

Supplemental Information

Patient-specific iPSCs model of a genetic vascular dementia syndrome reveals failure of mural cells to stabilise capillary structures

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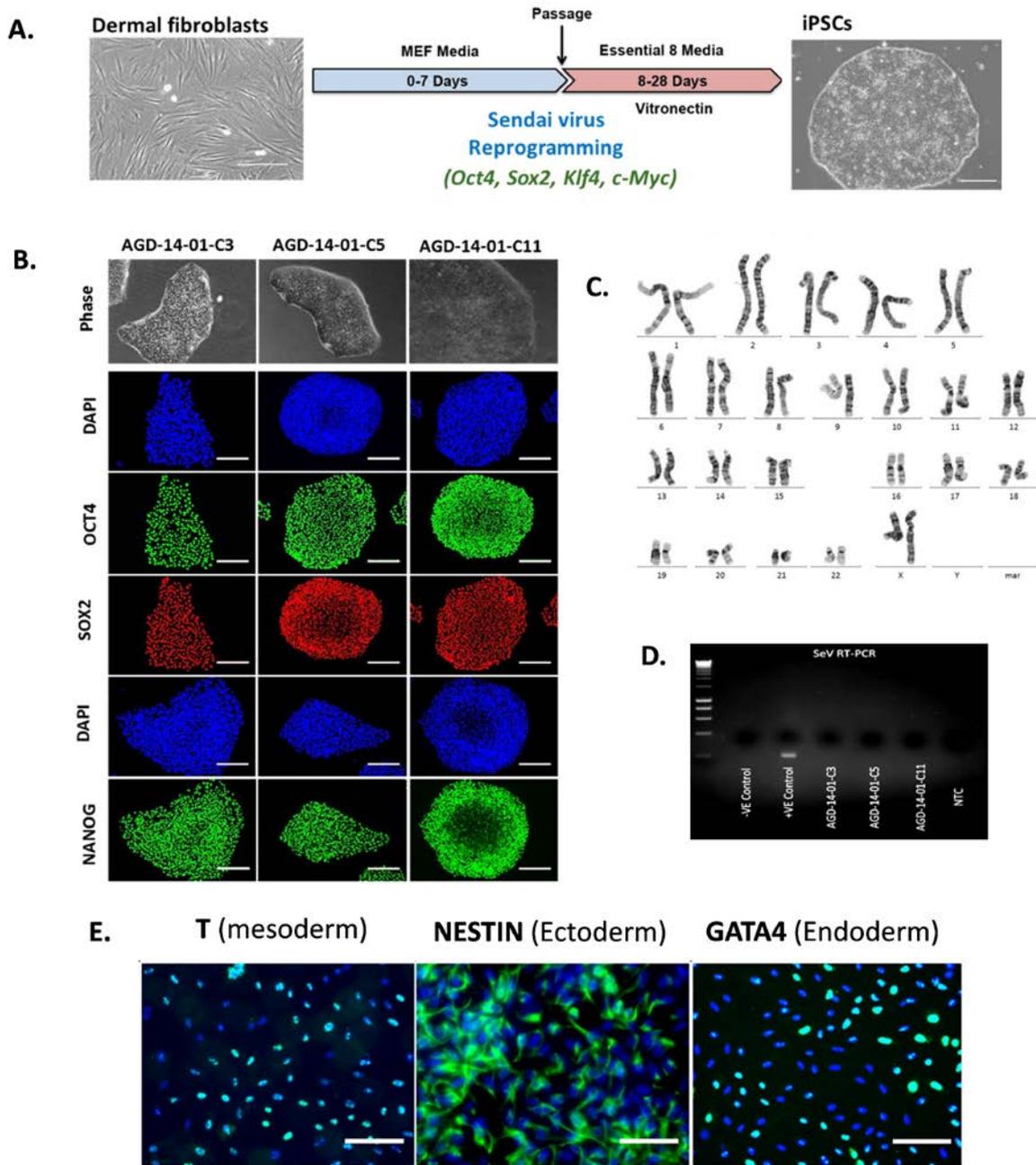


Figure S2. Establishment and characterisation of induced pluripotent stem cells (iPSCs). Related to Figures 1 and 2. Figures show representative data on samples from CADASIL patient 1 (AGD-14-01). **A.** Schematic illustrates Sendai virus mediated delivery of *OCT4*, *SOX2*, *KLF4* and *C-MYC* into dermal fibroblasts, and morphological changes of the dermal fibroblasts to form iPSCs after 28 days of the Sendai virus infection. **B.** Phase microscopy (top row) and immunofluorescence staining showing the three putative iPSC clones (AGD-14-01-C3, C5 and C11) chosen for subsequent experiments. All clones expressed pluripotency markers (Oct4, Sox2 and NANOG) with DAPI counterstain to show nuclei. **C.** Representative data from iPSC clone AGD-14-01-C3 shows a normal karyotype after reprogramming. **D.** PCR shows Sendai virus was undetectable in iPSCs after 10 passages. **E.** The iPSCs were able to form embryoid bodies (EB), and the EB outgrowths expressed markers of all three germ layers, the mesoderm (Brachyury), Neurectoderm (Nestin) and endoderm (GATA4) stained by immunofluorescence. Scale bars, 100 μ m.

