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Induction of Mesoderm and Neural Crest-Derived Pericytes from Human Pluripotent Stem Cells to Study Blood-Brain Barrier Interactions

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SUMMARY

In the CNS, perivascular cells ("pericytes") associate with endothelial cells to mediate the formation of tight junctions essential to the function of the blood-brain barrier (BBB). The BBB protects the CNS by regulating the flow of nutrients and toxins into and out of the brain. BBB dysfunction has been implicated in the progression of Alzheimer's disease (AD), but the role of pericytes in BBB dysfunction in AD is not well understood. In the developing embryo, CNS pericytes originate from two sources: mesoderm and neural crest. In this study, we report two protocols using mesoderm or neural crest intermediates, to generate brain-specific pericyte-like cells from induced pluripotent stem cell (iPSC) lines created from healthy and AD patients. iPSC-derived pericytes display stable expression of pericyte surface markers and brain-specific genes and are functionally capable of increasing vascular tube formation and endothelial barrier properties.

INTRODUCTION

Pericytes are a mural cell type defined by their "perivascular" association with endothelial cells (ECs) (Armulik et al., 2011). Found in microvessels throughout the body, pericytes play a vital role in the remodeling and stabilization of developing blood vessels during angiogenesis (Stratman et al., 2010; Zhao et al., 2015). Pericytes are also an essential component of the blood-brain barrier (BBB), which is a selectively permeable collection of blood vessels that is also comprised primarily of ECs and astrocytes (Obermeier et al., 2013; Winkler et al., 2011). The BBB protects the CNS from circulating insults such as pathogens and immune cells, and mediates the transportation of substances into and out of the brain. A healthy BBB is comprised of ECs that form continuous tight junctions, imparting barrier properties to this structure. Pericytes wrap around the vasculature formed by ECs and secrete extracellular matrix proteins into the shared basement membrane, which provides structural stability to the BBB (Armulik et al., 2010; Daneman et al., 2010). The abundance of pericytes in the BBB at a pericyte-to-endothelial ratio between 1:1 and 1:3, compared with peripheral blood vessels, underscores their importance in maintaining the health of the CNS. Indeed, pericyte dysfunction has been implicated in a number of neurodegenerative disorders including Alzheimer's disease (AD) (Zhao et al., 2015). Degeneration of pericytes has been observed in human postmortem tissue as well as mouse models of AD (Halliday et al., 2016; Sagare et al., 2013). Specifically, loss of pericyte coverage and resulting BBB breakdown was observed

in the cortex and hippocampus of AD mouse models (Nikolakopoulou et al., 2017; Winkler et al., 2012). Accumulation of perivascular beta-amyloid (Aβ), a hallmark of AD, has been shown to be toxic to pericytes (Winkler et al., 2014). In addition, the presence of *APOE4*, the single greatest genetic risk factor in late-onset AD, plays a role in pericyte degradation, accumulation of Aβ in the CNS, and breakdown of the BBB (Halliday et al., 2016; Tai et al., 2016).

Since their identification almost 150 years ago, pericytes have remained an elusive cell type to characterize due to their heterogeneous ontogeny. Quail-chick chimeras and lineage-tracing studies revealed a neural crest (NC) origin of pericytes destined for the forebrain, while mesoderm has been shown to give rise to pericytes of the brainstem, spinal cord, and mid-brain (Etchevers et al., 2001; Korn et al., 2002; Reyahi et al., 2015). While many cell types can be identified by expression of unique surface molecules (also known as "markers"), pericytes share expression of multiple markers with other closely related mural cell types such as smooth muscle cells (Obermeier et al., 2013). Despite this, a combination of PDGFRβ, CD13, CD146, and NG2 surface expression is commonly accepted to be suitable for distinguishing pericytes from other cell types (Armulik et al., 2011). Recently, markers to distinguish brain-resident pericytes from those of the periphery have been identified. These include *FOXF2*, *VTN*, and *FOXC1*, although none are entirely unique to pericytes (He et al., 2016; Reyahi et al., 2015; Siegenthaler et al., 2013).

