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Supplementary Materials for

Human pluripotent stem cell–derived brain pericyte–like cells induce blood-brain barrier properties

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Published 13 March 2019, *Sci. Adv.* **5**, eaau7375 (2019)
DOI: 10.1126/sciadv.aau7375

The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

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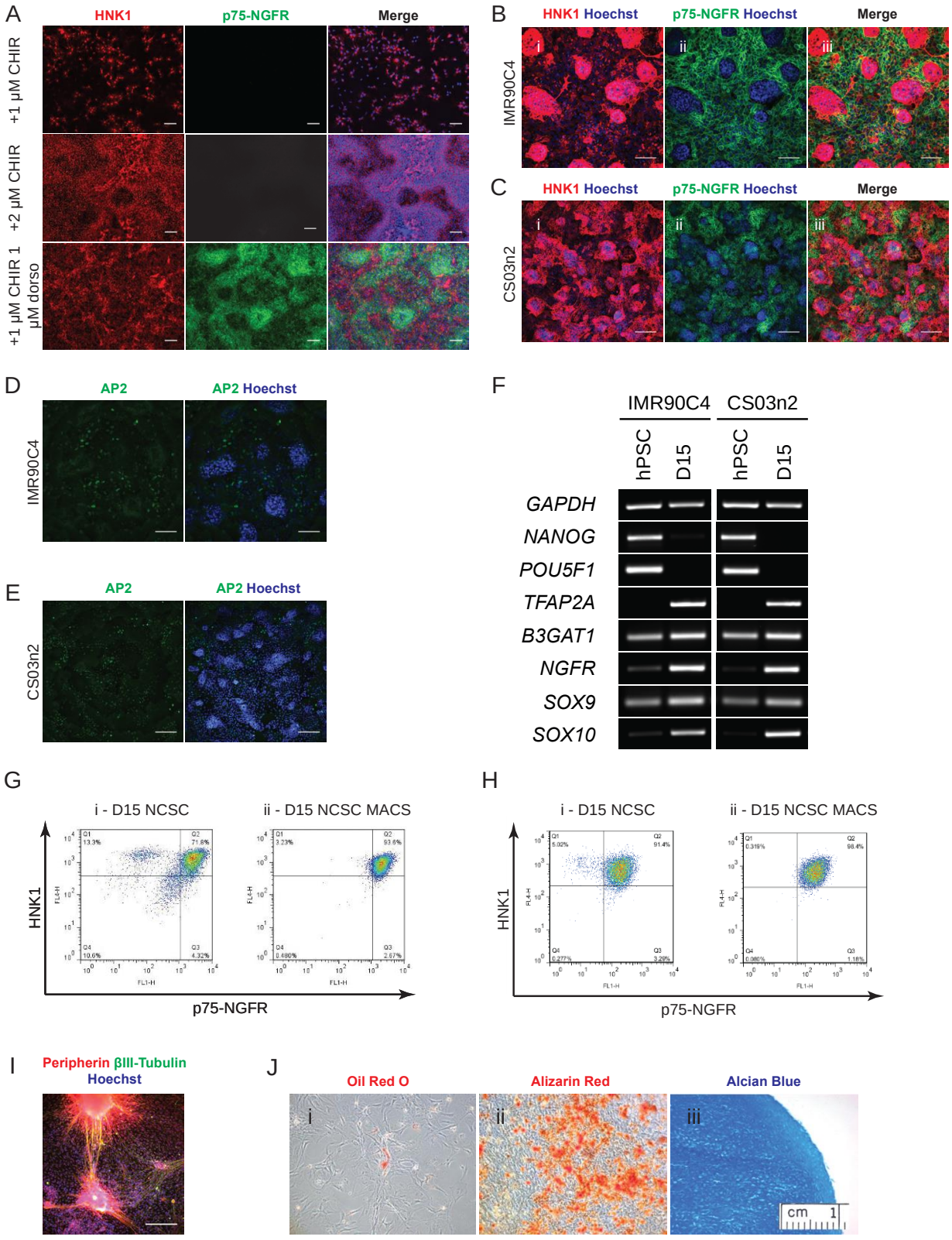
A
HNK1
p75-NGFR
Merge
+1 µM CHIR
+2 µM CHIR
+1 µM CHIR 1 µM dorso
B
HNK1 Hoechst
p75-NGFR Hoechst
Merge
IMR90C4
i
ii
iii
C
CS03n2
D
AP2
AP2 Hoechst
E
F
IMR90C4
CS03n2
hPSC
D15
GAPDH
NANOG
POU5F1
TFAP2A
B3GAT1
NGFR
SOX9
SOX10
G
i - D15 NCSC
ii - D15 NCSC MACS
HNK1
p75-NGFR
H
I
Peripherin βIII-Tubulin
Hoechst
J
Oil Red O
Alizarin Red
Alcian Blue
cm 1

Fig. S1. Generation of multipotent NCSC populations from multiple hPSC lines. A) Immunocytochemistry images of small molecule screen (n = 1) on HNK1 and p75-NGFR expression in cells differentiated from H9 hESCs. Cells were cultured fifteen days in E6 + 10 ng/mL FGF2 + 22.5 μg/mL heparin + 10 μM SB431542 + CHIR99012 ± dorsomorphin at the indicated concentrations. Hoechst nuclear counter stain (blue) is also included. Scale bars: 100 μm. B) Immunocytochemistry images of IMR90C4 iPSCs differentiated in E6-CSFD probed for the presence of HNK1 and p75-NGFR at D15. Hoechst nuclear counter stain (blue) is also included. Scale bars: 100 μm. C) Immunocytochemistry images of CS03n2 iPSCs differentiated in E6-CSFD probed for the presence of HNK1 and p75-NGFR at D15. Hoechst nuclear counter stain (blue) is also included. Scale bars: 100 μm. D) AP-2 immunocytochemistry images for IMR90C4-derived NCSCs at D15. Hoechst nuclear counter stain (blue) is also included. Scale bar: 100 μm. E) AP-2 immunocytochemistry images for CS03n2-derived NCSCs at D15. Hoechst DNA nuclear stain (blue) is also included. Scale bar: 100 μm. F) Temporal PCR analysis of pluripotency (*NANOG*, *POU5F1*) and NCSC (*TFAP2A*, *B3GAT1*, *NGFR*, *SOX9*, *SOX10*) transcripts in IMR90C4 and CS03n2 iPSCs and NCSC progeny. G) Flow cytometry analysis of IMR90C4-derived NCSCs. Panels include NCSC ($HNK1^+/p75\text{-}NGFR^+$) purity prior to MACS (panel i), and NCSC purity following MACS (panel ii). Inset percentages are included in each quadrant. Quantitation is shown in Table 1. H) Flow cytometry analysis of CS03n2-derived NCSCs. Panels include NCSC ($HNK1^+/p75\text{-}NGFR^+$) purity prior to MACS (panel i), and NCSC purity following MACS (panel ii). Inset percentages are included in each quadrant. Quantitation is shown in Table 1. I) Immunocytochemistry analysis of IMR90C4-derived NCSCs subsequently differentiated in peripheral neuron medium. Resultant cells were positive for βIII-tubulin and peripherin expression. Hoechst nuclear counter stain (blue) is also included. Scale bar: 100 μm. J) IMR90C4-derived NCSCs could be differentiated into mesenchymal derivatives, including Oil Red O stained adipocytes (panel i, red), Alizarin red stained osteocytes (panel ii, red), and Alcian blue stained chondrocytes (panel ii, blue).

