SUPPLEMENTAL MATERIAL

I. Supplementary Methods

Ethics Statement

The hESC lines H1 (WA01), H7 (WA07), H9 (WA09), and ES03 (ES03) used in these studies were obtained from the Wisconsin International Stem Cell Bank (WISCB). The use of all WISCB-donated hESC lines in these studies was approved by the Johns Hopkins Institutional Stem Cell Research Oversight (ISCRO) Committee. All animal surgical procedures were performed in accordance with protocols approved by the Johns Hopkins School of Medicine Institute of Animal Care and Use Committee (IACUC) and the Association for Research of Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Visual Research.

Episomal and Viral-Integrated hiPSC Lines

Detailed methods for generating four factor (4F) and seven factor (7F) nonviral, non-integrated hiPSC from human cord blood (CB) and fibroblasts with EBNA1-based episomes were previously described [1, 2]. Briefly, 4F (SOX2, OCT4, KLF, MYC: SOKM expressed from a single plasmid) or 7F (SOKM plus LIN28, NANOG, SV40T antigen expressed from three co-nucleofected plasmids) were nucleofected with the AMAXA nucleofector system (Lonza) in growth factor-expanded CD34+ CB progenitors, as described [1, 2]. Cells were co-cultured with irradiated (2000 rad) human bone marrow mesenchymal stromal cells (BMSC, Lonza) for three days. Stromal-primed (sp) 4F CB-iPSC lines (E5C3, E12C5, E17C6) and 7F CB-iPSC lines (6.2, 6.13, 19.11) were isolated on irradiated (5000 rad) mouse embryonic fibroblast (MEF, 5000 rad) in hESC medium supplemented with FGF-2 (40 ng/mL) and MEF-conditioned medium. Sp-CB-iPSC colonies were manually subcloned at ~ 3 weeks post-nucleofection in hESC medium supplemented with 10-20 ng/mL of bFGF. Non-integrated 7F episomal fibroblast-iPSC (7ta) and lentivirally-generated 4F fibroblast-iPSC lines (IMR90-1, IMR90-4[3] (WISCB), HUF3, HUF5[4]) were previously described.

hESC and hiPSC Culture

All hESC and hiPSC lines used in these studies were maintained and expanded in undifferentiated states on irradiated MEF, as previously described[5, 6]. MEF were derived from mouse embryos (E12.5-13.5) generated from CF1 (Charles River, Wilmington, MA) and DR4 (The Jackson Laboratory, Bar Harbor, ME) mouse matings. High quality, early passage (<P2) MEF were used for iPSC generation and thawing of hESC/iPSC. Human pluripotent stem cells (hPSC)

were grown in hESC medium comprising of DMEM/F12 supplemented with 20% Knockout Serum Replacer (KSR), 0.1 mM MEM-non-essential amino acid (MEM-NEAA), 1 mM L-glutamine, 0.1 mM beta-mercaptoethanol, and 4-20 ng/mL FGF-2 (all Life Technologies, Grand Island, NY) (**Table S1**). Medium was changed daily, and hPSC were maintained in >95% SSEA4+TRA-1-81+ undifferentiated states by rigorous manual and enzymatic passaging. Spontaneously differentiating colonies were routinely removed manually from cultures by micropipette-ablation using an inverted microscope (EVOS, Life Technologies) in a laminar flow cabinet (MidAtlantic Diagnostics Inc., Mount Laurel, NJ). Pluripotent stem cells were passaged in bulk enzymatically every 6-7 days using a solution of collagenase type-IV (1 mg/mL in DMEM/F12, 5 min, 37°C, Sigma, St. Louis, MO), or manually dissected using a 10 µL micropipette (Eppendorf, Hamburg, Germany) under EVOS microscope.

Generation of Luciferase Transgene+ hPSC Lines

A humanized luciferase cDNA optimized for expression was acquired from pGL4.13 (Promega), and subcloned into lentivector construct pWPT (Addgene) containing a modified EF-1 alpha promoter and a puromycin selection cassette for stable, constitutive expression in hESC/hiPSC. High titer lentiviral supernatants were prepared via standard methods. Prior to lentiviral transduction, hESC and hiPSC were plated onto Matrigel pre-coated plate (BD Biosciences, 1:100, 1 hr at 37°C) in MEF conditioned medium (CM) supplemented with 10 ng/ml FGF-2. When pluripotent stem cells were ~20-30% confluent (2^{nd} day of passage), medium was replaced with 1 mL fresh CM containing high titer luciferase lentivirus and diluted polybrene (final concentration 6 µg/mL, Life Technologies), incubated for 8 hours, and then replaced with fresh CM, and changed daily until hESC/hiPSC were ~70-80% confluent (5^{th} day of passage). Stably-integrated hESC/hiPSC clones were selected with puromycin (0.5-1.0 µg/mL) for 2 days, and puromycin-resistant colonies were expanded on DR4-MEF (9^{th} day). Stable luciferase transgene+ hESC/hiPSC clones were routinely maintained in hESC medium containing 0.5 µg/mL puromycin until vascular differentiation.

Generation of Vascular Progenitors (VP) from hPSC

Human embryoid body (hEB) differentiation: Hemato-endothelial differentiations of hEB with BMP4, VEGF, FGF2/heparin (BVF2H) were performed essentially as described[7, 8] which is modified and optimized from our original method[5]. hESC and hiPSC lines with >95% undifferentiated morphology were expanded on gelatinized 6-well tissue culture plates to 80-90% confluency. Culture medium was switched to adaptation medium (AM, **Table S3**) for one day prior

to hEB generation. On the next day (day 1; see **Figure 1A**), hPSC were treated with 2 mg/mL dispase (Sigma Aldrich) in DMEM/F12 for 5 min at 37°C. The dispase solution was removed, and 1 mL of DMEM/F12 was added to collect hPSC with a cell scraper (Sarstedt, Newton, NC) and hPSC clumps were resuspended in an ample amount of DMEM/F12 medium. Cell suspensions were centrifuged (300 g, 5 min, room temperature), and cell pellets were resuspended in AM (2 wells to 0.5mL). 0.5 mL of hESC/hiPSC suspensions were gently deposited over 2 mL of semi-solid methylcellulose medium (MM, **Table S4**) in a well of ultra-low attachment 6-well plates (Corning, Lowell, MA). Plates were tightly wrapped with Saran Wrap (SC Johnson, Racine, WI) to create semi-hypoxic conditions, and incubated at 37°C. Two days later, small aggregated hEB clumps were collected in ample amount of phosphate buffer saline (PBS, Life Technologies), centrifuged at 300 g for 5 min at room temperature, washed once more in PBS, and re-suspended in liquid differentiation medium (LDM, **Table S5**). The hEB/LDM suspension was returned to the original ultra-low attachment plate, and further incubated in semi-hypoxic conditions. hEB were collected every 2-3 days, centrifuged at 200g, 1 min and re-suspended in fresh LDM.

Vascular lineage differentiation: Day 8 hEBs generated as above were collected in PBS, and centrifuged 200g, 1 min. hEB were resuspended in 2-3 mL collagenase type-IV (1 mg/mL) for 5 min at 37°C, and enzymatically digested into clumps. hEB clumps were collected using a p1000 Pipetman following gentle disaggregation in MEF medium, centrifuged 200g, 5 min, then plated (~$1x10^5$ cells/well) onto fibronectin (10 µg/mL/well)-coated plates in endothelial growth medium 2 (EGM2, Lonza) supplemented with 25 ng/mL VEGF. The next day (day 1), cells were replenished with fresh EGM2 supplemented with 25 ng/mL VEGF and the medium was changed every 2 days. Adherent hEB cells were harvested from EGM2 cultures for FACS purification of VP populations with 0.05% trypsin-EDTA (Life Technologies) 4-5 days later.

Flow Cytometry

EGM2-cultured hEB cells were washed once in PBS, and enzymatically digested with 0.05% trypsin-EDTA (5 min, 37°C). Enzymatic activity was neutralized using MEF medium, and the cell suspension was filtered through a 40 µm cell-strainer (Fisher Scientific, Pittsburgh, PA) to eliminate large cell clusters. hEB cells were centrifuged (200 g, 5 min, room temperature) and resuspended in staining buffer (EBM alone or EMG2:PBS=1:1) at a concentration or lower of $1x10^7$ cells/mL. Single cell suspensions (<$1x10^6$ cells in 100 µL per tube) were incubated for 20 min on ice with directly conjugated mouse monoclonal anti-human antibodies (**Table S6**) in solo or duo combinations. Isotype controls matching each immunoglobulin subtypes were stained

analogously to reveal non-specific binding. Cells were washed with 3 mL of PBS, centrifuged (300g, 5 min, room temperature), and resuspended in 300 µL of staining buffer prior to acquisition. Viable cells were analyzed (10,000 events acquired for each sample) using the BD CellQuest Pro analytical software and FACSCalibur™ flow cytometer (BD Biosciences). For intracellular staining (e.g., anti-luciferase antibody), cells were fix and permeabilized using FIX & PERM reagents (Life Technologies) according to the manufacturer's protocol. All data files were subsequently analyzed offline using Flowjo analysis software (Tree Star Inc., Ashland, OR).

Matrigel and Collagen Gel Assays

Four hEB populations based on expression of CD31 and CD146 were FACS-purified on day 4 of EGM2 differentiation as described in Methods. FACS-purified cells were plated on fibronectin-coated tissue culture plates (10 µg/mL) and further expanded. Cells were harvested into single cell suspensions with 0.05% trypsin-EDTA for 5 min. $2x10^5$ cells/well were cultured in EGM2 medium in 48 well plates pre-coated with Matrigel (BD, 200 µL/well) or collagen gel (ECMatrix, EMD Millipore). The next day, phase contrast pictures of vascular tube formations were imaged with an inverted Eclipse Ti-u Nikon microscope (Nikon Instruments Inc., Melville, NY) and Eclipse imaging software.

Transmission Electron Microscopy (TEM) of Vascular Tubes

Tubes in collagen gels or Matrigel were prepared for TEM as follows, and visualized with a Hitachi TEM instrument. Tubes grown in either collagen or Matrigel in 6 well or 96 well tissue culture plates were fixed at room temperature in 2.5% paraformaldehyde/2%gluteraldehyde in 0.1M cacodylate buffer, pH 7.4 for a minimum of 3 hours then stored in fix overnight at 4˚C. They were washed in the culture dishes with 0.05M cacodylate buffer, pH 7.4 to remove the fix. The gel drops were teased from the wells using a small spatula. Areas containing tubes were dissected out and placed in small vials of 0.05M cacodylate buffer. The tubes were post fixed for 1.5 hours in 1% osmimum in 0.05M cacodylate, pH 7.4 and washed in cacodylate buffer. Dehydration was accomplished using a grade series of ethyl alcohols followed by 1hr en bloc staining with 1%uranyl acetate in absolute ethanol. Propylene oxide washes were used to enhance resin infiltration. Final embedding was done in LX112 resin (Ladd Research Industries, Inc., Williston, VT). The blocks were polymerized at 60˚C for 48 hours. One micron semi-thin and 72nm ultrathin sections were prepared using a Leica UC7 (Leica Microsystems, Wetzlar, Germany). Sections

were imaged with a Hitachi H7600 TEM at 80KV (Gaithersburg, MD) and a side mount AMT CCD camera (Woburn, Mass).

DiI-Acetylated Low Density Lipoprotein (DiI-Ac-LDL) Uptake

FACS-purified populations based on expression of CD31 and CD146 were expanded for 5-7 days prior to DiI-Ac-LDL (Life Technologies, Cat No. L-3484) uptake assay. Each population was plated on fibronectin pre-coated 6-well plates (1-1.5 x 10^5 cells/well), and cultured in EGM2 until they reached 60 to 70% confluency. Medium was then switched to fresh EGM2 supplemented with 10 µg/mL DiI-Ac-LDL, and incubated for 4 hours at 37°C. Cells were washed in PBS and DiI-Ac-LDL-positive cells were photographed using Nikon Eclipse Ti-u inverted microscope and Eclipse imaging software. Cells were treated with 0.05% trypsin-EDTA (5 min, 37°C) and processed for flow cytometry analysis to quantify DiI-Ac-LDL+ cells.

NOD-SCID Matrigel Plug Assay

Four FACS-purified populations based on expression of CD31 and CD146 were expanded in EGM2 for 5-7 days prior to Matrigel plug assays. Matrigel was mixed with 5 x 10^5 cells in a final volume of 200 µL, and the mixture was subcutaneously injected into the mid-lower abdominal region of immunodeficient NOD-SCID mice. After 14 days, the animals were euthanized and dissected to remove the Matrigel plugs. For the purpose of orientation, the abdominal skeletal muscle was removed intact with each Matrigel plug. Plugs were paraffin-embedded, sectioned, and stained with a polyclonal rabbit anti-CD31 (Abcam, Massachusetts, USA) followed by goat anti-mouse horseradish peroxidase secondary antibody and streptavidin horseradish peroxidase. For human specific CD31+ blood vessel detection, sections were treated with antigen retrieval step (Dako, Carpentaria, CA) and stained with mouse anti-human CD31 (Dako) followed by biotinylated goat anti-mouse secondary antibody and streptavidin-cy3.

Whole Genome Expression Microarrays

FACS-purified $CD31^+CD146^+$ VP cells were expanded for 5-7 days in EGM2 prior to collection in TRIZOL reagent for gene array samples. Total RNA was isolated with RNeasy Mini Kit (QIAGEN) with on-column DNase I digestion. All samples were processed at the Microarray Core Facility, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University. Briefly, 200 ng RNA from each sample was amplified and labeled using the Illumina Total Prep RNA Amplification Kit, AMIL1791 (Ambion, Austin, TX) as described in the manufacturer's manual. All arrays were hybridized at 58ºC for 16-20 hours followed by wash and stain procedures according to the Whole-Genome Gene Expression Direct Hybridization Assay Guide (Illumina, San Diego, CA).