Pluripotent stem cells (PSCs) provide an alternative approach to both model and treat diseases. AD patient
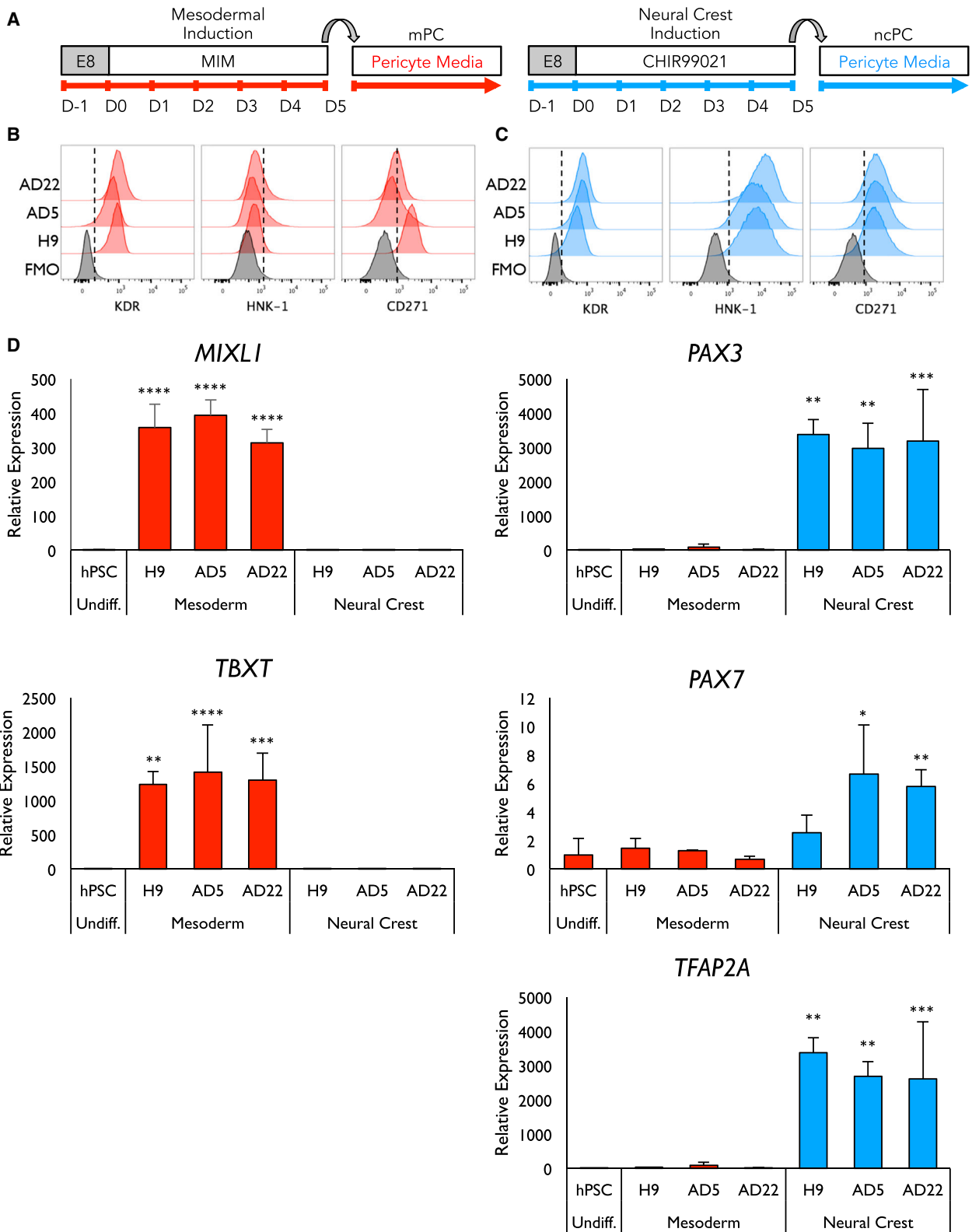
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induced PSCs (iPSCs) allow the development of *in vitro* models of the BBB to improve our understanding of AD-mediated breakdown of the BBB. While protocols exist to generate the cell types of the BBB (ECs, astrocytes, and pericytes) from iPSC lines, a method to generate *brain-specific* pericytes from iPSCs does not currently exist (Greenwood-Goodwin et al., 2016; Kumar et al., 2017; Orlova et al., 2014). To address this, we have developed two methods that rely on either mesoderm or NC induction to generate pericytes from iPSCs.

RESULTS

Differentiation of hPSCs into Mesoderm and NC

We developed two differentiation protocols to generate mesoderm- and NC-derived pericytes from human PSCs (hPSCs) including human embryonic stem cells (hESCs; H9) or human iPSCs (Figure 1A). Our iPSC lines are derived from adult AD patients bearing *APOE4* ("AD6") or *APOE3* ("AD22") alleles and also healthy patients bearing the *APOE3* allele ("AD5"), collectively referred to as "AD" lines (Table S1). To generate iPSC-derived pericytes, we first differentiated these lines into either mesoderm or NC (Figure 1A). hPSCs were grown in mesodermal induction medium (MIM) or a previously described NC induction medium containing the GSK3 inhibitor, CHIR 99021, to activate WNT signaling (Leung et al., 2016) (Figure 1A). After 5 days in culture, MIM-treated hPSCs expressed the mesodermal marker KDR and mesodermal genes *MIXL1* and Brachyury (*TBXT*), but did not express NC marker HNK-1 or genes *PAX3*, *PAX7*, or *TFAP2A* (Figures 1B and 1D). While MIM-treated H9 cells expressed the NC marker CD271, this marker is also known to be expressed in mesoderm-derived mesenchymal progenitors and, alone, is not sufficient to identify NC populations (Figure 1B) (Cattoretti et al., 1993; Kumar et al., 2017). Conversely, NC-derived cells expressed NC markers HNK-1 and CD271 with mild upregulation of KDR (Figure 1C). All NC-treated hPSC lines expressed NC genes *PAX3*, *PAX7*, and *TFAP2A*, and did not express mesodermal genes *MIXL1* and *TBXT* (Figure 1D). While NC-treated H9 hESCs only mildly upregulated