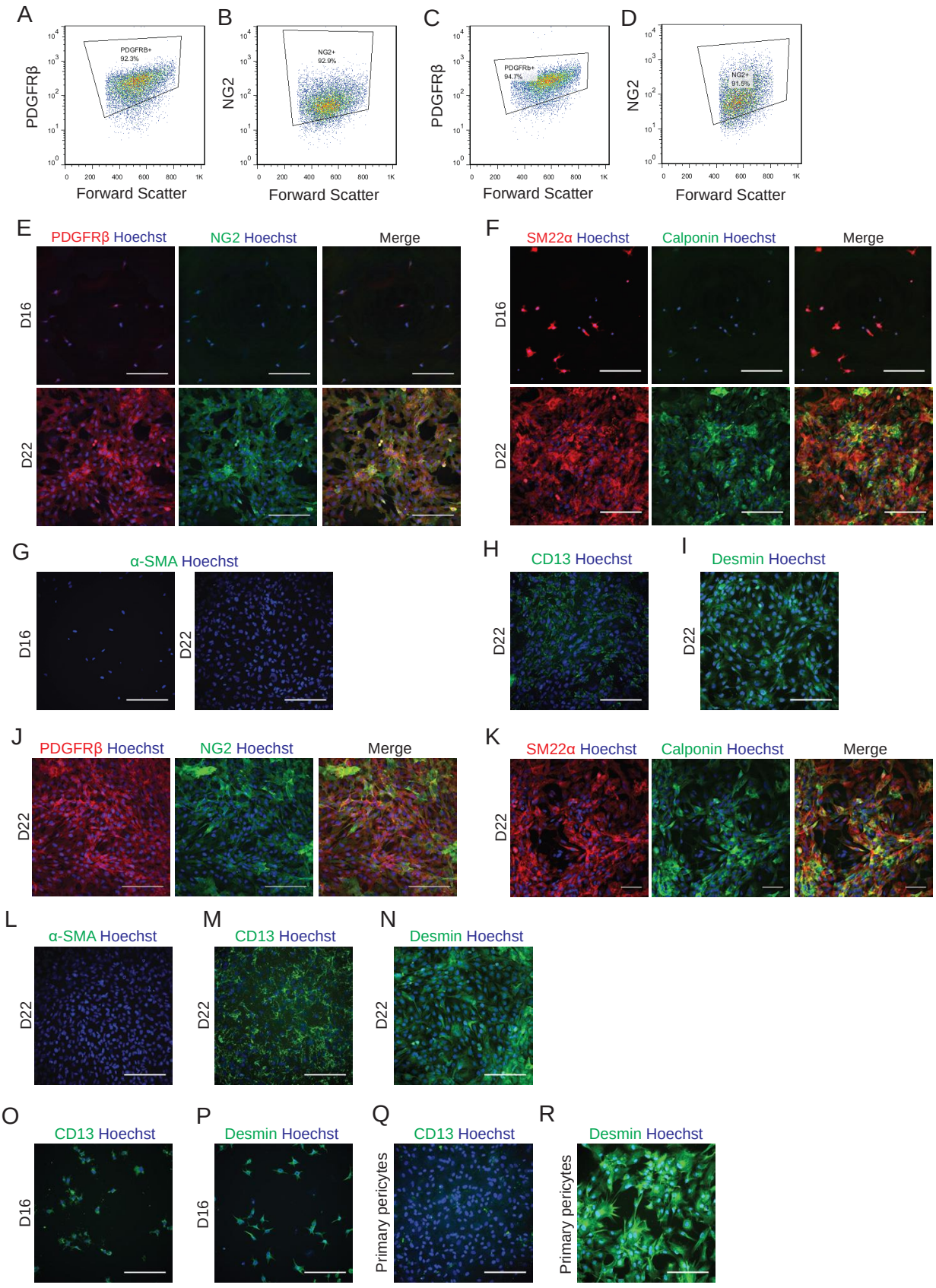
A
PDGFRβ
Forward Scatter
PDGFRB+
92.3%
B
NG2
Forward Scatter
NG2+
92.9%
C
PDGFRβ
Forward Scatter
PDGFRb+
94.7%
D
NG2
Forward Scatter
NG2+
91.5%
E
PDGFRβ Hoechst
NG2 Hoechst
Merge
D16
D22
F
SM22α Hoechst
Calponin Hoechst
Merge
D16
D22
G
α-SMA Hoechst
D16
D22
H
CD13 Hoechst
D22
I
Desmin Hoechst
D22
J
PDGFRβ Hoechst
NG2 Hoechst
Merge
D22
K
SM22α Hoechst
Calponin Hoechst
Merge
D22
L
α-SMA Hoechst
D22
M
CD13 Hoechst
D22
N
Desmin Hoechst
D22
O
CD13 Hoechst
D16
P
Desmin Hoechst
D16
Q
CD13 Hoechst
Primary pericytes
R
Desmin Hoechst
Primary pericytes