Fluorescent signals were obtained by scanning with the iScan System and data were extracted with GenomeStudio and signal intensities from multiple chips were quantile-normalized without background subtraction.

Gene microarrays bioinformatics data from Illumina Human HT-12 arrays were analyzed with the Partek Genomics Suite (Partek Inc., St. Louis, MO) and Spotfire DecisionSite for Functional Genomics™ (TIBCO Software Inc., Somerville, MA) platforms. Signal data was imported into Partek and log2 transformed. Gene identifiers were updated to NCBI builds as of July 2012. Where indicated, expression values were mean-normalized to better demonstrate how expression differed across the examined cell types. In mean normalization, each gene's mean log2 signal value is determined for all cell types and then subtracted (division in log space) from each cell type's value for that gene. The normalized values underwent unsupervised hierarchical clustering in Spotfire (Correlation algorithms) to compare cell types' gene expression in a heatmap-dendrogram wherever indicated (color spectrum indicates where lower=blue; higher=red).

Microarray Accession Numbers

The NIH Gene Expression Omnibus has issued the accession number GSE44926. The GEO-supplied link for Reviewers for anonymous, read-only access use of the microarray data related to this manuscript is:
http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=rlmbzgewskggyvs&acc=GSE44926

Immuno-fluorescence Staining

FACS-purified populations based on expression of CD31 and CD146 were plated onto fibronectin (10 μg/mL, 4 °C, overnight)-coated plates in EGM2. Five to 7 days later, cells were fixed in 2% paraformaldehyde/1x Tris-buffered saline (TBS), and incubated in blocking solution (2% normal goat serum). Cells were stained with Dil-Ac-LDL, α-SMA, CXCR4, and anti-luciferase antibodies as described in **Table S6**, and imaged with an inverted fluorescent microscope as described above.

Flat-mount Immuno-histochemistry of NOD-SCID Retinas

Engraftment of luciferase transgene-positive human cells into NOD-SCID mouse retinas was verified directly with anti-luciferase immunohistochemistry and murine vascular marker co-localization (murine CD31 and collagen IV). Animals were euthanized for retinal harvests at the

following time points after human VP injection (2 days post-ischemia-reperfusion): 3, 7, 14, 21, and 45 days after cell injections. Retinal whole mounts were used to map retinal cell counts at 7, 14, and 21 days after ischemia-reperfusion and cell injection. After euthanasia, eyes were enucleated and fixed in 2% paraformaldehyde in TBS for 60 min at room temperature. After removing cornea and lens, the retina was carefully separated from the choroid and sclera and permeabilized via incubation with 0.1% Triton-X-100 in TBS solution for 15 min at 4°C. Following thorough washing with TBS, free floating retinas were blocked with 2% normal goat serum in TBS with 1% bovine serum albumen and incubated overnight at 4°C in a primary antibody solution, using rabbit anti-mouse Collagen IV (ab756P, Millipore, 1:100) and rat anti-mouse CD31 (550274, BioSciences, 1:50) primary antibodies in 0.1% Triton-X-100 in TBS solution to label basement membrane and EC of blood vessels, respectively. On the next day, retinas were further washed with TBS, and incubated with secondary antibodies for 6 h at 4°C. A goat anti-rabbit Cy3 secondary antibody (111-165-003, Jackson Immuno Research, 1:200) was used to detect collagen IV primary antibody, and a goat anti-rat Alexafluor-647 secondary antibody (A21247, Invitrogen, 1:200) was used to detect the anti-CD31 primary antibody. Human cells were detected using anti-luciferase-FITC antibody (1:10, Abcam, Cambridge, MA, Cat no: ab34506, Lot no: 831617 or 765206). After washing in TBS, the flatmount retinas were observed with a confocal microscope LSM510 Meta (Carl Zeiss Inc., Thornwood, NY) in the Wilmer Eye Institute Imaging Core Facility.

For quantification of luciferase-positive cells in both superficial and deep vascular plexus of retinal vessels, immunostained I/R and no I/R-treated eyes were visualized with a Stereo Investigator microscope using Nero explorer software. Values were expressed as a mean ± SEM. P values were calculated with unpaired two-tailed Student t tests. Differences were considered statistically significant at *p*-values less than 0.05. After confocal microscopy, whole-mount retinas were cryo-embedded in a solution consisting of a 2:1 mixture of 20% sucrose in 0.1 mol/L phosphate buffer/OCT compound (TissueTek, Baxter Scientific, Columbia, MD). Eight-μm-thick frozen cross sections were cut with a Microm HM500M cryostat (Global Medical Instrumentation, Ramsey, MI) at -25°C.

Quantification of Migrating Cells and Human Cell Engraftment

For quantification of human cell migration into the retina, frozen whole mount retinas were serially cross-sectioned and three slides of 8 μm sections with 32 μm apart were used for quantification.

From each section, 3 to 4 microscopic fields at 20x were examined. The total height of retina (D_{retina}) was obtained by averaging 10 different areas throughout the retinal layer. The distance of human cell migration ($D_{human\ cells}$) was obtained using the mean value of the 10 human cells, which migrated furthest in each microscopic field. Migration rate (MR) was calculated by the following equation, $MR = (D_{retina}/D_{human\ cells})*100$. The quantification of human cell retinal engraftment used the same retinas as the migration assessment. All human cells were detected by green signal of luciferase staining and counted in each photograph, and mean ± SEM values were plotted.

TUNEL method

Retinas for TUNEL analysis were fixed in 2% paraformaldehyde overnight at 40C, washed and incubated with Rabbit anti-collagen IV as described above, and goat anti rabbit-Alexifluor 647 was used as the second step antibody. After immunohistochemistry, the retinas were cryopreserved as described above. Eight mm cryosections were processed for detection of TUNEL-positive cells using the In situ Death Detection kit with TM R red indicator (Roche Applied Science, Mannheim, Germany), as suggested by the manufacturer. TUNEL+ cells were detected with a 710 LSCM microscope in relationship to retinal blood vessels.

Senescence Assays

FACS-purified populations were plated onto 6-well tissue culture plates pre-coated with fibronectin (10 μg/mL), and passaged in EGM2 3-4 times up to 30 days. Prior to the analysis, cells were plated onto fibronectin coated 12-well plate and expanded to ~70-80% confluency. Cells were fixed in 2% paraformaldehyde and stained for β-galactosidase expression per manufacturer's protocol (Cell Signaling Technology, Danvers, MA) to detect senescent cells. Senescence-positive cells stained blue and were enumerated with inverted microscopy and Nikon NS3.1 software. Three individual wells were photographed at three similar locations (left, middle and right of the each wells) at 20x for each individual sample. Mean ± SEM were determined and plotted.

Western Blotting

FACS-purified populations were plated onto 6-well tissue culture plates pre-coated with fibronectin (10 μg/mL), and passaged in EGM2 3-4 times up to 30 days. Whole cell protein extracts were prepared using the 1X RIPA lysis buffer containing Complete™ mini protease inhibitor cocktail (Roche Life Sciences, NJ, USA), and the phosphatase inhibitors sodium orthovanadate and

sodium fluoride (Sigma-Aldrich Co., St. Louis, MO). Protein lysates were separated with SDS–polyacrylamide gel electrophoresis (PAGE) with 4-20% TGX precast gel (Bio-Rad, Hercules, CA) and transferred onto a PVDF membrane. After blocking with 5% BSA in TBS-T, membranes were probed with primary and secondary antibodies. Enhanced chemiluminescence (ECL; GE Healthcare, Pittsburgh, PA) was used for detection of immunoblotted proteins. The antibodies used were monoclonal anti-p53 (1:1000) (Cell Signaling Technology, Danvers, MA), anti-β-Actin (1:2000) (Abcam, Cambridge, MA), and anti-RAD51(1:200) (Santa Cruz Biotechnology, Santa Cruz, CA). Western blot band densities were quantified using the ImageJ software (NIH).

II. Supplementary Tables

Table S1. hESC and iPSC Medium

Element (Conc. of stock solution)	Volume in total 500mL (final conc.)	Commercial sources	Catalog Number
DMEM-F12	400 mL	Life Technologies	11330-032
KnockOut™ Serum Replacement	100 mL	Life Technologies	10828-028
L-Glutamine (200 mM)	2.5 mL (1 mM)	Life Technologies	25030-081
MEM Non-essential amino acid (MEM NEAA) (10 mM)	5 mL (0.1 mM)	Life Technologies	11140-050
β-mercaptoethanol* (55 mM)	910 μL (0.1 mM)	Life Technologies	21985-023
FGF-2** (100 ng/μL)	20 μL (4 ng/mL)	Life Technologies	13256-029

* Light sensitive

**Reconstitute in 5% BSA, aliquot, freeze at -20°C.

**Add bFGF after 0.22 μm filter sterilize the medium.

Table S2. Mouse Embryonic Fibroblast (MEF) Medium

Element (Conc. of stock solution)	Volume in total ~570mL (final conc.)	Commercial sources	Catalog Number
DMEM	500 mL	Life Technologies	11995-065
FBS	60 mL	Thermo Scientific	SH30071.03
L-Glutamine (200mM)	2.85 mL (1 mM)	Life Technologies	25030-081
MEM Non-essential amino acid (MEM NEAA) (10 mM)	5.7 mL (0.1 mM)	Life Technologies	11140-050
Penicillin/streptomycin (100x)	2.85 mL (0.5x)	Life Technologies	15140-122
β-mercaptoethanol* (55 mM)	570 µL (0.055 mM)	Life Technologies	21985-023

*Light sensitive

Table S3. Adaptation Medium (AM)

Element (Conc. of stock solution)	Volume in total 100mL (final conc.)	Commercial sources	Catalog Number
StemSpan SFEM*	82mL	STEMCELL Technologies	09650
ES-Cult FBS	15 mL	STEMCELL Technologies	06950
Ascorbic Acid** (5 mg/mL)	1 mL (50 µg/mL)	Sigma-Aldrich	A4403
Ex-Cyte***	1 mL	Millipore	81-129-1
Penicillin/Streptomycin	1 mL	Life Technologies	15140-122
ITS-X (Insulin-transferrin-selenium-ethanolamine)	0.5 mL	Life Technologies	51500-056

* Thaw it at 4°C

** Dissolve 100 mg in 20 mL of PBS, make 2mL aliquots and store at -20˚C

*** Light sensitive

Table S4. Methylcellulose Medium (MM)

Element (Conc. of stock solution)	Volume in total 100mL (final conc.)	Commercial sources	Catalog Number
Methocult SF H4236*	80 mL	STEMCELL Technologies	H4236
ES-Cult FBS**	15 mL	STEMCELL Technologies	06950
Ascorbic Acid (5 mg/mL)**	1 mL (50 µg/mL)	Sigma-Aldrich	A4403
PFHM II** (Protein-free hybridoma medium)	3.5 mL	Life Technologies	12040-077
Ex-Cyte**	0.5 mL	Millipore	81-129-1

* Thaw it overnight at 4°C.

** Mix all components, 0.22 µm filter sterilize, and add to Methocult SF H4236.

Table S5. Liquid Differentiation Medium (LDM)

Element (Conc. of stock solution)	Volume in total 100mL (final conc.)	Commercial sources	Catalog Number
StemPro-34 SFM*	98 mL	Life Technologies	16039-011
L-Glutamine	1 mL	Life Technologies	25030-081
Ascorbic Acid (5 mg/mL)	1 mL (50 µg/mL)	Sigma-Aldrich	A4403
1-Thioglycerol	3.5 µL	Sigma-Aldrich	M1753
BMP4 (100 ng/µL)**	50 µL (50 ng/mL)	R&D	314-BP-010
VEGF A_{165} (100 ng/µL)**	50 µL (50 ng/mL)	R&D	293-VE-50
FGF-2 (100 ng/µL)**	50 µL (50 ng/mL)	R&D	233-FB-025
Heparan Sulfate (2 mg/mL)**	250 µL (5 µg/mL)	Sigma-Aldrich	H7640

* Thaw the supplement at 4°C and add to basal medium.

