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P450 oxidoreductase regulates barrier maturation by mediating retinoic acid metabolism in a model of the human BBB

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SUMMARY

The blood-brain barrier (BBB) selectively regulates the entry of molecules into the central nervous system (CNS). A crosstalk between brain microvascular endothelial cells (BMECs) and resident CNS cells promotes the acquisition of functional tight junctions (TJs). Retinoic acid (RA), a key signaling molecule during embryonic development, is used to enhance *in vitro* BBB models' functional barrier properties. However, its physiological relevance and affected pathways are not fully understood. P450 oxidoreductase (POR) regulates the enzymatic activity of microsomal cytochromes. POR-deficient (PORD) patients display impaired steroid homeostasis and cognitive disabilities. Here, we used both patient-specific POR-deficient and CRISPR-Cas9-mediated POR-depleted induced pluripotent stem cell (iPSC)-derived BMECs (iBMECs) to study the role of POR in the acquisition of functional barrier properties. We demonstrate that POR regulates cellular RA homeostasis and that POR deficiency leads to the accumulation of RA within iBMECs, resulting in the impaired acquisition of TJs and, consequently, to dysfunctional development of barrier properties.

INTRODUCTION

The blood-brain barrier (BBB) is formed as a multicellular neurovascular unit (NVU) in which pericytes and astrocytes come in direct contact with brain microvascular endothelial cells (BMECs) (Pardridge, 2015). In turn, BMECs form specialized barrier properties created by (1) tight junctions (TJs) limiting the paracellular passage of molecules (Sasson et al., 2021) and (2) polarized efflux transporters, which form a transport barrier (Pardridge, 2005). Thus, the BBB controls brain homeostasis by facilitating the passage of nutrients and metabolic necessities—while restricting the penetrability of ions, neurotoxic agents, and most drugs (Itoh and Suzuki, 2012). Increasing evidence suggests that BBB dysfunctions are involved in central nervous system (CNS)-related pathologies (Abbott et al., 2010; Ballabh et al., 2004; Lim et al., 2017; Vatine et al., 2017).

Studies using animal models have contributed toward understanding the mechanisms underlying BBB development (Ben-Zvi et al., 2014; Lee et al., 2003). However, differences across species limit their relevance for the human BBB (Warren et al., 2009). Animal models are also limited in their ability to resolve the spatial and temporal dynamics of the NVU. To resolve these limitations, *in vitro* models of the human BBB were generated (Hajal et al., 2021; Herland et al., 2016; Lippmann et al., 2012, 2014; Maoz et al., 2018; Park et al., 2019). These models are based on compartmentalized platforms such as transwells or microfluidic chips, seeded with various NVU cell types, originating from primary (Maoz et al., 2018; Stone et al., 2019), immortalized (Förster et al., 2005; Hatherell et al., 2011), or pluripotent cells (Lim et al., 2017; Park et al., 2019; Vatine et al., 2017, 2019). These models allow coupling and uncoupling of the different cell types. Both *in vivo* and *in vitro* studies focus primarily on the barrier and transporter properties of the BBB, largely overlooking its possible metabolic roles (Lee et al., 2022; Potente and Carmeliet, 2017).

Cytochromes P450 (CYPs) are enzymes involved in the metabolism of steroids, sex hormones, and xenobiotics. The catalytic reactions of microsomal CYPs are dependent on P450 oxidoreductase (POR)-mediated electron transfer from nicotinamide adenine dinucleotide phosphate (NADPH; Pandey and Flück, 2013). Homozygous inactivating mutations in *POR* cause a spectrum of deficiencies that include defects of aromatase, 17α-hydroxylase, and 21-hydroxylase of the CYP family. POR-deficient (PORD) patients present imbalanced steroid homeostasis leading

to delayed sexual development and cognitive disabilities (Hershkovitz et al., 2008; Idkowiak et al., 1993; Pandey and Flück, 2013), suggesting a possible POR-mediated metabolic role in the development of the CNS.

Por knockout mice are embryonically lethal, possibly due to toxic all-trans retinoic acid (RA) accumulation (Otto et al., 2003; Ribes et al., 2007). RA is a signaling molecule involved in the development of tissues and organs, where it affects patterning along the anterior-posterior axis (Ghyselinck and Duester, 2019; Katsuyama and Saiga, 1998; Theodosiou et al., 2010). Interestingly, RA promotes vascular growth by regulating endothelial cell proliferation (Lai et al., 2003). Moreover, RA was shown to promote BBB properties in rodent (El Hafny et al., 1997; Lechardeur et al., 1995) and human immortalized BMEC lines (Mizee et al., 2013, 2014) and in iPSC-derived BMECs (iBMECs) (Lippmann et al., 2014). Interestingly, RA is catabolized by the CYP26 enzymes (Isoherranen and Zhong, 2019; Lutz et al., 2009).

Here, we interrogated the role of POR in the functional development of barrier properties using iBMECs derived from both PORD patients and CRISPR-Cas9-mediated POR-depleted iPSCs. PORD and depleted iBMECs displayed impaired RA-dependent acquisition of functional barrier properties. We suggest a mechanism through which POR regulates intrinsic RA metabolism and in whose absence RA accumulates, resulting in dysregulated molecular pathways and, consequently, in the impaired functional maturation of the BBB.

RESULTS

POR is expressed at the BBB and in iBMECs

To test a possible role of POR in the BBB, we first examined its expression *in vivo*. A transcriptome database of purified human brain cells (Zhang et al., 2016) shows that *POR* is expressed in astrocytes and at a lower level in brain endothelial cells (Figure 1A). In adult mouse brains (He et al., 2018; Vanlandewijck et al., 2018), *por* is expressed in various brain endothelial cells (Figure S1). To verify POR protein expression, we performed an immunohistochemical (IHC) analysis of adult mouse cortical sections. Por was observed in perivascular cortical vasculature and partly co-localized with the endothelial specific marker CD31 (Figure 1B), demonstrating that POR is expressed in endothelial cells at the NVU *in vivo*. Higher magnification imaging indicated a perinuclear pattern (Figure 1C), in agreement with the role of POR as an endoplasmic resident, interacting with endoplasmic reticulum (ER) membrane-bound CYPs (Pandey and Flück, 2013).