Fig. S2. Serum treatment directs iPSC-derived NCSCs toward mural cells. A, B) Representative PDGFRβ and NG2 flow cytometry plots for IMR90C4-derived NCSCs treated for 9 days with E6 + 10% FBS medium. C, D) Representative PDGFRβ and NG2 flow cytometry plots for CS03n2-derived NCSCs treated for 9 days with E6 + 10% FBS medium. Quantitative results can be found in Fig. 1J. E) PDGFRβ and NG2 immunocytochemistry of IMR90C4-derived NCSCs (D16) and mural cells (D22). Hoechst nuclear counter stain (blue) is also included. Scale bar: 200 μm. F) Calponin and SM22α immunocytochemistry of IMR90C4-derived NCSCs (D16) and mural cells (D22). Hoechst nuclear counter stain (blue) is also included. Scale bar: 200 μm. G) α-SMA immunocytochemistry of IMR90C4-derived NCSCs (D16) and mural cells (D22). Hoechst nuclear counter stain (blue) is also included. Scale bars: 200 μm. H,I) CD13 and desmin immunocytochemistry of IMR90C4-derived mural cells (D22). Hoechst nuclear counter stain (blue) is also included. Scale bars: 200 μm. J) PDGFRβ and NG2 immunocytochemistry of CS03n2-derived mural cells (D22). Hoechst nuclear counter stain (blue) is also included. Scale bar: 200 μm. K) Calponin and SM22α immunocytochemistry of CS03n2-derived mural cells (D22). Hoechst nuclear counter stain (blue) is also included. Scale bar: 200 μm. L) α-SMA immunocytochemistry of CS03n2-derived mural cells (D22). Hoechst DNA nuclear stain (blue) is also included. Scale bars: 200 μm. M,N) CD13 and desmin immunocytochemistry of CS03n2-derived pericyte-like cells (D22). Hoechst nuclear counter stain (blue) is also included. Scale bars: 200 μm. O,P) CD13 and desmin immunocytochemistry of H9-derived NCSCs (D16). Hoechst nuclear counter stain (blue) is also included. Scale bars: 200 μm. Q,R) CD13 and desmin immunocytochemistry of primary brain pericytes. Hoechst nuclear counter stain (blue) is also included. Scale bars: 200 μm.

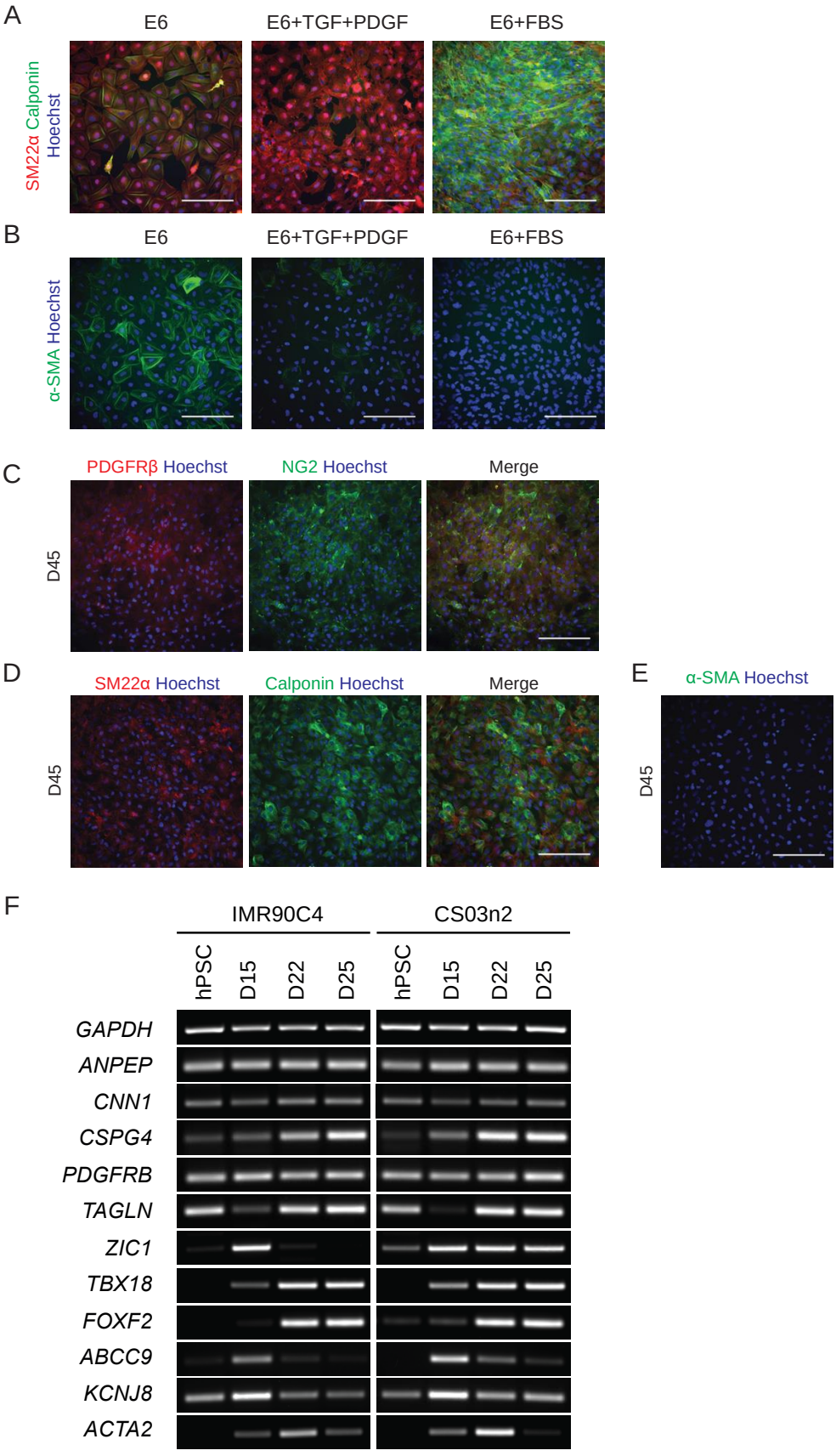
A
E6
E6+TGF+PDGF
E6+FBS
SM22α Calponin Hoechst
B
E6
E6+TGF+PDGF
E6+FBS
α-SMA Hoechst
C
PDGFRβ Hoechst
NG2 Hoechst
Merge
D45
D
SM22α Hoechst
Calponin Hoechst
Merge
D45
E
α-SMA Hoechst
D45
F
IMR90C4
CS03n2
hPSC
D15
D22
D25
hPSC
D15
D22
D25
GAPDH
ANPEP
CNN1
CSPG4
PDGFRB
TAGLN
ZIC1
TBX18
FOXF2
ABCC9
KCNJ8
ACTA2