PAX7, they expressed high levels of *PAX3* and *TFAP2A* (Figure 1D). These data indicate that mesoderm and NC cells can be generated using MIM and NC media, respectively.

Pericyte Induction of hPSC-Derived Mesoderm and NC Cells

Following mesoderm and NC induction, cells were passaged and maintained in pericyte medium, which is a proprietary medium that supports pericyte growth, to initiate pericyte differentiation. After 5 days in pericyte medium, mesoderm-derived pericytes (mPCs) and NC-derived PCs (ncPCs) exhibited high expression of pericyte cell-surface markers PDGFRβ, NG2, CD13, and CD146 at levels comparable with primary human brain vascular pericytes (HBVPs) (Figure 2A). All three pericyte populations were negative for expression of the hemato-endothelial marker CD34 (Figure 2A), and expressed only low levels of the smooth muscle marker, α-smooth muscle actin (Figure S1A), further confirming the pericyte-like identity of the iPSC-PCs. Both mPCs and ncPCs maintained consistent growth rates (Figure S1B) and stable expression of pericyte markers throughout early to late passages (Figures S1C and S1D).

To determine the brain specificity of mPCs and ncPCs, we examined the expression of three recently identified brain pericyte genes: vitronectin (*VTN*), and forkhead transcription factors *FOXF2* and *FOXC1*. Pericytes secrete VTN, which upregulates vascular endothelial growth factor A signaling in ECs, and both *FOXC1* and *FOXF2* are required for proper brain pericyte differentiation and angiogenesis during embryonic development (Reyahi et al., 2015; Siegenthaler et al., 2013; Sweeney et al., 2016). qRT-PCR analysis of these candidate brain-specific pericyte genes revealed that all three pericyte populations expressed *VTN* and *FOXC1*, although only ncPCs weakly expressed *FOXF2* (Figure 2B). By western blot, VTN and FOXF2 protein were both expressed across all mPC and ncPC lines (Figure 2C). Together this indicates that cells bearing expression of pericyte and *brain-specific* pericyte genes can be generated from iPSCs through both mesodermal and NC routes.


Figure 1. Differentiation and Characterization of hPSCs into Mesoderm and NC-Derived Pericytes

(A) Schematic diagram of mesoderm (MIM) and NC differentiation protocols. Five days following MIM and NC induction, cells were passaged and maintained in pericyte medium (PM) to produce mesoderm-derived pericytes (mPC) and neural crest-derived pericytes (ncPC).

(B and C) Representative flow cytometry analyses for surface expression of mesodermal marker KDR, and NC markers HNK-1 and CD271 in hPSCs after 5 days in MIM (B) or NC media (C) compared with fluorescence minus one (FMO) control stain.

(D) qRT-PCR analysis of mesodermal genes *TBXT* and *MIXL1* (left panel) and NC genes *PAX3*, *PAX7*, and *TFAP2A* expression (right panel) in hPSCs after 5 days in MIM (red) or NC media (blue). Gene expression was calculated relative to undifferentiated H9 hPSCs. Undifferentiated AD5 iPSCs showed similar expression as H9 hPSCs (data not shown).