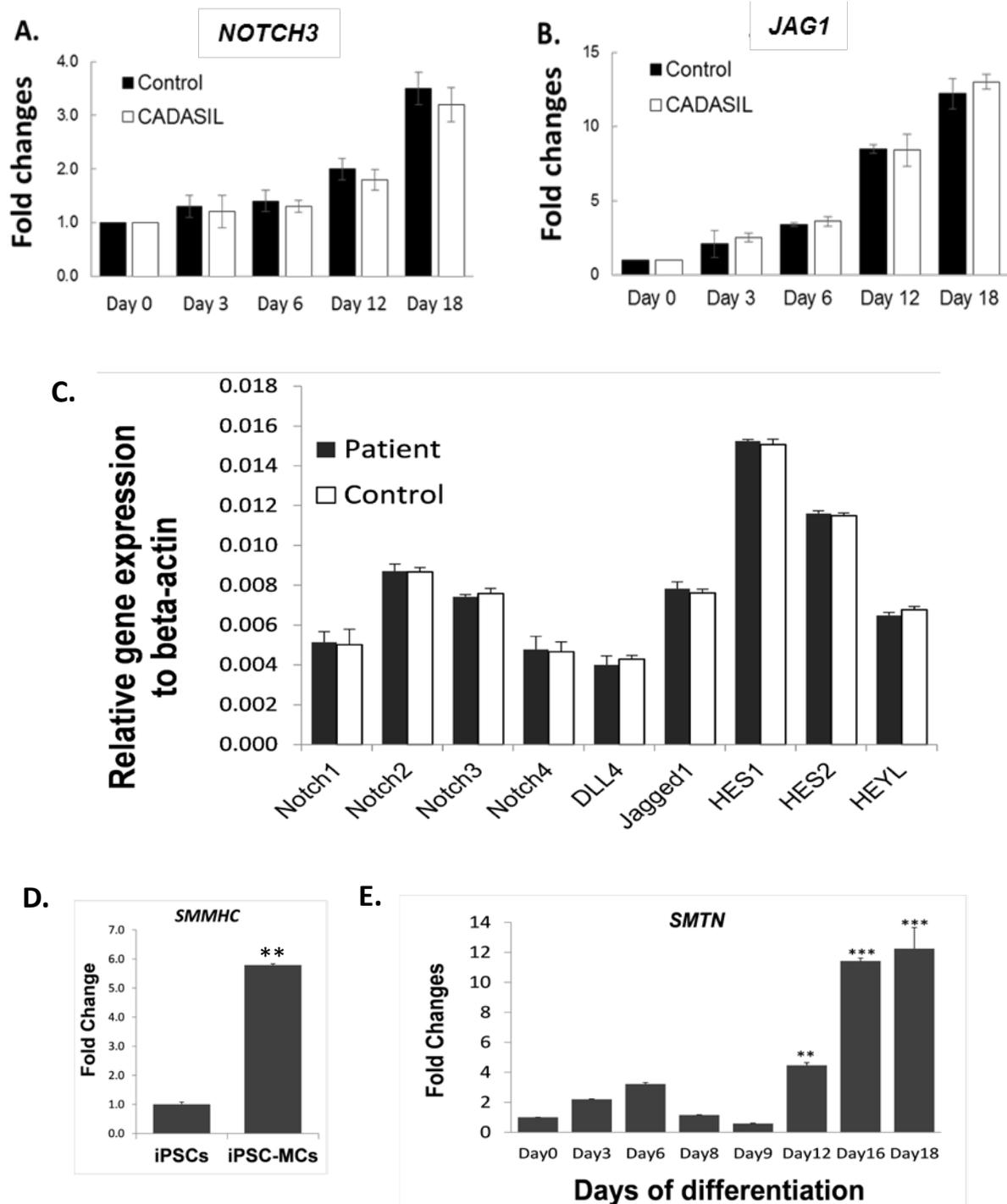


Figure S3. A-C. RT-qPCR determination of NOTCH-related gene expressions in the iPSC-MCs. Related to Figures 1 and 2. Gene expressions of *NOTCH3* (A.) and Notch ligand *JAG1* (B.) during the course of iPSC-MC differentiation. Data are presented as mean \pm SEM of 6 clones, n = 6. C. Expression of Notch receptor subtypes (*NOTCH* 1-4) and target genes (*HES1*, *HES2* & *HEYL*) in the differentiated iPSC-MCs from 3 CADASIL and 3 control clones. Data are presented as mean \pm SEM, n = 3 independent experiments. Two-way ANOVA and Turkey's post-hoc test showed no statistical differences between data from the CADASIL and controls. D & E. Additional marker gene expression in iPSC derived MCs. D. RT-qPCR determination of the expression of SMC marker, *SMMHC*, in the iPSCs and iPSC-derived MCs (iPSC-MCs). Related to Figure 2. Data are mean \pm SEM of representative samples from 3 iPSC clones from 3 independent experiments (n=3). E. RT-qPCR determination of the expression of SMC marker, *SMTN*, during MC differentiation. Data are mean \pm SEM of a representative iPSC clone with 3 replications.

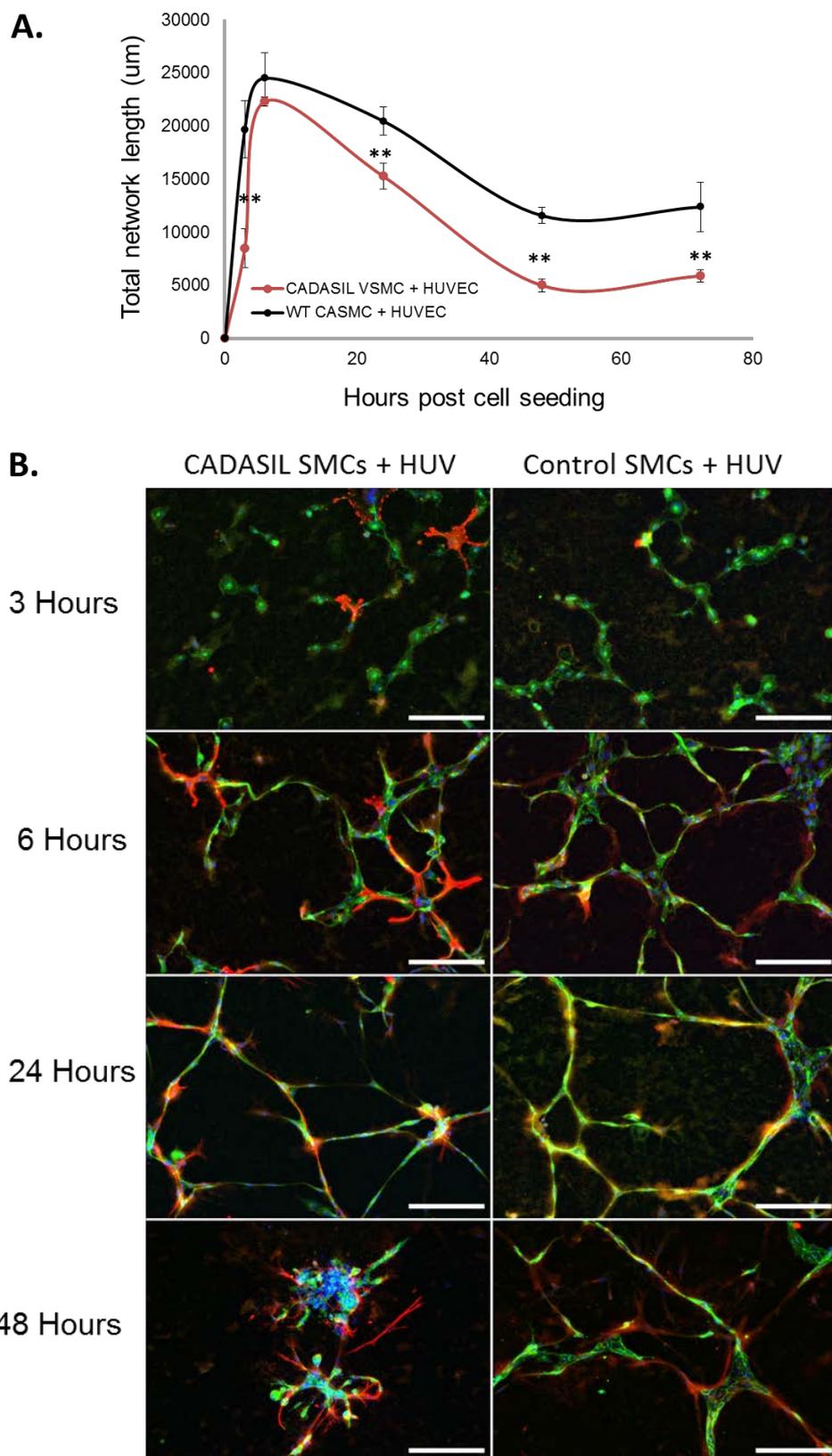


Figure S4. Primary SMCs isolated from the CADASIL patient failed to support angiogenic capillary structure *in vitro*. Related to Figures 3. SMCs isolated from small arteries of a CADASIL patient were co-cultured with HUVECs (HUV) in Geltrex on cover-glasses for *in vitro* angiogenesis assay. Human coronary artery SMCs (hCASMCs) were used as control. **A.** The capillary network was quantified and presented as mean total network length \pm SEM, $n=3$. Statistical significance was determined by two-way ANOVA and Tukey's post-hoc test. $**p<0.01$. **B.** The network structures were also double stained for α -SMA (red) and VE-cadherin (green), and counterstained by DAPI (blue). Scale bars, 200 μ m