To test POR expression in iBMECs, we differentiated iPSCs from a healthy control (CTR) donor (Falik et al., 2020) as previously described (Jagadeesan et al., 2020; Lippmann et al., 2014; Vatine et al., 2017; Figure 1D). Immunocytochemistry (ICC) analysis showed that the iBMECs formed a monolayer expressing the TJ protein Zona occludens-1 (ZO-1), the BBB glucose transporter (GLUT-1), and the TJ protein Claudin-5 (CLDN-5; Figure 1E), indicating successful differentiation. Similarly to the expression observed *in vivo* (Figure 1C), POR expression in iBMECs was perinuclear (Figure 1F). Western blot (WB) analysis resulted in a specific 75 kDa band in both iBMECs and undifferentiated iPSCs (Figure 1G), suggesting that iBMECs can provide a platform to study the role of POR in barrier formation.

Generation and characterization of PORD iPSCs

To functionally study the role of POR in iBMECs, we used a previously described set of iPSCs derived from PORD patients carrying a homozygous inactivating G539R mutation in POR (Zlotnik et al., 2020) and their healthy heterozygous family relative (Figure S2). Additionally, we used the CRISPR-Cas9 system to introduce insertion or deletion (indel) mutations in a CTR background (Figure S3). Together, these cells provide a comprehensive set of iPSCs suitable for studying POR functions.

POR is enzymatically active in CTR iBMECs and severely attenuated in PORD iBMECs

WB analysis of iPSCs and iBMECs showed the production of POR as a 75 kDa band in the CTR line, the heterozygous G539R line (POR^{01het}), and the homozygous G539R PORD lines ($POR^{02G539R}$ and $POR^{03G539R}$). The CTR^{mut1} line did not produce POR protein, and the CTR^{mut2} line expressed a shorter 55 kDa band (Figure 2A). ICC analysis showed POR expression in iBMECs from the POR^{01het} and $POR^{02G539R}$ lines but not in the CTR^{mut1} line (Figure 2B).

To functionally test POR activity, iBMEC lysates were subjected to a colorimetric enzymatic activity assay. CTR and POR^{01het} iBMECs exhibited similar enzymatic activities (Figure 2C), indicating that a single copy of functional *POR* is sufficient to drive POR activity, in agreement with the lack of clinical symptoms in heterozygous individuals (Hershkovitz et al., 2008). Decreased POR activity was observed in the homozygous PORD lines ($POR^{02G539R}$ and $POR^{03G539R}$) and was further reduced in POR mutated (PORM) lines (Figure 2C), confirming that POR is active in iBMECs. The differences between PORD and PORM iBMECs suggest knockdown and full depletion of POR, respectively.

PORD iBMECs exhibit impaired acquisition of barrier functions

To test a possible role of POR in the formation of TJs, we differentiated CTR, PORD, and PORM iPSCs to iBMECs

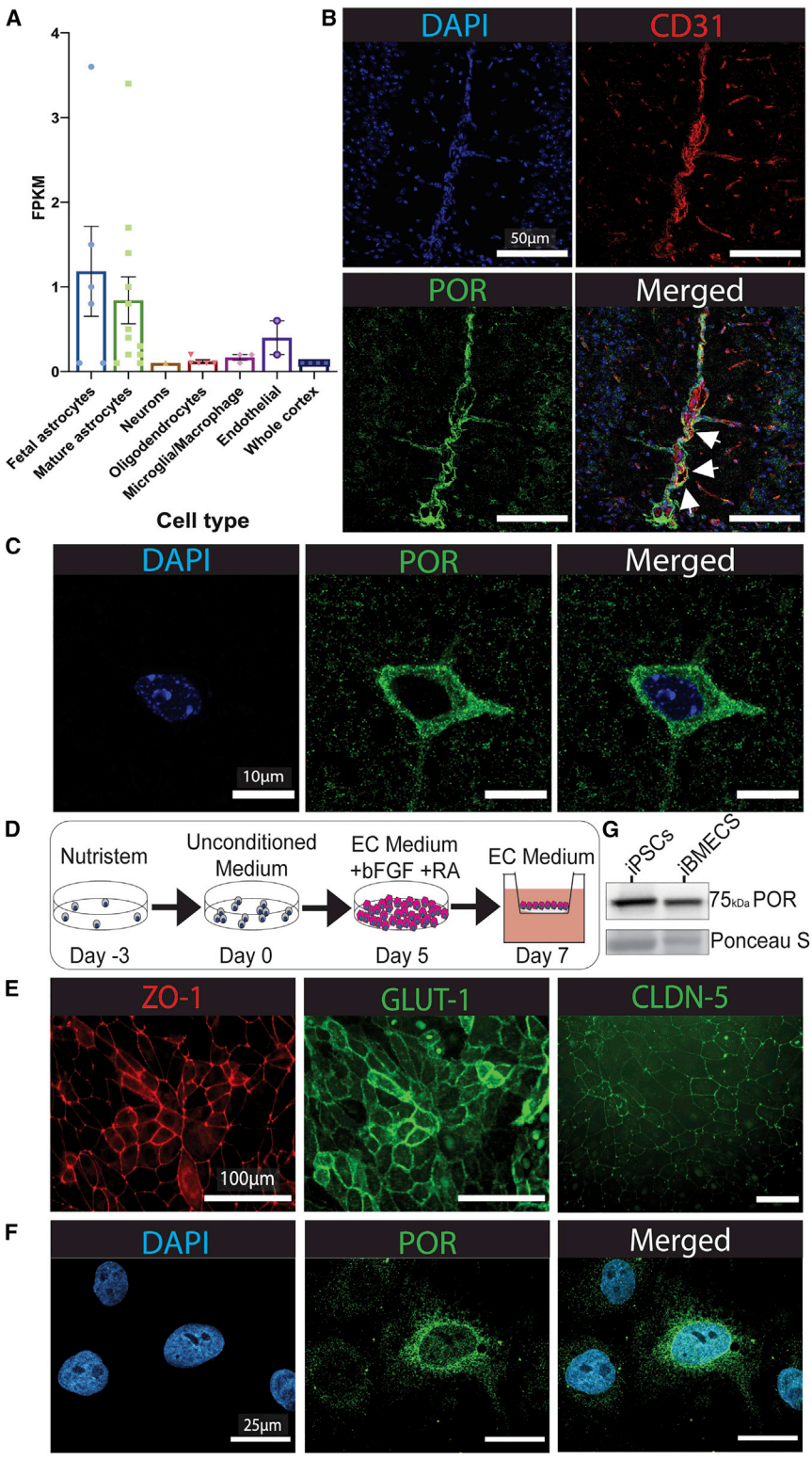

Figure 1. POR is expressed in human and mouse NVU and in iBMECs

(A) *POR* expression in acutely purified human brain cells from the Barres lab database (Zhang et al., 2016).