Mean ± SD was calculated from triplicate reactions of three to six biological replicates. Statistical significance in was determined using the Student's unpaired t test (**p < 0.05, ***p < 0.01, ****p < 0.001).
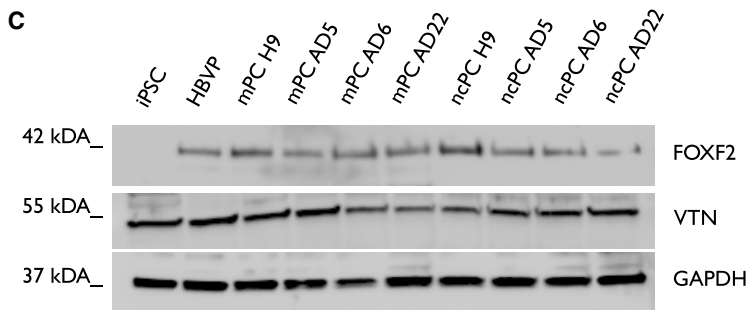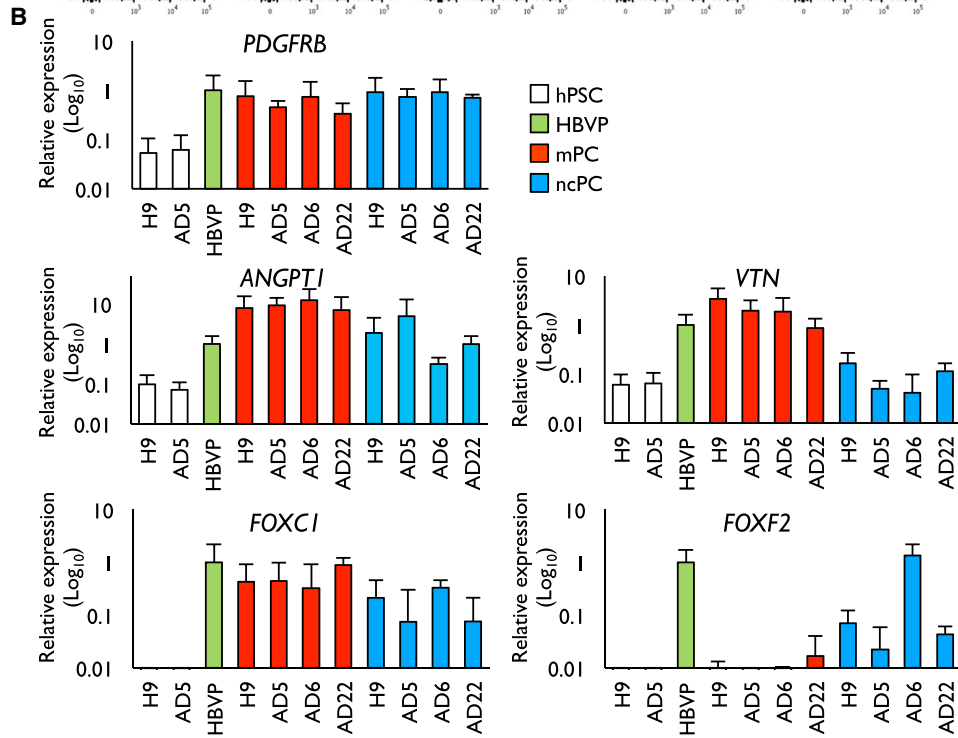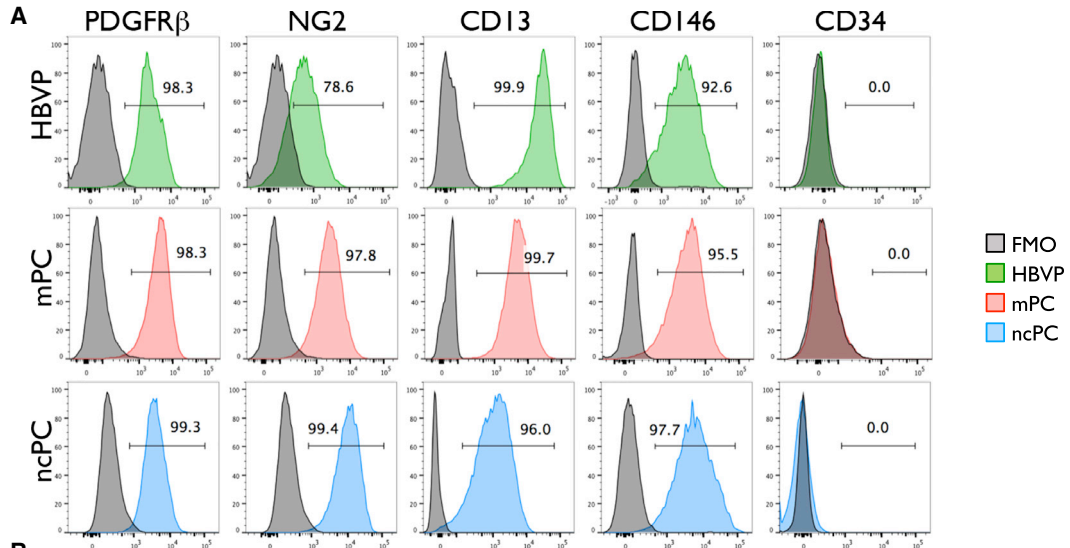
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Functional Validation of Pericyte Interaction with ECs

During embryonic development, brain ECs recruit pericytes to the developing BBB, resulting in their tight association which drives maturation of the developing vessel (Obermeier et al., 2013). Thus, a key functional property of pericytes is their ability to physically associate with ECs to support formation of lumenized vascular structures (Sweeney et al., 2016). To test this capability in hPSC-derived PCs, we cultured HBVPs, ncPCs, or mPCs in a semi-solid fibrin gel matrix in a 3D culturing system with human endothelial colony-forming cell-ECs (ECFC-ECs) (Figure 3A). ECFC-ECs were chosen as the source of ECs because these cells are plastic and robust in adapting to a tissue-specific EC phenotype in the proper native environment (Sobrino et al., 2016; Wang et al., 2016). ECFC-ECs cultured alone were unable to form extensive, lumenized vascular structures. However, when co-cultured with HBVPs, mPCs, or ncPCs, ECs formed elongated tube-like structures in close association with the PCs (Figure 3A). Quantification of EC tube length confirmed observations that mPCs and ncPCs contribute to vasculogenic tube assembly over ECFC-ECs alone (Figure 3B). These data demonstrate that both mesoderm- and NC-derived PCs support vessel growth and maturation.

