contrasts with the marked increase observed when CHIR treatment was initiated immediately after MACS (Figure 6B-D). Furthermore, CHIR treatment in matured ECs did not increase claudin-5 expression and did not increase EC number (Figure 6B-D), in contrast to the increases observed in both properties when treatment was initiated immediately after MACS (Figure 6B-D). We observed a similar lack of robust GLUT-1 induction in an additional differentiation and an additional hPSC line in which CHIR treatment was carried out at Passage 4 (Figure 6–figure supplement 1). Together, these data suggest that early, naïve endothelial progenitors are more responsive to Wnt activation than more mature ECs derived by the same differentiation protocol.
Comprehensive profiling of the Wnt-regulated endothelial transcriptome

We turned next to RNA-sequencing as an unbiased method to assess the impacts of Wnt activation on the EC transcriptome. We performed four independent differentiations and analyzed Passage 1 ECs treated with DMSO or CHIR, using fluorescence-activated cell sorting (FACS) to isolate CD31+ ECs from the mixed EC/SMLC cultures. We also sequenced the SMLCs from DMSO-treated cultures at Passage 1 from two of these differentiations. DMSO- and CHIR-treated ECs at Passage 3 from three of these differentiations were also sequenced. Principal component analysis of the resulting whole-transcriptome profiles revealed that the two cell types (ECs and SMLCs) segregated along principal component (PC) 1, which explained 57% of the variance. In ECs, the effects of passage number and treatment were reflected in PC 2, which explained 21% of the variance (Figure 7A). We next validated the endothelial identity of our cells; we observed that canonical endothelial marker genes (including CDH5, CD34, PECAM1, CLDN5, ERG, and FLI1) were enriched in ECs compared to SMLCs and had high absolute abundance, on the order of 100–1,000 transcripts per million (TPM) (Figure 7B; Supplementary file 1). SMLCs expressed mesenchymal (mural/fibroblast)-related transcripts (including PDGFRB, CSPG4, PDGFRA, TBX2, CNN1, and COL1A1), which ECs generally lacked, although we did observe slight enrichment of some of these genes in Passage 1 DMSO-treated ECs, likely reflective of a small amount of SMLC contamination despite CD31 FACS (Figure 7B). SMLCs also expressed SLC2A1 (Supplementary file 1) consistent with protein-level observations (Figure 1D). We also observed little to no expression of the epithelial genes CDH1, EPCAM, CLDN1, CLDN3 (Castro Dias et al., 2019), CLDN4, and CLDN6, reflecting the definitive endothelial nature of the cells (Figure 7B; Supplementary file 1).
First comparing CHIR- and DMSO-treated ECs at Passage 1, we identified 1,369 significantly upregulated genes and 2,037 significantly downregulated genes (Figure 7C; Supplementary file 2). CHIR-upregulated genes included SLC2A1, CLDN5, LSR, and CAV1, consistent with protein-level assays. PLVAP was downregulated, as were a number of mesenchymal genes (TAGLN, COL1A1), again reflective of slight contamination of SMLC transcripts in the DMSO-treated EC samples (Figure 7C-D). Additionally, important downstream effectors of Wnt signaling were upregulated, including the transcription factors LEF1 and TCF7, the negative regulator AXIN2, and the negative regulator APCDD1, which is known to modulate Wnt-regulated barrierogenesis in retinal endothelium (Mazzoni et al., 2017) (Figure 7C-D). We also identified upregulated transcription factors: ZIC3, which is highly enriched in brain and retinal ECs in vivo and downstream of Frizzled4 signaling (Wang et al., 2012; Sabbagh et al., 2018), and SOX7, which acts cooperatively with SOX17 and SOX18 in retinal angiogenesis (Zhou et al., 2015), were upregulated by CHIR in our system (Figure 7D). MSX1 and EBF1, which are expressed by murine brain ECs in vivo (Vanlandewijck et al., 2018) were also CHIR-upregulated (Figure 7D). Additional CHIR-upregulated genes included ABCG2 (encoding the efflux transporter Breast Cancer Resistance Protein, BCRP), APLN, a tip cell marker enriched in postnatal day 7 murine brain ECs compared to those of other organs, and subsequently downregulated in adulthood (Sabbagh et al., 2018; Sabbagh and Nathans, 2020), and FLVCR2, a disease-associated gene with a recently-identified role in brain angiogenesis (Santander et al., 2020) (Figure 7C-D). Finally, we detected CHIR-mediated downregulation of the fatty acid-binding protein-encoding FABP4, which is depleted in brain ECs compared to those of peripheral organs (Sabbagh et al., 2018). We also observed similar downregulation of SMAD6, which is depleted in brain ECs compared to lung ECs and is a putative negative regulator of
BMP-mediated angiogenesis (Mouillesseaux et al., 2016; Vanlandewijck et al., 2018) (Figure 7D).

In Passage 3 ECs, many of the CHIR-mediated gene expression changes observed at Passage 1 persisted, including SLC2A1, LSR, LEF1, AXIN2, APCDD1, ZIC3, EBF1, FLVCR2 and ABCG2 upregulation and PLVAP downregulation (Figure 7E; Figure 7–figure supplement 1). Additional concordantly CHIR-upregulated genes encoding secreted factors, transcription factors, and transmembrane proteins are shown in Figure 7–figure supplement 2, and include REEP1, a gene enriched in brain versus non-brain ECs (Sabbagh et al., 2018; Vanlandewijck et al., 2018) that encodes a regulator of endoplasmic reticulum function, and the Notch ligand-encoding gene JAG2. On the other hand, at Passage 3, CLDN5 was not upregulated in CHIR-treated cells compared to DMSO-treated cells, but was highly expressed (~2,500 TPM).

Similarly, CAV1 abundance remained high, but was not CHIR-upregulated in Passage 3 cells (Figure 7–figure supplement 1). Conversely, JAM2, which encodes junctional adhesion molecule 2, a component of EC tight junctions (Aurrand-Lions et al., 2001; Tietz and Engelhardt, 2015), was upregulated by CHIR at Passage 3, but not at Passage 1, as was the retinol-binding protein-encoding gene RBP1 (Figure 7–figure supplement 1).

We used Weighted Gene Correlation Network Analysis (WGCNA) (Zhang and Horvath, 2005; Langfelder and Horvath, 2008) to identify modules containing genes with highly correlated expression across the 14 EC samples (Figure 7–figure supplement 3A; Supplementary file 3). One such module (the green module, containing 441 genes) had a representative gene expression profile (module eigengene) with a strong, positive correlation with CHIR treatment (Figure 7–figure supplement 3B). Importantly, genes central to this module included canonical transcriptional targets of Wnt/β-catenin signaling, including AXIN2 and APCDD1, further
supporting the key role of β-catenin signaling in transcriptional changes observed in CHIR-treated ECs. Additional central (highly correlated) genes within the green module included SLC2A1, ZIC3, and FLVCR2, consistent with pairwise differential expression analysis, transcription factors (CASZ1, PRRX1), and genes with putative roles in vesicle trafficking (SNX4, ARL8B, AP1AR, VTI1A, VPS41) and lipid metabolism (AGPAT5, ASAHI) (Figure 7–figure supplement 3C).

To determine the effects of extended culture, we next compared control (DMSO-treated) ECs at Passage 3 versus Passage 1 (Figure 7–figure supplement 4; Supplementary file 2). Extended culture to Passage 3 in the absence of exogeneous Wnt activation led to 1,521 upregulated genes, including CLDN5 and CAV1, consistent with previously-reported protein-level observations in EECM-BMEC-like cells (Nishihara et al., 2020), which are analogous to Passage 3 DMSO-treated cells. We also observed 1,625 downregulated genes, including marked downregulation of PLVAP (Figure 7–figure supplement 4). SLC2A1, however, was not upregulated at Passage 3 (Figure 7–figure supplement 4), concordant with absence of GLUT-1 protein expression in the control ECs (Figure 6B), nor was LSR. Further, despite some similarly-regulated genes between the passage number and CHIR treatment comparisons (e.g., CLDN5, CAV1, PLVAP), the transcriptional responses to these two experimental variables were globally distinct as assessed by gene correlation network analysis (Figure 7–figure supplement 3B). We also evaluated transcript-level expression of components of the Wnt signaling pathway in Passage 3 control (DMSO-treated) ECs as a first step towards understanding the relative lack of responsiveness observed when CHIR treatment was initiated in matured (Passage 4) ECs (Figure 7–figure supplement 4). While CTNNB1, GSK3B, and genes encoding components of the
destruction complex were not significantly different between Passage 3 and Passage 1, \textit{LEF1} and \textit{TCF7} were strongly downregulated in Passage 3 cells (Figure 7–figure supplement 4).

Finally, to further understand the strengths and limitations of this model system both as a readout of early developmental changes in CNS ECs (Passage 1 cells) or as a source of CNS-like ECs for use in downstream modeling applications, we evaluated absolute transcript abundance and effects of treatment or passage number on 53 characteristic CNS EC genes encompassing tight junction components, vesicle trafficking machinery, solute carriers, and ATP-binding cassette (ABC) efflux transporters selected based on high expression in human brain endothelial cells from a meta-analysis of single cell RNA-seq data (Gastfriend et al., 2021) (Figure 7–figure supplement 5). While ECs expressed \textit{CLDN5}, \textit{TJP1}, \textit{TJP2}, \textit{OLCN}, and \textit{LSR}, they lacked \textit{MARVELD2} (encoding tricellulin) under all conditions. ECs under all conditions also lacked \textit{MFSD2A} and, despite CHIR-mediated downregulation of \textit{PLVAP}, retained high absolute expression of this and other caveolae-associated genes. Finally, while many solute carriers and ABC transporters were expressed (\textit{SLC2A1}, \textit{SLC3A2}, \textit{SLC16A1}, \textit{SLC38A2}, \textit{ABCG2}), others expressed at the \textit{in vivo} human BBB were not (\textit{SLC5A3}, \textit{SLC7A11}, \textit{SLC38A3}, \textit{SLCO1A2}, \textit{ABCB1}) (Figure 7–figure supplement 5). Thus, while CHIR treatment yields ECs with certain elements of CNS-like character, additional molecular signals are likely necessary to impart other aspects of the \textit{in vivo} CNS EC phenotype.