(B) IHC analysis of mouse cortical brain section shows that CD31 (red)-positive endothelial cells express Por (green). Nuclei were stained using DAPI (blue). White arrowheads denote the co-localization of CD31 and Por.

(C) Por (green) shows a perinuclear pattern of expression.

(D) Schematic illustration of iBMEC differentiation. EC, endothelial cells; bFGF, basic fibroblast growth factor; RA, all-trans retinoic acid.

(E) ICC analysis confirms that CTR iBMECs express GLUT-1, ZO-1, and CLDN-5.

(F) ICC analysis of iBMECs shows that POR is localized to the perinuclear area.

(G) WB analysis showed expression of POR as a 75 kDa band both in undifferentiated iPSCs and iBMECs. Ponceau S staining shows total protein levels.

See also Figure S1.

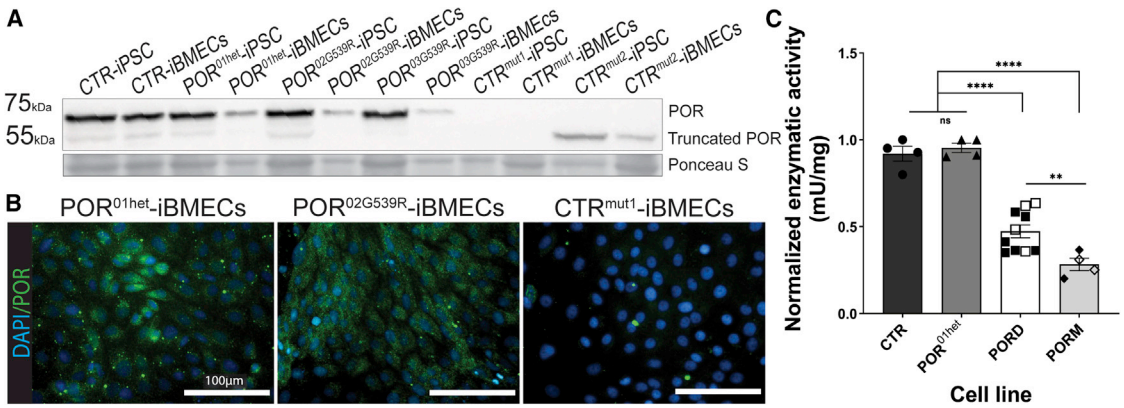

Figure 2. POR is enzymatically active in CTR and heterozygous iBMECs and severely attenuated in PORD and PORM iBMECs

(A) WB analysis of POR in undifferentiated iPSCs and iBMECs. CTR, POR^{01het}, and POR^{G539R} (PORD) cells express POR as a 75 kDa band in both undifferentiated iPSCs and iBMECs. CTR^{mut1} (PORM) did not express POR, and CTR^{mut2} produced a shorter, 55 kDa band, as predicted in Figure S2.

(B) ICC showed POR (green) expression in POR^{01het} and $POR^{02G539R}$ but not in CTR^{mut1} iBMECs.

(C) POR enzymatic activity is decreased in PORD and further reduced in the PORM lines compared with the CTR and POR^{01het} lines. One-way ANOVA; **p < 0.005, ****p < 0.0001, activity was normalized to the maximum level of each experiment. Different symbols refer to different cell lines (n = 4–10).

See also Figures S2 and S3 and Table S1.

(Figure 1D). PORD iBMECs showed CTR-like expression of the BBB-relevant markers ZO-1, GLUT-1, and CLDN-5. PORM iBMECs exhibited aberrant, non-continuous expression of ZO-1, decreased expression of GLUT-1, and non-detectable CLDN-5 (Figure 3A), indicating abnormal development of TJs in POR-depleted iBMECs and suggesting that POR plays a role in the *in vitro* differentiation of iBMECs and in the formation of TJs.

POR is the electron donor to CYP26 enzymes, which are known to act as the main catabolizing enzymes of RA (Isoherranen and Zhong, 2019). Since iBMEC differentiation includes the supplementation of RA (Lippmann et al., 2014), we hypothesized that POR mediates TJ formation through the regulation of RA metabolism. We therefore differentiated each iPSC line into iBMECs either without RA (vehicle [Ve]) or with a gradient of RA concentrations (0.25, 1, or 10 μM). iBMEC lines were then seeded on transwell inserts, and the trans-endothelial electrical resistance (TEER) was measured daily (Figure S4). Maximum TEER levels did not significantly differ between CTR, PORD, and PORM cells when iBMECs were differentiated without RA or at the lowest 0.25 μM concentration. However, when differentiated with 1 or 10 μM, TEER values of the CTR lines were significantly higher than both PORD and PORM lines (Figure 3B). 48 h post seeding, we applied a sodium fluorescein paracellular permeability assay. iBMECs that were differentiated with 10 μM RA had significantly increased paracellular permeability in the PORD cells and further significantly increased permeability in the PORM iBMECs. Together, these results demonstrate that PORD and PORM iBMECs fail to develop functional barrier properties under higher RA concentrations and suggest that POR is necessary for the RA-dependent acquisition of barrier functions.