Effects of Pericytes on Barrier Properties of ECs

Electrical resistance across an endothelial layer is a crucial physiological property that can be used to examine the strength of various barrier systems (Srinivasan et al., 2015). Specifically, the blood-brain barrier exhibits elevated transendothelial electrical resistance (TEER) values due to the tight junctions that form between ECs in the BBB. Multiple groups have shown that TEER values are significantly elevated in brain ECs cultured with HBVPs, suggesting that pericytes are essential to the integrity of an *in vitro* BBB system (Lippmann et al., 2014). To test whether ncPCs and mPCs improve TEER values in a brain vascular context, brain microvessel endothelial cells (BMECs) were differentiated from AD5 iPSCs according to a previously described protocol (Lippmann et al., 2014). We focused on the healthy patient

AD5 iPSC line to have a consistent and healthy source of BMECs across all PC lines. AD5 BMECs, which express endothelial markers CD144 and GLUT1 (Figure 3C), were then monocultured or co-cultured with mPCs, ncPCs, or HBVPs in a transwell system and TEER was measured over the course of several days (Figure 3D). All PC lines appeared healthy and morphologically normal during the co-culture in EC medium (EGM2, data not shown). BMECs cultured alone displayed lower TEER values and, therefore, weaker barrier properties compared with BMECs co-cultured with mPCs or ncPCs (Figure 3E). Peak TEER values were observed 2 days following initiation of co-culture (Figure 3F). These observations were consistent across BMECs derived from different hPSC lines including AD22 iPSCs and H9 hESCs (Figure S2). While BMEC quality, as measured by BMEC-only TEER values, varied across different experiments, the trends remained the same; maximal TEER is achieved when co-cultured with pericytes. Furthermore, maximal BMEC TEER was observed at day 2 of co-culture with pericytes, which supports the findings of previous studies (Appelt-Menzel et al., 2017; Lippmann et al., 2014).

Effect of WNT Inhibition on NC-Derived Pericytes

Our results suggest that brain pericytes can be generated through two distinct pathways. To further investigate the differences in these two routes, we focused on WNT signaling, which is required for NC induction but not mesoderm. Given the important role of WNTs in both NC induction, brain-specification of ECs, and BBB development, we reasoned that inhibition of WNT signaling would also have an impact on NC-derived pericyte induction. The WNT inhibitor DKK1 was added during mesoderm (Figure S3) and NC induction of hPSCs (Figure 4). We found that while WNT inhibition had no effect on mesoderm induction (Figure S2B), expression of *PAX7* appeared dramatically reduced in NC differentiation when WNT was inhibited (Figure 4B). However, after 5 days in pericyte medium, pericyte-like cells with expression of the pericyte markers PDGFRβ and CD146 were observed regardless of whether WNT was inhibited (DKK1) or activated (CHIR)

**Figure 2. Gene Expression Analysis of Pericyte Genes in ncPCs and mPCs**

(A) Representative flow cytometry analysis of pericyte (PDGFRβ, NG2, CD13, and CD146) and hemato-endothelial (CD34) markers in human brain vascular pericytes (HBVPs) (green, top row), mPC (red, middle row), and ncPC (blue, bottom row). The percentage of differentiated cells positive for each marker is shown for the stained cell (colored histograms) compared with the FMO controls (gray histograms). mPCs and ncPCs shown were derived from AD5 iPSCs and are representative of all hPSC lines.

(B) qRT-PCR of pericyte genes *PDGFRB*, *ANGPT1*, *VTN*, *FOXC1*, and *FOXF2* in undifferentiated hPSCs (white), HBVPs (green), mPCs (red), and ncPCs (blue). Gene expression was normalized to *RPLP0* and calculated relative to HBVPs. Mean ± SD was calculated from triplicate reactions of three to six biological replicates.

(C) Western blot of FOXF2 (top row) and VTN (middle row) protein in undifferentiated iPSCs, HBVPs, mPCs, and ncPCs. GAPDH protein (bottom row) was used as a loading control.