The \textit{Wnt-regulated endothelial transcriptome in multiple contexts}

To globally assess whether CHIR-mediated gene expression changes in our system are characteristic of the responses observed in ECs \textit{in vivo} and similar to those observed in other \textit{in vitro} contexts, we compared our RNA-seq dataset to those of studies that employed a genetic
strategy for β-catenin stabilization (the \textit{Ctnnb1}^{\text{flex3}} allele) in adult mouse ECs in several contexts: (i) pituitary ECs, which acquire some BBB-like properties upon β-catenin stabilization (Wang et al., 2019); (ii) liver ECs, which exhibit little to no barriergenic response to β-catenin stabilization (Munji et al., 2019); (iii) brain ECs briefly cultured \textit{in vitro}, which rapidly lose their BBB-specific gene expression profile even with β-catenin stabilization (Sabbagh and Nathans, 2020), and offer the most direct comparison to our \textit{in vitro} model system. Upon recombination, the \textit{Ctnnb1}^{\text{flex3}} allele produces a dominant mutant β-catenin lacking residues that are phosphorylated by GSK-3β to target β-catenin for degradation (Harada et al., 1999); as such, this strategy for ligand- and receptor-independent Wnt activation by β-catenin stabilization is similar to CHIR treatment, although GSK-3 phosphorylates targets other than β-catenin (discussed below).

We first used literature RNA-seq data from postnatal day 7 murine brain, liver, lung, and kidney ECs (Sabbagh et al., 2018) to define core sets of genes in brain ECs that are differentially expressed compared to all three of the other organs (Figure 8A-B). Using the resulting sets of 1094 brain-enriched and 506 brain-depleted genes, we asked how many genes in our Passage 1 ECs were concordantly-regulated by CHIR: 130 of the brain-enriched genes were CHIR-upregulated and 116 of the brain-depleted genes were CHIR-downregulated (Figure 8C). At Passage 3, 61 genes were concordantly upregulated and 46 downregulated (Figure 8–figure supplement 1). In pituitary ECs with β-catenin stabilization, 102 of the brain-enriched genes were upregulated and 48 of the brain-depleted genes were downregulated (Figure 8D).

Compared with the pituitary ECs, there were far fewer concordantly-regulated genes in liver ECs with β-catenin stabilization, with 25 upregulated and 1 downregulated (Figure 8E). Finally, cultured primary mouse brain ECs with β-catenin stabilization exhibited 72 concordantly upregulated and 16 downregulated genes (Figure 8F). The only gene concordantly-regulated in
all four comparisons was the canonical Wnt target $AXIN2$. Several additional genes were concordantly upregulated in three of four, including $TCF7$, $FAM107A$, $NKD1$, $TNFRSF19$, $GLUL$, $SLC30A1$, and $ABCB1$, which was the only gene concordantly regulated in all comparisons except the hPSC-derived ECs (Figure 8G). Several canonical target genes were shared by the hPSC-derived EC and pituitary EC systems, including $APCDD1$, $LEF1$, $CLDN5$, and $SLC2A1$; also in this category were $LSR$, the zinc/manganese transporter $SLC39A8$, and 12 additional genes (Figure 8G). Notably, the caveolae inhibitor $MFSD2A$ was robustly upregulated by β-catenin in pituitary ECs, but not in any other context (Figure 8C-F), suggesting other brain-derived factors may cooperate with Wnt to regulate expression of this important inhibitor of caveolín-mediated transcytosis. Complete gene lists from this comparative analysis are provided in Supplementary file 4. In sum, the data suggest that the hPSC-derived ECs responded to Wnt activation in a fashion that led to modest induction of CNS transcriptional programs and that the response was most similar to the pituitary β-catenin stabilization model. Importantly, this analysis also supports the hypothesis that immature endothelium is highly responsive to Wnt activation where mature (adult) endothelium is largely refractory except in regions proximal to barrier-forming regions.

Last, because GSK-3 is a component of numerous signaling pathways in addition to Wnt/β-catenin (Eto et al., 2005; Beurel et al., 2015; Hermida et al., 2017), we used RNA-seq data to infer pathways that might be differentially regulated by the two strategies for activating Wnt/β-catenin signaling employed in the experiments above: CHIR treatment, which increases β-catenin stability by inhibiting GSK-3, or direct stabilization of β-catenin. We tested lists of upregulated genes in (i) our Passage 1 ECs treated with CHIR versus DMSO, (ii) Passage 3 ECs treated with CHIR versus DMSO, and (iii) pituitary ECs with β-catenin stabilization versus
controls (Wang et al., 2019), against the Hallmark gene set collection (Liberzon et al., 2015) (Figure 8–figure supplement 2; Supplementary file 5). In all three comparisons, the Wnt/β-catenin signaling gene set was significantly enriched (Figure 8–figure supplement 2A).

Similarly, the Notch signaling, TNFα signaling via NF-κB, KRAS signaling up, and several additional gene sets were consistently enriched in all three comparisons (Figure 8–figure supplement 2A-B; Supplementary file 5), suggesting similar regulation by GSK-3 inhibition and direct β-catenin stabilization. In contrast, the PI3K AKT mTOR signaling gene set was enriched in Passage 1 ECs, but not in Passage 3 ECs or pituitary ECs. Similarly, the gene set mTORC1 signaling was enriched in Passage 1 ECs and pituitary ECs, but genes driving this enrichment were distinct (Figure 8–figure supplement 2C), and this gene set was not enriched in Passage 3 ECs. Thus, given the known, bidirectional interactions of GSK-3 and AKT/mTOR pathway components (Hermida et al., 2017), these results suggest that CHIR-mediated inhibition of GSK-3 may transiently activate this pathway in Passage 1 ECs. Conversely, the gene set TGF-β signaling was enriched only in pituitary ECs with β-catenin stabilization (Figure 8–figure supplement 2; Supplementary file 5). Taken together, these results, coupled with those of our CTNNB1 knockdown experiments and gene correlation network analysis, suggest a central role for β-catenin as a key effector of CHIR-mediated signaling, but also highlight some potential differences in the pathways activated in response to CHIR treatment versus β-catenin stabilization. Differences in other aspects of these two experimental paradigms (in vitro versus in vivo, naïve versus CNS-proximal, human versus mouse), however, caution against over-interpretation of these results.
The Wnt/β-catenin signaling pathway plays a central role in CNS angiogenesis and in establishing the unique properties of CNS ECs (Liebner et al., 2008; Stenman et al., 2008; Daneman et al., 2009; Kuhnert et al., 2010; Cullen et al., 2011; Vanhollebeke et al., 2015; Cho et al., 2017). In this work, we investigated the role of Wnt/β-catenin signaling in induction of BBB properties in a human EC model, using naïve endothelial progenitors derived from hPSCs. We reasoned that these immature EPCs (Lian et al., 2014) would be similar to the immature endothelium in the perineural vascular plexus and thus competent to acquire CNS EC phenotypes in response to Wnt activation. To activate Wnt signaling, we evaluated the widely used ligand Wnt3a (Liebner et al., 2008) and the GSK-3 inhibitor CHIR.

We found that CHIR treatment robustly induced several canonical CNS EC molecular phenotypes, including a marked induction of GLUT-1, upregulation of claudin-5, and downregulation of PLVAP, which correlated with differential gene expression in RNA-seq data. We also observed a functional decrease in paracellular permeability. Further, using RNA-seq and Western blotting, we identified LSR (angulin-1) as CHIR-induced in this system, supporting the notion that this highly CNS EC-enriched tricellular tight junction protein (Daneman et al., 2010a; Sohet et al., 2015) is Wnt-regulated. In RNA-seq data, we observed differential expression of known CNS EC-enriched/depleted and Wnt-regulated genes including upregulated LEF1, AXIN2, APCDD1, ABCG2, SOX7, ZIC3, FLVCR2, JAM2, and RBP1, and downregulated PLVAP, FABP4, SMAD6, and SLIT2. These RNA-seq data should therefore be useful in generating hypotheses of BBB-associated genes regulated by Wnt activation in ECs for future functional studies. Our work also defines an important set of phenotypes for which Wnt activation in ECs is not sufficient in our system: in the context of vesicle trafficking, we
observed caveolin-1 (CAV1) upregulation, no change in mean functional endocytosis, virtually no expression of MFSD2A, and high absolute PLVAP abundance in RNA-seq data despite CHIR-mediated downregulation. Given roles of brain pericytes in regulating PLVAP, MFSD2A, and functional transcytosis (Armulik et al., 2010; Daneman et al., 2010b; Ben-Zvi et al., 2014; Stebbins et al., 2019), and the observation that MFSD2A is Wnt-regulated in pituitary ECs in vivo (Wang et al., 2019), where pericytes are present, it is plausible that pericyte-derived cues are necessary in addition to Wnts to achieve the characteristically low rate of CNS EC pinocytosis.

Next, while ABCG2 (BCRP) was Wnt-induced in our system, other hallmark efflux transporters were not Wnt-regulated and either expressed at low levels (e.g., ABCC4, encoding MRP-4) or not expressed (e.g. ABCB1, encoding P-glycoprotein). Notably however, Abcb1a was Wnt-regulated in the three other β-catenin stabilization experiments from the literature that we evaluated (Munji et al., 2019; Wang et al., 2019; Sabbagh and Nathans, 2020). Thus, pericyte-derived cues, astrocyte-derived cues, and/or activation of the pregnane X or other nuclear receptors may be important for complete acquisition of the complement of CNS EC efflux transporters (Bauer et al., 2004; Berezowski et al., 2004; Praça et al., 2019).

CHIR is widely used to activate Wnt/β-catenin signaling in cell culture (Lian et al., 2012, 2014; Patsch et al., 2015; Sakaguchi et al., 2015; Gomez et al., 2019; Pellegrini et al., 2020; Guo et al., 2021). It remains unknown, however, to what extent CHIR-mediated inhibition of GSK-3 in ECs mimics the effects of Wnt ligand-induced inhibition of GSK-3 or direct stabilization of β-catenin. In our system, although the GLUT-1-inductive effect of CHIR was partially inhibited by β-catenin knockdown and our RNA-seq data revealed a transcriptional response characteristic of canonical Wnt signaling, it is possible that CHIR affects other signaling pathways, as suggested by pathway enrichment analysis. Thus, employing ligand-based strategies to activate Wnt
signaling will be an important next step. Our RNA-seq data suggests that the receptors and
coreceptors necessary to transduce Wnt7 and Norrin signaling (e.g., FZD4, LRP6, RECK,
ADGRA2 [GPR124], TSPAN12, DVL2) are expressed by hPSC-derived ECs (Figure 7–figure
supplement 6). Given evidence that Wnt ligands have poor solubility (Janda et al., 2012) and our
preliminary data suggesting that supplementation of culture medium with Wnt7a and Wnt7b is
largely ineffective in activating Wnt/β-catenin signaling in this system, special emphasis should
be placed on strategies that present Wnt7a, Wnt7b, and/or Norrin in a manner that concentrates
ligands at the cell surface, for example, by using direct cocultures of endogenously Wnt-
producing cells (neural progenitors or astrocytes) or Wnt-overexpressing cells. Importantly,
novel progenitor cells and astrocytes likely would also contribute other yet-unidentified ligands
important for acquisition of CNS EC phenotype. Finally, it would also be informative to directly
compare CHIR and/or Wnt ligand treatment to direct stabilization of β-catenin in this system, for
example, by generating a hPSC line with inducible expression of a dominant active β-catenin.