POR regulates RA-dependent transcriptional programs

RA exerts its canonical molecular action through binding to nuclear receptors, which in turn regulate gene transcription (Blumberg et al., 1992). Thus, RA-dependent gene expression provides a reliable readout reflecting intracellular RA concentrations (Armstrong et al., 2005; Åström et al., 1990). To assess POR-dependent cellular RA concentrations, CTR, PORD, and PORM iBMECs that were differentiated under the various RA concentrations were collected for bulk RNA sequencing (RNA-seq) analysis. CTR iBMECs showed a gradual dose-dependent increase in the number of differentially expressed genes (DEGs) with the increase in RA concentration (Figures S5 and 4A). Contrarily, PORD iBMECs showed an elevated amount of DEGs that was already apparent at the low (0.25 and 1 μM) RA concentrations. This was further accentuated in the PORM iBMECs. We next performed a bidirectional hierarchical clustering of normalized expression values of all genes differentially expressed in at least one condition (Figure 4B). All iBMECs cultured without RA clustered together. CTR iBMECs differentiated with lower RA concentrations (0.25 or 1 μM RA) clustered together and separately from CTR iBMECs differentiated with 10 μM RA. Contrarily, PORD and PORM iBMECs that were differentiated with any RA concentration

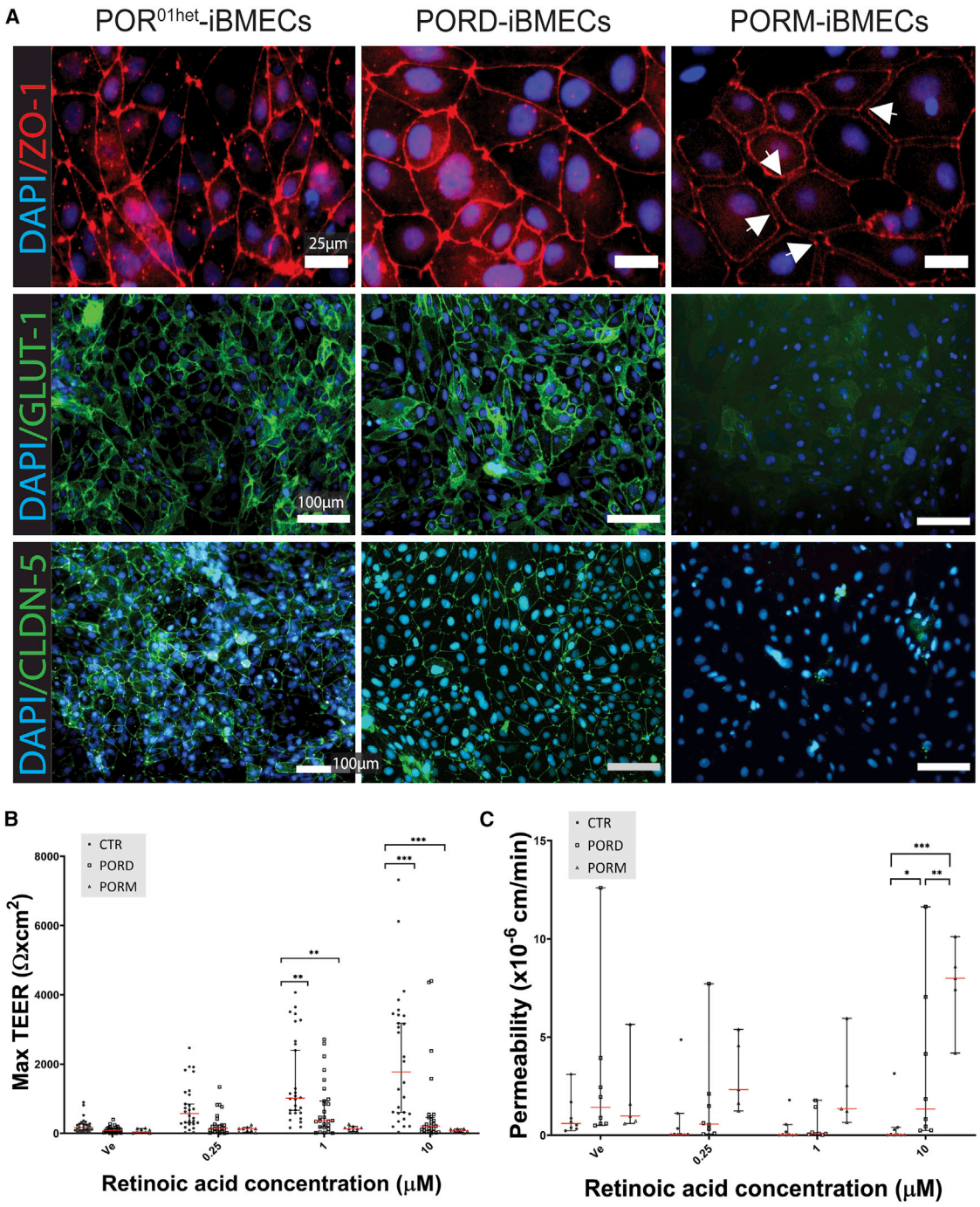

Figure 3. POR is necessary for the RA-dependent development of functional TJs

(A) ICC analysis of the TJ relevant markers ZO-1 (red), GLUT-1 (green), and CLDN-5 (green) for iBMECs differentiated with 10 μM RA from the healthy (POR^{01het}), PORD, and PORM lines. White arrowheads denote gaps between cells in which ZO-1 was expressed.

(B) Scatterplots of maximum (max) TEER values for the CTR, PORD, and PORM lines differentiated with 0 (vehicle [Ve]), 0.25, 1, or 10 μM RA (CTR n = 28; PORD, n = 27; PORM, n = 9). Two-way ANOVA with Tukey's multiple comparison test; **p < 0.005, ***p < 0.0001.

(C) Quantification of the paracellular permeability of the fluorescent tracer sodium fluorescein. When iBMECs were differentiated with 10 μM RA, the PORD (n = 8) and PORM (n = 5) lines showed a significant increase in paracellular permeability compared with CTR lines (n = 9). PORM iBMECs had higher permeability compared with PORD iBMECs. Two-way ANOVA with Tukey's multiple comparison test; *p < 0.05, **p < 0.005, ***p < 0.0001. Bars represent 95% confidence intervals; the solid red line represents the median.

See also Figure S4.