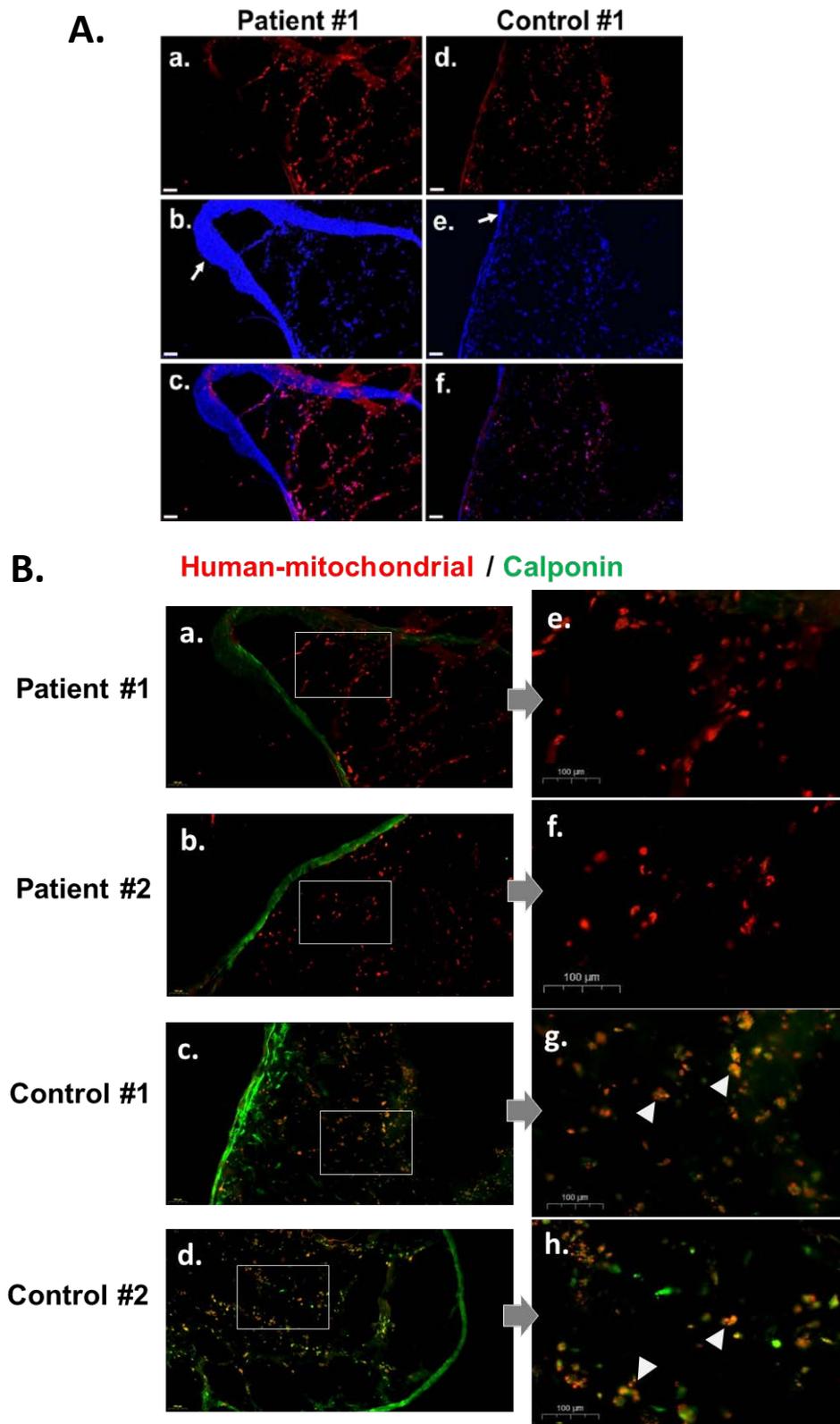


Figure S5. *In Vivo* angiogenesis analysis of iPSC derived endothelial cells and mural cells. Related to Figures 3. A mixture of the CADASIL or control iPSC-MCs and iPSC-ECs in Matrigel were injected subcutaneously into SCID mice. Two weeks after the Matrigel plugs were harvested and cryo-sectioned for immunostaining. Sections were stained using human specific anti-mitochondrial antibody (red), antibody against smooth muscle cell marker calponin (green), and counterstained with DAPI (blue). **A.** Arrows indicated capsules formed by host cells surrounding Matrigel plug. The majority of cells within the Matrigel plugs were positive for the antihuman antibody and of human origin, i.e., either iPSC-MCs or iPSC-ECs. **B.** Images in the right column (**e-h**) are the magnified regions highlighted by a square in their corresponding figures on the left (**a-d**). Human iPSC-MCs (mitochondria⁺/calponin⁺ cells) were abundant in the control plugs (arrow heads in g and h), but the mitochondria⁺/calponin⁺ cells had almost disappeared within the patient plugs. The host MCs (human mitochondria⁺/calponin⁺) were located mainly in the capsules of the plugs (white arrows in **A**). Scale bars, 100 μ m.

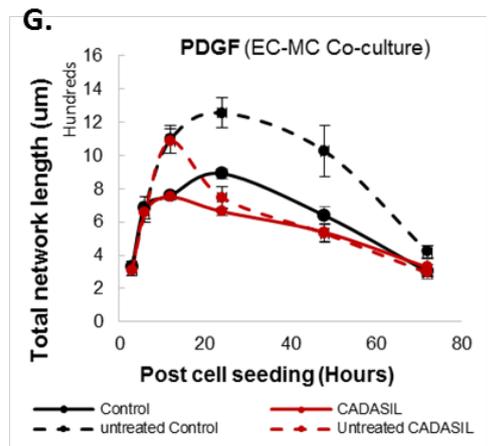
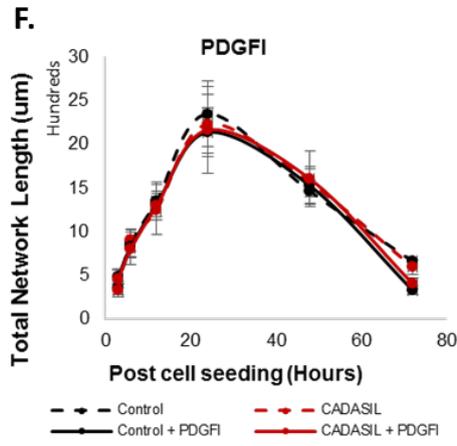
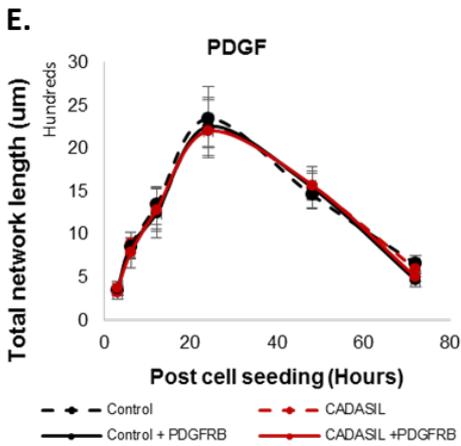
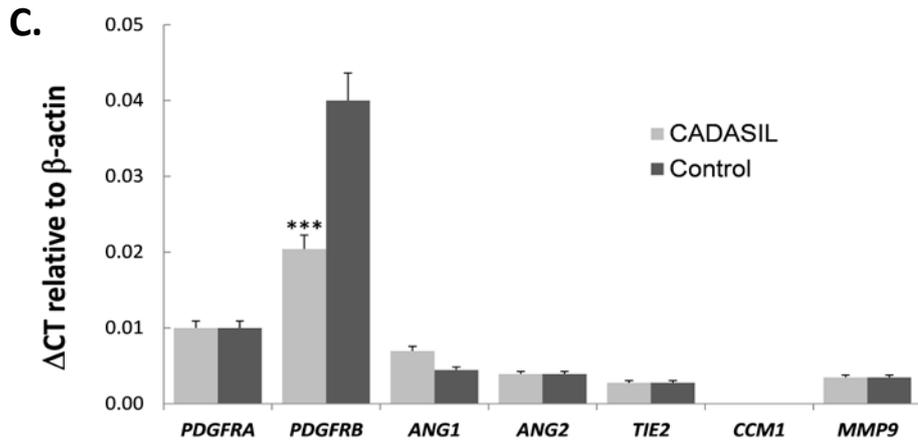
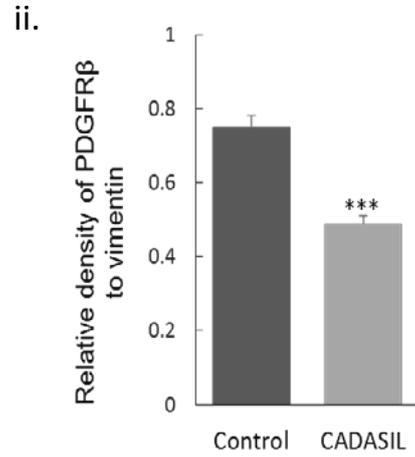
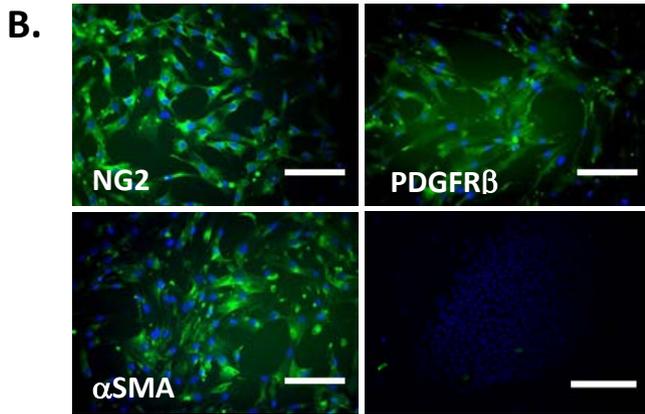
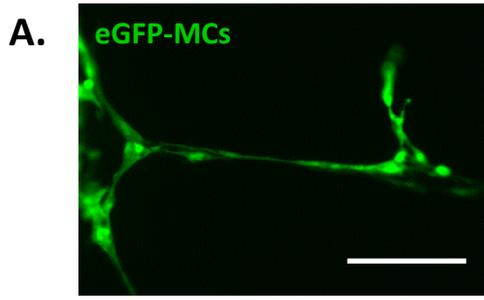