** Abbreviated as BVF2H; avoid freezing and thawing; add growth factors after 0.22µm filter sterilize medium

Table S6. List of Antibodies to Human Antigens

Name	Other name	Clone	Company	Catalog no.	Concentration (< $1x10^6$ cells)	Purpose
CD31-APC	PECAM-1	WM-59	eBioscience	17-0319-42	2 µL / 1:20	FC/FACS
CD34-PE	-	581	BD Biosciences	555822	5 µL	FC
CD44-PE	H-CAM	515	BD Biosciences	550989	5 µL	FC
CD90-APC	Thy-1	5E10	BD Biosciences	559869	1 µL	FC
CD90-PE	Thy-1	5E10	BD Biosciences	555596	1 µL	FC
CD105-PE	Endoglin	SN6	Invitrogen	MHCD10504	1 µL	FC
CD117-PE	c-Kit	YB5.B8	BD Biosciences	555714	1 µL	FC
CD133-PE	130-080-801	AC133	Miltenyi Biotec	130-080-801	10 µL	FC
CD143-APC	ACE, BB9	BB9	BD Biosciences	557929	5 µL	FC
CD144-PE	Ve-Cadherin	55-7H1	BD Biosciences	560410	5 µL	FC
CD146-PE	Mel-CAM	P1H12	BD Biosciences	550315	5 µL / 1:10	FC/FACS
CD184-APC	CXCR4	12G5	BD Biosciences	555976	5 µL	FC
Rabbit anti-human CD184-pure	CXCR4		Novus Biologicals	NLS1380	1:500	ICS
KDR-APC	VEGFR2	89106	R&D System	FAB357A	5 µL	FC
Luciferase-FITC	-	-	Abcam	Ab34506	1:1000	FC/ICS
aSMA-pure	-	1A4	Dako	M0851	1:100	ICS
UEA-biotin	-	-	Vector Lab	B-1065	1:100	ICS
Goat anti-mouse-biotin	-	-	Dako	E0433	1:1000	ICS
Goat anti-rabbit-Cy3	-	-	Jackson Immunoresearch	111-165-003	1:100	ICS
Streptavidin-Cy3	-	-	Sigma-Aldrich	S6402	1:500	ICS

FC: flow cytometry, FACS: fluorescent activated cell sorting,

ICS: immuno-cyto staining

Table S7. List of Human Primers for Quantitative RT-PCR

Human Gene	Forward Primer 5′ →3′	Reverse Primer 5′ →3′	Amplicon size (bp)	Annealing temp, °C
Actin	GCA CAG AGC CTC GCC TTT	GGA ATC CTT CTG ACC CAT GC	213	59-65°
CD31	GAG TCC TGC TGA CCC TTC TG	ATT TTG CAC CGT CCA GTC C	107	61
CD34	TGG ACC GCG CTT TGC T	CCC TGG GTA GGT AAC TCT GGG	167	61
vWF	CCC GAA AGG CCA GGT GTA	AGC AAG CTT CCG GGG ACT	288	61-63
FLT1	GAC TAG ATA GCG TCA CCA GCA G	GAA ACC GTC AGA ATC CTC CTC	101	65
TIE1	GTG CCC CCT TTC TTG CTC	GAT ACG GTC GTC CTT CTC CA	227	60
TIE2	ACC ATC CAC CGG ATC CTC	TTG CCA AGC CTC ATA GTG ATT	252 bp	59-60

Table S8. List of vascular genes analyzed in Figure 3B and C

Gene name	Relate to	Gene name	Relate to
AAMP AMOT AMOTL1 AMOTL2 ANGPT1 ANGPTL2 APOLD1 CAV1 CDH5 COL18A1 CTGF EDF1 EPAS1 F2R HIF1A MCAM MALL MMRN1 NOS3 PGF PPAP2B PROCR ROBO4 S1PR1 SCARF1 SERPINE1 TNFAIP1 VWF	Endothelial[5, 9]	CD34 CD44 EMCN ESAM FLI1 FLT1 HHEX KDR PECAM1 TEK TIE1	Angio-hematopoietic[5, 9, 10]
C10ORF10 DLL4 EPHB2 HEY1 NOTCH4 NRP1	Arterial[9, 11]	ACTA2 CSPG4 PDGFRB RGS5	Pericyte/Perivascular[12, 13]
APLNR EPHB4 FLT4 NR2F2 NRP2	Venous[9, 11]	CD55 CXCL12 CXCR4 ITGA5 ITGB3 MMP2 MMP9	Adhesion/Migration[14]

Table S9 (.xls file): Gene Ontology (GO) Categories Overrepresented Among Genes Significantly Differentially Expressed in VP-hiPSC vs. VP-hESC**.** GO-Elite[15] was used to identify GO categories over-represented (Z score > 2, permutation $p < 0.05$) among genes significantly differentially expressed in each VP-hiPSC subtype vs. VP-hESC ($p < 0.05$, FC ≥ 1.5) for which an Entrez GeneID was available. Only those categories with at least five genes are reported.

III. Supplementary Figures

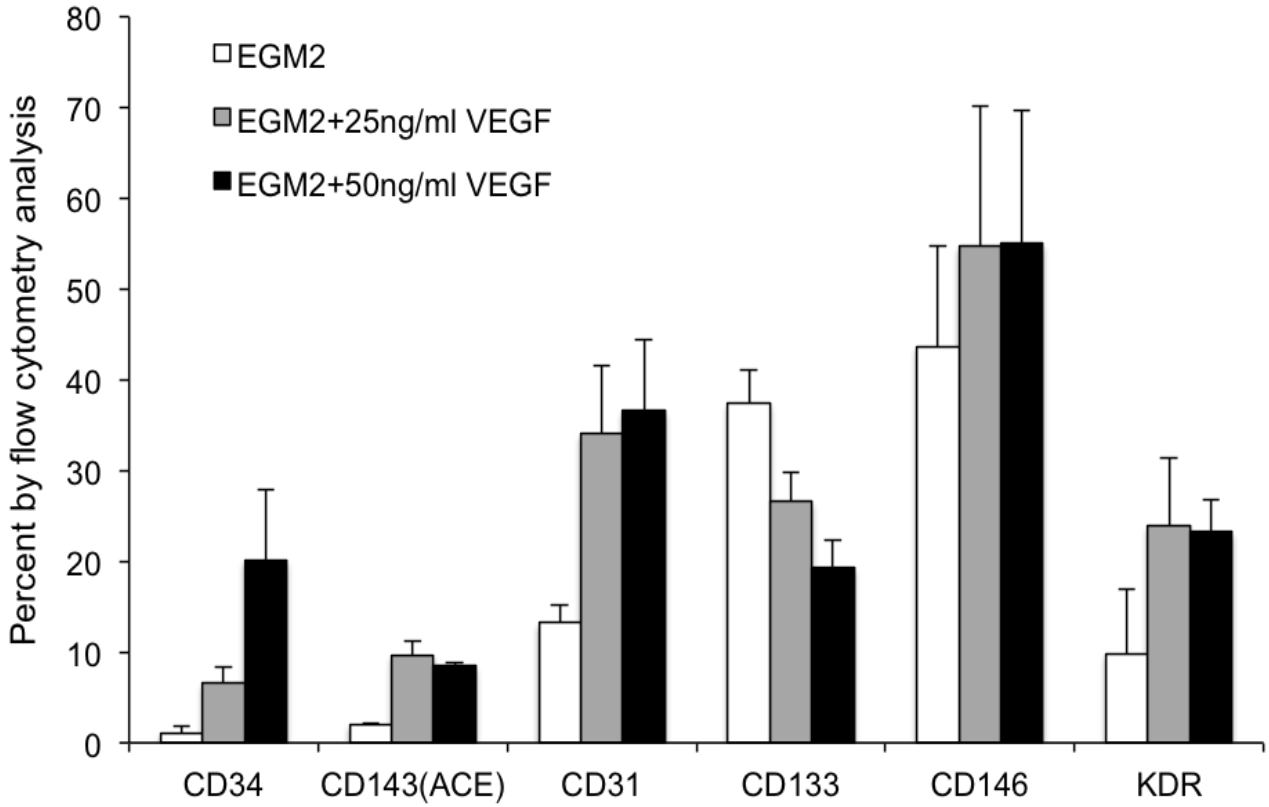

Figure S1. Expression of surface hemato-endothelial markers in EGM2-expanded hEB cells. Day 8 hEB cells from H9 hESC were differentiated in hemato-endothelial differentiation conditions as described in Supplemental Method. Whole hEB in suspension were disaggregated into cell clumps with collagenase type IV and further plated onto fibronectin-coated tissue culture plate in EGM2 medium supplement with VEGF (25 and 50 ng/mL). Expressions of human CD31, CD34, CD146, CD143 (ACE aka BB9), KDR (VEGFR2), and CD133 on single hEB cells were quantitated by flow cytometry at 12-13 days of differentiation (see Fig 1A). EGM2 supplemented with 25 ng/mL $VEGF_{165}$ was found optimal for differentiation to the vascular lineage based on marker expressions.

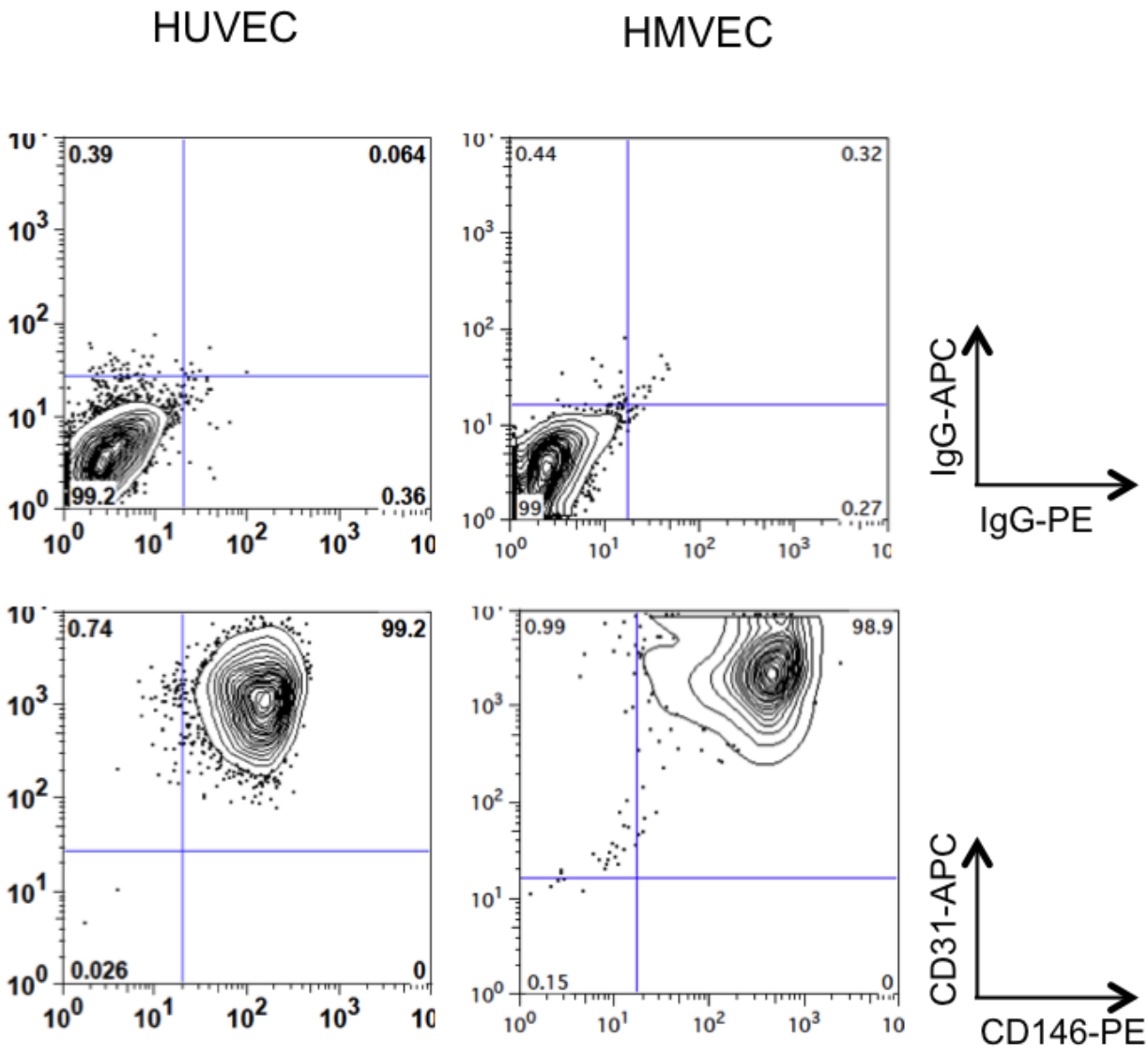

Figure S2. Expression of CD31 and CD146 on human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HMVEC). Surface CD146 is co-expressed with CD31 in HUVEC and HMVEC, but is also expressed on pericytic mesenchymal stem/progenitor cell (MSC) with non-endothelial phenotype (see **Fig. S3** below).

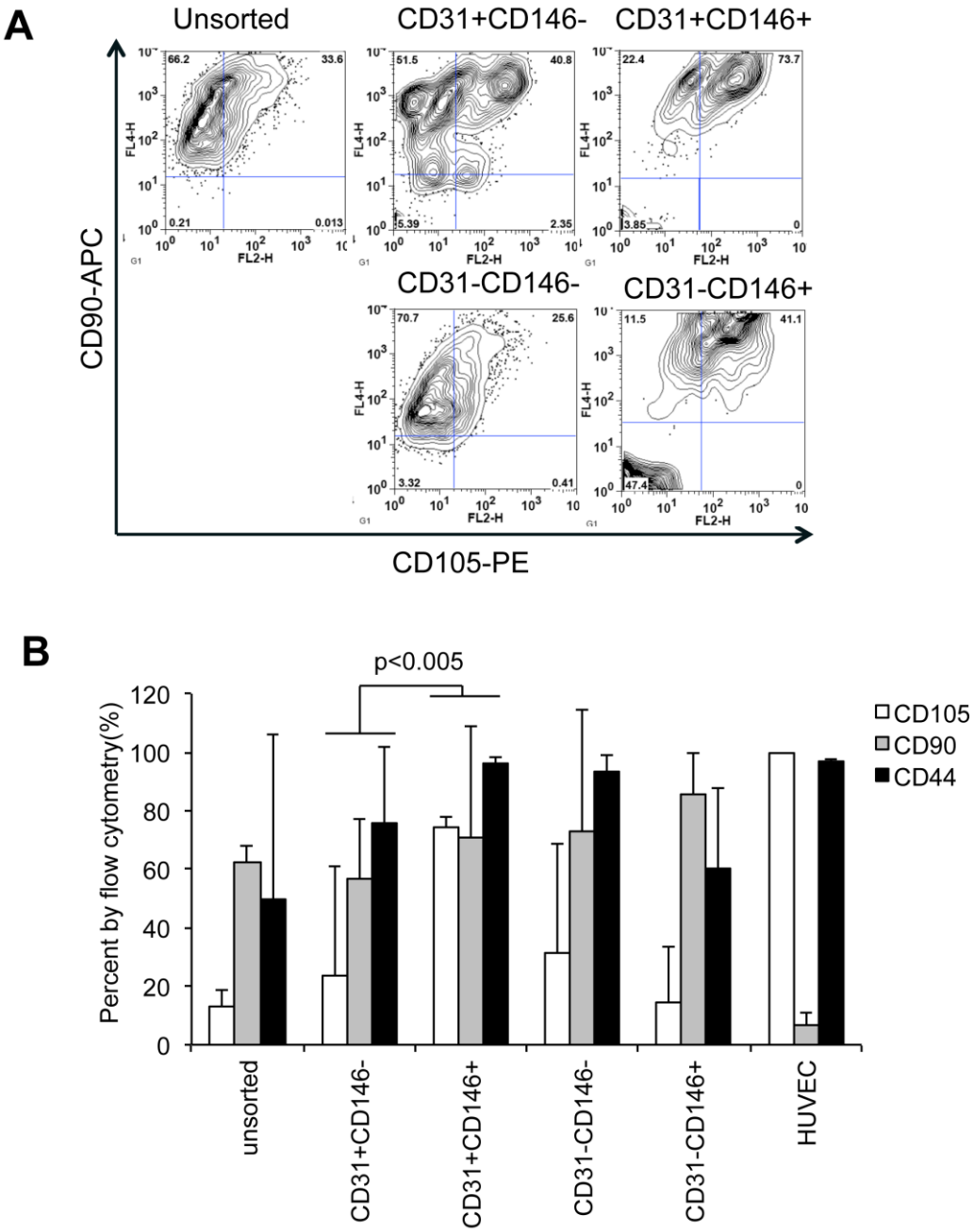

Figure S3. Mesenchymal stem/progenitor cell (MSC) marker expression in FACS-purified hEB populations. (**A**) Representative flow cytometry analysis of H9 hEB-derived populations. Shown are surface expressions of CD90 and CD105 of FACS-purified $CD31^{+}CD146^{-}$, $CD31^{+}CD146^{+}$, $CD31^{-}CD146^{-}$, $CD31^{-}CD146^{+}$ EGM2-cultured hEB populations, as well as non-purified (unsorted) EGM2-cultured hEB cells. (**B**) Expression of MSC markers CD44, CD90, and CD105 on FACS-purified and unsorted populations. Shown are mean percentages of hEB cell expression ± standard deviation.