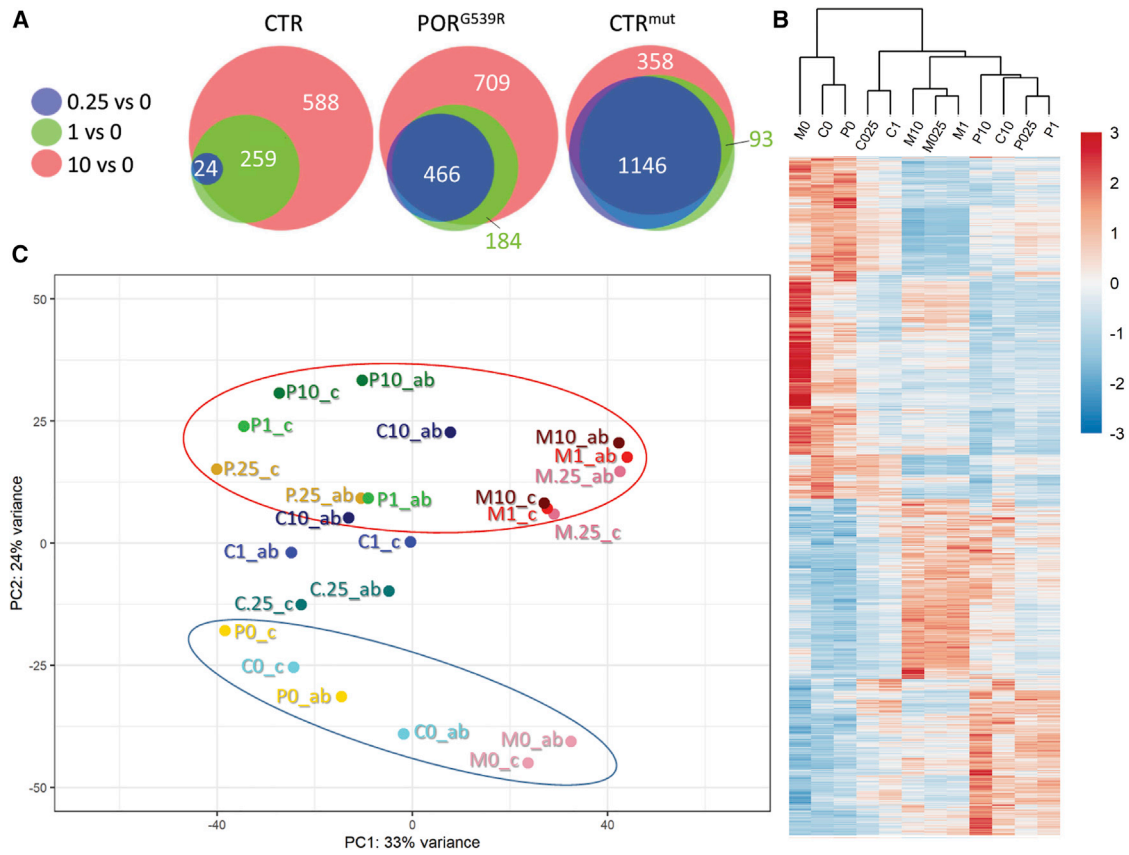

Figure 4. POR regulates RA-dependent gene expression

(A) Venn diagrams showing significant DEG profiles for each line in response to the RA concentration gradient. The size of the circle is proportional to the number of DEGs indicated within each line.

(B) Heatmap of hierarchical cluster analysis for genes that were defined as DEGs in at least one condition in any cell line.

(C) A PCA plot of normalized counts. Each dot represents a different sample. Technical repeats (a and b) were averaged and merged (ab). All lines cluster along the PC2 axis when differentiated without RA (encircled in blue). POR-deficient lines differentiated with all RA concentrations clustered along PC2 with the CTR line differentiated with 10 μM (encircled in red). C, CTR; M, PORM; P, PORD. 0, 0.25, 1, 10: RA concentrations in μM.

See also Figure S5.

clustered together with CTR iBMECs differentiated with 10 μM RA. These findings indicate that PORD and PORM iBMECs display an increased response to RA at lower concentrations. We next subjected all datasets to principal-component analysis (PCA; Figure 4C). CTR iBMECs separated along principal component 2 (PC2), which represents 24% of total variance across all genes, in an RA-dependent manner. CTR, PORD, and PORM iBMECs differentiated without RA clustered together along PC2. However, all PORD and PORM iBMECs that were differentiated with RA clustered together with CTR iBMECs that were differentiated with 10 μM RA, suggesting that cellular RA concentrations are POR-dependent.

We next asked which CYPs are RA-induced in iBMECs. Interestingly, both *CYP26A1* and *CYP26B1* were significantly upregulated in response to RA (Figures 5 and S6). These results suggest that RA is involved in transcriptional regulation of these genes.

RA-dependent pathways are involved in BBB functions through upregulation of TJs

While the role of RA in promoting barrier properties in brain endothelial cells *in vitro* is well established, its physiological relevance has been questioned (Bonney and Siegenthaler, 2017) due to relatively high concentrations (5–10 μM) used *in vitro* compared with lower physiological concentrations estimated as below 0.6 μM (Napoli et al., 1991). Thus, our experimental setup provides an opportunity to study RA-induced gene expression in a range spanning both physiological and pharmacological concentrations. We therefore analyzed the transcriptome of CTR iBMECs differentiated under the various RA concentrations. Gene set enrichment

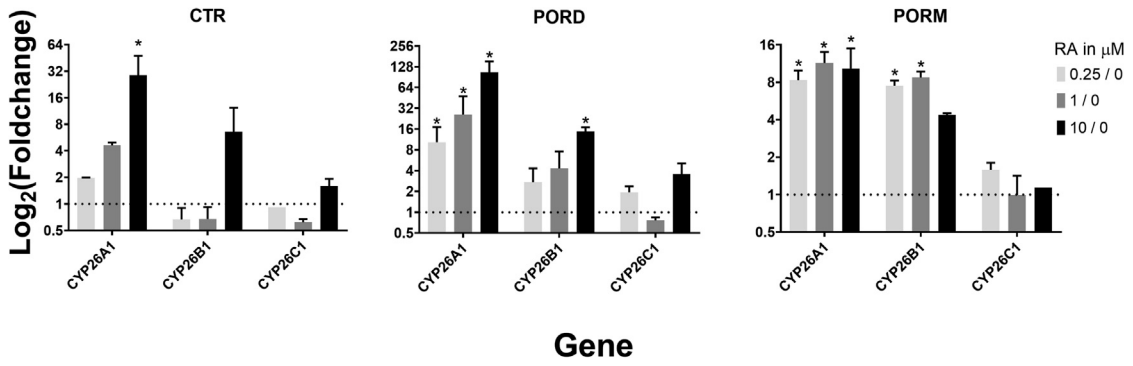

Figure 5. Expression of the CYP26 gene family in response to RA

Log_2(fold change) of each *CYP26* gene in response to RA for CTR, PORD, and PORM iBMECs. Significance threshold, market with asterisks was taken as adjusted $p < 0.1$.