See also Figure S1.
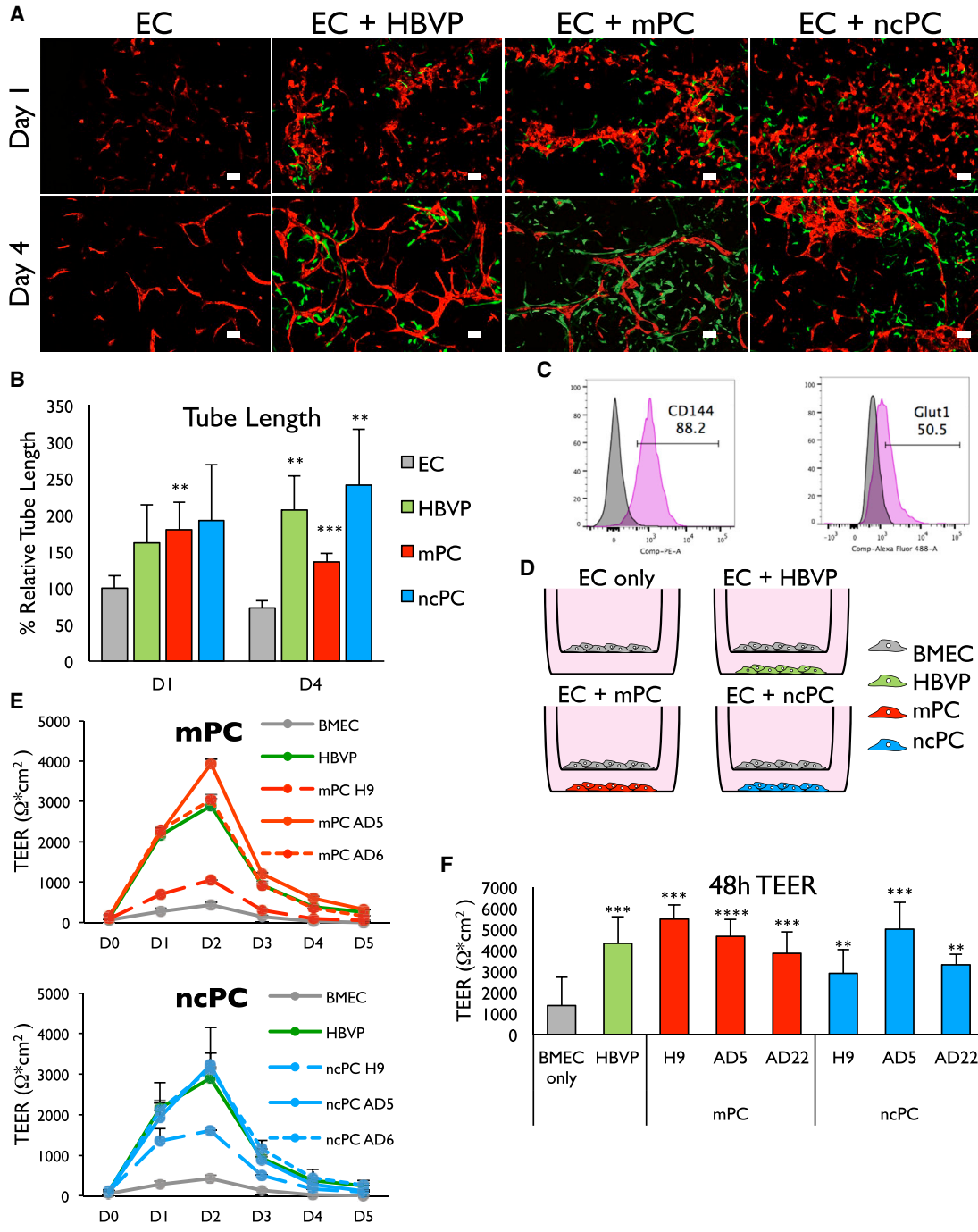
Figure 3. iPSC-Derived Pericytes Promote EC Function

(A) ECFCs (EC, red) and pericytes (PC, green) co-cultured in 3D tube formation assay. ECFCs were cultured alone or co-cultured with HBVPs, mPCs, and ncPCs and imaged after 1 day (top row) or 4 days (bottom row) in culture. Scale bars, 100 μm.

(B) Percent (%) relative tube length of ECs cultured alone (gray) or with HBVPs (green), mPCs (red), or ncPCs (blue) was calculated relative to the tube length at day 1 of ECs cultured alone. Mean ± SD was calculated from three to five biological replicates. mPCs and ncPCs were derived from AD5 iPSCs and are representative of all hPSC-PCs.

(C) Representative flow cytometry analysis of endothelial markers CD144 and GLUT1 in iPSC-derived brain microvessel endothelial cells (BMECs).

