

Supplementary Materials for

**Specialized Retinal Endothelial Cells Modulates Blood-Retina- Barrier in
Diabetic Retinopathy**

Short Running Title: Specialized EC modulates retinal function in DR

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Materials and Methods

Single-cell RNA-seq

We harvested retina tissue from 20-week-old male diabetic mice (db/db) and normal mice (db/m). The retina was dissected and digested to harvest single live endothelial (CD31+) cells enriched by using CD31 microbeads for single-cell RNA sequencing.

Two samples were included in each group. The cell suspensions were mixed with filtered trypan blue to evaluate cell viability and concentration. Using the cell count and recommended cell concentration, we performed the following steps on a 10x Genomics chromium single-cell platform, and libraries were constructed according to the manufacturer's instructions of 10x Chromium Single Cell 3' Regent Kit v3 protocol (10X Genomics, 1000121). Briefly, single-cell suspensions were loaded to 10x Chromium. cDNA amplification and library construction were performed according to standard protocols. Libraries were sequenced using an Illumina NovaSeq 6000 sequencing system (paired-end multiplexing run, 150bp) by LC-Bio Technology Co., Ltd. (Hangzhou, China), at a minimum depth of 20,000 reads per cell.

Single-cell RNA-seq Processing

We used the Cell Ranger software suite (version 7.0.0) to perform single-cell barcode processing, read alignment, and feature barcode matrix generation. Specifically, we used the Mus musculus genome mm10 as the reference genome and applied STAR (v.2.7.10b) software (15) for FASTQs alignment. The cell barcodes were automatically determined based on the distribution of the UMI counts. A preliminary count matrix was used for downstream analysis.

Cell Filtering

Several criteria were applied for cell filtering. Specifically, genes that were expressed in fewer than 200 cells were removed. Cells with fewer than 150 detected genes or expressing more than 5% of mitochondrial genes were filtered out. We used Scrublet (version 0.2.3) (16) to identify potential doublets. Cells with doublet scores larger than 0.1 were filtered out. Overall, 18,376 cells were retained after quality control.

Single-cell Clustering

Cell clustering was performed using Scanpy (version 1.8.2) (17). The cells were normalized to the total counts of all genes, followed by logarithmic transformation.

Overall, 1,500 highly variable genes were detected using the *pp.highly_variable_genes* function. Specifically, the normalized dispersion was obtained by scaling with the mean and standard deviation of the dispersions for genes falling into 20 bins for the mean expression of genes. For each bin of mean expression, we then z-normalized the dispersion measure of all genes. After excluding genes with average expression lower than 0.0125 or higher than 3, we identified 1500 genes with the top z-score, indicating the top variable genes. We calculated a principal component analysis (PCA) matrix with 20 components using the informative genes (*pp.pca* function). The resulting PCA matrix was used to build the nearest-neighbor graph (*pp.neighborhood* function). The nearest-neighbor graph was then implemented to identify clusters using the Louvain algorithm (*tl.louvain* function) (18). Cluster-specific marker genes were identified using the *rank_genes_groups* function. Specifically, each cell group was compared with other groups using

two-sided, unpaired Wilcoxon tests, and a Benjamini–Hochberg adjusted P -value <0.05 was considered significantly expressed. In the first round of clustering (resolution = 0.3), six major cell types were identified: endothelial cells, pericytes, microglia, Müller cells, neurons, and photoreceptors. We performed a second round of clustering on the endothelial cells with a clustering resolution range of 0.1 to 0.5.

Differential Gene Analysis, EC Subtypes Annotation and Gene Ontology Enrichment

For each EC subtype, we identified and compared the expression level of genes with other cell subtypes and differentially expressed genes with the rank_genes_groups method. Each cell group was compared with other groups using two-sided, unpaired Wilcoxon tests, and a Benjamini-Hochberg adjusted P -value <0.05 was considered significantly expressed. The resulting subtypes were annotated based on known marker genes from the top 30 cluster-specific significantly highly expressed genes. For each EC subtype, genes with z-scores > 0 and Benjamini-Hochberg adjusted P -value < 0.05 were retained for Gene Ontology (GO) enrichment analysis. The R package clusterProfiler (version 4.2.2) (19) was used to obtain GO biological process terms-enriched in each cell subtype. A Benjamini-Hochberg adjusted P -value < 0.05 was considered significant. R package “org.Mm.eg.db” (version 3.14.0) was used to map gene identifiers.