We also directly addressed the hypothesis that immature ECs are more plastic, that is,
more competent to acquire BBB properties upon Wnt activation, than mature ECs. This
hypothesis is supported by existing observations that ectopic expression of Wnt7a is sufficient to
induce GLUT-1 expression in non-CNS regions of the mouse embryo (Stenman et al., 2008), but
β-catenin stabilization in adult mouse liver and lung ECs produces only a slight effect (Munji et
al., 2019). We repeated our CHIR treatment paradigm in hPSC-derived ECs after an extended
period of in vitro culture (Passage 4 ECs), and observed much weaker induction of GLUT-1 and
no pro-proliferative effect. Thus, our results support this hypothesis and suggest that the loss of
BBB developmental plasticity in ECs is an intrinsic, temporally-controlled process rather than a
result of the peripheral organ environment. The molecular mechanisms underlying this loss of

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plasticity remain poorly understood. While previous studies have demonstrated that the level of Wnt/β-catenin signaling in CNS ECs peaks early in development and subsequently declines (Corada et al., 2018; Hübner et al., 2018), this finding does not address mechanisms underlying the competence of ECs (CNS and non-CNS) to respond to Wnt signals. In RNA-seq data of Passage 3 control (DMSO-treated) ECs, \textit{LEF1} and \textit{TCF7} were strongly downregulated compared to Passage 1 cells. This result suggests that low baseline expression of these transcription factors, which form a complex with nuclear β-catenin to regulate Wnt target genes, may partially explain the poor efficacy of CHIR in matured ECs, although additional work is necessary to assess the functional relevance of these differences. Interestingly, ECs in non-BBB-forming regions of the CNS (i.e., CVOs), and in the anterior pituitary, which is directly proximal to the CNS, retain some of their plasticity in adulthood (Wang et al., 2019), possibly as the result of a delicate balance between Wnt ligands and Wnt-inhibitory factors in these regions. Our model should facilitate additional systematic examination of factors that may enhance or attenuate EC Wnt responsiveness.

Finally, our work establishes an improved hPSC-based model for investigating mechanisms of BBB development in naïve ECs. hPSCs are an attractive model system to complement \textit{in vivo} animal studies because they (i) are human, (ii) permit investigation of developmental processes in contrast to primary or immortalized cells, (iii) are highly scalable, (iv) can be derived from patients to facilitate disease modeling and autologous coculture systems, and (v) are genetically tractable. While widely used hPSC-based BBB models are useful for measuring molecular permeabilities and have been employed to understand genetic contributions to barrier dysfunction (Vatine et al., 2016, 2019; Lim et al., 2017), they have not been shown to proceed through a definitive endothelial progenitor intermediate (Lippmann et al., 2012; Lu et
al., 2021) and express epithelial-associated genes (Qian et al., 2017; Delsing et al., 2018; Vatine et al., 2019; Lu et al., 2021). Thus, new models with developmentally relevant differentiation trajectories and definitive endothelial phenotype are needed for improved understanding of developmental mechanisms. Motivated in part by prior use of endothelial cells derived from hematopoietic progenitors in human cord blood to generate BBB models (Boyer-Di Ponio et al., 2014; Cecchelli et al., 2014), we and others recently showed that hPSC-derived naïve endothelial progenitors or ECs are good candidates for such a system (Praça et al., 2019; Nishihara et al., 2020; Roudnicky et al., 2020a, 2020b). For example, Praça et al. showed that a combination of VEGF, Wnt3a, and retinoic acid directed EPCs to brain capillary-like ECs with moderate transendothelial electrical resistance (TEER) similar in order of magnitude to that reported here. We previously showed that BBB-like paracellular barrier characteristics are induced in hPSC-EPC-derived ECs after extended culture in a minimal medium. These so-called EEKM-BMEC-like cells had TEER and small molecule permeability similar to primary human brain ECs, well-developed tight junctions, and an immune cell adhesion molecule profile similar to brain ECs in vivo (Nishihara et al., 2020). In this study, we showed it was possible to use the small molecule Wnt agonist CHIR to induce additional hallmarks of CNS EC phenotype in hPSC-EPC-derived ECs, including canonical GLUT-1, claudin-5, and PLVAP effects (both Passage 1 and 3 CHIR-treated ECs). However, it is important to note that despite the improvements in CNS EC character with CHIR treatment, further improvements to functional endocytosis, and efflux transporter and solute carrier phenotype should be targets of future study and may be facilitated by cocultures and/or additional molecular factors. Along these lines, the Passage 1 CHIR-treated CNS-like ECs would be at a differentiation stage well suited to investigate cues subsequent to Wnt signaling that may be key for the induction of additional CNS EC properties. Alternatively,
the Passage 3 CHIR-treated CNS-like ECs may be suitable for other BBB modeling applications.

In summary, our work has defined the EC response to Wnt activation in a simplified, human system and established a new hPSC-derived \textit{in vitro} model that will facilitate improved understanding of endothelial barrier genesis.
### Key resources table

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hPSC maintenance

Tissue culture plates were coated with Matrigel, Growth Factor Reduced (Corning, Glendale, AZ). A 2.5 mg aliquot of Matrigel was thawed and resuspended in 30 mL DMEM/F-12 (Life Technologies, Carlsbad, CA), and the resulting solution used to coat plates at 8.7 µg/cm² (1 mL per well for 6-well plates; 0.5 mL per well for 12-well plates). Plates were incubated at 37°C for at least 1 h prior to use. hPSCs were maintained on Matrigel-coated plates in E8 medium (STEMCELL Technologies, Vancouver, Canada) at 37°C, 5% CO₂. hPSC lines used were: IMR90-4 iPSC, WTC11 iPSC, H9-CDH5-eGFP hESC, H9-7TGP-ishcat2 hESC, and 19-9-11-7TGP-ishcat3 iPSC. Medium was changed daily. When hPSC colonies began to touch, typically at approximately 70–80% confluence, cells were passaged using Versene (Life Technologies). Briefly, cells were washed once with Versene, then incubated with Versene for 7 min at 37°C. Versene was removed and cells were dissociated into colonies by gentle spraying with E8 medium. Cells were transferred at a split ratio of 1:12 to a new Matrigel-coated plate containing E8 medium. hPSC cultures were routinely tested for mycoplasma contamination using a PCR-based assay performed by the WiCell Research Institute (Madison, WI).

Endothelial progenitor cell differentiation

EPCs were differentiated according previously published protocols (Lian et al., 2014; Bao et al., 2016; Nishihara et al., 2020) with slight modifications. On day -3 (D-3), hPSCs were treated with Accutase (Innovative Cell Technologies, San Diego, CA) for 7 min at 37°C. The resulting single cell suspension was transferred to 4× volume of DMEM/F-12 (Life Technologies) and centrifuged for 5 min, 200×g. Cell number was quantified using a hemocytometer. Cells were resuspended in E8 medium supplemented with 10 µM ROCK inhibitor Y-27632 dihydrochloride (Tocris, Bristol, United Kingdom) and seeded on Matrigel-
coated 12-well plates at a density of (1.5–2.5)×10⁴ cells/cm², 1 mL per well. Cells were maintained at 37°C, 5% CO₂. On the following two days (D-2 and D-1), the medium was replaced with E8 medium. The following day (D0), differentiation was initiated by changing the medium to LaSR medium (Advanced DMEM/F-12 [Life Technologies], 2.5 mM GlutaMAX [Life Technologies], and 60 µg/mL L-ascorbic acid 2-phosphate magnesium [Sigma-Aldrich, St. Louis, MO]) supplemented with 7–8 µM CHIR 99021 (Tocris), 2 mL per well. The following day (D1), medium was replaced with LaSR medium supplemented with 7–8 µM CHIR 99021, 2 mL per well. On the following three days (D2, D3, and D4), the medium was replaced with pre-warmed LaSR medium (without CHIR), 2 mL per well.

On D5, EPCs were isolated using CD31 magnetic activated cell sorting (MACS). Cells were treated with Accutase for 15–20 min at 37°C. The resulting cell suspension was passed through a 40 µm cell strainer into an equal volume of DMEM (Life Technologies) supplemented with 10% FBS (Peak Serum, Wellington, CO) and centrifuged for 5 min, 200×g. Cell number was quantified using a hemocytometer. Cells were resuspended in MACS buffer (Dulbecco’s phosphate buffered saline without Ca and Mg [DPBS; Life Technologies] supplemented with 0.5% bovine serum albumin [Sigma-Aldrich] and 2 mM EDTA [Sigma-Aldrich]) at a concentration of 10⁷ cells per 100 µL. The CD31-FITC antibody (Miltenyi Biotec, Auburn, CA) was added to the cell suspension at a dilution of 1:50. The cell suspension was incubated for 30 min at room temperature, protected from light. The cell suspension was brought to a volume of 15 mL with MACS buffer and centrifuged for 5 min, 200×g. The supernatant was aspirated and the pellet resuspended in MACS buffer at a concentration of 10⁷ cells per 100 µL. The FITC Selection Cocktail from the EasySep Human FITC Positive Selection Kit (STEMCELL Technologies) was added at a dilution of 1:10 and the cell suspension was incubated for 20 min
at room temperature, protected from light. The Dextran RapidSpheres (magnetic particles) solution from the Selection Kit was added at a dilution of 1:20 and the cell suspension was incubated for an additional 15 min at room temperature.

The cell suspension was brought to a total volume of 2.5 mL with MACS buffer (for total cell number less than $2 \times 10^8$, the approximate maximum yield from two 12-well plates; for a larger number of plates/cells, a total volume of 5 mL was used). 2.5 mL of cell suspension was transferred to a sterile 5 mL round-bottom flow cytometry tube and placed in the EasySep magnet (STEMCELL Technologies) for 5 min. The magnet was inverted to pour off the supernatant, the flow tube removed, the retained cells resuspended in 2.5 mL of MACS buffer, and the flow tube placed back in the magnet for 5 min. This step was repeated 3 times, and the resulting cell suspension transferred to a centrifuge tube, and centrifuged for 5 min, 200×g. Cell number was quantified using a hemocytometer. Resulting EPCs were used directly for experiments as described below or cryopreserved in hECSR medium supplemented with 30% FBS and 10% DMSO for later use. hECSR medium is Human endothelial serum-free medium (Life Technologies) supplemented with 1× B-27 supplement (Life Technologies) and 20 ng/ml FGF2 (Waismann Biomanufacturing, Madison, WI).