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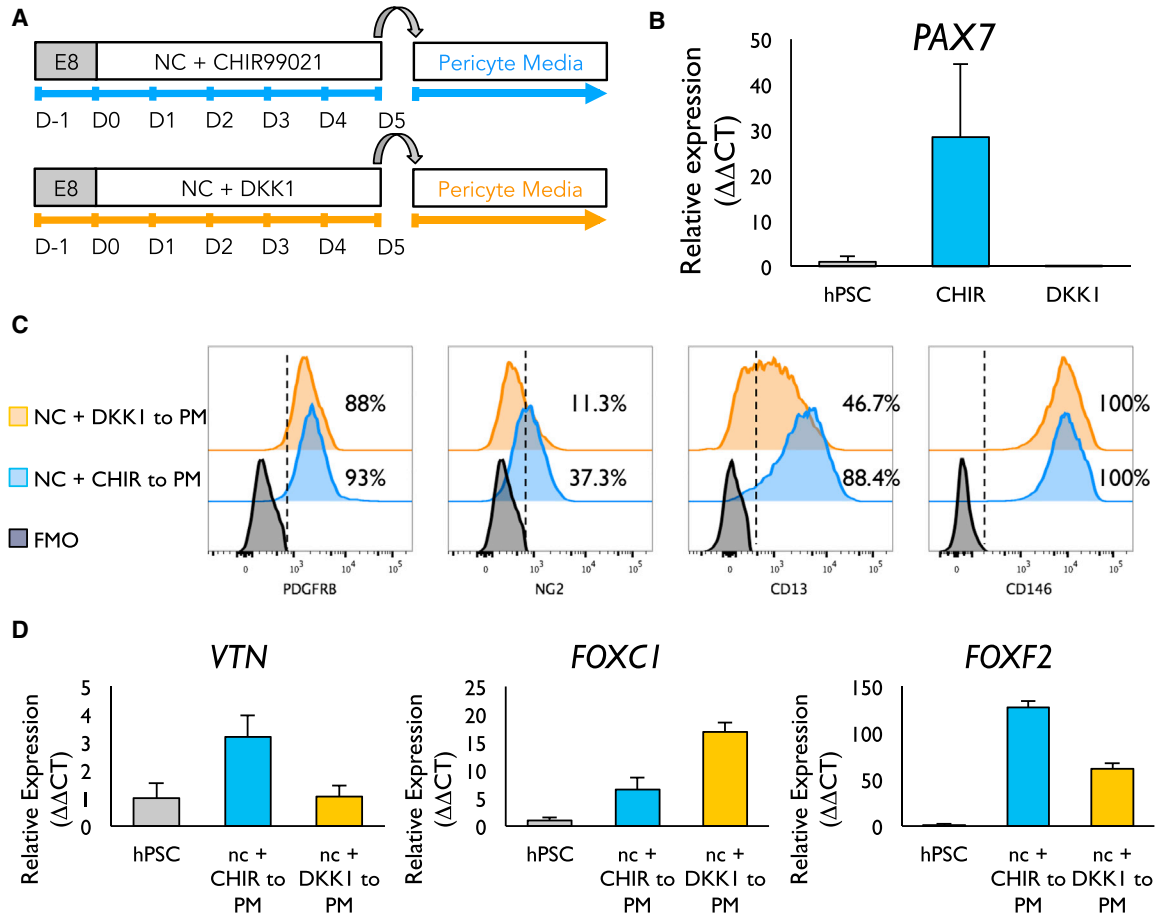
Figure 4. Effect of WNT Inhibition on NC-Derived Pericyte Induction

(A) Schematic diagram of NC differentiation protocol supplemented with the WNT activator CHIR99021 (top row) or with the WNT inhibitor DKK1 (bottom row) for 5 days, then passaged and maintained in pericyte medium (PM).

(B) qRT-PCR analysis of NC gene *PAX7* expression in undifferentiated hPSCs (gray) or hPSCs after 5 days in NC supplemented with CHIR99021 or DKK1. Mean ± SD was calculated from triplicate reactions of two biological replicates.

(C) Representative flow cytometry analysis of pericyte marker expression in pericytes derived from NC supplemented with CHIR99021 (blue) or DKK1 (orange) after passage into PM for 3–7 days. The FMO control (gray histogram) was used to define the gate (dashed line) used to calculate the percentage of cells positive for each marker, which is shown.

(D) qRT-PCR of pericyte genes *VTN*, *FOXC1*, and *FOXF2* in cells derived from NC supplemented with CHIR99021 (blue) or DKK1 (orange) after passage into PM. Gene expression was normalized to *RPLP0* and calculated relative to undifferentiated hPSCs (gray). Mean ± SD was calculated from triplicate reactions of two biological replicates.

See also Figure S3.

during NC induction, although other pericyte markers CD13 and NG2 were downregulated after WNT inhibition (Figure 4C). DKK1-treated cells also appeared to exhibit lower expression of the brain-specific pericyte genes *VTN* and *FOXF2*, but higher expression of *FOXC1* compared with CHIR-treated cells (Figure 4D). This suggests that

(D) Schematic diagram of transwell system used to co-culture BMECs with pericytes to determine transendothelial electrical resistance (TEER).