Fig. S3. Supplemental analysis of hPSC-derived pericyte-like cells. A,B) Analysis of cells obtained by culturing NCSCs in E6, E6 + TGFβ1 + PDGF-BB, or E6 + 10% FBS for 6 days. Calponin, SM22α, and α-SMA immunocytochemistry. Hoechst nuclear counter stain (blue) is also included. Scale bars: 200 μm. C,D,E) Long-term maintenance of hPSC-derived pericyte-like cells. PDGFRβ, NG2, calponin, SM22α, and α-SMA immunocytochemistry of H9-derived pericyte-like cells maintained in E6 + 10% FBS to D45. Hoechst nuclear counter stain (blue) is also included. Scale bars: 200 μm. F) Temporal PCR analysis of mural and pericyte transcripts for the differentiating IMR90C4 and CS03n2 iPSC lines.

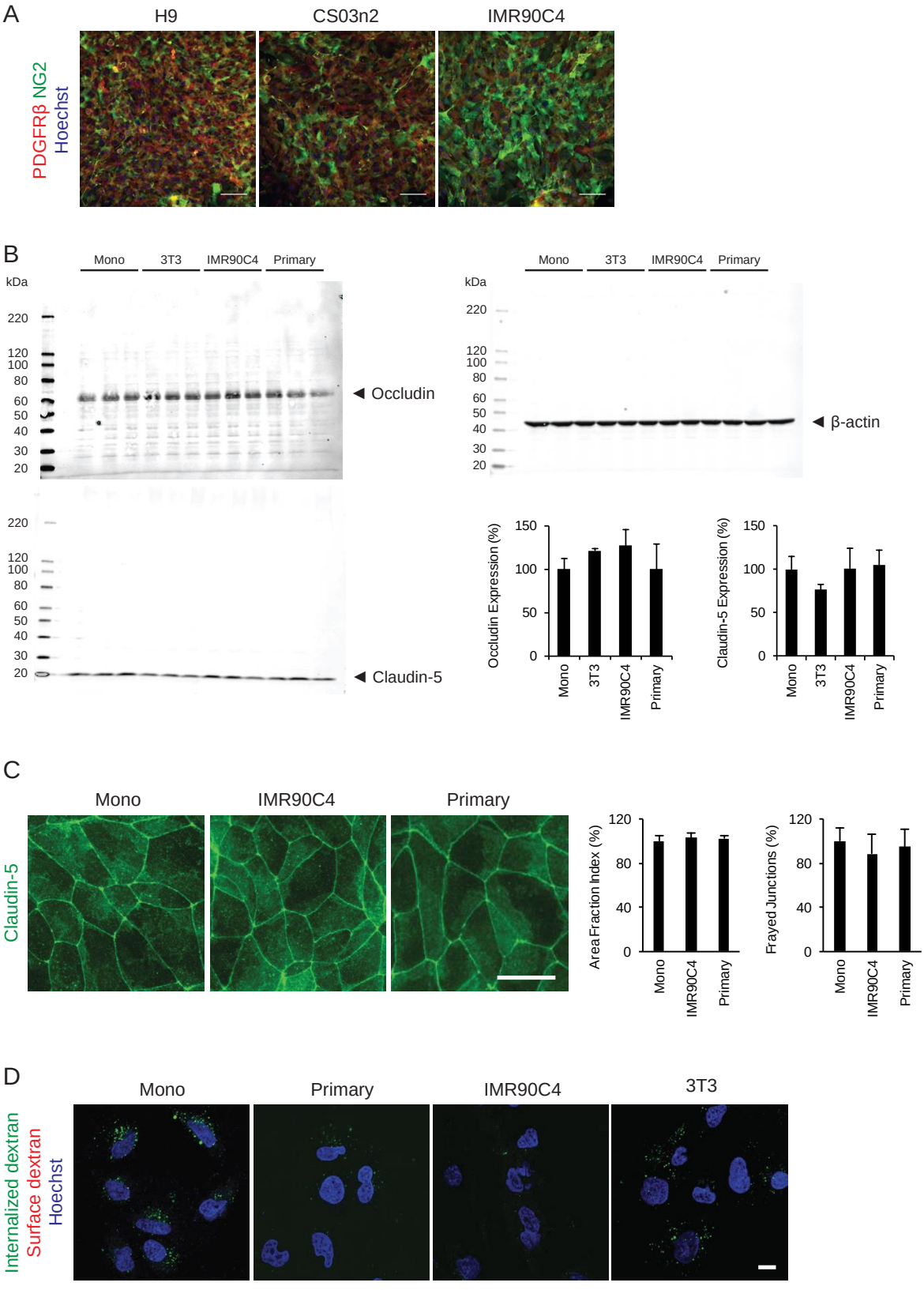
A
H9
CS03n2
IMR90C4
PDGFRβ NG2
Hoechst
B
Mono
3T3
IMR90C4
Primary
kDa
220
120
100
80
60
50
40
30
20
◀ Occludin
◀ Claudin-5
◀ β-actin
Occludin Expression (%)
Claudin-5 Expression (%)
150
100
50
0
C
Claudin-5
Area Fraction Index (%)
Frayed Junctions (%)
D
Internalized dextran
Surface dextran
Hoechst