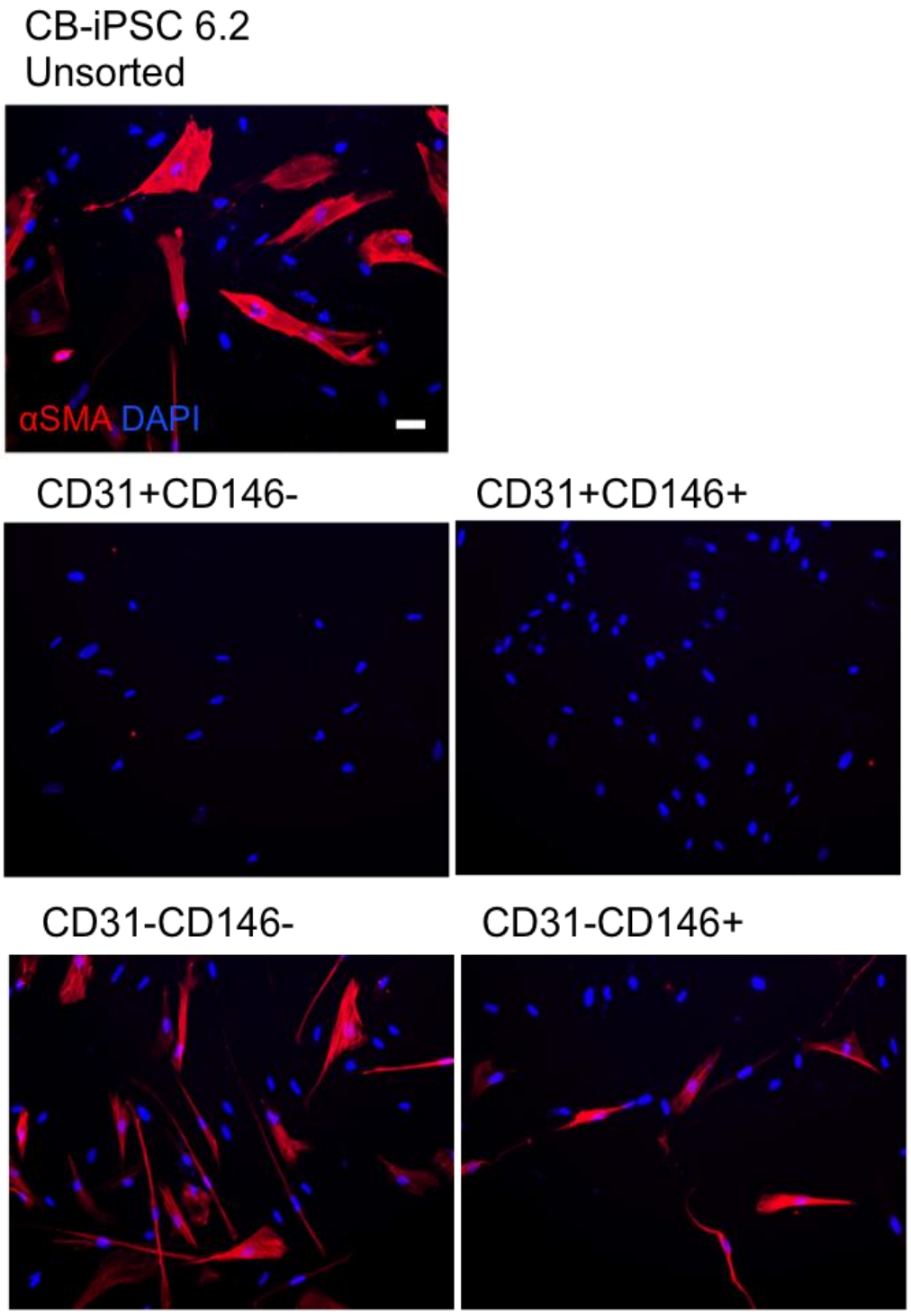

Figure S4. Immunofluorescence staining of FACS-purified CB-iPSC-6.2 hEB populations for expression of CD31 and CD146. Staining of human alpha-smooth muscle actin (α-SMA; red, DAPI nuclear stains, blue) of FACS-purified cells cultured in EGM2. Scale bar is 50 μm.

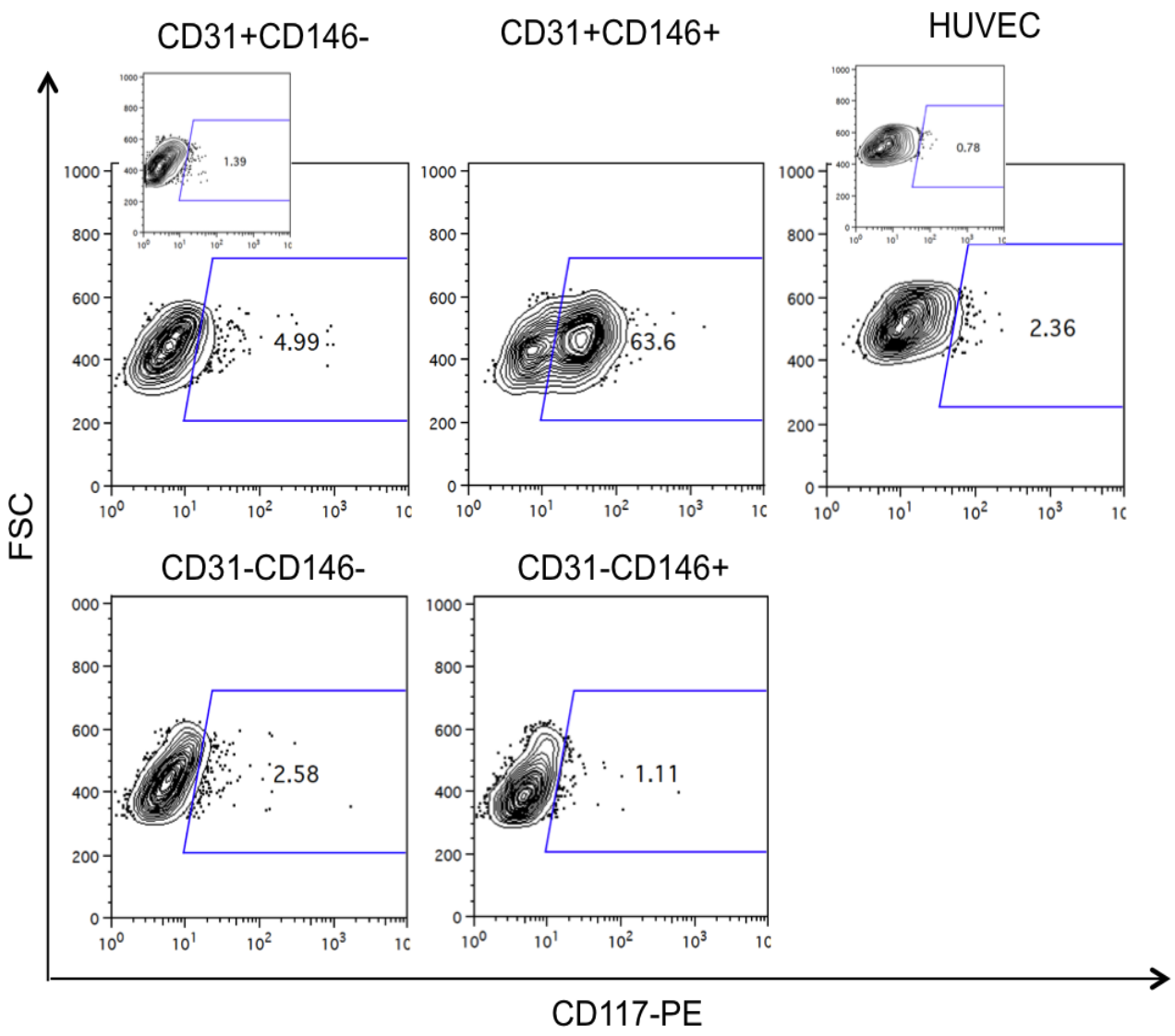

Figure S5. Surface CD117 expression in FACS-purified hEB populations. Inset plots show isotype control stainings. hEB cells were differentiated from H9 hESC and expanded in EGM2 culture as described in Methods. Shown in right panel are HUVEC control cells. Inserts are isotype IgG controls.

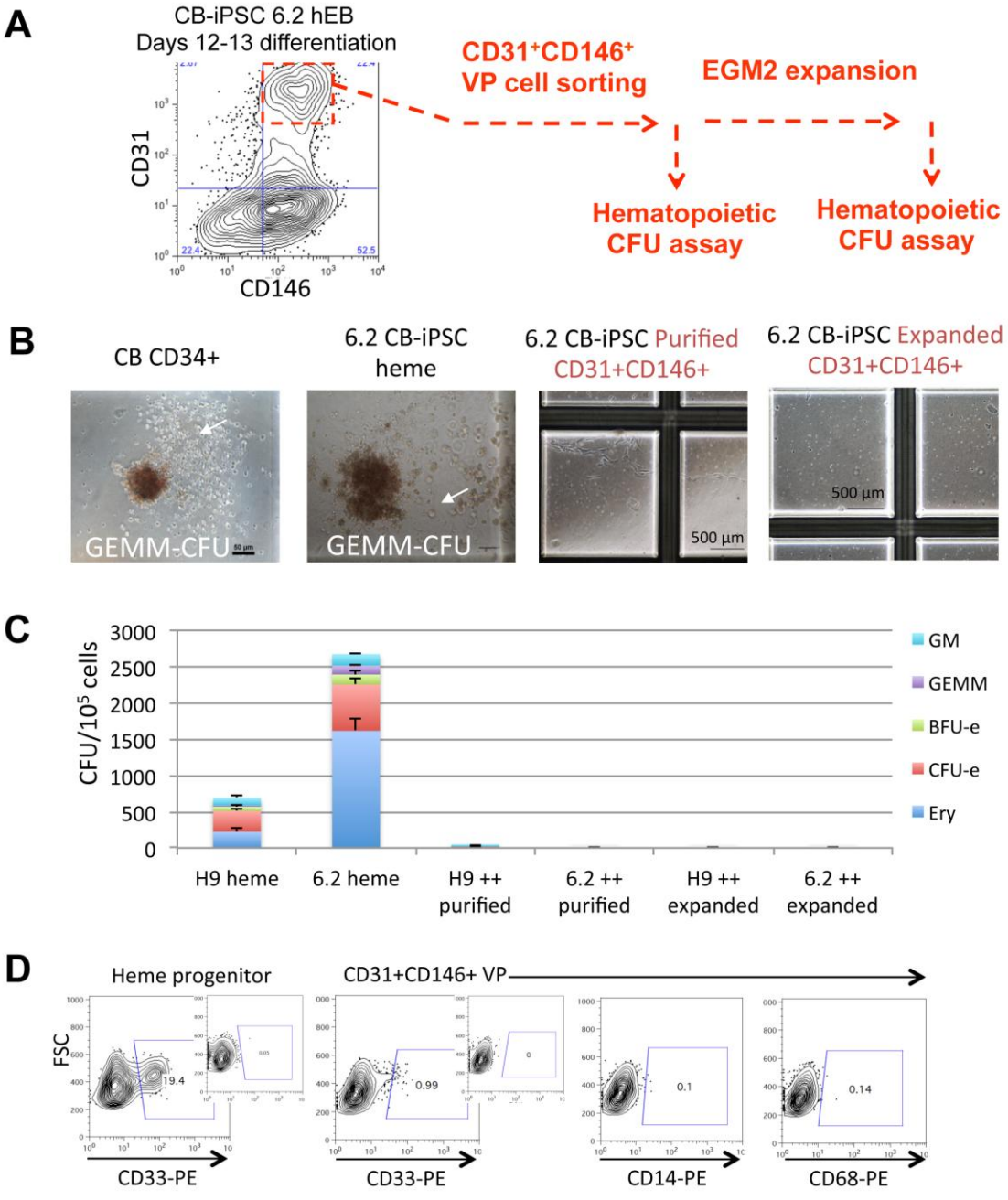

Figure S6. Hematopoietic potential of purified CD31+CD146+ VP generated from hESC and CB-iPSC. (**A**) HESC H9 and CB-iPSC 6.2 were differentiated to VP and FACS-purified at day 13. On the same day, non-adherent hematopoietic hEB cells (heme) were assayed (see Park et al, Cytometry Part A 2013) for (**B**, **C**) hematopoietic potential in methylcellulose CFU assays. Single purified VP cells did not possess Ery (erythroid colony), CFU-e (colony forming unit-erythroid cells), BFU-e (burst forming unit-erythroid cells), GEMM-CFU (granulocyte-erythroid-macrophage-

megakaryocyte colony forming units), and GM-CFU (granulocyte-macrophage-colony forming units) activity. (**B**) Representative images of erythro-myeloid GEMM-CFU of CD34+ cord blood cells and CB-iPSC hEB hematopoietic progenitor cells. White arrows show typical morphology of myeloid (monocyte-macrophage) cells produced by CB and hEB cells. Scale bars: 100 μm. (**C**) Purified and expanded CD31+CD146+ VP from differentiated H9 hESC and CB-iPSC were incapable of generating hematopoietic or myeloid CFU, and (**D**) did not express myeloid-specific markers (e.g., CD33, CD14, CD68, CD45 (not shown)). Inserts are isotype controls. Scale bars: 100 μm.