See Figure S6 and supplemental experimental procedures for details.

analysis (GSEA) was performed on a list of RA-related genes, revealing upregulation of well-established RA-induced genes such as *RARs*, *RXRs*, and several *HOX* genes (Figure 6A). Moreover, cellular RA-binding protein 2 (CRABP2) was also upregulated, indicating increased transport of RA into to the nucleus. Interestingly, while some genes are expressed in a dose-dependent manner, other genes were only induced at the higher RA concentration. In addition, GSEA revealed that barrier structural genes, including various CLDNs, other TJs and integrins were significantly enriched by RA treatment (Figure 6B). GSEA analysis also revealed downregulated gene sets that are related to cell cycle (Figure 6C), consistent with previous studies suggesting a role of RA in cell proliferation and differentiation (Chen and Ross, 2004; Liu et al., 2014). Together, this analysis reveals RA-dependent pathways that affect the acquisition of TJ-related genes and functional barrier formation.

PORD and PORM cells display increased inflammatory response to RA

To identify POR-affected pathways, we next ran GSEA comparisons of the different RA concentrations in each cell line using the MsigDB hallmark collection. Interestingly, the tumor necrosis factor alpha (TNF-α) signaling via nuclear factor kappa B (NF-κB) gene set was upregulated in response to all three concentrations of RA in both PORD and PORM (Figures 6D and 6E) but not in CTR iBMECs. These results suggest that POR dysfunction may promote an inflammatory response that may disturb barrier integrity.

DISCUSSION

The BBB is formed as a multicellular NVU in which endothelial cells communicate with other CNS cells. As a result, brain endothelial cells acquire TJs, which limit the paracellular passage of solutes from the blood into the CNS. BBB impairments were reported in various disorders including defective transporter systems (Ceballos et al., 2009; Mayerl et al., 2014; Vatine et al., 2017), dysfunctional barriers (Lim et al., 2017; Raut et al., 2022; Williams et al., 2022; Wu et al., 2021), or ischemia (Page et al., 2016). Here, we took advantage of our PORD cells to study a possible metabolic role of POR within barrier-forming cells. Since iPSCs are prone to variability across different lines (Vitale et al., 2012), we also used the CRISPR-Cas9 system to generate POR-depleted isogenic lines.

iBMECs are increasingly used to model various aspects of the BBB (Motallebnejad et al., 2022; Ohshima et al., 2019; Park et al., 2019; Piantino et al., 2022; Rapier et al., 2022; Vatine et al., 2019). Their strength lies in their scalability and functional properties and the opportunity to model disorders in a personalized manner (Lim et al., 2017; Vatine et al., 2017; Wu et al., 2021). However, we (Vatine et al., 2019) and others (Lu et al., 2021) have also shown that iBMECs display a mixed identity of endothelial and epithelial cells. Thus, these cells do not provide a *bona fide* source of BMECs and should be used with caution. Our results suggest that POR is expressed in BMECs *in vivo* (He et al., 2018; Vanlandewijck et al., 2018; Zhang et al., 2016) and in iBMECs with a similar perinuclear pattern of expression, in agreement with the role of POR as the unique electron donor to microsomal CYPs (Pandey and Flück, 2013). In addition, we confirmed that POR is enzymatically active in iBMECs.

POR deficiency is an autosomal recessive disorder of steroidogenesis with multiple clinical manifestations, including impaired development of genitals, bone malformation, and cognitive disability. The diversity in symptoms reflects POR regulation of the various CYPs (Scott and Miller, 2008). $por^{-/-}$ mice are embryonically lethal (Otto et al., 2003), which was associated with an increased