Fig. S4. Supplemental analysis of BMEC/hPSC-derived pericyte-like cell cocultures. A) PDGFRβ and NG2 immunocytochemistry of hPSC-derived pericyte-like cells following 48 hours of co-culture with iPSC-derived BMECs. Hoechst nuclear counter stain (blue) is also included. Images are representative of two independent differentiations. Scale bars: 100 μm. B) Western blot analysis of occludin and claudin-5 expression in iPSC-derived BMECs cultured alone or co-cultured with primary brain pericytes, IMR90C4-derived pericyte-like cells, or 3T3s. Quantification of occludin and claudin-5 expression after normalized to β-actin signal and to monoculture expression levels. Plotted are the means ± SD from 3 Transwells from a single differentiation. No significant differences by ANOVA. C) Representative images of claudin-5 immunocytochemistry of BMECs cultured for 48 h in EC medium (Mono) or EC medium conditioned by primary brain pericytes or IMR90C4-derived pericyte-like cells. Scale bar: 25 μm. Quantification of claudin-5 area fraction index and frayed junctions. Plotted are the means ± SEM of 3 independent differentiations. No significant difference by ANOVA. D) Confocal microscopy of monocultured iPSC-derived BMECs incubated with Alexa 488-tagged 10 kDa dextran (green) with EC medium (Mono) or conditioned medium from primary brain pericytes, IMR90C4-derived pericyte-like cells, or 3T3s. Total dextran is depicted in green. Surface dextran was labeled with Alexa 647 (red), with little observed signal. Thus, the observed green signal is a result of internalized dextran. Hoechst nuclear counter stain (blue) is also included. Scale bar: 10 μm.

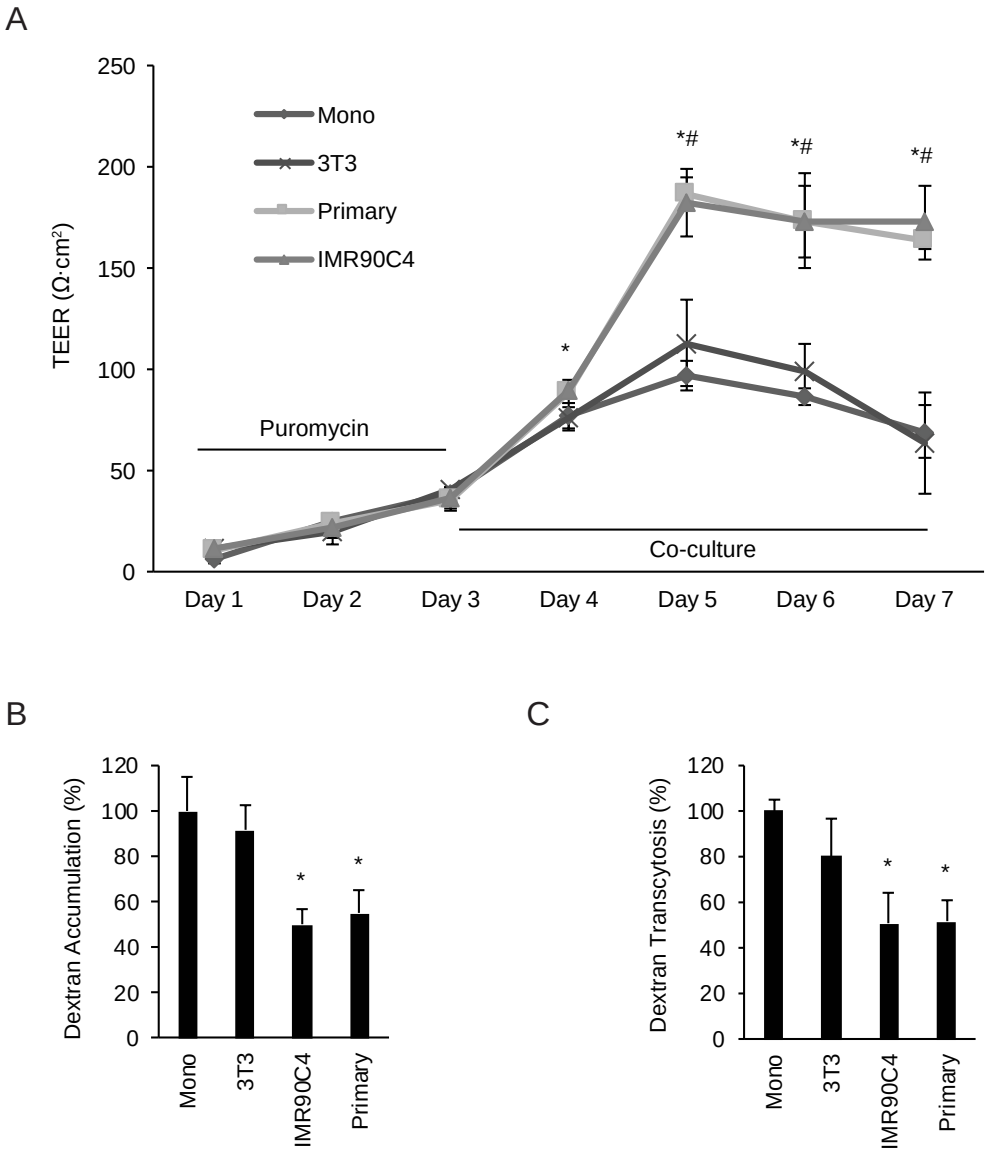

Fig. S5. Measurement of the effects of hPSC-derived pericyte-like cells on primary rat BMEC phenotypes. A) TEER profile of primary rat BMECs either in monoculture or co-culture with primary brain pericytes, IMR90C4-derived pericyte-like cells, or 3T3s. Plotted are means ± SD of three Transwells from a single rat BMEC isolation. * $P < 0.05$ IMR90C4-derived pericyte-like cell co-culture vs. monoculture; # $P < 0.05$ primary pericyte co-culture vs. monoculture; ANOVA followed by Dunnett's test. B,C) Accumulation (B) or transcytosis (C) of Alexa 488-tagged 10 kDa dextran in primary rat BMECs following co-culture with cell types as described in A. All results normalized to BMEC monoculture control. Plotted are the means ± SD of 3 Transwells from a single rat BMEC isolation. * P <0.05 vs. monoculture; ANOVA followed by Dunnett's test.