Figure S6. iPSC-MCs possess pericyte features and the role of PDGF-BB in angiogenesis. Related to Figures 3.

A. eGFP labelled iPSC-MCs wrapping around unlabelled endothelial tubular structures in an *in vitro* Matrigel angiogenesis assay, indicative of pericyte function. Scale bar, 50 μ m. **B.** Immunocytochemistry staining showing that iPSC-MCs express a set of common markers of vascular pericytes. Scale bars, 200 μ m. **C.** Expression of angiogenesis-associated genes in CADASIL and control iPSC-MCs determined by RT-qPCR. Data are mean \pm SEM from 3 clones of a patient line and 3 clones of a control line, respectively, with triplicate assays for each sample, n = 3. Result showed downregulation of PDGFR β in CADASIL iPSC-MCs as compared to the control. **D.** Western blotting of PDGFR β in CADASIL and control iPSC-MCs (i.), and quantification of the band density (ii.) confirmed the reduced PDGFR β protein level in CADASIL iPSC-MCs. Vimentin was used as sample loading control. Data are mean \pm SEM, n = 3. Student *t*-test, ***p<0.001. **E-G.** *In vitro* tubule formation by CADASIL and control iPSC-ECs in the presence of exogenous PDGF-BB (**E**) or PDGFR β inhibitor (**F**). **G.** Effect of exogenous PDGF-BB on the angiogenic tubule stability (solid lines) formed by co-culture of CADASIL (dotted red line) and control (dotted blue line) iPSC-ECs and iPSC-MCs. Data are mean \pm SEM from 3 independent experiments using 3 iPSC clones from one patient and 3 iPSC clones from one control iPSC line, n = 3. Two-way ANOVA with Tukey's post-hoc test, ***p \leq 0.001, vs controls.