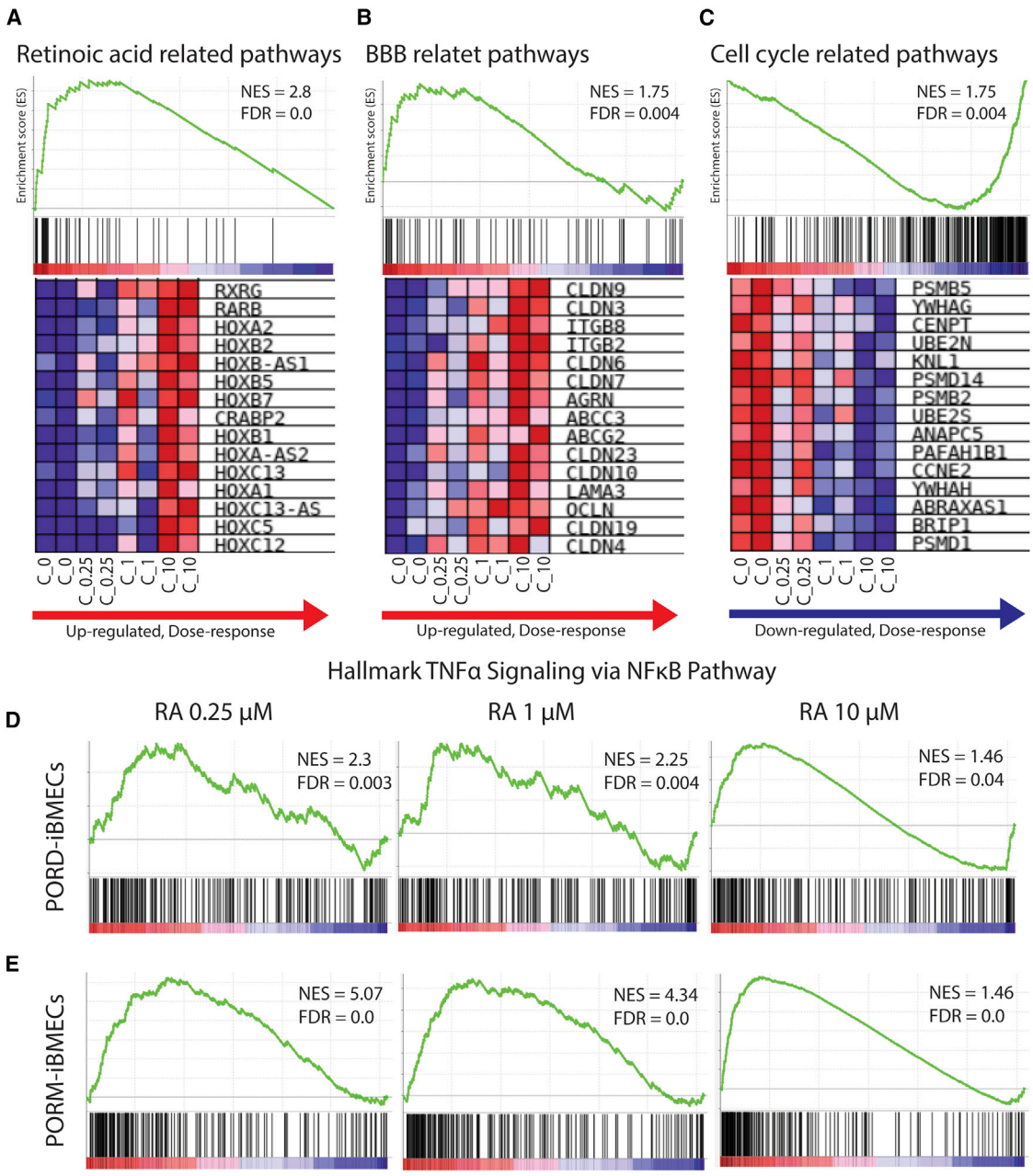

Figure 6. RA dose response in CTR iBMECs

To detect gene sets that are altered in response to RA in a dose-response manner, gene set enrichment analysis (GSEA) was performed on all CTR samples with all RA concentrations (continuous GSEA).

(A) Upregulated RA-related genes (literature curated gene set).

(B) Upregulated blood-brain-barrier-related genes (gene set taken from PathCards database).

(C) Downregulated genes of cell-cycle checkpoints (gene set taken from REACTOME database). Fifteen top altered genes are presented in each heatmap.

(D and E) GSEA analysis using the MsigDB hallmark collection was used to compare gene expression within each cell line in response to the various RA concentrations. The gene set of TNFα signaling via NF-κB is upregulated in (D) PORD and (E) PORM iBMECs. NES, normalized enrichment signal; FDR, false discovery rate.

RA/retinol ratio and could be partially rescued by a vitamin A-depleted diet (Ribes et al., 2007), linking POR activity to RA homeostasis. Interestingly, when POR was specifically depleted from the liver, mice were viable and fertile (Wang et al., 2005), suggesting that the lethality is the result of a POR role in non-liver tissue.

Some of the intracellular communication in the NVU is mediated by RA signaling (Bonney and Siegenthaler, 2017; Mizee et al., 2014; Pollock et al., 2018). RA is the active metabolite of vitamin A, locally synthesized by the retinaldehyde dehydrogenase (RALDH) enzyme. Thus, RA signaling is dependent on cells that can metabolize retinol to RA. RA-generating cells release RA, which is taken up by neighboring cells. Within target cells, RA is transported into the nucleus by CRABP1 and CRABP2, which facilitate RA ligation to RA receptors (RARs) (Majumdar et al., 2011; Napoli et al., 1991). Thus, RA controls gene transcriptional programs through activating nuclear RARs that bind to RA response elements (RAREs) (Maden, 1982; Petkovich et al., 1987). Astrocytes and neural progenitors were shown to express RALDHs (Chattopadhyay and Brown, 2001; Kane et al., 2008; McCaffery et al., 2004; Wang et al., 2011) and locally produce RA (Wuarin et al., 1990), thus providing a possible RA source for BMECs. In the development of the BBB and the blood-retina barrier (BRB), RA was shown to promote barrier properties in mouse (Lai et al., 2003) and human sources of BMECs (Mizee et al., 2013), as well as in iBMECs (Lippmann et al., 2014).

By testing the effect of a range of RA concentrations, we show a dose-dependent acquisition of TJs and the development of a functional barrier in CTR cells. GSEA analysis revealed that RA promotes pathways that are associated with TJs and BBB maturation. These hits were driven by RA-induced expression of the TJ marker OCCLUDIN and several CLDNs. Interestingly, central TJ components such as ZO-1 and CLDN-5 were expressed in iBMECs; however, they were not dependent on RA. These results suggest that other TJ proteins may be involved in the RA-mediated role of functional barrier development.

The main mechanism of action of RA is mediated through regulating gene transcription. Thus, transcriptome analysis provides an indirect proxy of cellular RA concentrations. Our RNA-seq analysis reveals that PORD and PORM iBMECs show increased RA-induced gene expression, suggesting that POR plays a role in cellular RA homeostasis. The catabolism of RA was previously shown to be facilitated by the activity of monooxygenase CYP26 enzymes (Snyder et al., 2020), for which POR acts as a crucial regulator. Interestingly, Cyp26b1 is known to be involved in learning and memory processes (Maclean et al., 2009; Shearer et al., 2012). We show that *CYP26A1* and *CYP26B1* mRNA levels were increased in PORD and PORM cells, suggesting that RA may also contribute to its transcriptional regulation. These conclusions are supported by a study showing that *cyp26a1* is upregulated by RA in zebrafish (Dobbs-McAuliffe et al., 2004). The augmented expression of *CYP26* genes may be part of a mechanism by which cells attempt to dampen RA levels. However, without functional POR, CYPs are unable to carry out these functions, with a resulting detrimental cellular RA accumulation. Interestingly, a study in zebrafish showed that CYP26 expression maintains a constant inflammatory-like state in endothelial cells of the pituitary, leading to the fenestration of blood vessels crucial for pituitary functions in the sensing of hypothalamic peptides (Anbalagan et al., 2018). It is thus possible that the enhanced expression of CYP26 in PORD and PORM cells attenuates functional barrier formation through similar mechanisms.

Our study supports a mechanism by which POR regulates RA homeostasis within BMECs through the action of CYP26 enzymes. In turn, RA promotes transcription of TJ proteins and integrins, which results in the acquisition of functional barrier properties (Figure 7). In the absence of POR, the action of CYP26 in degrading RA is abolished, leading to the accumulation of RA and therefore to impaired RA homeostasis and to a dysfunctional barrier. The dysfunctional barrier may be mediated by TNF-α-mediated inflammatory response, previously shown to promote BBB leakage in iBMECs (Vatine et al., 2019). The affected pathways may in part explain some of the cognitive disabilities observed in PORD patients; however, since POR regulates the activity of a variety of CYPs, other contributions cannot be excluded.