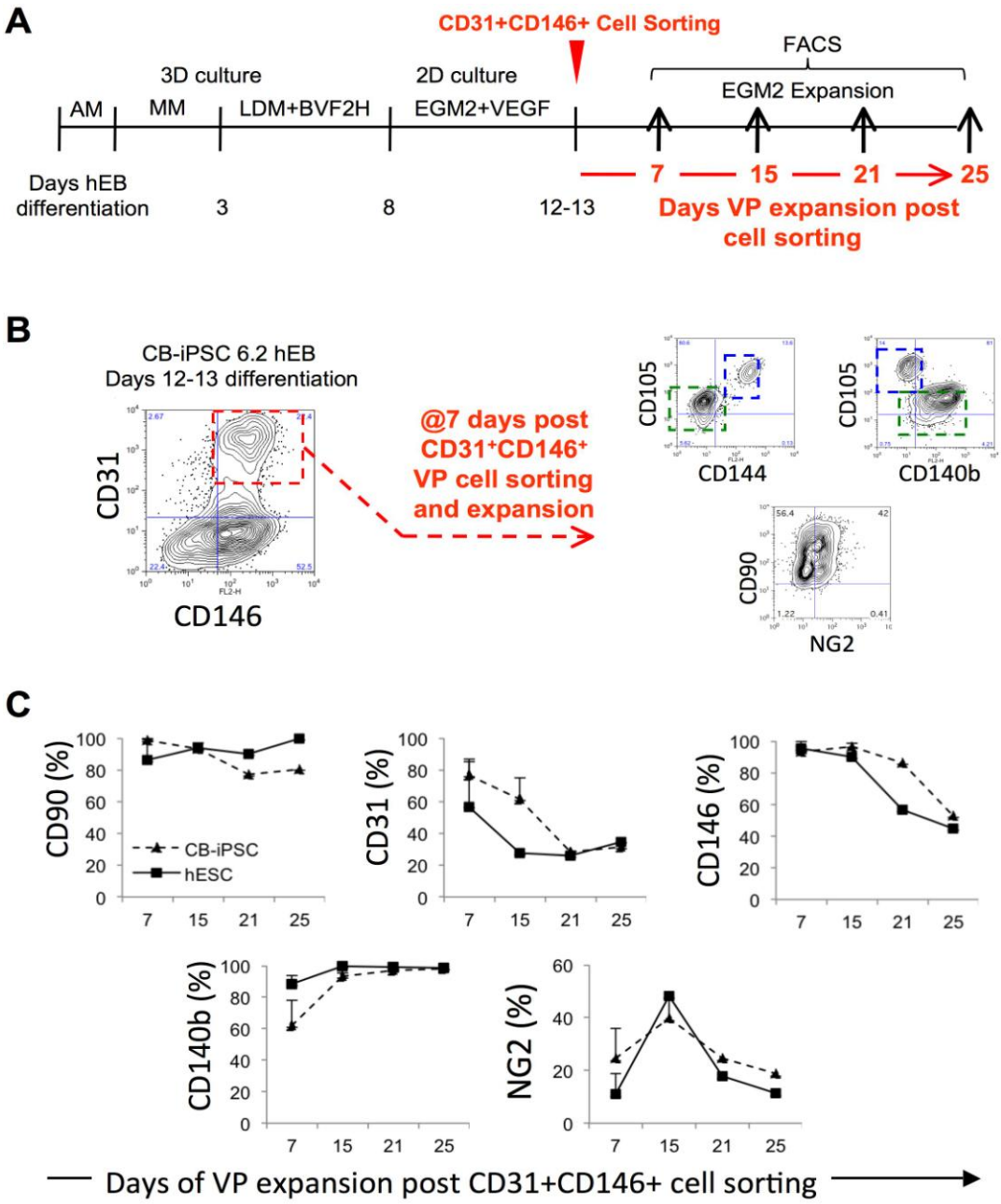

Figure S7. Kinetics of vascular endothelial and pericytic markers during CB-iPSC VP differentiation and expansion. (A) Human IPSC line 6.2 and hESC H9 were differentiated to FACS-purified VP and further expanded for an additional 7-25 days in EGM2. (**B**) Vascular-pericytic surface markers of FACS-purified 6.2 CD31+CD146+ VP further expanded in EGM2 for 7 days revealed two discrete $CD105^{hi}CD144+$ and $CD105^{dim}CD140b^{+}$ populations. (**C**) 6.2 and H9 CD31+CD146+ VP expanded for 7-25 days. VP cells were injected into I/R-injured eyes following 7-12 days of EGM2 expansion (n=2-5 experiments; ±SEM)

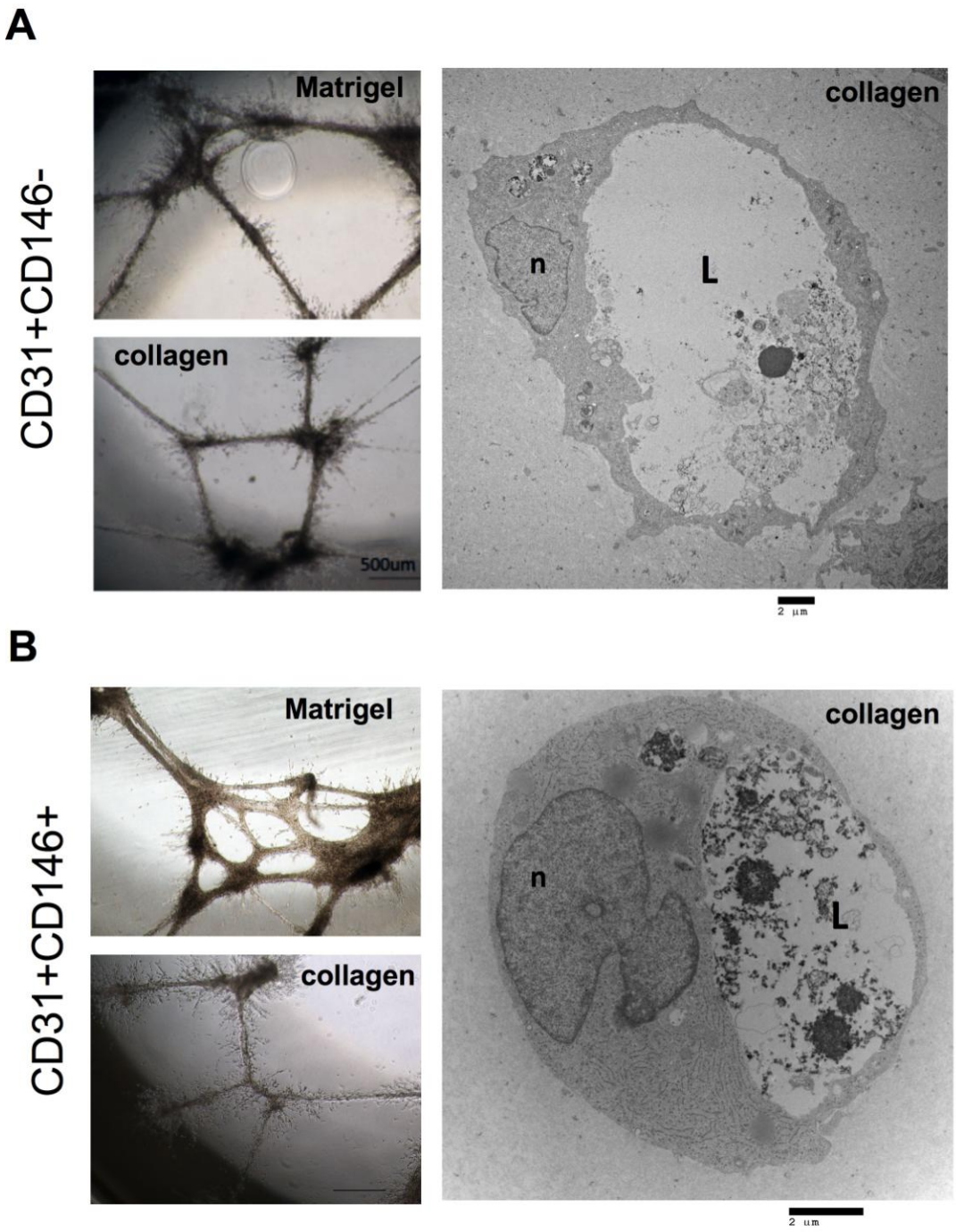

Figure S8. Generation of capillary-like structures with endothelial cell-like lumens by CB-iPSC-derived VPs. Vascular tubes formed by **(A)** CB-iPSC 6.2-derived purified and expanded CD31+CD146- cells and **(B)** CB-iPSC 6.2-derived CD31+CD146+VP cells. (Left panels) Matrigel and collagen gel assays; (right panels) TEM images of fixed vascular tube structures in collagen gel; n=nucleus, L=lumen.

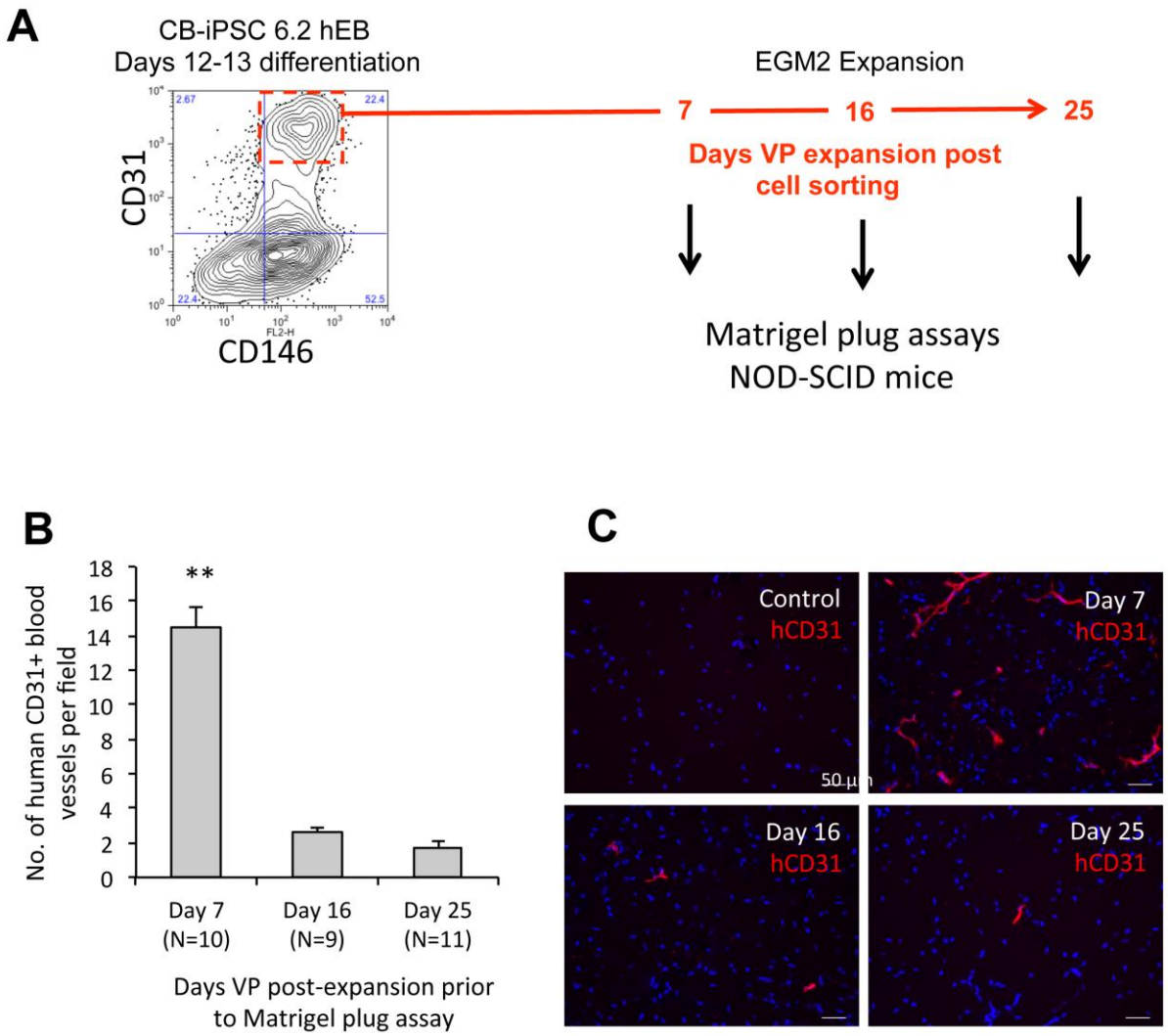

Figure S9. Vascular potency of CB-iPSC-derived VP following EGM2 expansion (A) FACS-purified CD31+CD146+ VP were expanded on fibronectin-coated plates in EGM2 medium for an additional 7, 16, and 25 days. Expanded cells were harvested at each time points, and tested for the *in vivo* ability to generate chimeric mouse-human vasculature structures in Matrigel plugs in NOD-scid mice. 14 days following Matrigel/VP injections, plug samples were harvested, paraffin embedded/sectioned, immunofluorescent-stained with anti-human CD31 antibody (hCD31), and the formation of human vessels was quantitated. **(B)** Bar graphs represent average numbers of blood vessels quantified per section in x20 microscopic fields ± standard error of mean, P-value < 0.005 when comparing day 7 vs. day 16 and day 7 vs. day 25. **(C)** Representative immuno-fluorescent images of control plugs (no human cells, Matrigel with bFGF 20 ng/mL), and Matrigel plugs following 7, 16, and 25 days of EGM2 expansion. Bar= 100 um