**Endothelial cell culture and treatment**

Collagen IV (Sigma-Aldrich) was dissolved in 0.5 mg/mL acetic acid to a final concentration of 1 mg/mL. Collagen IV-coated plates were prepared by diluting a volume of this stock solution 1:100 in water, adding the resulting solution to tissue culture plates, or #1.5 glass bottom plates (Cellvis, Sunnyvale, CA) for cells intended for confocal imaging (1 mL per well for 6-well plates, 0.5 mL per well for 12-well plates, 0.25 mL per well for 24-well plates), and incubating the plates for 1 h at RT. Collagen IV coating solution was removed and EPCs
obtained as described above were suspended in hECSR medium and plated at approximately 3×10^4 cells/cm^2. In some experiments, ligands and small molecules were added to hECSR medium: CHIR 99021 (Tocris) was used at 4 µM except where indicated; DMSO (Sigma-Aldrich) was used as a vehicle control for CHIR; Wnt3a (R&D Systems) was used at 20 ng/mL; doxycycline was used at 1, 2, or 4 µg/mL. The hECSR medium, including any ligands or small molecules, was replaced every other day until confluent (typically 6 days). We denote this time point “Passage 1.”

For extended culture, ECs were selectively dissociated and replated as previously described (Nishihara et al., 2020). Cells were incubated with Accutase until endothelial cells appeared round, typically 2–3 min at 37°C. The plate was tapped to release the ECs while SMLCs remained attached, and the EC-enriched cell suspension transferred to 4× volume of DMEM/F-12 and centrifuged for 5 min, 200×g. Cells were resuspended in hECSR medium and seeded on a new collagen IV-coated plate at approximately 3×10^4 cells/cm^2. hECSR medium was replaced every other day until confluent (typically 6 days). The selective dissociation and seeding described above was repeated, and hECSR medium was again replaced every other day until confluent (typically 6 days). We denote this time point “Passage 3.” In one experiment, these steps were repeated for another two passages. Except where indicated, CHIR 99021 or vehicle (DMSO) was included in the hECSR medium for the entire duration of culture.

**RNA-seq**

RNA-seq was performed on ECs and SMLCs from the IMR90-4 hPSC line. Four independent differentiations were performed, with DMSO- and CHIR-treated ECs at Passage 1 analyzed from all four differentiations. DMSO- and CHIR-treated ECs at Passage 3 were analyzed from three of the four differentiations. DMSO-treated SMLCs at Passage 1 were
analyzed from two of the four differentiations. Fluorescence-activated cell sorting (FACS) was used to isolate CD31^+ ECs and CD31^- SMLCs from mixed Passage 1 cultures. Cells were incubated with Accutase for 10 min at 37°C, passed through 40 µm cell strainers into 4× volume of DMEM/F-12, and centrifuged for 5 min, 200×g. Cells were resuspended in MACS buffer and incubated with CD31-APC antibody (Miltenyi Biotec) for 30 min at 4°C, protected from light. The cell suspension was brought to a volume of 15 mL with MACS buffer and centrifuged at 4°C for 5 min, 200×g. Cells were resuspended in MACS buffer containing 2 µg/mL 4',6-diamidino-2-phenylindole (DAPI; Life Technologies). A BD FACS Aria III Cell Sorter (BD Biosciences, San Jose, CA) was used to isolate DAPI CD31^+ cells (live ECs) and DAPI CD31^- cells (live SMLCs). The resulting cell suspensions were centrifuged at 4°C for 5 min, 200×g, and cell pellets immediately processed for RNA extraction as described below.

RNA was isolated using the RNeasy Plus Micro Kit (Qiagen, Germantown, MD). Buffer RLT Plus supplemented with 1% β-mercaptoethanol was used to lyse cells (pellets from FACS of Passage 1 cells, or directly on plates for Passage 3 ECs). Lysates were passed through gDNA Eliminator spin columns, loaded onto RNeasy MinElute spin columns, washed with provided buffers according to manufacturer instructions, and eluted with RNase-free water. Sample concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) and RNA quality assayed using an Agilent 2100 Bioanalyzer with Agilent RNA 6000 Pico Kit (Agilent, Santa Clara, CA). First-strand cDNA synthesis was performed using the SMART-Seq v4 Ultra Low Input RNA kit (Takara Bio, Mountain View, CA) with 5 ng input RNA followed by 9 cycles of PCR amplification and library preparation using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA). Sequencing was performed on a NovaSeq
6000 (Illumina), with approximately 40–60 million 150 bp paired-end reads obtained for each sample.

FASTQ files were aligned to the human genome (hg38) and transcript abundances quantified using RSEM (v1.3.3) (Li and Dewey, 2011) calling bowtie2 (v2.4.2) (Langmead and Salzberg, 2012). Estimated counts from RSEM were input to DESeq2 (v1.26.0) (Love et al., 2014) implemented in R (v3.6.3) for differential expression analysis. Elsewhere, transcript abundances are presented as transcripts per million (TPM). Differentiation pairing as described above was included in the DESeq2 designs. The Wald test with Benjamini-Hochberg correction was used to generate adjusted P-values. Principal component analysis was performed on counts after the DESeq2 variance stabilizing transformation. Transcription factor annotations were based on the list available at http://humanfts.ccbr.utoronto.ca/ (Lambert et al., 2018); secreted and transmembrane annotations were based on the UniProt database (UniProt Consortium, 2021). Weighted Gene Correlation Network Analysis (WGCNA) (v1.70-3) (Zhang and Horvath, 2005; Langfelder and Horvath, 2008) was performed on the 14 EC datasets. Genes with an average of fewer than 50 estimated counts across these datasets were excluded, and the DESeq2 variance stabilizing transformation was used to generate the expression matrix for input to WGCNA. The topological overlap matrix (TOM) was constructed using the signed network type and a power of 20. Hierarchical clustering was performed on dissimilarity (1 – TOM) with average linkage. Gene modules were detected by a constant height (0.99) cut of the hierarchical clustering dendrogram with a minimum module size of 30 genes. Module eigengenes (the first principal component of the expression matrix for genes in each module) were computed as described, and the Pearson correlation between module eigengenes and experimental variables (CHIR v. DMSO: CHIR = 1, DMSO = 0; Passage 3 v. Passage 1: Passage 3 = 1, Passage 1 = 0)
was used to identify modules of interest. Cytoscape (v3.8.3) (Shannon et al., 2003) was used to visualize the 30 genes in the green module (strong positive correlation with CHIR treatment) with the highest intramodular connectivity. The list of genes, corresponding modules, and correlations to experimental variables and module eigengenes is provided in Supplementary file 3.

Bulk RNA-seq data from the literature (FASTQ files; see Previously published datasets used) were obtained from the Gene Expression Omnibus (GEO). These FASTQ files were aligned to the mouse genome (mm10) and transcript abundances quantified as described above. DESeq2 was used for differential expression analysis as described above. For direct comparison of human and mouse data, the biomaRt package (v2.42.1) (Durinck et al., 2009) and Ensembl database (Yates et al., 2019) was used to map human gene names to mouse homologs. Venn diagrams were generated using the tool available at http://bioinformatics.psb.ugent.be/webtools/Venn/. To identify solute carrier and efflux transporter genes highly expressed at the human BBB in vivo, we used five human brain scRNA-seq datasets (see Previously published datasets used) integrated in a previous meta-analysis (Gastfriend et al., 2021). SLC and ABC genes with average expression greater than 100 TPM in endothelial cells across the five independent datasets were selected. For pathway enrichment analysis, lists of upregulated genes (log2(fold change) > 0, adjusted P-value < 0.05, DESeq2 Wald test with Benjamini-Hochberg correction) were tested against the Hallmark gene sets collection (Liberzon et al., 2015) using the tool available at http://www.gsea-msigdb.org/gsea/msigdb/annotate.jsp.
**Immunocytochemistry**

Immunocytochemistry was performed in 24-well plates. Cells were washed once with 500 µL DPBS and fixed with 500 µL cold (−20°C) methanol for 5 min, except cells intended for calponin/SM22α and CD31/Ki67 detection, which were fixed with 500 µL of 4% paraformaldehyde for 15 min. Cells were washed three times with 500 µL DPBS and blocked in 150 µL DPBS supplemented with 10% goat serum (Life Technologies) for 1 h at room temperature, except cells intended for calponin/SM22α detection, which were blocked and permeabilized in DPBS supplemented with 3% BSA and 0.1% Triton X-100, or cells intended for CD31/Ki67 detection, which were blocked and permeabilized in DPBS supplemented with 5% non-fat dry milk and 0.4% Triton X-100. Primary antibodies diluted in 150 µL of the above blocking solutions (see Key Resources Table for antibody information) were added to cells and incubated overnight at 4°C on a rocking platform. Cells were washed three times with 500 µL DPBS. Secondary antibodies diluted in 150 µL of the above blocking solutions (see Key Resources Table for antibody information) were added to cells and incubated for 1 h at room temperature on a rocking platform, protected from light. Cells were washed three times with 500 µL DPBS, followed by 5 min incubation with 500 µL DPBS plus 4 µM Hoechst 33342 (Life Technologies). Images were acquired using an Eclipse Ti2-E epifluorescence microscope (Nikon, Tokyo, Japan) with a 20× or 30× objective or an A1R-Si+ confocal microscope (Nikon) with a 100× oil objective. Confocal images were acquired with 1 µm slice spacing.

Images were analyzed using FIJI (ImageJ) software. For epifluorescence images, 5 fields (20× or 30×) were analyzed per well, with 3–4 wells per treatment condition. For quantification of cell number, EC colonies were manually outlined, and the Analyze Particles function was used to estimate the number of nuclei within the EC colonies. Nuclei outside the EC colonies...
were manually counted. EC purity (% EC) was calculated as the number of nuclei within EC colonies relative to total nuclei. To estimate % GLUT-1+ ECs, cells within the EC colonies with membrane-localized GLUT-1 immunoreactivity (e.g., arrowheads in Figure 2B) were manually counted. To estimate % Ki67+ ECs, cells within the EC colonies with at least one nuclear-localized Ki67 punctum were manually counted. For quantification of fluorescence intensity in epifluorescence images, EC colonies were manually outlined, and the Measure function was used to obtain the mean fluorescence intensity for each image channel (fluorophore). A cell-free area of the plate was similarly quantified for background subtraction. Following background subtraction, the mean fluorescence intensity of each protein of interest was normalized to the mean fluorescence intensity of Hoechst to correct for effects of cell density. For confocal images, 3–4 fields (100×) containing only VE-cadherin+ ECs were analyzed per well, with 4 wells per treatment condition. The first slice with visible nuclei (closest to glass) was defined as Z = 0, and the Measure function was used to obtain the mean fluorescence intensity for each image channel (fluorophore) in each slice from Z = 0 to Z = 7 µm. A cell-free area of the plate was similarly quantified for background subtraction. After background subtraction, to approximate total abundance (area under the fluorescence versus Z curve, AUC) for each channel, mean fluorescence intensities were summed across all slices. AUC for the proteins of interest were normalized to Hoechst AUC.