Cell-Cell Interaction Analysis

CellPhoneDB (version 0.8.0) (20) were applied to illustrate the cell-cell communication among pericyte and EC subtypes. Specifically, CellPhoneDB was

applied to the search for interacted ligand-receptor pairs among each of the interacting pairs of cell types. Cell annotation information and a count matrix were used for the CellPhoneDB statistical analysis. The enrichment of the interacting ligand-receptor pair in each of the interacting pairs of cell types was calculated, and a ligand-receptor pair with a Benjamini-Hochberg adjusted P -value < 0.05 was considered significantly enriched.

Cell Culture

Human umbilical vein ECs (HUVECs) were isolated and cultured as per the protocol described elsewhere (21). Human retinal endothelial cell (HRECs) were grown in the Endothelial Cell Medium (ECM, ScienCell Research Laboratories, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin, and 1% endothelial cell growth factors at 37 °C with 5% CO₂ and 95% air. Cell passages 4–7 were used for all the experiments. For stimulation experiments, cells were starved for 12 h with ECM (5.5 mM Glucose) and 0.5% FBS before treatment.

Quantification of Gap Index

The gap index (number of intercellular gaps/cell number) was calculated using the following formula: $([\text{intercellular gap number}/\text{total cell area}] \times 1000)/\text{cell number}$. In each case, a minimum of five fields were quantified (≈ 30 cells per field) per experiment, and the data represent the mean of at least three independent experiments (22).

1 **Metabolomics Profiling**

2 One million of cultured cells or vitreous humor was homogenized in 100 uL water by
3 using a tissue tearor. An aqueout of 50 uL sample homogenate or 20 uL of plasma
4 were added to a clean glass tube and mixed with 300 uL of methanol containing 0.1
5 μ M of each internal standard. The mixture was vortexed well and centrifuged at 3,000
6 g for 10 minutes. The clear liquid phase was transferred to a second clean glass tube
7 using a Pasteur pipette followed by evaporation under nitrogen. The extracted lipids
8 were reconstituted in 50 μ L of methanol:acetonitrile (v:v=1:1) for ceramide assay, or
9 in 50 uL of methanol:water (v:v=1:1) for assay of sphingosine and its phosphate form.
10 Then the reconstituted sample was transferred to an LC/MS autosampler vials (Waters,
11 P/N 600000670CV) for injection. All LC/MS assays were carried out on a Waters
12 Xevo TQ-S micro MS ACQUITY UPLC system (Waters, Milford, MA). The system
13 was controlled by Mass Lynx Software version 4. Positive ESI-MS/MS mass
14 spectrometry was performed using the following parameters: capillary voltage, 3.8 kV;
15 source temperature, 150 °C; desolvation temperature, 500 °C; desolvation gas flow,
16 1000 L/h; and collision gas flow, 0.15 mL/min. The optimized cone voltage was 22 V,
17 collision energy for multiple reactions monitoring mode (MRM) was 26 eV. Different
18 species were confirmed by comparing the retention times of experimental compounds
19 with those of authentic standards. Concentrations of species in the samples were
20 quantified by comparing integrated peak areas for those of each ceramide against
21 those of known amounts of purified standards. Loss during extraction was accounted
22 for by adjusting for the recovery of the internal standards. All solvents for sample

1 extraction and LC/MS were LC/MS grade (or LC grade when LC/MS grade was not
2 available) and were purchased from Fisher Scientific (Pittsburgh, PA, USA).
3 Ceramide, sphingosine, shingosine-1-phosphate and other calibration standards and
4 internal standards were purchased from Avanti Polar Lipid, Inc. (Alabaster, AL,
5 USA).

6 **siRNA Transfection**

7 HUVECs or HRECs were transfected with siRNAs using the Lipofectamine RNAi
8 Max Kit following the manufacturer's instructions (Cat. No. L 13778-150). Human
9 siRNAs were constructed using GenePharma (Shanghai, China) and the sequences
10 were as follows: siRNA-ACER2 5'- UGACCGAGCUUUCUGCGAGUU -3';
11 siRNA-negative control 5'- UAAGGCUAUGAAGAGAUACUU -3'.