(E) Representative TEER values of BMECs that were cultured alone (gray) or with HBVPs (green), mPC (red, left graph), or ncPCs (blue, right graph). Mean ± SD was calculated from triplicate transwell reactions. mPCs and ncPCs were derived from AD5 iPSCs.

(F) Quantification of peak TEER values at 48 h post co-culture of BMEC monocultures compared with co-culture with pericyte lines derived from multiple iPSC lines (H9, AD5, and AD22) and averaged across multiple experiments.

Mean ± SD was calculated from three to six biological replicates in triplicate transwell reactions. Statistical significance in (B) and (F) was determined using the Student's unpaired t test (**p < 0.05, ***p < 0.01, ****p < 0.001). See also Figure S2.

WNT signaling is not necessary for pericyte specification, although WNT signaling appears to play a role in completing pericyte development through the NC pathway.

DISCUSSION

In this study, we developed two methods to generate brain pericyte-like cells from hPSCs through mesodermal (mPC) or neural crest (ncPC) routes. While both mPCs and ncPCs exhibited similar pericyte gene expression and functional properties, subtle differences between them were observed. Both populations displayed robust marker expression of PDGFRβ, NG2, CD13, and CD146, but *FOXF2* gene expression was higher in ncPCs. During development, *FOXF2* is expressed in the NC, which might explain its higher expression in ncPCs as opposed to mPCs (Ormestad et al., 2004). We find that, while mPCs and ncPCs display slight differences in the expression of certain genes, they are functionally similar. Surprisingly, despite the requirement for WNT signaling in NC induction, pericytes could still be generated via this route when WNT was inhibited. This raised the possibility that the ncPCs may derive from a rare population during NC differentiation that is independent of WNT. We hypothesized this might be mesoderm, but there was no *TBXT* expression in DKK1-treated NC cells (data not shown). Future RNA sequencing studies are necessary to determine precisely how similar these two PC cell types are.

In summary, our study shows that human pericyte-like cells can be generated from hESCs and iPSCs using two distinct protocols. Both mesoderm and NC induction routes are easy to perform, use fully defined serum-free components, and produce pericytes with consistent marker expression and functional properties through multiple passages and freeze-thaw cycles. Both PC types are easy to transfect, and can be sorted by fluorescence-activated cell sorting (FACS) if necessary. We have multiple, independently generated mPC and ncPC lines from a variety of healthy and AD patient iPSC lines representing *APOE3/3* and *APOE4/4* genotypes (Table S1). These pericyte-like cells can be used as a tool to investigate the role of pericytes in healthy and diseased BBB contexts. Future studies will compare the impact of *APOE* polymorphism on BBB function, which could identify new therapeutic targets to restore BBB function and mitigate AD pathology.

EXPERIMENTAL PROCEDURES

PSC Culture

hPSC lines were cultured in TeSR-E8 medium (STEMCELL Technologies) on Matrigel-coated plates (Corning). Human iPSC lines were provided by the University of California Irvine Alzheimer's Disease Research Center (UCI-ADRC). All procedures using pluripotent stem cell lines were approved by UCI's human stem cell research oversight committee. See Supplemental Experimental Procedures for more details.

Primary Cell Culture

Primary HBVPs (ScienCell) were grown in pericyte medium (1201, ScienCell) according to the manufacturer's instructions. ECFC-ECs were a gift from Dr. Christopher Hughes' laboratory, and are cultured in EGM-2 medium (Lonza). See Supplemental Experimental Procedures for more details.

Differentiation of hPSCs into Mesoderm and NC-Derived Pericyte-like Cells

hPSCs were passaged as single cells onto Matrigel-coated plates in either MIM (STEMCELL Technologies) or a previously described NC induction medium (Leung et al., 2016) for 5 days to initiate mesoderm and NC, respectively. Resulting cells were passaged into pericyte medium (1201, ScienCell) for pericyte specification. See Supplemental Experimental Procedures for more details.

Differentiation of hPSCs into BMECs

BMEC differentiation was performed according to a previously established protocol (Lippmann et al., 2014). See Supplemental Experimental Procedures for more details.

3D Tube Formation Assay

ECFC-ECs were combined with or without HBVPs, mPCs, or ncPCs in a semi-solid fibrin matrix at a cell ratio of 50:1. Cells were imaged everyday using an Olympus FV3000 Laser-Scanning Confocal and tube length was quantified using Angiotool software. See Supplemental Experimental Procedures for more details.

Transwell TEER Assay

iPSC-derived BMECs were seeded onto transwell filters (Corning) that were placed into 12-well plates containing HBVPs, ncPCs, or mPCs. TEER measurements were made every 24 h. See Supplemental Experimental Procedures for more details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2019.01.005.

AUTHOR CONTRIBUTIONS

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