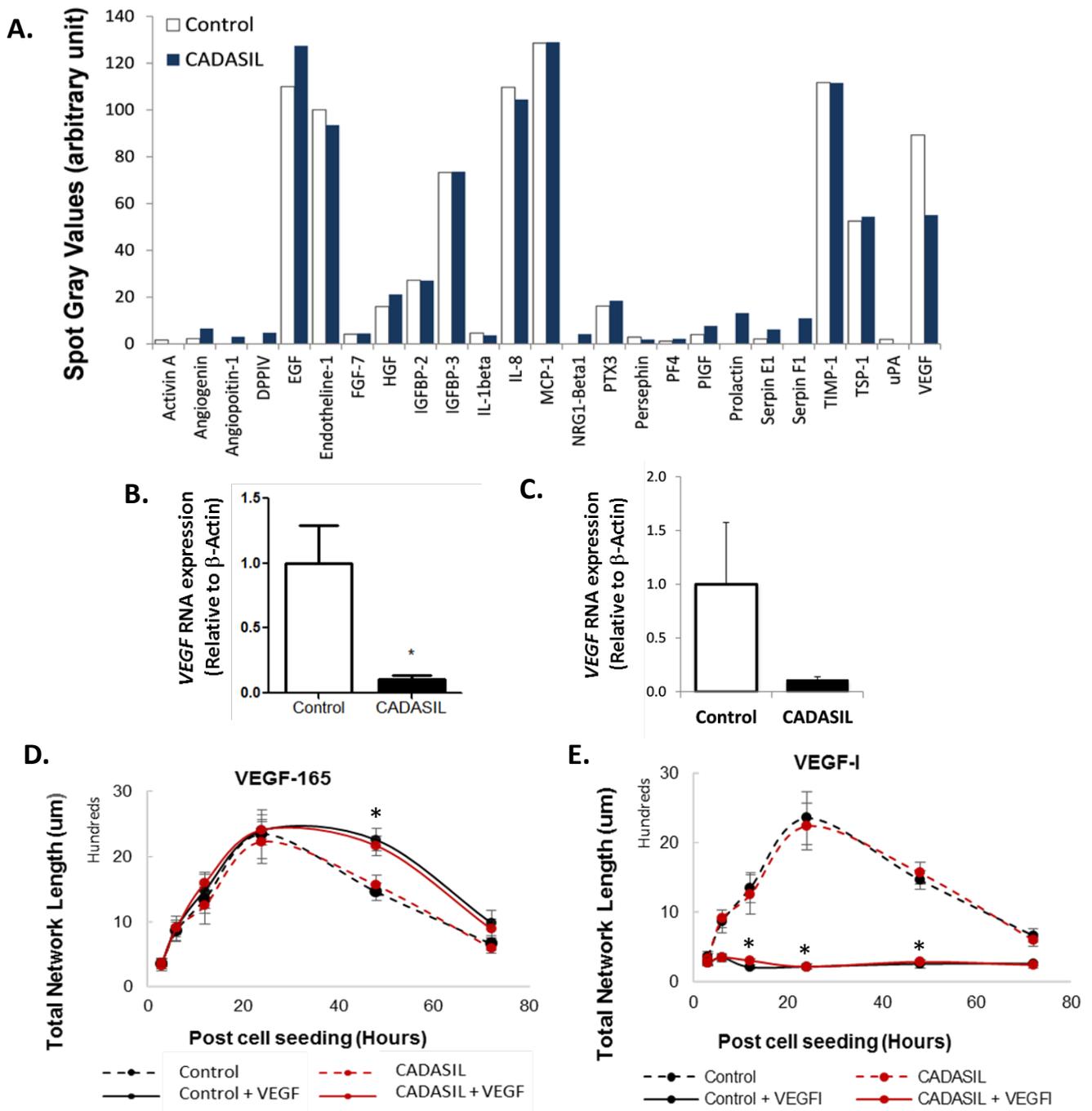


Figure S7. Quantification of the Proteome Profiler Human Angiogenesis Array and VEGF expression and effect on angiogenesis. Related to Figures 6 and 7. **A.** The Proteome Profiler Human Angiogenesis Array Kit (ARY007, R&D systems) was used to screen angiogenesis related proteins secreted from the control and CADASIL iPSC-MCs in the conditioned media according to the manufacturer's protocol. The density of the dots on the array films were quantified using ImageJ software. Data presented for each factor were the average of two separate blots using pooled culture media for the control and CADASIL iPSC-MCs, respectively. **B & C.** The expression of *VEGF* mRNA in primary VSMCs. RT-qPCR was performed on VSMCs obtained from CADASIL and control individuals in two separate studies (**B** and **C**). **B.** Studies performed in cells from 3 patients and 5 controls showed a significant decrease in *VEGF* expression ($*p < 0.05$). **C.** In a second set of experiments ($n = 4/\text{group}$), *VEGF* expression was reduced, but did not reach significance ($p = 0.121$) due to the heterogeneity in the control group. Data are mean \pm SEM. Based on these differential results, we can conclude that there is a trend of decreased *VEGF* expression in the primary VSMCs in CADASIL. **D & E.** Effects of VEGF on iPSC-EC capillary tubule formation. *In vitro* Matrigel assisted angiogenic tubule formation were carried out using CADASIL or control iPSC-ECs in the presence of recombinant VEGF-165 (**D**) or VEGF receptor inhibitor (**E**). Data are presented as mean \pm SEM of 6 clones, $n = 6$. Statistical significance was determined by two-way ANOVA and Turkey's post-hoc test, $*p < 0.05$, vs control.

Table S1. Primers used in RT-qPCR, related to Experimental Procedures.

Gene	Forward (5'-3')	Reverse (5'-3')
<i>OCT4/POU5F1</i>	AGACCATCTGCCGCTTTGAG	GCAAGGGCCGCAGCTT
<i>NANOG</i>	TTAATAACCTTGGCTGCCGT	GCAGCAAATACGAGACCTCT
<i>SOX-2</i>	GGAGCTTTGCAGGAAGTTTG	GCAAGAAGCCTCTCCTTGAA
<i>SOX-1</i>	CCTCCGTCCATCCTCTG	AAAGCATCAAACAACCTCAAG
<i>CNN1</i>	GTCCACCCTCCTGGCTTT	AAACTTGTGGTGCCCATCT
<i>α-SMA</i>	ACTGCCTTGGTGTGTGACAA	TCCCAGTTGGTGATGATGCC
<i>SM22a</i>	CGCGAAGTGCAGTCCAAAAT	CAGCTTGCTCAGAATCACGC
<i>SMMHC</i>	GACTTCCCTGCTCAATGCCT	GGACCTCTTCTCGTGGTTGG
<i>SMTN</i>	CGAAGCGCTGGTGAGTATGA	CTCTGGCACCTCACACTGTT
<i>PDGFRβ</i>	ACCTGCAATGTGACGGAGAG	GGGTGCGGTTGTCTTTGAAC
<i>ANG1</i>	GAACACGATGGCAACTGTCTG	GCTGTATCTGGGCCATCTCC
<i>ANG2</i>	CTAAGGACCCACTGTTGCT	CCATCCTCACGTCGCTGAATA
<i>VEGF-165</i>	CTACGTCCACCATGCCAAGT	GCACTAGCTGCGCTGATAGA
<i>KDR</i>	TGATGCCAGCAAATGGGAAT	GCACCACGGCCAAGAGGCTTA
<i>ISL-1</i>	AGATTATATCAGGTTGTACGGGATCA	ACACAGCGGAAACACTCGAT
Brachyury (T)	GGGTCCACAGCGCATGAT	TGATAAGCAGTCACCGCTATGAA
<i>PECAM1 (CD31)</i>	ATTTTGCACCGTCCAGTCC	GAGTCCTGCTGACCCTTCTG
<i>VE-CADHERIN</i>	ATCAAGCCCATGAAGCCTCT	GGTCCTGCGGATGGAGTATC
<i>MESP1</i>	AAGAAGAGCATGGGAGAGGTG	CAGTTCCCCACCAGGTTCAAA
<i>PDGFRα</i>	GCGCAGGGAGTTTGAGAGAA	CCACGGCCTCCAATGATCTC
<i>TEK (Tie2)</i>	TCCATGGAGAAACAGAGGCTGA	TAGACCTCTTGAGAGGGGAG
<i>KRIT1 (CCM1)</i>	AGCGCCTGTGAAGGAGATTC	AGAACATGCGCTGAAGGTGA
<i>MMP9</i>	TTTGAGTCCGGTGGACGATG	GCTCCTCAAAGACCGAGTCC
<i>NOTCH3</i>	CATCTCCGACCTGATCTGCC	GTCTGTAGAGCGGTTTCGGA
<i>NOTCH1</i>	AAGAATGGTGCCAAGTGCCT	GAAGCAGAGGTAGGCGTTGT
<i>NOTCH2</i>	GGAGGGACCTGCTCTGACTA	TACCCAGGCCATCAACACAC
<i>NOTCH4</i>	ATGTGTGTGTGACGTGGGTT	GACATGGCCCTGAGTGACAA
<i>HES1</i>	GAAAGATAGCTCGCGGCATT	TGATCTGGGTGATGCAGTTGG
<i>HEY1</i>	TCTGAGCTGAGAAGGCTGGT	GATAACGCGCAACTTCTGCC
<i>JAG1 (JAGGED1)</i>	GCCTGTCAGTGATGTGCAAG	TTCATTTGTTCTGCCTGTGC

Table S2. Antibodies used in immunofluorescence staining and western blotting, related to Experimental procedures.

Antigen	Antibody name	Supplier	Working concentration
OCT4	Human/Mouse Oct-3/4 Antibody (AF-1759-SP)	R&D Systems	1 µg/ml
SOX2	Human/Mouse SOX2 Antibody (MAB2018-SP)	R&D Systems	1 µg/ml
SOX1	Anti-SOX1 antibody (ab22572)	Abcam	2 µg/ml
Brachyury (T)	Human/Mouse Brachyury Antibody (2085-SP)	R&D Systems	1 µg/ml
Nestin	Anti-Nestin antibody [10C2] (ab22035)	Abcam	500 ng/ml
GATA4	Human GATA-4 Antibody (AF2606-SP)	R&D Systems	2 ug/ml
PECAM1	Human CD31/PECAM-1 Antibody (BBA7)	R&D Systems	500 ng/ml
VE-Cadherin	Human VE-Cadherin Antibody (MAB9381-SP)	R&D Systems	500 ng/ml
VE-Cadherin conjugated (FACS)	Human VE-Cadherin PE-conjugated Antibody (FAB93811P-025)	R&D Systems	1 µg/ml
Calponin	Anti-Calponin antibody [EP798Y] (ab46794)	Abcam	1 µg/ml
SM22 α	Anti-SM22 alpha antibody (ab14106)	Abcam	1 µg/ml
α SMA	Anti-alpha smooth muscle Actin antibody [1A4] (ab7817)	Abcam	1 µg/ml
NG2	Anti-NG2 antibody [132.38] (ab50009)	Abcam	1 µg/ml
PDGFR β	Anti-PDGF Receptor beta antibody [Y92] (ab32570)	Abcam	500 ng/ml
Vimentin	Vimentin (V9) anti-Mouse (SC-6260)	Santa Cruz Biotechnology	500 ng/ml
Human mitochondria	Anti-Mitochondria (MAB1273)	EMD Millipore	20 µg/ml