Cell cycle analysis

Passage 1 cultures were dissociated by treatment with Accutase for 10 min at 37°C. Cell suspensions were passed through 40 µm cell strainers into 4× volume of DMEM/F-12 and centrifuged for 5 min, 200×g. Approximately 5×10^5 cells per replicate were resuspended in MACS buffer and incubated with the CD31-APC antibody (Miltenyi Biotec) for 30 min at 4°C,
protected from light. Cell suspensions were brought to a volume of 5 mL with MACS buffer and centrifuged at 4°C for 5 min, 200×g. Cells were resuspended in 500 µL MACS buffer containing 2 µg/mL DAPI and 0.5 µL Vybrant DyeCycle Green Stain (Invitrogen) and incubated at room temperature for 1 h, protected from light. Cells were analyzed on an Attune NxT flow cytometer (Invitrogen). FlowJo software (BD Biosciences) was used to gate CD31⁺ cells and quantify the percentage of S/G2/M phase cells.

**Western blotting**

To enrich samples from Passage 1 cultures for ECs, the Accutase-based selective dissociation method described above was employed. Dissociated cells were centrifuged for 5 min, 200×g, and resulting cell pellets were lysed in RIPA buffer (Rockland Immunochemicals, Pottstown, PA) supplemented with 1× Halt Protease Inhibitor Cocktail (Thermo Scientific). Passage 3 cells were lysed with the above buffer directly on plates. Lysates were centrifuged at 4°C for 5 min, 14,000×g, and protein concentration in supernatants quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein were diluted to equal volume with water, mixed with sample buffer, and heated at 95°C for 5 min, except lysates intended for GLUT-1 Western blotting, which were not heated. Samples were resolved on 4–12% Tris-Glycine gels and transferred to nitrocellulose membranes. Membranes were blocked for 1 h in tris-buffered saline plus 0.1% Tween-20 (TBST) supplemented with 5% non-fat dry milk. Primary antibodies (see *Key Resources Table* for antibody information) diluted in TBST plus 5% non-fat dry milk were added to membranes and incubated overnight at 4°C on a rocking platform. Membranes were washed five times with TBST. Secondary antibodies (see *Key Resources Table* for antibody information) diluted in TBST were added to membranes and incubated for 1 h at room temperature on a rocking platform, protected from light. Membranes
were washed five times with TBST and imaged using an Odyssey 9120 (LI-COR, Lincoln, NE).

Band intensities were quantified using Image Studio software (LI-COR).

**Dextran accumulation assay**

A fixable, Alexa Fluor 488-conjugated dextran with an average molecular weight of 10 kDa (Invitrogen) was used as a tracer to estimate total fluid-phase endocytosis. Dextran was added at 10 µM to the medium of Passage 1 cultures. Plates were incubated on rotating platforms at 37°C or 4°C for 2 h. For inhibitor experiments, 20 µM chlorpromazine (Sigma), 100 U/mL nystatin (Sigma), or 2 µM rottlerin (Tocris) were added to the medium 30 min prior to addition of dextran. Medium was removed and cells were washed once with DPBS, and then incubated with Accutase for 10 min at 37°C. Cell suspensions were passed through 40 µm cell strainers into 4× volume of DMEM/F-12 and centrifuged for 5 min, 200×g. Cells were resuspended in MACS buffer and incubated with the CD31-APC antibody (Miltenyi Biotec) for 30 min at 4°C, protected from light. Cell suspensions were brought to a volume of 5 mL with MACS buffer and centrifuged at 4°C for 5 min, 200×g. Pellets were resuspended in DPBS supplemented with 4% paraformaldehyde and incubated for 15 min at room temperature, protected from light. Cells were centrifuged for 5 min, 200×g. Pellets were resuspended in MACS buffer and analyzed on a BD FACSCalibur flow cytometer (BD Biosciences). FlowJo software was used to gate CD31+ cells and quantify geometric mean fluorescence intensity and coefficient of variation (CV) of dextran. For imaging, the dextran accumulation assay was performed on cells cultured on #1.5 glass bottom plates. After 2 h of dextran treatment, medium was removed and cells washed with DPBS. Cells were fixed with 4% paraformaldehyde for 15 min. Cells were washed three times with 500 µL DPBS and blocked and permeabilized with DPBS supplemented with 10% goat
serum and 0.1% Triton X-100 for 1 h at room temperature. Cells were stained with the caveolin-
1 primary antibody and imaged on a confocal microscope as described above.

**Transendothelial electrical resistance and sodium fluorescein permeability**

Transwell inserts (6.5 mm diameter with 0.4 µm pore polyester filters) (Corning) were
coated with 50 µl of a solution of collagen IV (400 µg/mL) and fibronectin (100 µg/mL) in water
for 4 h at 37°C. Passage 3 DMSO- and CHIR-treated ECs were seeded on Transwell inserts at
10^5 cells/cm^2 in hECSR medium supplemented with DMSO or CHIR. Medium volumes were
200 µL for the apical chamber and 800 µL for the basolateral chamber. Beginning the day after
seeding, TEER was measured daily for 6 days using an EVOM2 epithelial voltohmmeter with
STX2 chopstick electrodes (World Precision Instruments, Sarasota, FL). Medium was replaced
every other day. TEER values were corrected by subtracting the resistance of a collagen
IV/fibronectin-coated Transwell insert without cells and multiplying by the filter surface area of
0.33 cm^2. Permeability of endothelial monolayers to sodium fluorescein was assessed 6 days
after seeding cells on Transwell inserts. Medium in both apical and basolateral chambers was
replaced and cells returned to the incubator for 1 h. Medium in apical chambers, including the
apical chamber of a collagen IV/fibronectin-coated Transwell insert without cells, was then
replaced with medium supplemented with 10 µM sodium fluorescein (Sigma Aldrich), and plates
placed on an orbital platform in an incubator. At 15, 30, 45, and 60 min, an 80 µL sample of the
basolateral chamber medium was withdrawn from each Transwell, transferred to a 96-well plate,
and 80 µL fresh medium replaced in the basolateral chamber of each Transwell. At 60 min, an 80
µL sample of apical chamber medium was also withdrawn from each Transwell and transferred
to the 96-well plate. 80 µL of medium lacking sodium fluorescein was also transferred to the 96-
well plate for background subtraction. Fluorescence intensity of all samples was measured using
an Infinite M1000 PRO plate reader (Tecan, Männedorf, Switzerland) with 485 nm excitation and 530 nm emission wavelengths. Background-subtracted fluorescence intensity values at the 30, 45, and 60 min timepoints were corrected for sampling-induced dilution as previously described (Stebbins et al., 2016). The endothelial permeability coefficient ($P_e$), which is a concentration-independent parameter corrected for the permeability of a cell-free Transwell insert, was calculated as previously described (Stebbins et al., 2016).

Statistics

Individual wells of cultured cells that underwent identical experimental treatments are defined as replicates, and all key experiments were repeated using multiple independent hPSC differentiations. Detailed information about replication strategy is provided in figure legends. Student’s $t$ test was used for comparison of means from two experimental groups. One-way analysis of variance (ANOVA) was used for comparison of means from three or more experimental groups, followed by Dunnett’s post-hoc test for comparison of multiple treatments to a single control, or Tukey’s honest significant difference (HSD) post-hoc test for multiple pairwise comparisons. When data from multiple differentiations were combined, two-way ANOVA (one factor being the experimental treatment and one factor being the differentiation) was used for comparison of means to achieve blocking of differentiation-based variability, followed by post-hoc tests as described above if more than two experimental treatments were compared. For fluorescence intensities (a.u.), two-way ANOVA was performed prior to normalization of these values to the control group within each differentiation (for visualization in plots). Statistical tests were performed in JMP Pro (v15.0.0). For RNA-seq differential expression analysis, the DESeq2 Wald test with Benjamini-Hochberg correction was used to calculate P-values. Descriptions of the statistical tests used are provided in figure legends.
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Competing interests

BDG, HN, BE, SPP, and EVS are inventors on a provisional US patent application (63/185815) related to this work.

Supplementary files

- **Supplementary file 1. RNA-sequencing gene expression data for hPSC-derived ECs and SMLCs.** Abundances are provided in transcripts per million (TPM).
- **Supplementary file 2. RNA-sequencing differential expression analysis of hPSC-derived ECs.** (A-C) DESeq2-derived average expression (baseMean), log2(fold change), Wald statistic, P-value (Wald test), and adjusted P-value (Benjamini-Hochberg correction) are shown. (A) Passage 1 CHIR-treated ECs versus Passage 1 DMSO-treated ECs. (B) Passage 3 CHIR-treated ECs versus Passage 3 DMSO-treated ECs. (C) Passage 3 DMSO-treated ECs versus Passage 1 DMSO-treated ECs. (D) Lists of upregulated and downregulated genes comprising the intersection of the comparisons in (A) and (B), used to generate Venn diagram in Figure 7E.
- **Supplementary file 3. Gene correlation network analysis.** For each gene, module assignment, correlation with experimental variables (CHIR treatment and passage number), and module membership (correlation between gene and module eigengene) are shown. Genes with an average of fewer than 50 estimated counts were excluded from this analysis.

- **Supplementary file 4. Wnt-regulated EC genes in multiple contexts.** (A) Differential expression analysis of P7 murine brain, liver, lung, and kidney ECs (Sabbagh et al., 2018). DESeq2-derived average expression (baseMean), log2(fold change), Wald statistic, P-value (Wald test), and adjusted P-value (Benjamini-Hochberg correction) are shown. (B) Lists of brain-enriched and brain-depleted genes comprising the intersection of the comparisons in (A). (C-E) Differential expression analysis of adult murine ECs with β-catenin-stabilization versus controls from pituitary (Wang et al., 2019) (C), liver (Munji et al., 2019) (D), and brain ECs cultured in vitro (Sabbagh and Nathans, 2020) (E). DESeq2-derived average expression (baseMean), log2(fold change), Wald statistic, P-value (Wald test), and adjusted P-value (Benjamini-Hochberg correction) are shown. (F) Lists of concordantly Wnt-regulated genes in Passage 1 hPSC-derived ECs and the three comparisons shown in (C-E), from the set of brain-enriched and brain-depleted genes identified in (B).

- **Supplementary file 5. Pathway analysis of ECs with Wnt activation.** (A-C) Enriched gene sets from the Hallmark gene set collection are shown. For each gene set, the total number of genes in the gene set (K), the number of genes enriched in a given comparison (k), the enrichment ratio (k/K), and the false discovery rate (FDR), are shown. (A) Passage 1 CHIR-treated ECs versus Passage 1 DMSO-treated ECs. (B) Passage 3 CHIR-
treated ECs versus Passage 3 DMSO-treated ECs. (C) Adult mouse pituitary ECs with β-
catenin-stabilization versus controls (Wang et al., 2019).