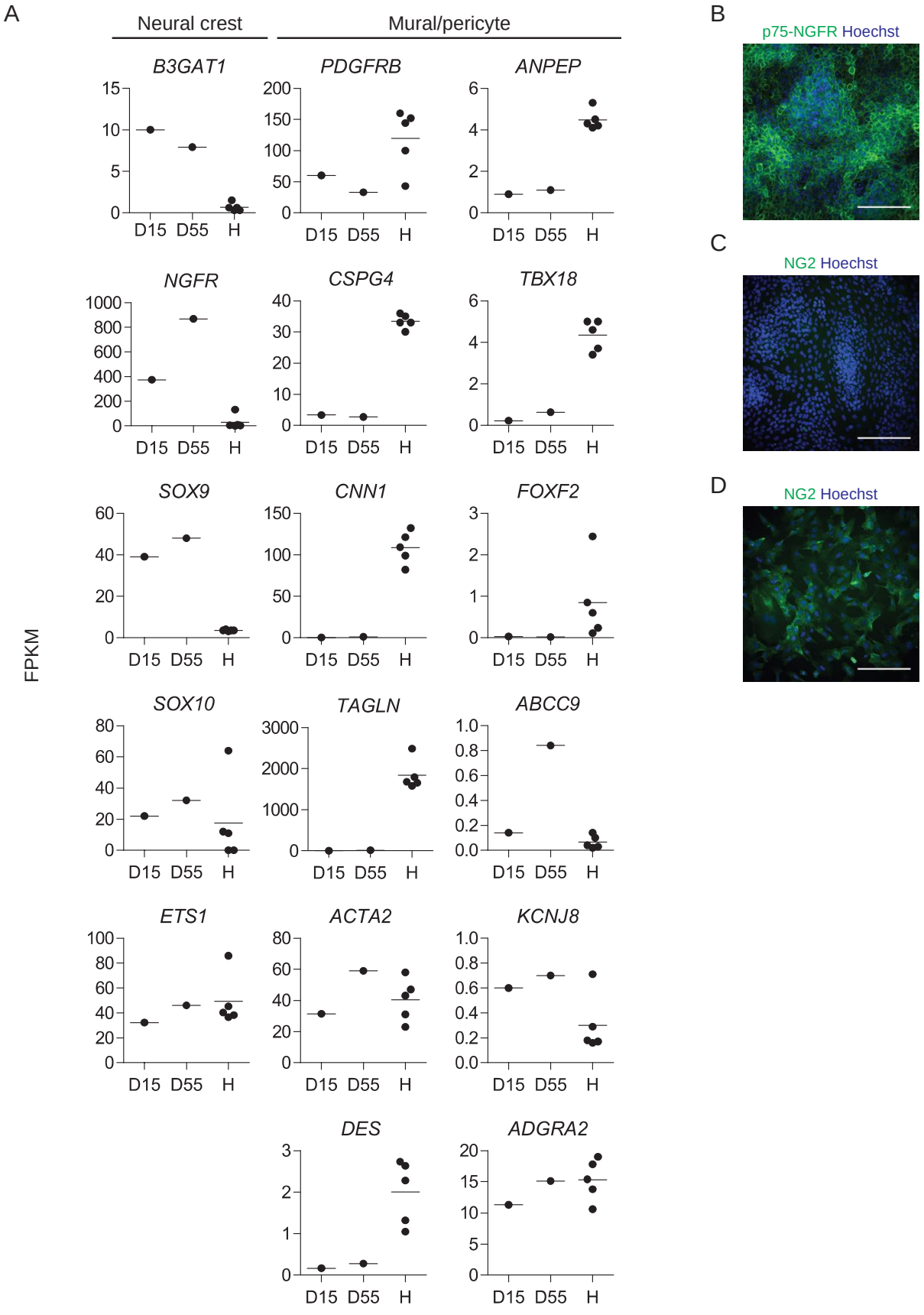
A
Neural crest
Mural/pericyte
B3GAT1
PDGFRB
ANPEP
NGFR
CSPG4
TBX18
SOX9
CNN1
FOXF2
SOX10
TAGLN
ABCC9
ETS1
ACTA2
KCNJ8
DES
ADGRA2
FPKM
D15 D55 H
B
p75-NGFR Hoechst
C
NG2 Hoechst
D
NG2 Hoechst

Fig. S6. NCSCs maintained in E6-CSFD retain neural crest marker expression and do not develop pericyte marker expression. A) Expression (FPKM) of selected transcripts in D15 NCSCs, D55 NCSCs (maintained in E6-CSFD for an additional 40 days), and all D25 pericyte-like cell samples ("H"). B) p75-NGFR immunocytochemistry analysis of NCSCs maintained in E6-CSFD for 3 months. Hoechst nuclear counter stain (blue) is also included. Scale bar: 200 μm. C) NG2 immunocytochemistry analysis of NCSCs maintained in E6-CSFD for 3 months. Hoechst nuclear counter stain (blue) is also included. Scale bar: 200 μm. D) NG2 immunocytochemistry analysis of H9-derived D22 pericyte-like cells, processed alongside and identically to the NCSC sample above. Hoechst nuclear counter stain (blue) is also included. Scale bar: 200 μm.