Supplemental Experimental Procedures

Establishment of patient-derived iPSCs

Punch skin biopsies were taken from two CADASIL patients carrying *NOTCH3* mutations Arg153Cys (73 years old) and Cys224Tyr (58 years old), respectively, and a non-affected control individual (62 years old). The procedure for patient recruitment and iPSC generation was approved by the North West – Greater Manchester East branch of the National Research Ethics Service (REC reference NO 12/NW/0533). Both patients clinically presented with recurrent strokes and cognitive decline. Adult human dermal fibroblasts (HDFs) were purchased from Invitrogen as an additional non-CADASIL control. A 2-4 mm full-thickness skin plug was added to 0.25% Trypsin (Invitrogen) made up in sterile water and incubated for 10 minutes at 38°C. The sample was centrifuged at 500xg for 10 mins and the pellet was then treated with 0.5 mg/ml of collagenase at 38°C for 1 hour and centrifuged at 500g for 10 minutes and the supernatant discarded. The digested cell pellet was re-suspended in Chang D culture medium (Irvinesci) in a T25 cm² flask and cultured in a CO₂ incubator at 37°C with medium replacement every 3 days. Once cells had reached confluence, they were treated with a trypsin-EDTA solution (0.3 µM versene and 0.125% trypsin, Invitrogen) to dissociate the cells for sub culture.

HDF reprogramming was achieved by infecting passage 2-4 HDFs with a commercial non-integrative Sendai virus (SeV) Cytotune-iPSC 2.0 kit (Life Technologies), containing three reprogramming plasmid vectors (carrying *OCT*, *C-MYC*, *KLF4* and *SOX-2*) according to the manufacturer's instructions. Over the course of 28 days the HDFs were monitored for morphological changes. Identified colonies were excised and cultured in feeder free conditions as described below.

iPSC culture

iPSCs were routinely cultured on truncated, recombinant vitronectin (VTN-N, Life technologies) coated Costa 6-well cell culture plastic plates (Corning). The 6-well plates were incubated with 1.0 ml/cm² VTN-N for 30 minutes at room temperature or 4°C overnight prior to iPSC seeding. iPSCs were routinely cultured in Essential 8 (E8, Life Technologies) pluripotent cell culture medium replaced every 24 hours. For sub-culture, iPSC colonies were incubated with 0.5 µM EDTA in phosphate buffered saline (PBS) for about 1 minute. The colonies were then aspirated and replated into fresh E8 medium supplemented with 10µM Y-27632 (Sigma Aldrich) and gently pipetted repeatedly to dissociate the iPSC colonies into smaller cell clusters of around 10-20 cells. The iPSC clusters were then seeded into new VTN-N coated 6-well plates at a 1:3 ratio. iPSC colonies were routinely imaged using Leica DM IL LED inverted microscope (Leica).

Chromosome karyotype analysis and DNA sequencing

iPSCs were cultured in Costa 6-well plates until confluent. The iPSCs in the plates were sent to the Genomic Diagnostics Laboratory, Manchester Centre for Genomic Medicine, Central Manchester University Hospitals NHS Foundation Trust, Saint Mary's Hospital, for karyotyping using a standard Giemsa banding protocol. For DNA sequencing, genomic DNA from iPSCs were extracted using ISOLATE II Genomic DNA Kit (BIOLINE). Sanger DNA sequencing was conducted by the DNA sequencing Service in the University of Manchester using intronic primers flanking *NOTCH3* exon 4 (PCR primers: sense: 5'-TAGTCGGGGGTGTGGTCAGT-3', antisense: 5'-CCTCTGACTCTCCTGAGTAG-3'). Sequencing primer was the same as the sense primer.

Endothelial cell differentiation from iPSCs

iPSCs were seeded onto a VTN-N coated Costa 6-well plate at around 1 cell cluster (10-20 cells) per cm² in E8 medium supplemented with 10 µM Y-27632 and cultured for 24 hours at 37°C. The adherent cells were then washed with PBS without calcium and magnesium (Sigma Aldrich), and cultured in fresh E8 media in the absence of Y-27632 for 24 hours before changing to Essential 6 (E6, Life technologies) medium supplemented with 3 µM CHIR99021 (Calbiochem), 10 ng/ml recombinant BMP4 (Peprotech) and 10 ng/ml recombinant FGF2 (Peprotech). After a further 24 hours of culture, the medium was replaced with E6 supplemented with 50 ng/ml BMP4 and 10 ng/ml FGF2 and renewed every 24 hours until day 6 of differentiation. At day 7 of differentiation, BMP was reduced to 25 ng/ml, and 25 ng/ml VEGF-165 (Peprotech) included in the medium. The cells were cultured for a further 24 hours before VEGF-165 was increased to 50 ng/ml and BMP4 withdrawn. The medium was replaced every 24 hours until day 12 of differentiation.

Fluorescence associated cell sorting (FACS)

At day 12 of iPSC-endothelial differentiation, the differentiated cell population was disassociated by incubation with TrypLE (Life Technologies) for 3.5 minutes, and then suspended into 10 ml PBS and washed 3 times by centrifugation at 200xg. The cells were then incubated with 5% fetal calf serum (FCS) in PBS containing a PE-Conjugated Antibody to human VE-Cadherin (clone #123413, cat no: FAB9381P, R&D systems) for 1 hour. The cells were washed 3 times with PBS and sorted by FACS to obtain VE-Cadherin⁺ EC cell population using the FACSAria fusion (BD Biosciences) running Diva 8.0.1 software (BD Biosciences). Forward scatter and side scatter gating was used to distinguish live from dead cells using a 488 nm laser.

Mural cell (MC) differentiation from iPSCs

iPSCs were passaged onto VTN-N coated Costar 6-well plates at around 1-2 clusters/cm² in E8 medium supplemented with 10 μ M of Y-27632 and cultured for 24 hours. The iPSCs were then washed with PBS and cultured in E6 medium with 10 μ M SB-431542 (Sigma Aldrich) and 10 ng/ml FGF2 for 24 hours. The medium was replaced every 24 hours until day 5 of differentiation when the cells were subcultured into new VTN-N coated Costar 6-well plates at a density of 0.5x10⁴ cells/cm². At day 6, the supplements were replaced with 2 ng/ml TGF- β (Peprotech) and 5 ng/ml PDGF-BB (Peprotech), and medium replaced every 24 hours until day 18 of differentiation. The iPSC-derived MCs (iPSC-MCs) were characterised by immunofluorescence staining, as described below, for alpha-smooth muscle actin (α -SMA), transgelin (TNN1), calponin (CNN1), smoothelin (SMTN), smooth muscle myosin heavy chain 11 (SMMHC), Neuroglial antigen 2 (NG2) and platelet derived growth factor receptor beta (PDGFR β).