**Data availability**

RNA-seq data have been deposited in GEO under accession number GSE173206.

To review GEO accession GSE173206:


Enter token mfipkyywjtchpux into the box.
### Previously published datasets used

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Figure 1. hPSC-derived endothelial progenitors as a model for studying Wnt-mediated barriergenesis.

(A) Overview of the endothelial differentiation and Wnt treatment protocol. (B) Immunocytochemistry analysis of CD34 and CD31 expression in D5 EPCs prior to MACS. Hoechst nuclear counterstain is overlaid in the merged image. Scale bars: 200 μm. (C) Flow cytometry analysis of CD34 and CD31 expression in D5 EPCs prior to MACS. (D) Immunocytochemistry analysis of β-catenin and GLUT-1 expression in Passage 1 ECs treated with Wnt3a or control. Hoechst nuclear counterstain is overlaid. Arrowheads indicate smooth muscle-like cells (SMLCs). Scale bars: 200 μm. (E) Quantification of the percentage of GLUT-1+ ECs in control- and
Wnt3a-treated conditions. Points represent replicate wells from 2 independent differentiations of the IMR90-4 line, each differentiation indicated with a different color. Bars indicate mean values. P-value: Two-way ANOVA.
Figure 1–figure supplement 1. Smooth-muscle like cells (SMLCs). Immunocytochemistry analysis of calponin and smooth muscle protein 22-α (SM22α) in Passage 1 cultures containing ECs and SMLCs. Hoechst nuclear counterstain is overlaid in the merged image. Dashed area indicates an EC colony. Scale bars: 200 μm.
Figure 2. Effect of CHIR on endothelial properties. (A) Immunocytochemistry analysis of claudin-5, caveolin-1, and GLUT-1 expression in Passage 1 ECs treated with DMSO or 4 µM CHIR. Hoechst nuclear counterstain is overlaid in the merged images. Dashed lines indicate the border between an EC colony and SMLCs in the DMSO condition. Scale bars: 200 µm. (B) Quantification of images from the conditions described in (A) for number of ECs per 20× field, percentage of ECs (claudin-5⁺ cells relative to total nuclei), percentage of GLUT-1⁺ ECs (relative to total claudin-5⁺ ECs), and mean fluorescence intensity of claudin-5, caveolin-1, and GLUT-1 normalized to Hoechst mean fluorescence intensity within the area of claudin-5⁺ ECs only. Points represent replicate wells from 2–6 independent differentiations of the IMR90-4 line, each differentiation indicated with a different color. Bars indicate mean values. For the fluorescence intensity plots, values were normalized within each differentiation such that the mean of the DMSO condition equals 1. P-values: Two-way ANOVA on unnormalized data.
Figure 2—figure supplement 1. Dose-dependent effects of CHIR on endothelial properties. (A)

Immunocytochemistry analysis of claudin-5, caveolin-1, and GLUT-1 expression in Passage 1 ECs treated with 2 μM, 4 μM, or 6 μM CHIR, or DMSO vehicle control. Hoechst nuclear counterstain is overlaid in the merged images. Scale bars: 200 μm. (B) Quantification of images from the conditions described in (A) for number of ECs per 20× field and percentage of ECs (claudin-5+ cells relative to total nuclei). Points represent replicate wells from one differentiation of the IMR90-4 line and bars indicate mean values. P-values: ANOVA followed by Tukey’s HSD test. (C) Quantification of claudin-5, caveolin-1, and GLUT-1 mean fluorescence intensity
normalized to Hoechst mean fluorescence intensity within the area of claudin-5+ ECs only. Points represent replicate wells from one differentiation of the IMR90-4 line. Bars indicate mean values, with values normalized such that the mean of the DMSO condition equals 1. P-values: ANOVA followed by Tukey’s HSD test.
Figure 2—figure supplement 2. CHIR-mediated effects in an additional hPSC line. (A)

Immunocytochemistry analysis of claudin-5, caveolin-1, and GLUT-1 expression in Passage 1 ECs differentiated from the WTC11 iPSC line treated with 2 μM, 4 μM, or 6 μM CHIR, or DMSO vehicle control. Hoechst nuclear counterstain is overlaid in the merged images. Scale bars: 200 μm. (B) Quantification of images from the conditions described in (A) for number of ECs per 20× field and percentage of ECs (claudin-5+ cells relative to total nuclei). Points represent replicate wells from 1–2 differentiations of the WTC11 line, each differentiation indicated with a different color. Bars indicate mean values. P-values: Two-way ANOVA
followed by Tukey’s HSD test. (C) Quantification of claudin-5, caveolin-1, and GLUT-1 mean fluorescence intensity normalized to Hoechst mean fluorescence intensity within the area of claudin-5+ ECs only. Points represent replicate wells from 1–2 differentiations of the WTC11 line, each differentiation indicated with a different color. Bars indicate mean values, with values normalized within each differentiation such that the mean of the DMSO condition equals 1. P-values: Two-way ANOVA followed by Tukey’s HSD test on unnormalized data.
Figure 2–figure supplement 3. β-catenin-dependence of CHIR-mediated GLUT-1 induction. (A) Immunocytochemistry analysis of GLUT-1 expression in Passage 1 ECs treated with DMSO, CHIR, or CHIR + doxycycline (Dox) at 1, 2, or 4 μg/mL. Images from the H9-7TGP-ishcat2, 19-9-11-7TGP-ishcat3, and IMR90-4 lines are shown. Hoechst nuclear counterstain is overlaid. Dashed lines indicate borders between EC colonies.
and SMLCs in the DMSO condition. Scale bars: 200 μm. (B) Quantification of images from the conditions described in (A) for GLUT-1 mean fluorescence intensity normalized to Hoechst mean fluorescence intensity within the area of ECs only. At left, points represent replicate wells from one differentiation of the H9-7TGP-ishcat line (green) and one differentiation of the 19-9-11-7TGP-ishcat3 line (orange). Bars indicate mean values, with values normalized within each differentiation such that the mean of the DMSO condition equals 1.

P-values: Two-way ANOVA followed by Tukey’s HSD test on unnormalized data. At right, points represent replicate wells from one differentiation of the IMR90-4 line. Bars indicate mean values, with values normalized such that the mean of the DMSO condition equals 1. P-values: ANOVA followed by Tukey’s HSD test.
Figure 2–figure supplement 4. Effect of CHIR on endothelial cell proliferation. (A) Immunocytochemistry analysis of CD31 and Ki67 expression in Passage 1 ECs treated with DMSO or CHIR. Hoechst nuclear counterstain is overlaid. Dashed lines indicate borders between the EC colony and SMLCs in the DMSO condition. Arrowheads indicate examples of Ki67+ ECs. Scale bars: 200 μm. (B) Quantification of the percentage of Ki67+ ECs in DMSO- and CHIR-treated conditions. Points represent replicate wells from one differentiation of the WTC11 line, and bars indicate mean values. P-value: Student’s t test. (C) Flow cytometry-based cell cycle analysis. Representative plots of Vybrant DyeCycle Green Stain abundance in CD31+ cells from Passage 1 cultures treated with DMSO or CHIR. (D) Quantification of the percentage of S/G2/M phase ECs. Points represent replicate wells from one differentiation of the WTC11 line, and bars indicate mean values. P-value: Student’s t test.
**Figure 3. Effect of CHIR on endothelial PLVAP expression.** (A) Confocal immunocytochemistry analysis of VE-cadherin and PLVAP expression in Passage 1 ECs treated with DMSO or CHIR. Hoechst nuclear counterstain is overlaid. Eight serial confocal Z-slices with 1 μm spacing are shown. Scale bars: 50 μm. (B) Quantification of PLVAP and VE-cadherin area under the curve (AUC) of mean fluorescence intensity versus Z-position normalized to Hoechst AUC. Points represent replicate wells from 3 independent differentiations of the IMR90-4 line, each differentiation indicated with a different color. Bars indicate mean values, with values normalized within each differentiation such that the mean of the DMSO condition equals 1. P-values: Two-way ANOVA on unnormalized data.
Figure 4. Effect of CHIR on protein expression in Passage 1 and Passage 3 ECs. (A) Western blots of Passage 1 ECs treated with DMSO or CHIR probed for GLUT-1, claudin-5, PLVAP, VE-cadherin, LSR, and β-actin. (B) Quantification of Western blots of Passage 1 ECs. GLUT-1, claudin-5, PLVAP, VE-cadherin, and LSR band intensities were normalized to β-actin band intensity. Points represent replicate wells from 2–3 independent differentiations of the IMR90-4 line, each differentiation indicated with a different color. Bars
indicate mean values, with values normalized within each differentiation such that the mean of the DMSO condition equals 1. **P-values:** Two-way ANOVA on unnormalized data. (C) Western blots of Passage 3 ECs treated with DMSO or CHIR probed for GLUT-1, claudin-5, PLVAP, VE-cadherin, LSR, and β-actin. (D) Quantification of Western blots of Passage 3 ECs. GLUT-1, claudin-5, PLVAP, VE-cadherin, and LSR band intensities were normalized to β-actin band intensity. Points represent replicate wells from 3 independent differentiations of the IMR90-4 line, each differentiation indicated with a different color. Bars indicate mean values, with values normalized within each differentiation such that the mean of the DMSO condition equals 1. **P-values:** Two-way ANOVA on unnormalized data.
**Figure 5. Functional properties of CHIR- and DMSO-treated ECs.** (A) Flow cytometry analysis of CD31 expression in Passage 1 ECs following the dextran internalization assay. CD31+ cells were gated for further analysis. (B) Flow cytometry analysis of 10 kDa dextran-Alexa Fluor 488 (AF488) abundance in CD31+ cells. Cells were treated with DMSO or CHIR for 6 d prior to the assay. Representative plots from cells incubated with dextran for 2 h at 37°C are shown. (C) Quantification of 10 kDa dextran-AF488 geometric mean
fluorescence intensity in CD31+ cells. Treatment and assay conditions were as described in (B). Points represent replicate wells from 3 independent differentiations of the IMR90-4 line, each differentiation indicated with a different color. Bars indicate mean values. P-value: Two-way ANOVA. (D) Quantification of the coefficient of variation (CV) of 10 kDa dextran-AF488 fluorescence intensity in CD31+ cells. Points represent replicate wells from 3 independent differentiations of the IMR90-4 line, each differentiation indicated with a different color. Bars indicate mean values. P-value: Two-way ANOVA. (E) Transendothelial electrical resistance (TEER) of Passage 3 ECs. The x-axis indicates the number of days after seeding cells on Transwell inserts. Points represent replicate wells from three independent differentiations of the IMR90-4 line, each differentiation indicated with a different shape. P-value: Two-way ANOVA. (F) Permeability of Passage 3 ECs to sodium fluorescein. Points represent replicate wells from two independent differentiations of the IMR90-4 line, each differentiation indicated with a different color. Bars indicate mean values. P-value: Two-way ANOVA.
Endocytosis-dependence of dextran uptake. (A-B) Flow cytometry analysis of 10 kDa dextran-Alexa Fluor 488 (AF488) abundance in DMSO-treated CD31\(^{+}\) cells. Representative plots from cells incubated with dextran for 2 h at 37°C (same plot shown in Figure 5B) or 4°C (A) or cells incubated with dextran and indicated inhibitors (B) are shown. (C) Quantification of 10 kDa dextran-AF488 geometric mean fluorescence intensity in CD31\(^{+}\) cells. Treatment and assay conditions were as described in (B). Points represent replicate wells from 2 independent differentiations of the IMR90-4 line, each differentiation indicated with a different color. Bars indicate mean values. P-values: Two-way ANOVA followed by Dunnett’s test. CPZ: chlorpromazine. (D) Confocal immunocytochemistry analysis of caveolin-1 expression and 10 kDa dextran internalization in Passage 1 DMSO-treated ECs. Hoechst nuclear counterstain is overlaid. Single confocal Z-slices from two representative fields are shown. XZ and YZ projections derived from serial Z-slices.
with 0.25 μm spacing are shown at right and below. Arrowheads indicate dextran$^+$ caveolin-1$^+$ puncta. Scale bars: 10 μm.
**Figure 6. Effect of CHIR treatment in EPCs and matured endothelium.** (A) Overview of the endothelial differentiation, extended culture, and CHIR treatment protocols. (B) Immunocytochemistry analysis of claudin-5 and GLUT-1 expression in ECs treated with DMSO or CHIR as outlined in (A). Images from the IMR90-4 line are shown. Hoechst nuclear counterstain is overlaid. Scale bars: 100 μm. (C) Quantification of images from the conditions described in (B) for number of ECs per 30x field. Points represent replicate wells from one differentiation of the IMR90-4 line. Bars indicate mean values. P-values: ANOVA followed by Tukey’s HSD.
test. (D) Quantification of images from the conditions described in (B) for GLUT-1 and claudin-5 mean fluorescence intensity normalized to Hoechst mean fluorescence intensity within the area of claudin-5+ECS only. Points represent replicate wells from one differentiation of the IMR90-4 line. Bars indicate mean values, with values normalized such that the mean of the DMSO condition equals 1. P-values: ANOVA followed by Tukey’s HSD test.
Figure 6–figure supplement 1. Effect of CHIR treatment in matured endothelium. (A)