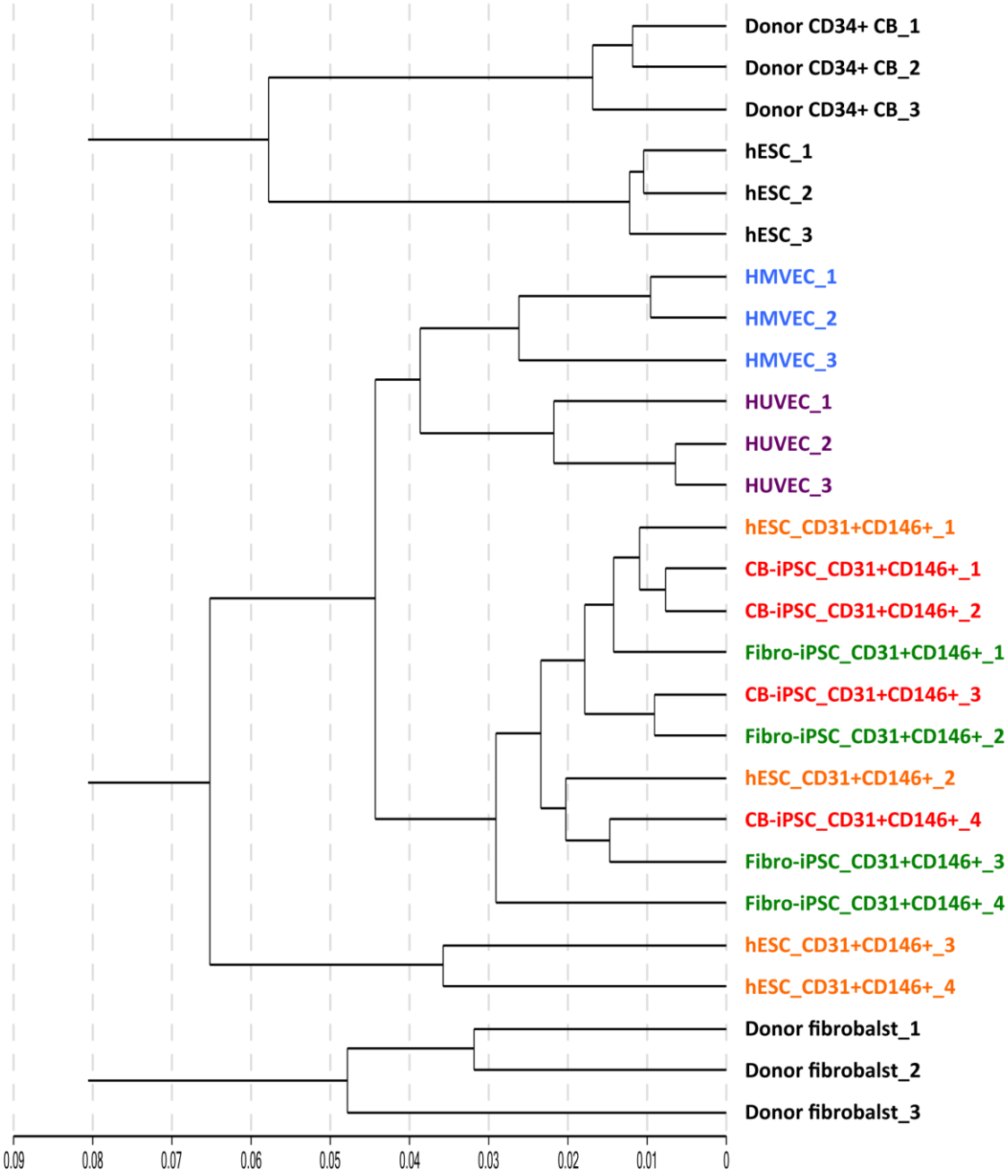

Figure S10. Unsupervised hierarchical correlation clustering of genome-wide expression signatures of hPSC-VP. Global RNA expression of FACS-purified $CD31^{+}CD146^{+}$ VP from hESC (gold), CB-iPSC (red), Fibroblast-iPSC (green), control HUVEC, HMVEC, donor growth factor-activated CB cells, donor fibroblasts, and hESC was evaluated with Illumina expression microarrays as detailed in Supplementary Methods.

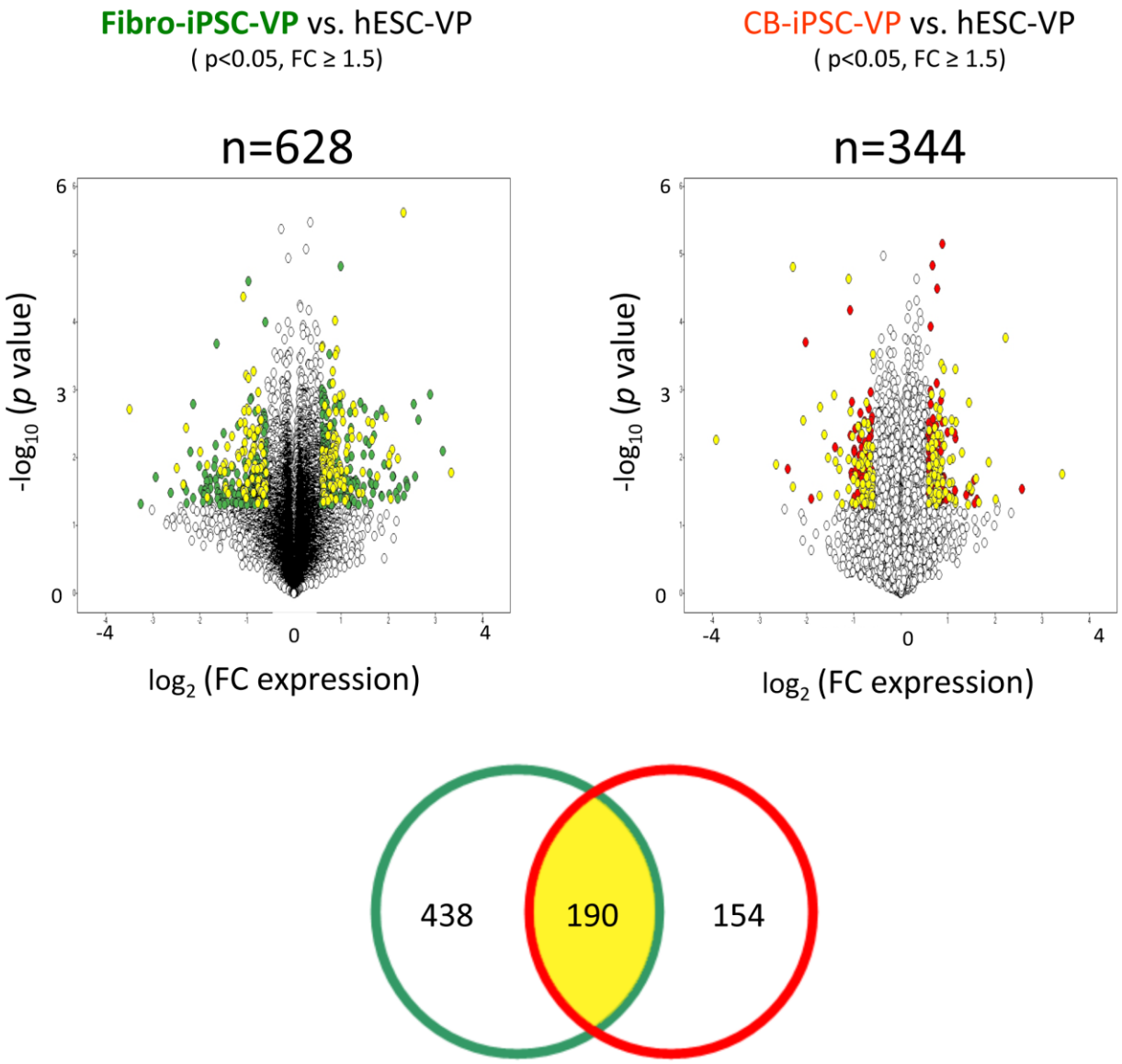

Figure S11. Volcano plots of genes differentially expressed genes between iPSC-VP and hESC-VP. Differentially-expressed genes were identified by ANOVA ($p < 0.05$), and fold-change ≥ 1.5x. 190 genes common to both comparisons are marked in yellow.

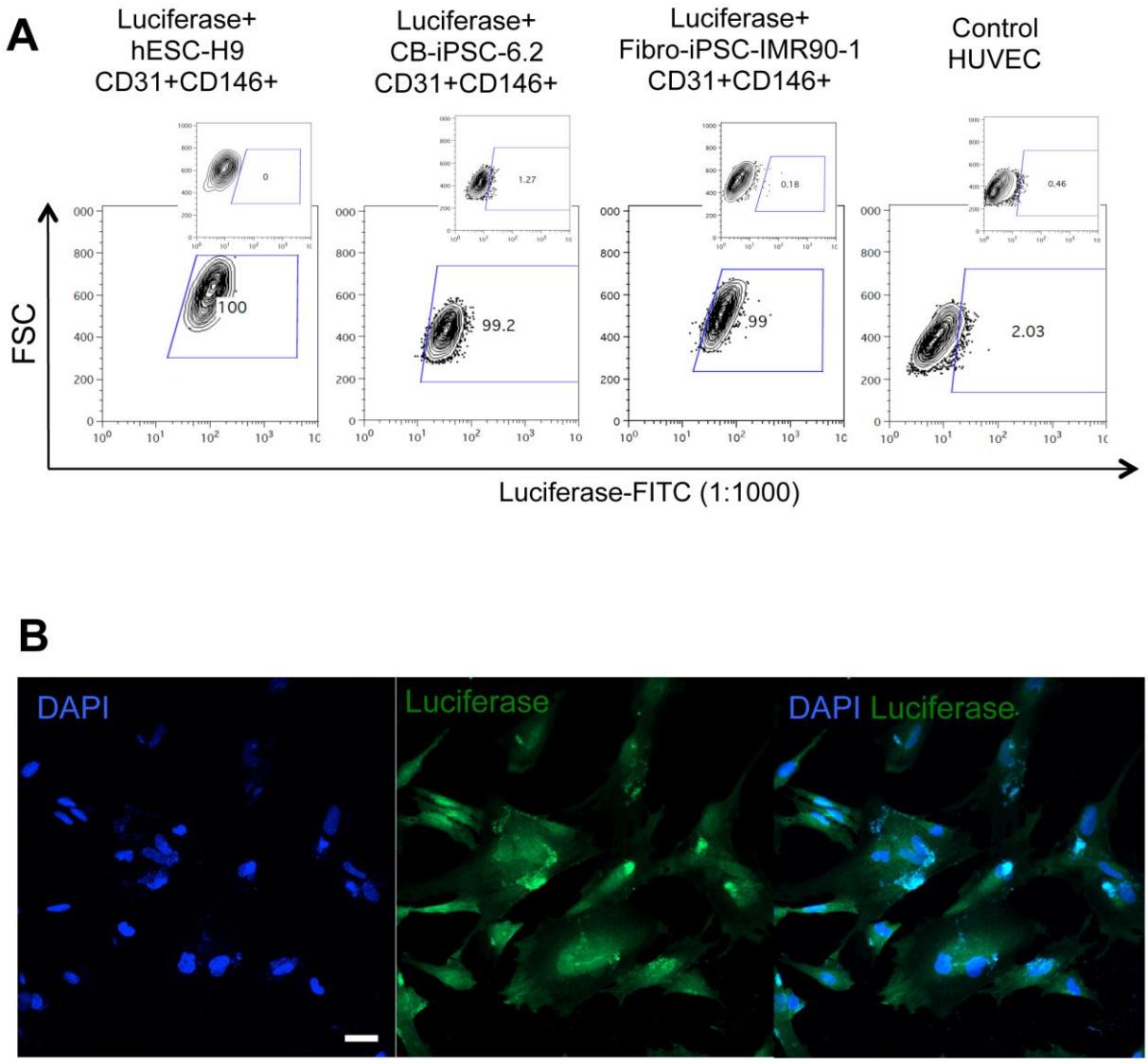

Figure S12. Expression of lentiviral luciferase transgene in FACS-purified CD31^{+}CD146^{+} VP prior to *in vivo* injection. (**A**) Flow cytometry of CD31^{+}CD146^{+} VP cells from hESC (H9) CB-iPSC (6.2), and fibroblast-iPSC (IMR90-1) expressing lentiviral luciferase with anti-luciferase-FITC antibody. Undifferentiated hESC/hiPSC cells were maintained in the presence of puromycin (0.5 µg/mL) for stable luciferase expression. Non-transduced HUVEC were used as negative controls. Inserts are isotype-FITC controls. (**B**) Representative *in situ* immunofluorescent luciferase staining (green) of FACS-purified and EGM2-expanded CD31^{+}CD146^{+} VP cells from luciferase+ H9 hESC, prior to *in vivo* eye injections. Viable nuclei were stained with DAPI (blue).