Primary Cell culture

Primary human coronary arterial endothelial cells (HCAECs) used as controls for the nitric oxide measurement, and primary human coronary artery smooth muscle cells (HCASMCs) used as controls for the *in vitro* angiogenesis, were supplied by Promocell. HCAECs were cultured in Endothelial Cell Growth Medium 2 (Promocell). HCASMCs were cultured in Smooth Muscle Cell Growth Medium 2 (Promocell) or in Medium 231 supplemented with Smooth Muscle Growth Supplement (SMGS, Invitrogen) and used between passages 3 and 8. For the *in vitro* angiogenesis and apoptosis confirmatory experiments on primary cultured VSMCs from CADASIL patients and healthy controls, ethics approval for recruiting CADASIL and health control individuals and isolation of primary SMCs from these individuals was obtained from the West of Scotland Research Ethics Service (WS/12/0294) and from the Ethics Board of the Ottawa Hospital Research Institute, Canada (#997392132), Canada. Written informed consent was obtained for all study participants in accordance with the Declaration of Helsinki. Patients with CADASIL were recruited from the Neurovascular Genetics clinic, Queen Elizabeth University Hospital, Glasgow, and healthy controls were volunteers at the OHRI. Subjects underwent a gluteal biopsy under local anaesthetic. Small arteries (<200 μ m diameter) were dissected from subcutaneous fat. Vessels were cleaned of adventitial tissue and placed in Ham's F-12 culture medium containing 1% gentamicin, collagenase (type 1), elastase, soybean trypsin inhibitor, and BSA and incubated for 1 hour at 37°C under constant agitation. The digested tissue was further dissociated by repeated aspiration through a syringe with 20G needle. The cell suspension was centrifuged (2000 rpm, 4 minutes) and the cell pellet was resuspended in Ham's F-12 culture medium containing 10% FBS. Cells were seeded onto 25mm round glass coverslips. For the first 48 hours, cells were incubated in Ham's F-12 culture medium containing 10% heat-inactivated FCS. Thereafter, VSMCs were maintained in 231 media containing smooth muscle growth supplement (Thermo Fisher, Glasgow, UK) with penicillin/streptomycin. Cells from 4 CADASIL patients (mutations Arg169Cys; Arg141Cys; Arg54Cys) and 4 controls were used between passages 2 and 6.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RNA was extracted from cells using the RNeasy mini kit (Qiagen) according to manufacturer's instruction. Lysate was purified by centrifugation through Qiashreder (Qiagen) columns, and samples were treated with RNase-Free DNase kits (Qiagen). Two hundred ng/ μ l of total RNA was reverse transcribed to cDNA using Tetro cDNA synthesis kit (Bioline). Ten ng/ml of cDNA and 0.25 μ M of forward and reverse primers were used for each qPCR reaction. SYBR green reagent (Applied Biosystems; Thermo Scientific) was used for the qPCR reaction. QPCR was carried out in a StepOnePlus real-time PCR system (Applied Biosystems) with reaction conditions as follows: 94°C for 15 seconds followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. All qPCR reactions were carried out in triplicate. Expression of specific genes was normalised against the internal control gene, β -actin. The results were presented either as fold changes compared to a control sample using 2^{- $\Delta\Delta$ CT} or Δ CT against internal control. The sequences of the primers used in qRT-PCR are presented in Suppl Table 1. The absence of the Cytotune 2.0 Sendai Reprogramming vectors in patient derived iPSCs was confirmed by RT-PCR using the manufacturer suggested primers.

Immunofluorescence staining

Cells were first fixed with 2-4% paraformaldehyde (PFA) in PBS at room temperature for 10-15 minutes and washed three times with PBS. Non-specific binding sites were blocked with 5% FCS in PBS for 1 hour at room temperature followed by incubation with primary antibody for 1 hour in 5% FCS in PBS. After a further wash with PBS, the cells were incubated with secondary antibody in 5% FCS in PBS for 45 minutes and then counterstained with a 10 μ g/ml DAPI solution. Samples were imaged immediately using a Leica DM IL LED inverted microscope.

Western blotting

Ten µg protein of cell lysate was loaded to 4-20% mini-protean TGX pre-cast gels (Biorad) and electrophoresed in mini-PROTEAN tetra vertical electrophoresis cells (Biorad) at 200V for about 1 hour before transfer to a nitrocellulose membrane using a Trans-Blot Turbo transfer system (Biorad). The membrane was then blocked in 5% Marvel milk in Tris-buffered saline (TBS), and incubated with primary antibody (table 2) in 5% Marvel in TBS supplemented with 0.1% Tween-20 (Biorad) (TBS-T) over night at 4°C. The blot was washed three times with TBS-T and incubated with HRP conjugated secondary antibody (Dako) at room temperature for 45 minutes. After a further wash with TBS-T, the protein bands were visualised using Amersham ECL Start western blotting detection reagent and Hyperfilm ECL (GE Healthcare).

In vitro angiogenesis assay

For the *in vitro* angiogenesis assay using iPSC-derived vascular cells, 70 µl of 10 mg/ml Matrigel (Corning) solution was added into each well of Costar 96-well cell culture plates and incubated for 1 hour at room temperature. Ten thousand iPSC derived ECs (iPSC-ECs) were plated into each well of the Matrigel coated plates in E6 medium supplemented with 5 ng/ml VEGF-165 and 2 ng/ml FGF2, and cultured in a CO₂ incubator at 37°C for 3, 6, 12, 24, 48 and 72 hours, respectively, for capillary network formation. For co-culture of iPSC-ECs/MCs used in *in vitro* angiogenesis assays, a mixture of 1x10⁴ iPSC-ECs and 0.5x10⁴ iPSC-MCs were seeded into each well of a Matrigel coated 96-well plate and then the same procedure followed for the iPSC-EC angiogenesis assay as described above. For determination of roles of VEGF and PDGF in angiogenesis, recombinant VEGF-165 (PeproTech), VEGFR2 inhibitor ZM 323881 hydrochloride (R&D Systems, 3 µM), or PDGFRβ inhibitor DMPQ dihydrochloride (Santa Cruz Biotechnology, 3 µM) were added to the angiogenesis assay, respectively, during cell seeding and replaced with the culture media every 24 hours. To quantify the angiogenesis assay, 5 phase contrast images taken from each experimental condition were analysed to measure the total network length formed by iPSC-ECs or iPSC-ECs/MCs using the ImageJ software [Fiji ImageJ (<http://imagej.net/Fiji/Downloads>)] with 'angiogenesis analyser' plugin. Cultures were imaged at 6, 12, 24, 48 and 72 hours using a Leica DM IL LED inverted microscope and a Leica DFC365 FX CCD microscope camera attachment. Results were presented as total network length.

For *in vitro* angiogenesis assay using primary CADASIL VSMCs, the cells were cultured in Medium 231 with Smooth Muscle Growth Supplement (Thermo Fisher Scientific). Glass cover slips (13 mm) were coated with Geltrex (Thermo Fisher Scientific). Either 1x10⁴ HCASMCs (control) or CADASIL VSMCs in 400 µl were added to wells containing the Geltrex-coated cover slips followed by the addition of 2x10⁴ human umbilical vein endothelial cells (HUVECs, Promocell). Both cell types were suspended in Endothelial Cell Growth Medium 2 (Promocell). Cells were then fixed at 3, 6, 24, 48 and 72 hours using 4% paraformaldehyde in PBS for 10 min followed by quenching and permeabilization using 0.2 M glycine 0.5% Triton x-100 for 5 minutes. Cell networks were stained with anti-human VE-Cadherin antibody (D87F2, Cell Signalling Technology) and anti-human α-SMA antibody (1A4, DAKO), followed by secondary antibodies Donkey anti-Mouse IgG (H+L) Alexa Fluor 555, (A31570, Thermo Fisher Scientific) and Donkey anti-Rabbit IgG (H+L) Alexa Fluor 488 (A21206, Thermo Fisher Scientific). Coverslips were mounted with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Images were collected on an Olympus BX51 upright microscope using 4x objectives and captured using a Coolsnap ES camera (Photometrics) through MetaVue Software (Molecular Devices). Images were processed and analysed using ImageJ and presented as the total network length.