Immunocytochemistry analysis of claudin-5 and GLUT-1 expression in ECs treated with DMSO or CHIR at Passage 4 (as outlined in Figure 6A). Images from the IMR90-4 and H9-CDH5-eGFP lines are shown. Hoechst nuclear counterstain is overlaid. Scale bars: 100 μm. (B) Quantification of images from the conditions described in (A) for number of ECs per 20× field. Points represent replicate wells from one differentiation of the IMR90-4 line (orange) and one differentiation of the H9-CDH5-eGFP line (blue). Bars indicate mean values. P-value: Two-way ANOVA. (C) Quantification of images from the conditions described in (A) for GLUT-1 and claudin-5 mean fluorescence intensity normalized to Hoechst mean fluorescence intensity within the area of claudin-5+ ECs only. Points represent replicate wells from one differentiation of the IMR90-4 line (orange) and one differentiation of the H9-CDH5-eGFP line (blue). Bars indicate mean values, with values normalized within each differentiation such that the mean of the DMSO condition equals 1. P-values: Two-way ANOVA on unnormalized data.
Figure 7. RNA-seq of DMSO- and CHIR-treated ECs. (A) Principal component analysis of EC and SMLC whole-transcriptome data subject to variance stabilizing transformation by DESeq2. Points from Passage 1 ECs represent cells from 4 independent differentiations of the IMR90-4 line, points from Passage 3 ECs represent cells from 3 independent differentiations of the IMR90-4 line, and points from SMLCs represent 2 independent differentiations of the IMR90-4 line. Points are colored based on treatment: DMSO (black), CHIR (red). Data
are plotted in the space of the first two principal components, with the percentage of variance explained by
principal component 1 (PC1) and principal component 2 (PC2) shown in axis labels. Dashed lines indicate
points from Passage 1 and Passage 3 ECs, and are not confidence ellipses. (B) Heat map of transcript abundance
[log₂(TPM+1)] for endothelial, mesenchymal, and epithelial genes across all samples. Abundance data for all
transcripts are provided in Supplementary file 1. (C) Differential expression analysis of Passage 1 CHIR-treated
ECs compared to Passage 1 DMSO-treated ECs. Differentially expressed genes (adjusted P-values < 0.05,
DESeq2 Wald test with Benjamini-Hochberg correction) are highlighted in green (upregulated) and red
downregulated). The number of upregulated, downregulated, and non-significant (ns) genes are shown in the
legend. Complete results of differential expression analysis are provided in Supplementary file 2. (D) Transcript
abundance (TPM) of Wnt-regulated, barrier-related genes in Passage 1 DMSO- and CHIR-treated ECs. Points
represent cells from 4 independent differentiations of the IMR90-4 line and lines connect points from matched
differentiations. All genes shown were differentially expressed (adjusted P-values < 0.05, DESeq2 Wald test
with Benjamini-Hochberg correction). P-values are provided in Supplementary file 2. (E) Venn diagram
illustrating the number of genes identified as upregulated or downregulated (adjusted P-values < 0.05, DESeq2
Wald test with Benjamini-Hochberg correction) in ECs treated with CHIR versus DMSO at Passage 1
compared to Passage 3. Gene lists are provided in Supplementary file 2 and selected genes are shown in Figure
7–figure supplement 1 and Figure 7–figure supplement 2.
Figure 7–figure supplement 1. Differential expression analysis of Passage 3 ECs treated with CHIR versus DMSO. (A) Differential expression analysis of Passage 3 CHIR-treated ECs compared to Passage 3 DMSO-treated ECs. Differentially expressed genes (adjusted P-values < 0.05, DESeq2 Wald test with Benjamini-Hochberg correction) are highlighted in green (upregulated) and red (downregulated). The number of upregulated, downregulated, and non-significant (ns) genes are shown in the legend. Complete results of differential expression analyses are provided in Supplementary file 2. (B) Transcript abundance (TPM) of selected genes in Passage 1 and Passage 3 DMSO- and CHIR-treated ECs. Points represent cells from 3–4 independent differentiations of the IMR90-4 line. Lines connect points from matched differentiations between the DMSO and CHIR conditions; for clarity, lines connecting points between Passage 1 and Passage 3 are omitted. Genes shown were differentially expressed (CHIR v. DMSO, adjusted P-values < 0.05, DESeq2 Wald test with Benjamini-Hochberg correction), except where indicated as ns. P-values are provided in Supplementary file 2.
Figure 7—figure supplement 2. Genes upregulated by CHIR at both Passage 1 and Passage 3. (A-C) Heat maps of transcript abundance \( \log_2(\text{TPM}+1) \) for genes encoding secreted factors (A), transcription factors (B), and transmembrane proteins (C) that are CHIR-upregulated (adjusted P-values < 0.05, DESeq2 Wald test with Benjamini-Hochberg correction) in both Passage 1 and Passage 3 ECs. P-values are provided in Supplementary
file 2. The log₂(fold change) (CHIR v. DMSO, average of Passage 1 and Passage 3 values) for each gene is shown at left.
Figure 7—figure supplement 3. Gene correlation network analysis. (A) Heatmap representation of the WGCNA topological overlap matrix (TOM). Red indicates high topological overlap. Dendrogram from hierarchical clustering of genes based on dissimilarity (1 – TOM) is shown above. Module assignments (colors) are shown above and at left. (B) Correlation between module eigengenes and experimental variables (Passage number and CHIR treatment). Each box shows the Pearson correlation coefficient $r$ and the P-value ($P$). (C) Network representation of the 30 genes in the green module with the highest intramodular connectivity. Edge color represents topological overlap. Edges with topological overlap less than 0.08 are not shown. For reference, maximum topological overlap (excluding the diagonal) within the entire network is 0.38, and topological overlap between $AXIN2$ (green module) and $ACTB$ (turquoise module) is $3.8 \times 10^{-4}$. 
Figure 7–figure supplement 4. Differential expression analysis of Passage 1 versus Passage 3 ECs. (A) Differential expression analysis of Passage 3 DMSO-treated ECs compared to Passage 1 DMSO-treated ECs. Differentially expressed genes (adjusted P-values < 0.05, DESeq2 Wald test with Benjamini-Hochberg correction).
correction) are highlighted in green (upregulated) and red (downregulated). The number of upregulated, downregulated, and non-significant (ns) genes are shown in the legend. Complete results of differential expression analyses are provided in Supplementary file 2. (B) Transcript abundance (TPM) of selected genes. Genes shown were differentially expressed (adjusted P-values < 0.05, DESeq2 Wald test with Benjamini-Hochberg correction) except those indicated as ns. P-values are provided in Supplementary file 2. (C) Flow cytometry analysis of CD34 and CD31 expression in D5 EPCs (prior to MACS) and in Passage 2 ECs.
Figure 7-figure supplement 5. Blood-brain barrier transcriptional profile. Heat map of transcript abundance [log$_2$(TPM+1)] for BBB genes encompassing tight junctions, vesicle trafficking components, solute carriers, and efflux transporters. Solute carrier and efflux transporter genes that were expressed in human brain ECs at an average of >100 TPM in a meta-analysis of scRNA-seq datasets (Gastfriend et al., 2021) are included.
Abundance data for all transcripts is provided in Supplementary file 1. At right, arrows indicate directionality of change for differentially expressed genes (adjusted P-values < 0.05, DESeq2 Wald test with Benjamini-Hochberg correction) for the three comparisons shown above. Changes with expected directionality for gain of CNS EC character have arrows highlighted in green.
Figure 7–figure supplement 6. Expression of Wnt pathway components in naïve ECs. Abundance of transcripts (in transcripts per million, TPM) encoding Wnt receptors, co-receptors, and other pathway components in Passage 1 DMSO-treated ECs. Points represent cells from 4 independent differentiations of the IMR90-4 line. Bars indicate mean values. ADGRA2 is also known as GPR124.
Figure 8. Identification of concordantly Wnt-regulated CNS EC-associated genes in RNA-seq data. (A) Differential expression analysis of P7 murine brain ECs compared to liver, lung, or kidney ECs (Sabbagh et al., 2018). Differentially expressed genes (adjusted P-values < 0.05, DESeq2 Wald test with Benjamini-Hochberg correction) are highlighted in green (up, brain-enriched) and red (down, brain-depleted). The number of up, down, and non-significant (ns) genes are shown in the legends. (B) Venn diagrams illustrating the number of genes identified as brain EC-enriched (left) or brain EC-depleted (right) versus liver, lung, or kidney ECs (adjusted P-values < 0.05, DESeq2 Wald test with Benjamini-Hochberg correction). The 1094 genes enriched in brain ECs compared to each other organ, and the 506 genes depleted in brain ECs compared to each other organ, were used for subsequent analysis of the effects of Wnt activation in the various experimental contexts. (C-F) In each plot, the x-axis indicates average log₂(fold change) of gene expression in brain ECs compared to liver, lung, and kidney ECs for the 1094 brain EC-enriched genes and 506 brain EC-depleted genes described in (B) with known mouse-human homology. Homologous human gene names are shown. The y-axes indicate differential expression [log₂(fold change)] in Passage 1 CHIR-treated ECs compared to Passage 1 DMSO-treated ECs (C), in adult mouse pituitary ECs with stabilized β-catenin (gain-of-function, GOF) compared to controls (Wang et al., 2019) (D), in adult mouse liver ECs with stabilized β-catenin compared to controls (Munji et al., 2020) (E), or in cultured adult mouse brain ECs with stabilized β-catenin compared to controls (Sabbagh et al., 2020) (F). Points are highlighted in blue if concordantly-regulated (upregulated in both comparisons or downregulated in both comparisons). The number of concordantly upregulated and concordantly downregulated genes is shown. Genes were identified as upregulated or downregulated based on adjusted P-values < 0.05, DESeq2 Wald test with Benjamini-Hochberg correction. (G) Venn diagrams illustrating the number of brain EC-enriched genes concordantly upregulated with β-catenin GOF (top) and the number of brain EC-depleted genes concordantly downregulated with β-catenin GOF (bottom) for the four comparisons shown in (C-F). Complete results of this analysis are provided in Supplementary file 4.
Figure 8–figure supplement 1. Concordantly Wnt-regulated CNS EC-associated genes in RNA-seq data of Passage 3 ECs. The x-axis indicates average log₂(fold change) of gene expression in brain ECs compared to liver, lung, and kidney ECs for the 1094 brain EC-enriched genes and 506 brain EC-depleted genes described in Figure 8B with known mouse-human homology. Homologous human gene names are shown. The y-axis indicates differential expression [log₂(fold change)] in Passage 3 CHIR-treated ECs compared to Passage 3 DMSO-treated ECs. Points are highlighted in blue if concordantly-regulated (upregulated in both comparisons or downregulated in both comparisons). The number of concordantly upregulated and concordantly downregulated genes is shown. Genes were identified as upregulated or downregulated based on adjusted P-values < 0.05, DESeq2 Wald test with Benjamini-Hochberg correction.
Figure 8—figure supplement 2. Pathway analysis of ECs with Wnt activation. (A) Enriched gene sets (false discovery rate < 0.05) in Passage 1 CHIR-treated ECs compared to Passage 1 DMSO-treated ECs (top), Passage 3 CHIR-treated ECs compared to Passage 3 DMSO-treated ECs (middle), or adult mouse pituitary ECs with Wnt activation (bottom). (B) Overlapping gene sets in Passage 1 CHIR-treated ECs compared to Passage 1 DMSO-treated ECs (top), Passage 3 CHIR-treated ECs compared to Passage 3 DMSO-treated ECs (middle), or adult mouse pituitary ECs with Wnt activation (bottom). (C) Up-regulated gene sets in Passage 1 CHIR-treated ECs compared to Passage 1 DMSO-treated ECs (top), Passage 3 CHIR-treated ECs compared to Passage 3 DMSO-treated ECs (middle), or adult mouse pituitary ECs with Wnt activation (bottom).
stabilized β-catenin (gain-of-function, GOF) compared to controls (Wang et al., 2019) (bottom). For each gene set, the ratio of the number of genes in the gene set that are upregulated (log$_2$(fold change) > 0, adjusted P-value < 0.05, DESeq2 Wald test with Benjamini-Hochberg correction) in the relevant comparison (k) to the total number of genes in the gene set (K) is shown. For each comparison, the 20 gene sets with the highest k/K ratio are shown. Supplementary file 5 lists all enriched gene sets, and values of k, K, and false discovery rate. (B) Venn diagram illustrating the number of enriched gene sets for the three comparisons in (A). (C) Genes driving gene set enrichment. Selected gene sets enriched in at least one comparison shown in (A) are shown, with squares highlighted in green indicating upregulation (log$_2$(fold change) > 0, adjusted P-value < 0.05, DESeq2 Wald test with Benjamini-Hochberg correction). Only genes upregulated in at least one of the three comparisons are shown.
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<th>D2</th>
<th>D5</th>
<th>+ 6 d (Passage 1)</th>
<th>+ ~12 d (Passage 3)</th>
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<tr>
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<td>+ 7-8 μM CHIR</td>
<td>CD31 MACS</td>
<td>± Wnt</td>
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<td>± Wnt</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Extended culture; passage every ~6 d</td>
</tr>
</tbody>
</table>