12 **Adenovirus or Adeno-associated Virus Production and Infection**

13 Human *ACER2* (NM_001010887) sequences were inserted into the GV138 vector
14 (CMV-MCS-3FLAG) to generate a recombinant adenovirus. Mouse *ACER2*
15 (NM_139306) sequences were inserted into the GV411 vector
16 (CMV-betaGlobin-MCS-3Flag-SV40 PolyA) to generate a recombinant
17 adeno-associated virus. All adenoviruses and adeno-associated viruses were
18 constructed using the GeneChem software (Shanghai, China). HUVECs or HRECs
19 were infected with the adenovirus at an MOI of 10, and no detectable cellular toxicity
20 was observed. For AAV9 infection studies, AAV9-ACER2/empty vectors (2.7×10^{11}
21 vector genomes/mice) were delivered by intravitreal injection.

Permeability Assays

Lysine-fixable Cadaverine conjugated to Alex Fluor-555 (Invitrogen) was injected intravenously into the lateral tail vein of db/db and db/m mice at a concentration of 100 µg Cadaverine/20 g of mice. It was circulated for 30 min, and the retinas were fixed and flat-mounted. For fundus fluorescein angiography, fluorescein sodium (5 mg: 0.5 g (10%), Alcon Laboratories, Inc) was delivered by intraperitoneal injection 100 µL/mice.

Immunofluorescence

ECs were seeded onto coverslips. After treatment, cells were washed twice with ice-cold phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 min. After washing with PBS, cells were permeabilized with 0.5% Triton-X100 for 30 min and blocked with normal goat serum for 1 h. Subsequently, cells were incubated with the primary antibodies: anti-VE-cadherin (sc-9989, Santa Cruz Biotechnology), anti-ACER2 (PA5-101415, Thermo Fisher Scientific) at 4°C overnight. Furthermore, the cells were rinsed with PBS and incubated with the corresponding secondary antibodies labeled with Alexa Fluor (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h. Fluoroshield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) was used to cover the slides and fluorescent signals were detected using a confocal fluorescence microscope (LSM 800; Carl Zeiss, Germany).

For frozen eyes sections, eyes were enucleated from mice and fixed in 4% paraformaldehyde (PFA) for 1 h at 4 °C. They were transferred to 30% sucrose/PBS

1 at 4 °C overnight and subsequently embedded in optimal cutting temperature
2 compound (Sakura, Japan) and frozen at -80 °C. Subsequently, sagittal cryosections
3 of eyes were obtained. Frozen sections with a size of 15 µm were fixed in 4%
4 paraformaldehyde for 15 min. After washing with PBS, the sections were
5 permeabilized with 0.5% Triton-X100 for 30 min and blocked with normal goat
6 serum for 1 h. The sections were incubated with the primary antibodies: anti-
7 VCAM-1 (sc-13160, Santa Cruz Biotechnology), anti-ACER2 (PA5-101415, Thermo
8 Fisher Scientific), anti-Flag (14793S, Cell Signaling Technology), anti-CD31
9 (MAB1398Z, Sigma-Aldrich), anti-GFAP (80788T, Cell Signaling Technology) at
10 4°C overnight. The sections were rinsed with PBS and then incubated with the
11 corresponding secondary antibodies labeled with Alexa Fluor (Thermo Fisher
12 Scientific, Waltham, MA, USA) for 1 h. One-step TUNEL Apoptosis Assay Kit (Cat.
13 No. KTA2010; Abbkine Scientific Co., Ltd.) was used after staining with secondary
14 antibodies. Fluoroshield mounting medium containing DAPI was used to cover the
15 slides. Fluorescent signals were detected using a confocal fluorescence microscope
16 (LSM 800; Carl Zeiss, Germany). Representative images were selected randomly
17 from each group.

18 For flat-mounted retinas, eyes were enucleated from mice and fixed in 4% PFA for 1
19 h at 4 °C. Retinas were dissected, washed with PBS, permeabilized with PBS
20 containing 1% TritonX-100 overnight at 4°C, and blocked in PBS containing 2%
21 bovine serum albumin and 0.3% TritonX-100 for 12 h at 4 °C. After blocking,
22 flat-mounted retinas were stained with isolectin GS-IB4 (1:100) for 2 h at room

1 temperature for visualization of retinal vasculature. For immunofluorescence staining
2 of Isolectin B4, ZO-1, Claudin-5, and CD31, flat-mounted retinas were incubated in
3 blocking solution with the following primary antibodies 4 °C overnight: anti-Isolectin
4 B4 (I21411, Invitrogen), anti-ZO-1 (339188, Invitrogen), anti-Claudin-5 (49564S,
5 Cell Signaling Technology), anti-CD31 (MAB1398Z, Sigma-Aldrich). After washing,
6 the retinas were incubated with the corresponding secondary antibodies (1:300,
7 Jackson ImmunoResearch) for 2 h at room temperature. Flat-mounted retinas were
8 analyzed using a Zeiss confocal laser scanning microscope.