For determination of the role of soluble factors secreted from the iPSC-MCs on EC angiogenic network formation, either a transwell set-up or use of conditioned medium from iPSC-MCs was employed. For the transwell experiment, polyester (PET) membrane transwell-clear insert plates (Corning) were coated with 150 µl of 10 mg/ml Matrigel for 1 hour at room temperature. iPSC-ECs were seeded at 1x10⁴ cells per cm² onto the Matrigel coated plates in 2 ml per well of Opti-MEM I media. The iPSC-ECs were allowed to adhere to the Matrigel coated surface for 2 hours. In the meantime, iPSC-MCs were seeded onto a 2 cm diameter, 0.4 µm pore size PET membrane transwell insert in 1 ml of Opti-MEM I medium and cultured for 3 hours to allow cells to adhere. The iPSC-MC-loaded transwell insert was then placed into the iPSC-EC culture well and cultured for up to 72 hours for capillary network formation. For the angiogenesis assay using conditioned medium, iPSC-ECs were directly placed in the conditioned medium from iPSC-MCs cultures as described above, and seeded into the Matrigel coated 96-well plate.

In vivo angiogenesis assay

All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals at Kings College of London. Differentiated iPSC-MCs and iPSC-ECs from two CADASIL and two control individuals (1x10⁶ cells per sample) were mixed with 100 µl of Matrigel and injected subcutaneously into the back or flank of NOD.CB17-Prkdc^{scid}/NcrCrl mice. Six injections were conducted for each group. Fourteen days later, the mice were killed and the plugs were harvested, frozen in liquid nitrogen, and cryosectioned. Cryosectioned slides were fixed with 4% paraformaldehyde in PBS at 4°C, and then stained with anti-human mitochondria (EMD Millipore) and anti-human calponin antibody (Abcam) as described in the

immunofluorescence staining section above.

Conditioned media collection from iPSC-MC cultures

iPSC-MCs were seeded at 1×10^4 cells/cm² onto tissue culture plastic plates in Opti-MEM I Reduced Serum Medium (Gibco, Life Technologies) and cultured for 24 hours. The medium was replaced with fresh Opti-MEM medium and the cells were cultured for a further 48 hours. The medium was then harvested and centrifuged at 500xg for 5 mins to be used either immediately or aliquotted and stored at -80°C.

Angiogenesis proteome profiler array analysis

The Proteome Profiler Human Angiogenesis Array Kit (ARY007, R&D systems) was used to assess differences in angiogenesis related proteins secreted from the control and CADASIL iPSC-MCs in the conditioned media collected from iPSC-MC cultures as described above according to the manufacturer's protocol. The kit consists of 4 nitrocellulose membranes which contain 55 capture antibodies absorbed onto each membrane, in duplicate. The blots were quantified by densitometry using the ImageJ software.

ELISA analysis

Conditioned medium collected from cultures of iPSC-MCs, primary VSMCs from CADASIL patient, or hCASCs were subject to ELISA assays using Human VEGF Quantikine ELISA kit (R&D Systems) to quantify the concentrations of VEGF. The assay was conducted according to the manufacturer's instruction.

Apoptosis assay

One thousand iPSC-MCs or primary microvascular SMCs from CADASIL patients, or 1.5×10^3 iPSC-ECs per well were seeded into white opaque 96-Well Microplates (Pierce, Thermo Scientific), respectively, and cultured for 3 hours to allow cells to adhere. The cells were then washed with PBS and the medium replaced with 50 μ l Opti-MEM containing different concentrations of hydrogen peroxide (1 – 1000 μ M) or Staurosporine (0.1 – 100 nM), respectively, and incubated for 24 hours. The Caspase3/7 activity was then measured using the Caspase-Glo 3/7 Assay kit (Promega). Briefly, an equal volume of the Caspase-Glo 3/7 substrate was added to each well of the cell culture plate and mixed well using a microplate vortex mixer. The plate was then incubated at room temperature for one hour before reading luminescence using GloMax-multi+ microplate multimode reader (Promega).

For measuring the impact of CADASIL iPSC-MCs on the survival of iPSC-ECs, iPSC-MCs and iPSC-ECs were mixed to a 1:1 ratio and seeded into single wells of a Costar 6-well plate at a density of 1×10^4 cells/cm² and co-cultured for 48 hours. The co-cultured iPSC-EC and iPSC-MCs were dissociated in TrypLE for 3 minutes, centrifuged at 200xg for 5 mins and washed with PBS. The cell pellets were suspended in 5% FCS in E6 medium containing anti-CD31 Dynabeads (Thermo Fisher Scientific) and incubated for 30 mins at room temperature. The iPSC-ECs and iPSC-MCs were then separated by magnetic associated cell sorting (MACS). Post sorted iPSC-ECs and iPSC-MCs were subject to the caspase3/7 assays as mentioned above.

Nitric oxide measurement using Griess reaction

Nitric oxide concentration was quantified using the Griess Reagent System (Promega). Fresh conditioned media were collected from cultured ECs and centrifuged at 500xg for 5 minutes to remove cell debris. The supplied sulphanilamide solution and N-1-naphthylethylenediamine dihydrochloride (NED) solutions were allowed to equilibrate at room temperature for 30 minutes. Fifty μ l of the conditioned media or a nitrite standard were added to each well of a 96-well microplate (ThermoFisher Scientific, Nunc). Fifty μ l of sulphanilamide solution was added to each well and incubated at room temperature for 10 minutes, 50 μ l of NED solution was then added to each well and incubated at room temperature, protected from light, for a further 10 minutes. The absorbance of each well was measured immediately at 530nm using GloMax-multi+ microplate multimode plate reader (Promega). Samples were quantified according to a standard curve of nitrite concentration plotted against absorbance.

Small interfering RNA (siRNA) NOTCH3 knockdown in iPSC-MCs

NOTCH3 specific siRNA sequences and a scrambled negative control siRNA were purchased from Qiagen (Venlo, Netherlands): 'FlexiTube GeneSolution GS4854' and 'Negative Control', respectively. *NOTCH3* siRNA SI00009513 (Seq 5' AAG GAA TAG TTA ACA CTC AAA 3') or scrambled siRNA (5' AAT TCT CCG AAC GTG TCA CGT 3') were delivered into 2×10^6 iPSC-MCs by the 4D-nucleofector system (AAF-1002B) + X unit (AAF-1002X) (Lonza) using the 4D p3 kit (Lonza) with programme CM138. After nucleofection, 2ml of pre-warmed Promocell Smooth Muscle Cell Growth Medium 2 (Promocell) was immediately added into the cell suspension, and the cells were reseeded into Costar 6-well plates (Corning) and cultured for 24 hours before total RNA extraction for qRT-PCR, cell lysis preparation for western blotting or reseeded into Matrigel coated Costa 96-well plates for *in vitro* angiogenesis analysis.

Statistics

Gaussian distribution of each data set was determined by the Shapiro-Wilks test using Microsoft excel utilising the Real statistics addon available from <http://www.real-statistics.com/free-download>. Data that displayed a P value greater than 0.05 was considered to be normally distributed and further statistical tests were then applied. Data were presented as mean \pm SEM. Unpaired Student *t*-test was used to compare differences between results from two groups of samples. Where more than two means were compared, one-way or two-way ANOVA was performed in Microsoft excel using the data analysis tool and 'ANOVA, single factor'. Tukey's post-hoc test or Bonferroni post-hoc test were then followed to confirm the differences between two groups. A p-value \leq 0.05 was considered as statistically significant.