In this study, we focused on RA for its pivotal role in embryonic development. Yet, since various CYPs are responsible for numerous metabolic processes, PORD and PORM cells provide the opportunity to study various endogenous and exogenous molecules in the CNS and other tissues. For example, CYP3A4 is an important oxidizing enzyme that eliminates xenobiotics. Underactivity of such an enzyme may lead to undesired consequences, especially when local metabolic homeostasis is disturbed, as shown in this study (Pandey and Flück, 2013).

EXPERIMENTAL PROCEDURES

Differentiation into iBMECs

iPSCs were differentiated into iBMECs as previously described (Lippmann et al., 2014). Briefly, cells were cultured in Nutristem (BI) for 3 days after passaging. When cells reached a density of 2×10^5 cells per well, medium was replaced with 3 mL unconditioned medium without bFGF (1:1 DMEM:F12) supplemented with 20% knockout serum-free replacement (KOSR; Gibco), 1% NEAA, 0.5% Glutamax (Gibco), 220 nM β-mercaptoethanol, and

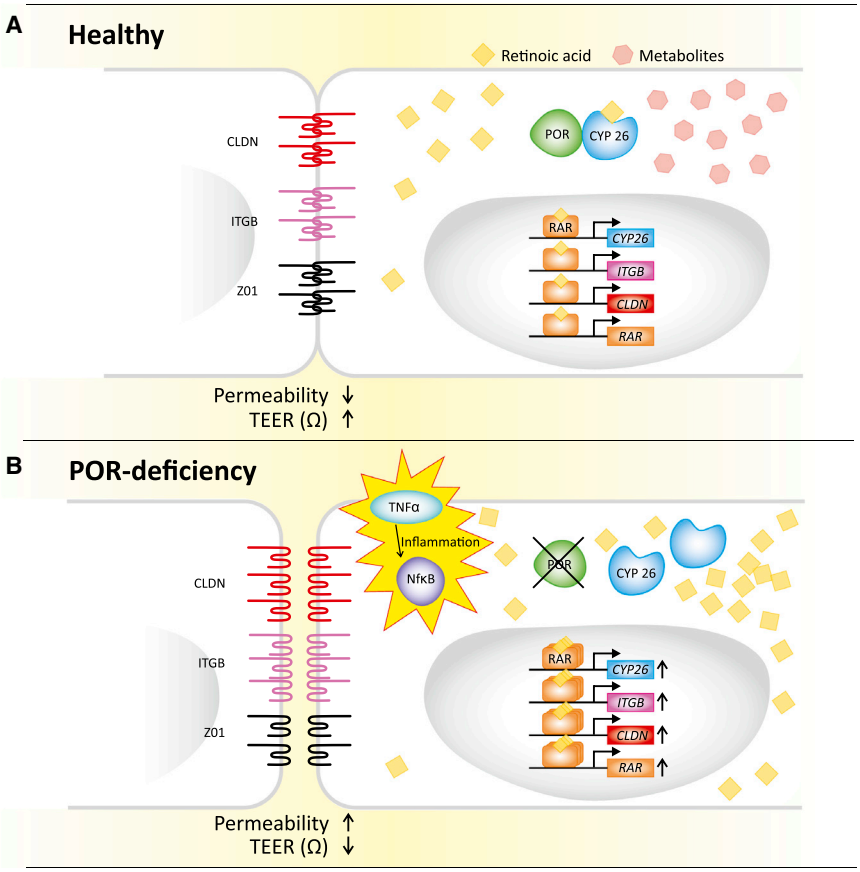

Figure 7. Schematic description of the suggested mechanism

(A) POR mediates the activity of CYP26s, which regulate cellular RA levels by catabolizing RA. In turn, RA regulates the expression of CYP26s, TJs, and integrin (*ITGB*) genes, which leads to the formation of a functional barrier.

(B) When POR is malfunctioning, CYP26s are inactive, causing accumulation of cellular RA. In turn, this leads to transcriptional alterations and an inflammatory response. As a result, TJ formation is impaired, leading to a malfunctioning barrier.

1% PSA. On day 6, the medium was replaced with human endothelial serum-free medium (ESFM; Gibco) supplemented with 20 ng/mL bFGF and 0, 0.25, 1, or 10 μM RA in DMSO. On day 8, transwells, plastic dishes, or coverslips were pre-coated with 100 μg/mL fibronectin (BI) and 400 μg/mL collagen IV (Sigma). On the day of seeding, the ECM solution was aspirated and left to dry for 30 min. iBMECs were harvested in Accutase (Gibco) for 30 min, then washed with ESFM, counted, and centrifuged. iBMECs were resuspended in ESFM at 1×10^6 cells/mL and plated on 12 mm with 0.4 μm pore polycarbonate membrane transwell inserts (Corning, cat# 3401); 1 mL fresh ESFM was supplemented to the bottom chamber, and 1 mL ESFM with cells was applied per transwell. 100 μL was added to 96-well plates and 500 μL was added to 24-well plates with coverslips.

POR enzymatic activity assay

iBMECs were harvested using Accutase for 30 min at 37°C, washed with DPBS, and centrifuged. The pellet was subjected to the Colorimetric Cytochrome P450 Reductase Activity Assay Kit (ab204704, Abcam) according to manufacturer's instructions.

Statistical analysis

All statistical analyses were performed on data from at least three independent experiments each with at least duplicates, except for the POR enzymatic activity assay for the CRISPR-Cas9 cell lines, in which data are taken from two experiments with technical duplicates. Each experiment included at least one healthy donor-derived sample and one G539R-derived sample. One- and two-way ANOVA followed by Dunnett's and Tukey's (respectively) multiple comparison tests were performed using GraphPad Prism v.8.0.2 for Windows (GraphPad). Error bars represent SEM unless indicated otherwise.

Data and code availability

The accession number for the RNA-seq data reported in this paper is GEO: GSE202485.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2022.07.010.

AUTHOR CONTRIBUTIONS

D.Z. and G.D.V. provided the conceptual framework for the study, designed the experiments, and wrote the manuscript. T.R. provided technical assistance with the generation and characterization of iPSCs. A.H. and A.B.-Z. performed the IHC analysis. S.A. participated in RNA preparation. Y.N., I.P., and H.B. performed the bioinformatical analysis. O.Y.G. and B.R. performed the flow sorting experiments. E.H. provided cells from PORD patients and family relatives as well as the Helsinki and IRB protocols.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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