**B**

- **CD34**
- **CD31**
- **Merge + Hoechst**

**C**

![Graph showing CD31-APC vs CD34-FITC with percentage distribution areas: 0.2% to 7.1%, 92.4% to 0.3%]

**D**

- **Control**
- **Wnt3a**

**E**

- ![Graph showing % GLUT-1+ ECs with statistical significance P = 0.0005]
A

VE-cadherin  PLVAP  Hoechst

DMSO

z = -1 μm  z = 0 μm  z = 1 μm  z = 2 μm
z = 3 μm  z = 4 μm  z = 5 μm  z = 6 μm

CHIR

z = -1 μm  z = 0 μm  z = 1 μm  z = 2 μm
z = 3 μm  z = 4 μm  z = 5 μm  z = 6 μm

B

$P = 0.0071$

$P = 0.81$
A. Flow cytometry plot showing CD31 expression and FSC-H.

B. Flow cytometry histogram of CD31+ cells normalized to mode.

C. Scatter plot showing 10 kDa dextran-AF488 IV geometric mean for DMSO and CHIR.

D. Scatter plot showing 10 kDa dextran-AF488 CV for DMSO and CHIR.

E. Line graph showing TEER over 6 days for DMSO and CHIR.

F. Line graph showing NaFlP e x 10^3 for DMSO and CHIR.
A

CD31^+ 

4°C  37°C 

Normalized to mode

10 kDa dextran-AF488

B

CD31^+ 

DMSO  CPZ  Nystatin  Rottlerin

10 kDa dextran-AF488

C

Geometric mean (a.u.)

P < 0.0001

P = 0.30

P = 0.0007

D

10 kDa dextran  Caveolin-1  Hoechst
A

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<tr>
<td>Passage 0</td>
<td>Passage 0</td>
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<tr>
<td>+ 6 d</td>
<td>+ 6 d</td>
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**hECSR** ± 4 μM CHIR

<table>
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<th>Analysis</th>
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<tr>
<td>CD31 MACS</td>
<td>CD31 MACS</td>
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<tr>
<td>Extended culture; passage every ~6 d</td>
<td>± 4 μM CHIR</td>
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B

<table>
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<tr>
<th>Treatment at Passage 0</th>
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<td><strong>DMSO</strong></td>
<td><strong>CHIR</strong></td>
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<th>CLAUDIN-5</th>
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<tr>
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<td>CHIR</td>
<td>DMSO</td>
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</table>

C

**P < 0.0001**  **P = 0.65**

**P = 0.015**  **P = 0.021**  **P = 0.19**

**P = 0.0033**  **P < 0.0001**  **P = 1.0**  **P = 0.51**
A. Claudin-5, GLUT-1, Hoechst

B. DMSO vs. CHIR

C. GLUT-1/Hoechst vs. DMSO vs. CHIR

D. Claudin-5/Hoechst vs. DMSO vs. CHIR

IMR90-4

H9-CDH5-eGFP

P = 0.0003

P < 0.0001

P = 0.55
A

Passage 3, CHIR v. DMSO, log_{2} (fold change)

B

- Passage 1, DMSO
- Passage 1, CHIR
- Passage 3, DMSO
- Passage 3, CHIR

- Up (508)
- Down (933)
- ns (16168)
### Gene Expression Changes

The table below compares gene expression changes across different conditions and cell passages. The expression levels are represented using a log2(TPM+1) scale, where TPM denotes transcripts per million.

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<tr>
<th>Efflux transporters</th>
<th>Solute carriers</th>
<th>Vesicle trafficking</th>
<th>Tight junctions</th>
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<td>SLC39A10</td>
<td>CAV1</td>
<td>CLDN5</td>
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<td>ABCG2</td>
<td>SLC38A5</td>
<td>CAVIN1</td>
<td>TJP2</td>
</tr>
</tbody>
</table>

**Legend:**
- **DMSO**: Dark blue shading indicates a decrease in expression.
- **CHIR**: Green shading indicates an increase in expression.

**Passages:**
- **Passage 1**
- **Passage 3**
- **Passage 1, CHIR v. DMSO**
- **Passage 3, CHIR v. DMSO**
- **DMSO, Passage 3 v. Passage 1**

**Note:**
- The **↑** symbol indicates an increase in expression.
- The **↓** symbol indicates a decrease in expression.

---

**Solute Carriers**

- **SLC39A10**
- **SLC44A1**
- **SLC44A4**

**Tight Junctions**

- **CDM5**
- **OCLN**
- **LEPR**

**Efflux Transporters**

- **ABCA1**
- **ABCB1**
- **ABCG2**

---

**Vesicle Trafficking**

- **Cavin1**
- **CAV2**
- **MEMD2A**
- **MARVELD2**
- **PLVAP**
- **PVR**
- **TFRC**
- **INSR**
- **LDLR**
- **LEPR**
- **LRP8**

---

**Solute Carriers**

- **SLC3A2**
- **SLC5A3**
- **SLC5A6**
- **SLC6A6**
- **SLC7A1**
- **SLC7A11**
- **SLC7A5**
- **SLC7A8**
- **SLC9A3R2**
- **SLC12A2**
- **SLC16A1**
- **SLC16A14**
- **SLC25A3**
- **SLC25A5**
- **SLC25A6**
- **SLC26A2**
- **SLC30A1**
- **SLC31A1**
- **SLC38A2**
- **SLC38A3**
- **SLC38A5**
- **SLC39A10**
- **SLC39A8**
- **SLC40A1**
- **SLC44A1**
- **SLC52A2**
- **SLCO1A2**
- **SLCO2A1**
- **SLCO2B1**
- **ABCA1**
- **ABCB1**
- **ABCG2**
Brain v. liver/lung/kidney, log$_2$(fold change)

hPSC EC, Passage 3, CHIR/DMSO, log$_2$(fold change)

61 up
46 down