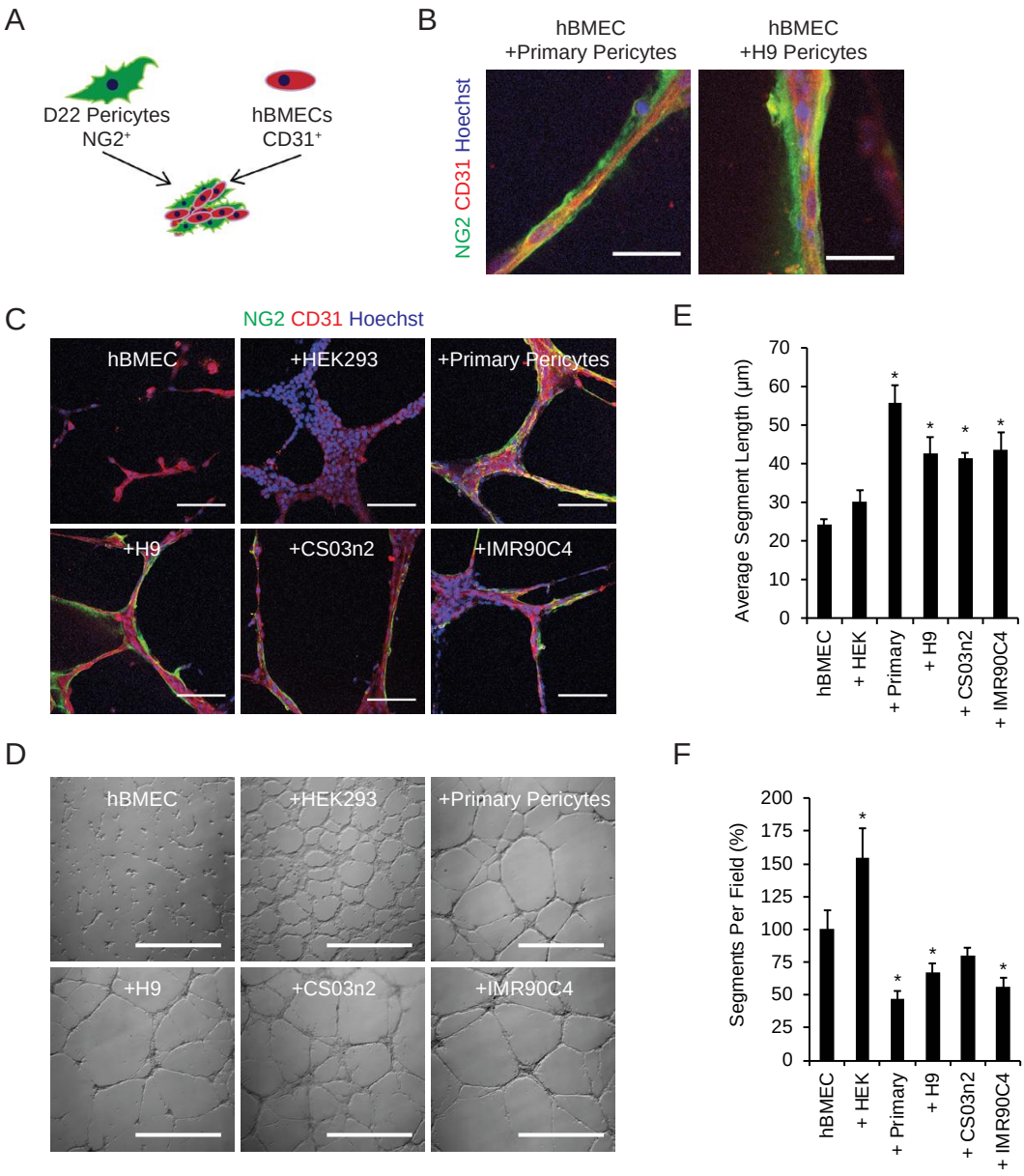

Fig. S7. hPSC-derived pericyte-like cell assembly with brain endothelial cells. A) Self-assembly schematic. hPSC-derived pericyte-like cells self-assemble with hBMECs to form vascular cords. B) Confocal immunocytochemistry images of primary pericytes and H9-derived pericyte-like cells (NG2) aligning with and extending processes along hBMEC cords (CD31). Hoechst nuclear counter stain (blue) is also included. Scale bars: 50 μm. C) Immunocytochemistry images of hBMECs alone or cultured with HEK293 fibroblasts (+HEK293), primary human brain pericytes (+Primary Pericytes), CS03n2-derived pericyte-like cells (+CS03n2), H9-derived pericyte-like cells (+H9), or IMR90C4-derived pericyte-like cells

(+IMR90C4). Hoechst nuclear counter stain (blue) is also included. Scale bars: 200 μm. D) Representative bright field images of HUVECs alone or cultured with the various cell types. Scale bars: 200 μm. E) Quantification of the average segment lengths from bright field images in panel D. Plotted are means ± SD of three imaging fields from one well. * $P < 0.05$ vs. hBMEC monoculture; ANOVA followed by Dunnett's test. F) Quantification of the number of segments per field normalized to hBMEC monoculture from bright field images in panel D. Plotted are means ± SD of three imaging fields from one well. * $P < 0.05$ vs. HUVEC monoculture; ANOVA followed by Dunnett's test.

Table S1A. Antibody staining conditions.

Marker	Fixative	App.	Vendor (Clone/Catalog #, Species)	Dilution	Blocking Buffer	Staining Buffer
p75-NGFR	4% PFA	ICC	Advanced Targeting Systems (ME20.4, Mouse IgG_1)	1:1000	1% BSA	1% BSA
		Flow	Advanced Targeting Systems (ME20.4, Mouse IgG_1)	0.2 µL/10^6 cells	N/A	PBS
HNK1	4% PFA	ICC	Sigma (C6608, Mouse IgM)	1:300	1% BSA	1% BSA
		Flow	Sigma (C6608, Mouse IgM)	0.2 µL/10^6 cells	N/A	PBS
AP2	4% PFA	ICC	DSHB (3B5, Mouse IgG_{2B})	1:50	1% BSA + 0.1% TX-100	1% BSA
Peripherin	4% PFA	ICC	Millipore (AB1530, Rabbit Polyclonal)	1:200	1% BSA + 0.1% TX-100	1% BSA
βIII-Tubulin	4% PFA	ICC	Sigma (T8860, Mouse IgG_{2b})	1:500	1% BSA + 0.1% TX-100	1% BSA
NG2	4% PFA	ICC	Millipore (MAB2029, Mouse IgG_{2a})	1:100	5% goat serum + 0.4% TX-100	PBS + 0.4% TX-100
		Flow	Millipore (MAB2029, Mouse IgG_{2a})	2 µL/10^6 cells	N/A	MACS Buffer
PDGFRβ	4% PFA	ICC	Cell Signaling Technology (28E1, Rabbit Monoclonal)	1:100	5% goat serum + 0.4% TX-100	PBS + 0.4% TX-100
		Flow	BD Biosciences (28D4, Mouse IgG_{2a})	1.25 µL/10^6 cells	N/A	MACS Buffer
CNN1	4% PFA	ICC	Sigma (C2687, Mouse IgG_1)	1:15000	3% BSA + 0.1% TX-100	3% BSA
SM22α	4% PFA	ICC	Abcam (ab14106)	1:1000	3% BSA + 0.1% TX-100	3% BSA
CD31	4% PFA	ICC	Thermo Fisher (RB-10333, Rabbit Polyclonal)	1:25	5% goat serum + 0.4% TX-100	PBS + 0.4% TX-100
aSMA	4% PFA	ICC	Lab Vision (MS-113-P)	1:100	5% milk + 0.1% TX-100	5% milk
β-actin	N/A	WB	Cell Signaling Technology (13E5, Rabbit Monoclonal)	1:1000	TBST + 5% milk	TBST + 5% milk
Occludin	MeOH	ICC	Invitrogen (OC-3F10, Mouse IgG_1)	1:200	10% goat serum	10% goat serum
	N/A	WB	Invitrogen (OC-3F10, Mouse IgG_1)	1:500	TBST + 5% milk	TBST + 5% milk
Claudin-5	N/A	WB	Invitrogen (4C3C2, Mouse IgG_1)	1:250	TBST + 5% milk	TBST + 5% milk
CD13	4% PFA	ICC	R&D Systems (MAB3815, Mouse IgG_{2a})	1:50	10% goat serum	10% goat serum
Desmin	MeOH	ICC	Thermo Fisher (RB-9014, Rabbit Polyclonal)	1:50	10% goat serum	10% goat serum