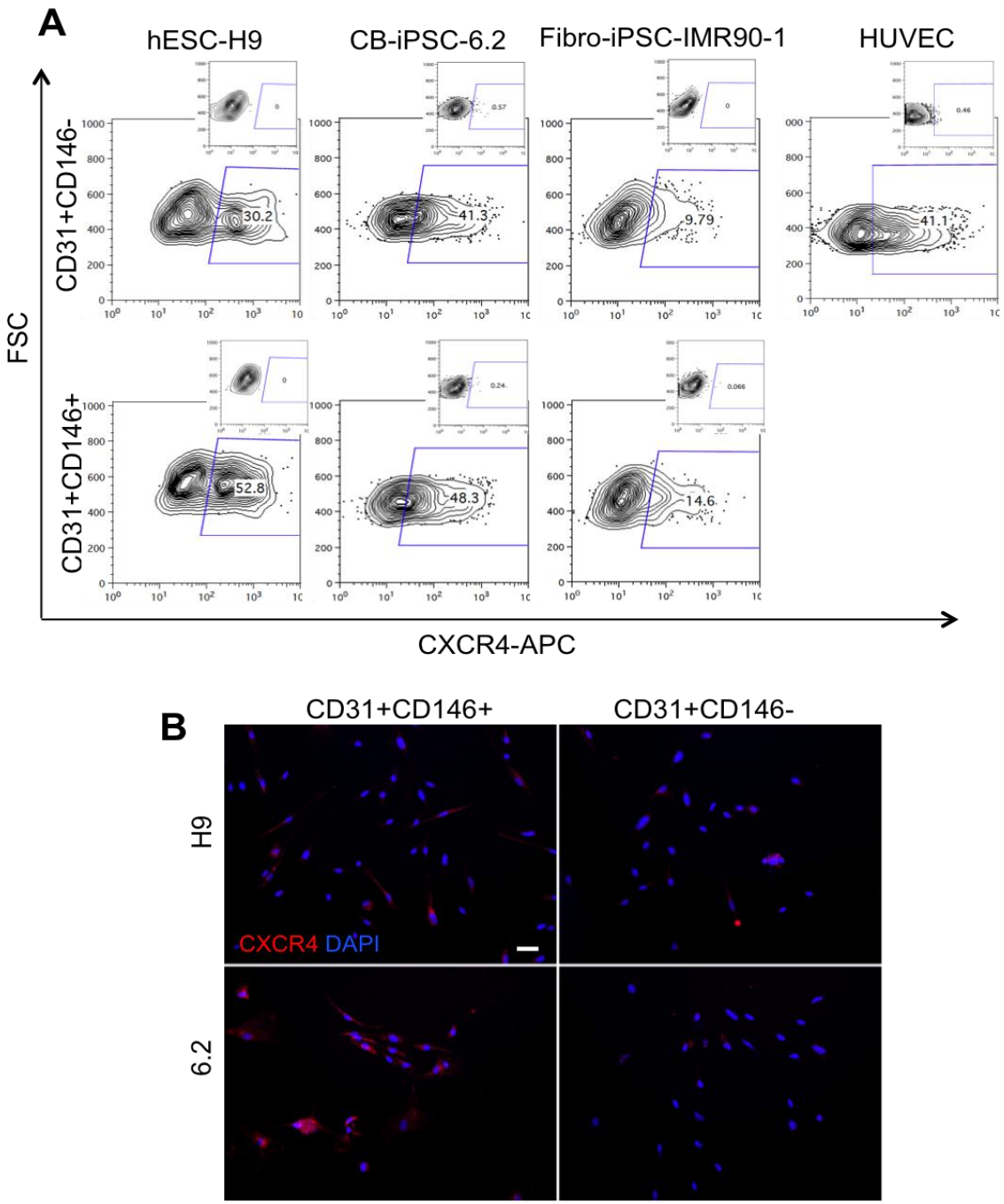

Figure S13. Expression of surface CXCR4 in FACS-purified hEB populations derived from hESC, CB-iPSC, and fibroblast-iPSC. (A) Flow cytometry staining of CXCR4 (CD184) on $CD31^{+}CD146^{+}$ VP cells and $CD31^{+}CD146^{-}$ hEB cells differentiated from hESC (H9), CB-iPSC (6.2), fibroblast-iPSC (IMR90-1), and HUVEC. Inserts are isotype IgG controls. (B**)** *In situ* immunofluorescent staining of CXCR4 on $CD31^{+}CD146^{+}$ VP cells and $CD31^{+}CD146^{-}$ cells from hESC (H9) and CB-iPSC (6.2). Scale bar is 50 µm.

A

Antibody	Damaged blood vessels	Normal blood vessels
mCD31	-	+
Coll-IV	+	+

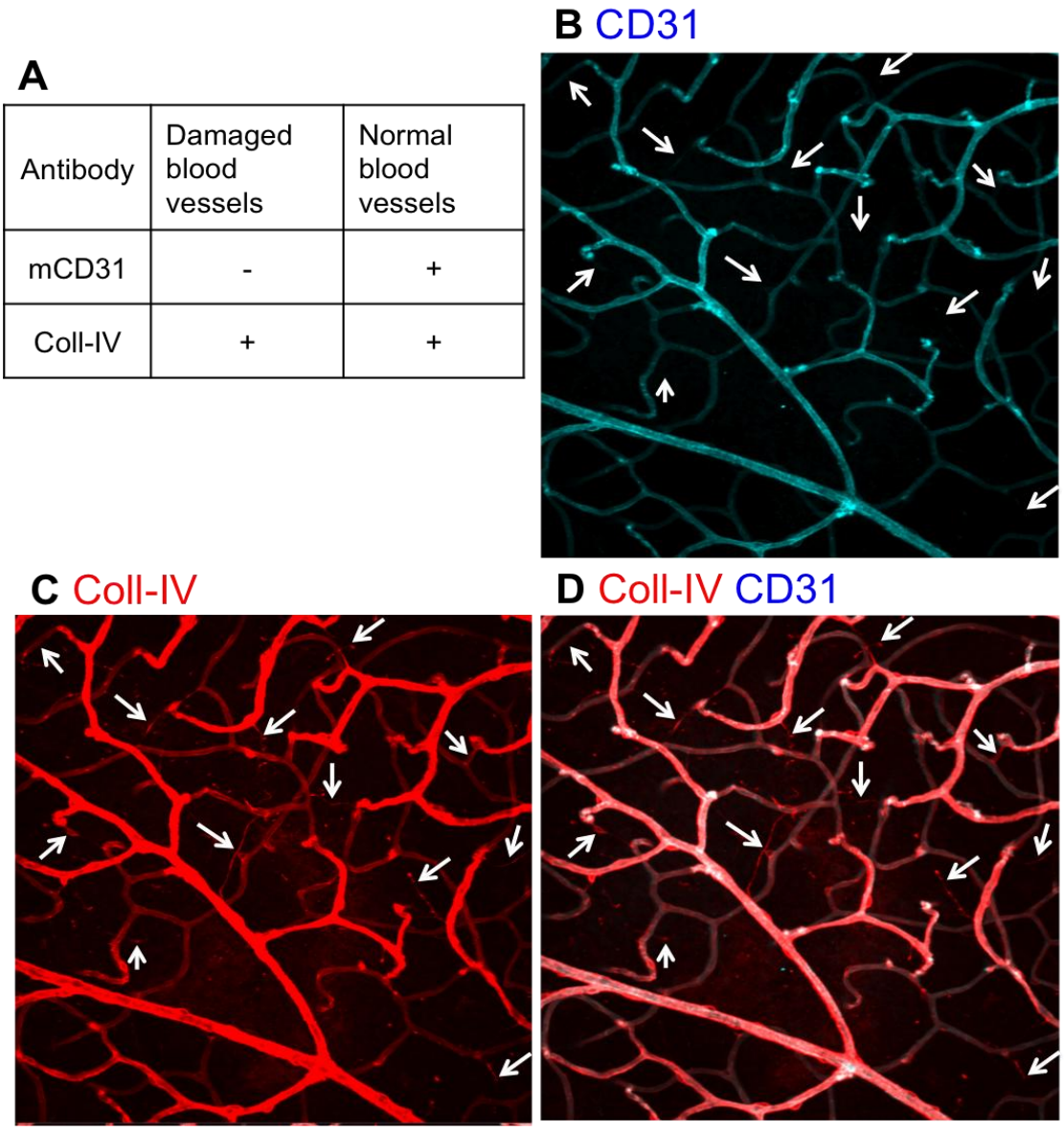

Figure S14. Damaged host retinal blood vessels following I/R injury. (**A**) A combination of mouse anti-CD31 (mCD31) and anti-collagen type-IV (Coll-IV) allowed recognition of viable and damaged blood vessels (which lacked viable CD31 endothelial cells) in murine retinal blood vessels. (**B**) Representative image using staining of CD31 (blue), (**C**) collagen type-IV (red), and (**D**) merged image demonstrating multiple damaged blood vessels (arrows) 7 days after I/R injury.

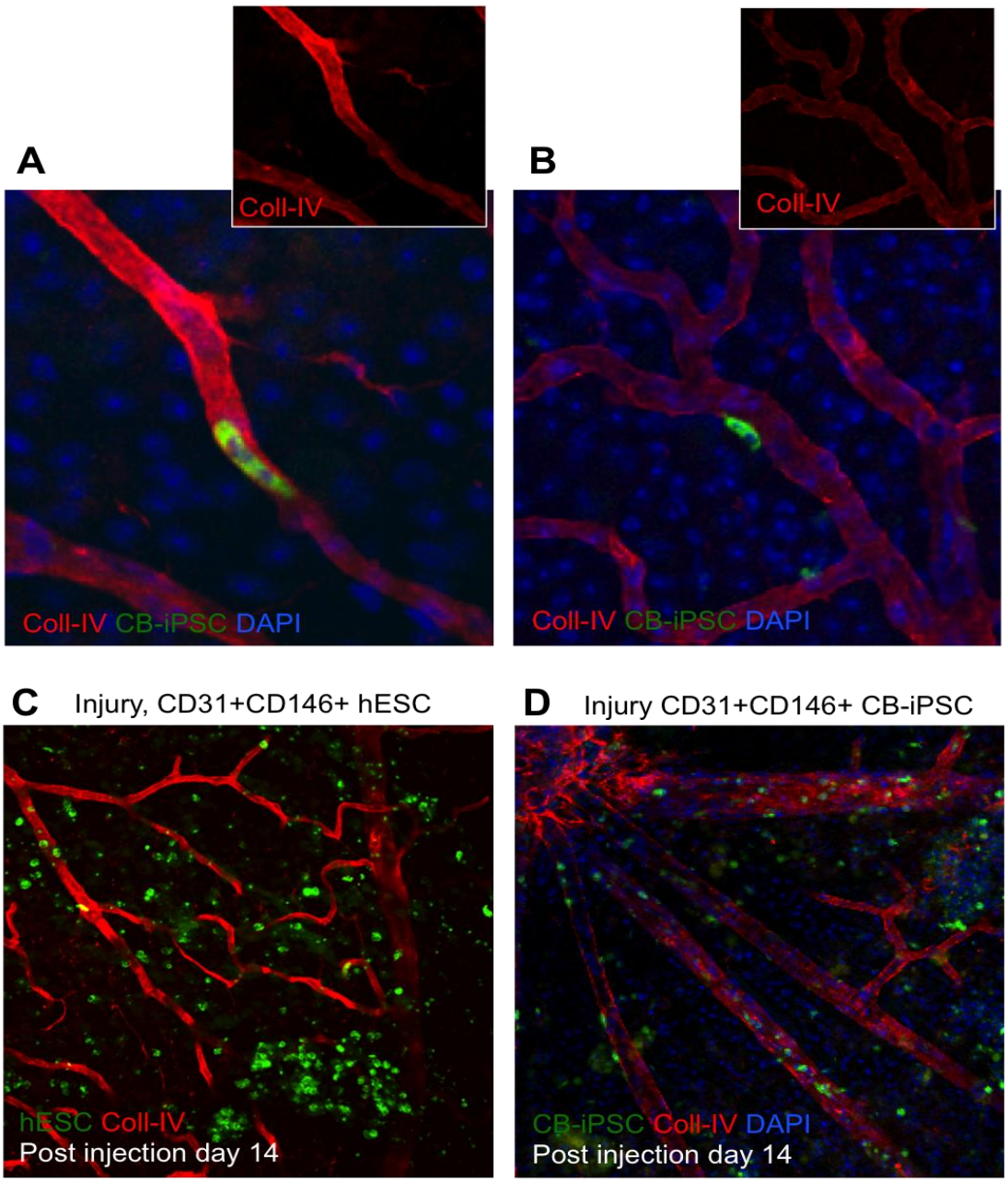

Figure S15. Migration, homing, and engraftment of CD31^{+}CD146^{+} VP cells derived from hESC-H9 and CB-iPSC-6.2 at 14 days post-injection. Luciferase transgene^{+}CD31^{+}CD146^{+} VP cells from CB-iPSC were detected at both luminal (**A**) and abluminal locations (**B**). Shown are immunofluorescent staining of collagen type-IV (red), DAPI (blue), and luciferase (green). Inserts are collagen type-IV only (red channel only). (**C)** Nonspecific scattered type of engraftment was occasionally observed using CD31^{+}CD146^{+} cells from the hESC-H9. (**D**) Consistently specific blood vessel engraftment was observed using CD31^{+}CD146^{+} cells from CB-iPSC-6.2.

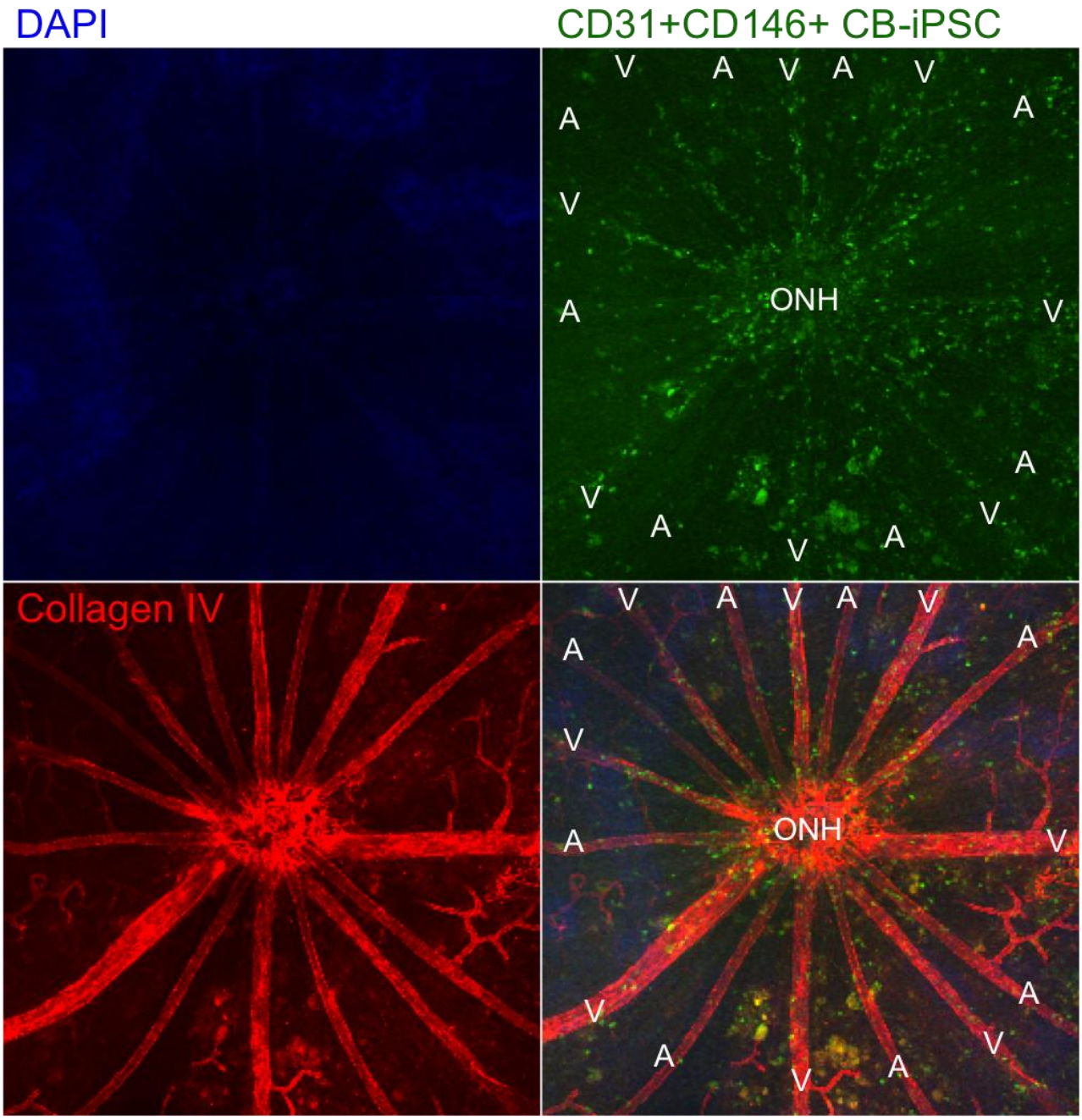

Figure S16. Specific homing of intravitreally-injected CD31$^+$CD146$^+$ VP cells derived from CB-iPSC-6.2 to retinal veins. At post-injection day 14, luciferase$^+$CD31$^+$CD146$^+$ VP cells derived from CB-iPSC 6.2 (green) homed and engrafted primarily to veins, but less into arteries. Homing of human cells into murine vessels was detected using anti-luciferase (green), collagen type-IV (red), and DAPI (nuclear staining, blue); A: artery; V: vein; ONH: optic nerve head.

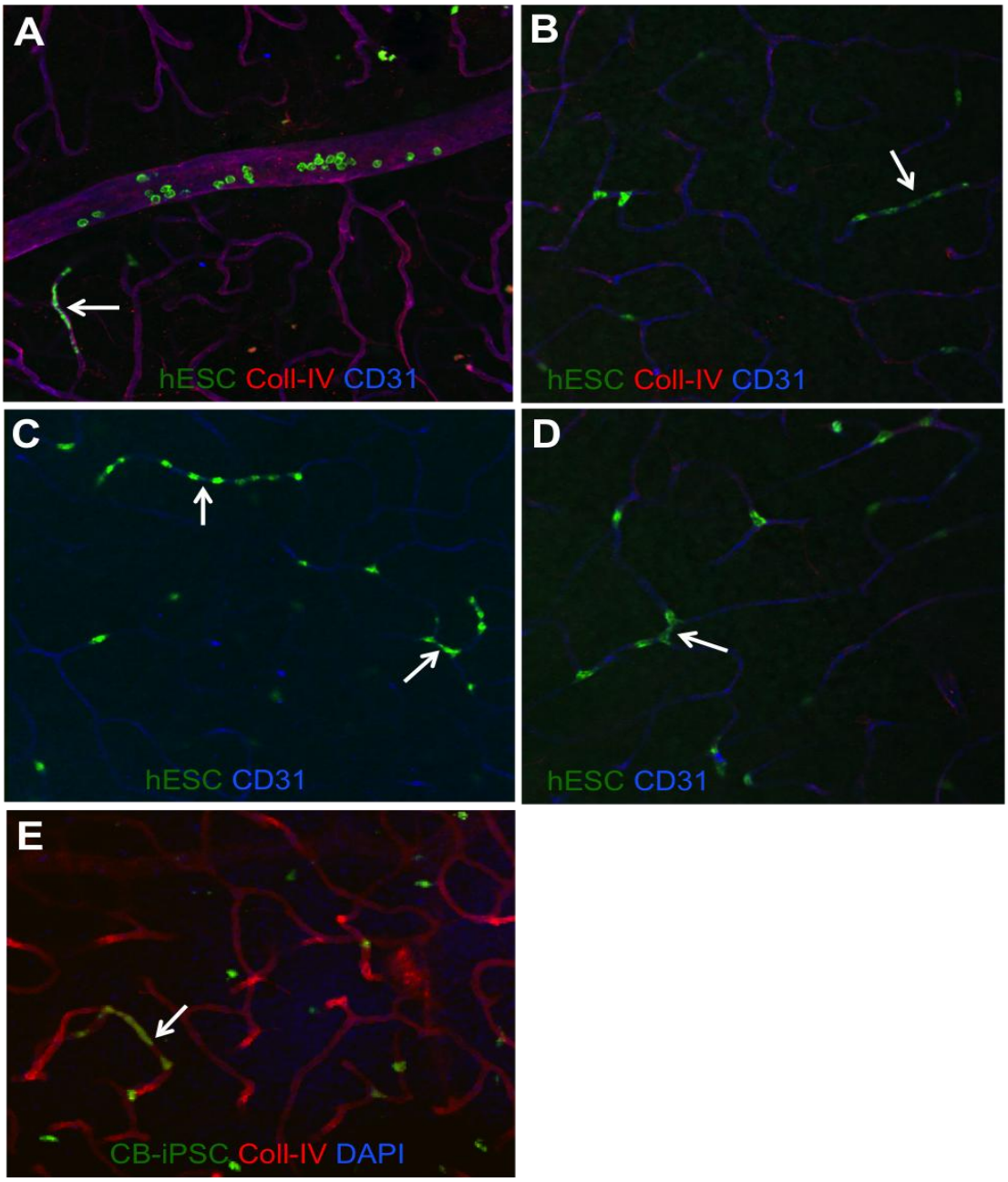

Figure S17. Regeneration of I/R-Damaged host retinal blood vessels by human CD31^{+}CD146^{+} VP cells. At post-injection day 21, luciferase^{+}CD31^{+}CD146^{+} VP cells derived from hESC-H9 (**A-D**, green) and CB-iPSC-6.2 (**E**, green) were observed to home, integrate and regenerate capillaries (arrows) in the mouse retina I/R model both linear (**A-C**, **E**) and branched (**C**, **D**) segments were repopulated. Staining of (**A**, **B**) collagen type-IV (red), mouse CD31 (blue), luciferase (green); (**C, D**) mouse CD31 (blue), luciferase (green); (**E**) collagen type-IV (red), DAPI (blue), luciferase (green)

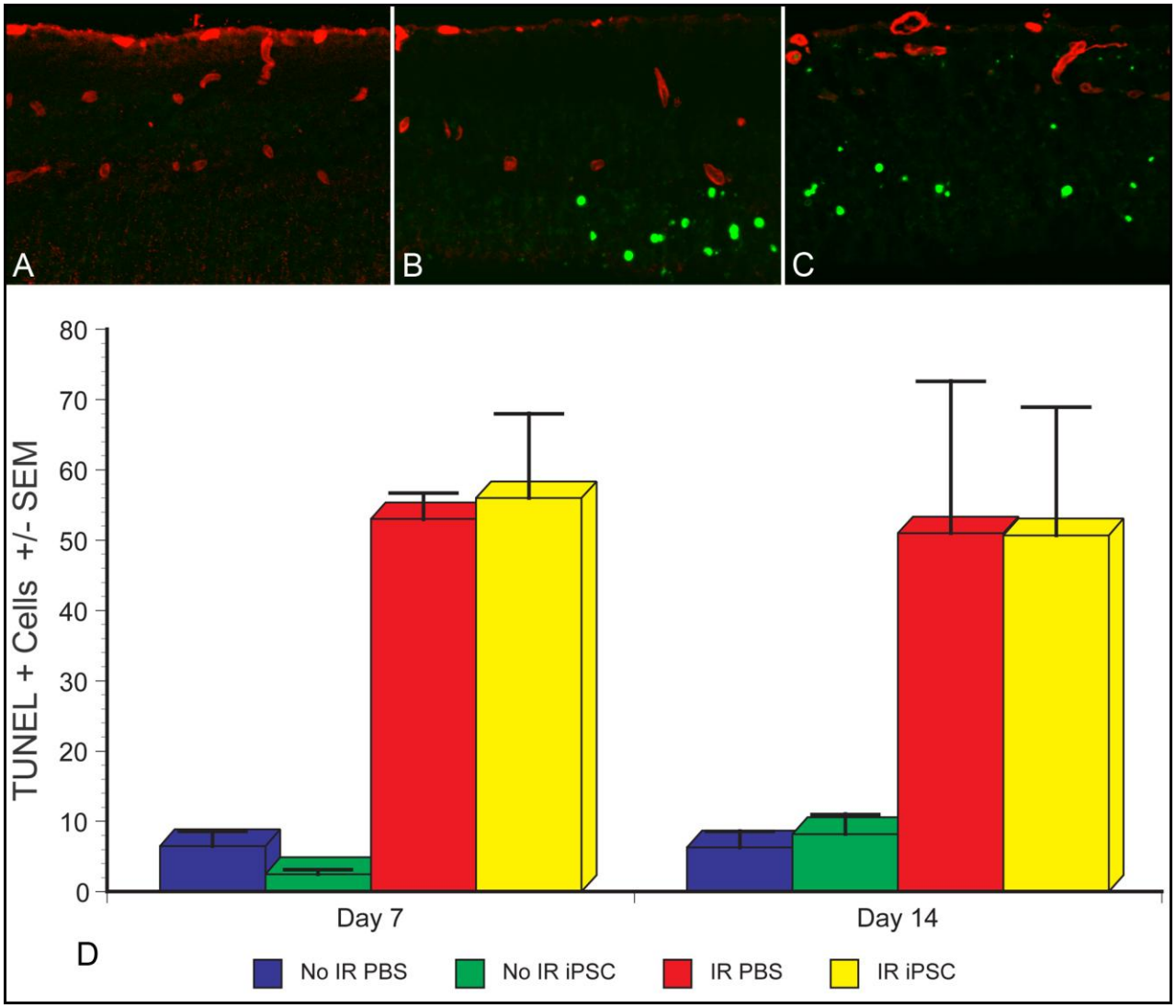

Figure S18. Neuronal viability: TUNEL labeling of retinal neuronal cells. A, Representative TUNEL stains. TUNEL$^+$ cells were scarce in retinas without I/R treatment, and injected with PBS only (red = collagen IV localization). However, there were abundant TUNEL$^+$ cells (green) present in the outer nuclear layer of retinas that experienced I/R injury and PBS (**B**), and also in retinas treated with I/R injury and injected with CB-iPSC VPs (**C**). Representative retinal TUNEL stains shown were from eyes 7 days post intravitreal injection of VP cells. **D**, The mean number of TUNEL+ cells (+/- SEM) from three retinas is shown for eyes receiving no I/R, no I/R and PBS injection, I/R with PBDS injection, and I/R and CB-iPSC-origin VP injection. The p values of I/R injected with PBS vs I/R injected with CB-iPSC-VP cells were not significant at both days 7 and 14.

Supplemental Movie S1 (mpeg file). Three-dimensional reconstruction of $CD31^+CD146^+$ CB-iPSC-VP homing to host murine retinal vessels at post-injection day 14. $CD31^+CD146^+$ CB-iPSC-VP cells (green), collagen type-IV (red), and DAPI (blue). Note that VP cells were observed at both endothelial and perivascular (pericytic) locations.

Supplemental Movie S2 (mpeg file). Three-dimensional reconstruction of $CD31^+CD146^+$ CB-iPSC VP engrafting into host murine retinal vessels at post-Injection day 14. Note the specific engraftment and incorporation of human VP cells directly into host retinal blood vessels**.** $CD31^+CD146^+$ CB-iPSC VP (green), collagen type-IV (red), DAPI (blue). A: artery; V: vein.

IV. Supplementary References

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