Table S1B. DNA primer sequences and running conditions.

Gene	T_a (°C)	Cycles	Fwd Sequence	Rev Sequence
ABCC9	60	40	5'- TCA ACC TGG TCC CTC ATG TCT -3'	5'- CAG GAG AGC GAA TGT AAG AAT CC -3'
ACTA2	60	30	5'- TGT TCC AGC CAT CCT TCA TC -3'	5'- GCA ATG CCA GGG TAC ATA GT -3'
ANPEP	49	40	5'- GAA GAG AAC TGG AGG AAG ATT CAG -3'	5'- CCA GGT TGA AGG CGT CAT TA -3'
B3GAT1	58	35	5'- TCG CCT GGA CTG GAC TGG GG -3'	5'- TGG CCT TGG CCT CCC TCC TC -3'
CNN1	53	40	5'- GTC CAC CCT CCT GGC TTT -3'	5'- AAA CTT GTT GGT GCC CAT CT -3'
CSPG4	54	35	IDT DNA Hs.PT.58.39417158 Predesigned Probe	
FOXF2	52	40	5'- ACC AGA GCG TCT GTC AGG ATA TT -3'	5'- GTG ACT TGA ATC CGT CCC AGT TTC -3'
GAPDH	60	30	5- GAA GGT GAA GGT CGG AGT CAA CG -3'	5'- TCC TGG AAG ATG GTG ATG GGA T -3'
KCNJ8	60	40	5'- GTG ATT GCC GTC CGA AAT GG -3'	5'- AGT TGG TGA ATA GGA ACC ACC T -3'
NANOG	58	30	5'- CGA AGA ATA GCA ATG GTG TGA CG -3'	5'- TTC CAA AGC AGC CTC CAA GTC -3'
NGFR	60	30	5'- GTG GGA CAG AGT CTG GGT GT -3'	5'- AAG GAG GGG AGG TGA TAG GA -3'
PDGFRB	53	40	5'- GCT CAC CAT CAT CTC CCT TAT C -3'	5'- CTC ACA GAC TCA ATC ACC TTC C -3'
POU5FI	58	30	5'- CAG TGC CCG AAA CCC ACA C -3'	5'- GGA GAC CCA GCA GCC TCA AA -3'
SOX10	61	40	5'- ATA CGA CAC TGT CCC GGC CCT AAA -3'	5'- TTC TCC TCT GTC CAG CCT GTT CTC -3'
SOX9	60	40	5'- AGC GAA CGC AACA TCA AGA C -3'	5'- CTG TAG GCG ATC TGT TGG GG -3'
TAGLN	51	40	5'- TCT TTG AAG GCA AAG ACA TGG -3'	5'- TTA TGC TCC TGC GCT TTC TT -3'
TBX18	60	40	5'- CCC AGG ACT CCC TCC TAT GT -3'	5'- TAG GAA CCC TGA TGG GTC TG -3'
TFAP2A	50	30	5'- TCC CTG TCC AAG TCC AAC AGC AAT -3'	5'- AAA TTC GGT TTC GCA CAC GTA CCC -3'
ZIC1	59	40	5'- TGG CCC GGA GCA GAG TAA T -3	5'- CCC TGT GTG CGT CCT TTT G -3'

Table S2. Pericyte-enriched genes identified by single cell RNA-seq in mouse (*40*) with human homologs.

Gene	FPKM	
	Primary	hPSC-derived*
ABCC9	0.3	0.1
AGAP2	0.0	0.0
ANK2	9.6	2.3
ANO4	0.2	2.5
APOD	0.2	0.6
APOE	42.9	25.6
ARHGAP31	3.4	7.6
CORO1B	68.4	56.1
ECE1	58.7	31.0
EMCN	3.3	1.7
FAM118B	0.0	0.0
FBLN1	24.7	112.0
FLT1	4.7	7.6
FOXF2	4.4	0.9
GGT1	104.8	181.9
GPR4	5.4	4.6
IFI30	18.4	17.2
IGF2	0.3	14.0
ITIH5	2.6	0.2
ITM2A	0.3	3.9
JUP	6.4	20.8
KCNJ8	0.8	0.3
LGALS9	5.1	3.4
NODAL	0.1	0.1
NRXN2	2.5	0.8
NXPH4	30.3	0.4
PCDHGC3	0.0	0.0
PDE2A	0.6	0.1
PDE8A	6.5	6.0
PHC1	0.0	10.8
PHLDB1	28.5	28.8
PLOD1	189.1	145.8
POR	33.4	28.4
PPP1CC	40.7	82.8
PREX2	0.0	0.0
SEPP1 (SELENOP)	19.8	1.4
SFRP2	0.0	16.7
SLC22A8	0.0	0.0
SLC6A13	0.4	0.1
SPP1	0.96	11.6
ST8SIA4	0.0	0.3
SYNE1.2 (SYNE1)	16.9	6.0
TNFRSF19	0.4	13.6
TRPC3	0.2	0.1
TTLL3	160.3	210.9
UCHL1	739.1	415.9
Number with FPKM ≥ 1	**26**	**29**

*Average of all D25 hPSC-derived pericyte-like cell differentiations (H9 A-C, CS03n2 and IMR90C4)