9 **Retinal Trypsin Digestion Assay**

10 The eyes were enucleated from the mice and fixed in 4% PFA. The retina was divided
11 into four quadrants on the optic disc. They were then incubated with 3% Trypsin, 0.1
12 M Tris (pH 7.8) for 1-3 h at 37 °C until the medium became cloudy. After digestion,
13 internal limiting membranes were carefully removed. The retina was separated from
14 the disintegrated neuronal tissue in the retinal vasculature and mounted on a glass
15 slide for air drying. The isolated retinal vasculature was treated with a periodic acid
16 solution and stained with Schiff's reagent and hematoxylin (glycogen periodic acid
17 Schiff stain kit; Beijing Solarbio Science & Technology Co., Ltd.). A digital imaging
18 system was used to observe histological changes in the four quadrants per retina. All
19 measurements were conducted in a masked fashion and observed under bright field
20 using an imaging system. Cellular capillaries are shown as the total number/mm² of
21 the retina area.

Electroretinogram (ERG) Assay

ERG was recorded using a Phoenix-Micron ERG system (Phoenix-Micron, Inc., Bend, OR, USA). The mice were dark-adapted for 24 h before obtaining ERG recordings. The mice were maintained under anesthesia with 1.2% Afodin for intraperitoneal injection and placed on a heated stage during testing to prevent hypothermia throughout the procedure. The pupils were dilated using tropicamide phenylephrine eye drops (Santen Pharmaceutical Co., Ltd., Shiga Plant). Oxybuprocaine hydrochloride eye drops (Santen) were used to ensure proper contact with corneal electrodes. Three electrodes were attached to record the transretinal electrical signals: the Ganzfeld corneal (positive) electrode was made of a gold conducting ring around the objective lens, the contact was attached to the cornea, and the eye was sealed. A reference (negative) electrode was inserted subcutaneously through the skin of the head positioned at the midline between the ears, such that the end of the needle was between the eyes. Finally, the ground electrode was inserted subcutaneously through the base of the tail. Following baseline signal stabilization, ERG signals were recorded according to the manufacturer's protocol. For each mouse, at least three sweeps of electrical responses were acquired at each flash intensity with varying stimulus intervals (-1, 1, and 2.8). Waveform amplitudes at each flash stimulus were acquired from three averaged sweeps and analyzed using the Labscribe software (LabScribeERG version 3.016800, iWorx Systems, Dover, NH, USA).

Angiogenesis Assay for Tube Formation

Twelve-well plates were coated with ice-cold Matrigel solution (BD Biosciences) and incubated at 37 °C for at least 30 min to allow the Matrigel to solidify. siRNA-transfected HRECs with or without VEGF (25 ng/mL) were harvested and suspended in FBS-reduced medium (2%). Subsequently, these cells were seeded in the Matrigel-coated wells at a density of 1×10^5 cells/well and pre-incubated for 30 min at 37 °C for cell attachment. Images were acquired using a Zeiss Axiovert microscope and analyzed with NIH ImageJ software.

Western Blot Analysis

Whole-cell lysates were prepared in lysis buffer containing a complete protease inhibitor cocktail, PhosSTOP, and phenylmethylsulfonyl fluoride. For western blot analysis, protein samples were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Cat. No. 10600001; GE Healthcare; Chicago, IL, USA). Membranes were then incubated with primary antibodies against VE-cadherin and pY685 (Cat. No. ab119785) antibodies were purchased from Abcam (Cambridge, UK). The antibodies against *ACER2* (Cat. No. PA5-39016) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). VE-cadherin and pY731 (Cat. no. 310306) were obtained from Zen BioScience (Research Triangle Park, NC, USA). VE-cadherin (Cat. No. sc-9989) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Cat. no. 60004-1-Ig) was purchased from Proteintech (Wuhan, China). Membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (1:5000 dilution) and

1 visualized using an Enhanced Chemiluminescent Western Blotting Detection kit (Cat.
2 No. 34580; Thermo Fisher Scientific, Waltham, MA, USA) in the ChemiScope3600
3 Mini chemiluminescence imaging system (Clinx Science Instruments; Shanghai,
4 China). Band intensity was quantified by scanning autoradiogram densitometry using
5 the ImageJ software.

6 **Total RNA Isolation and Real-time Polymerase Chain Reaction (PCR) Analysis**

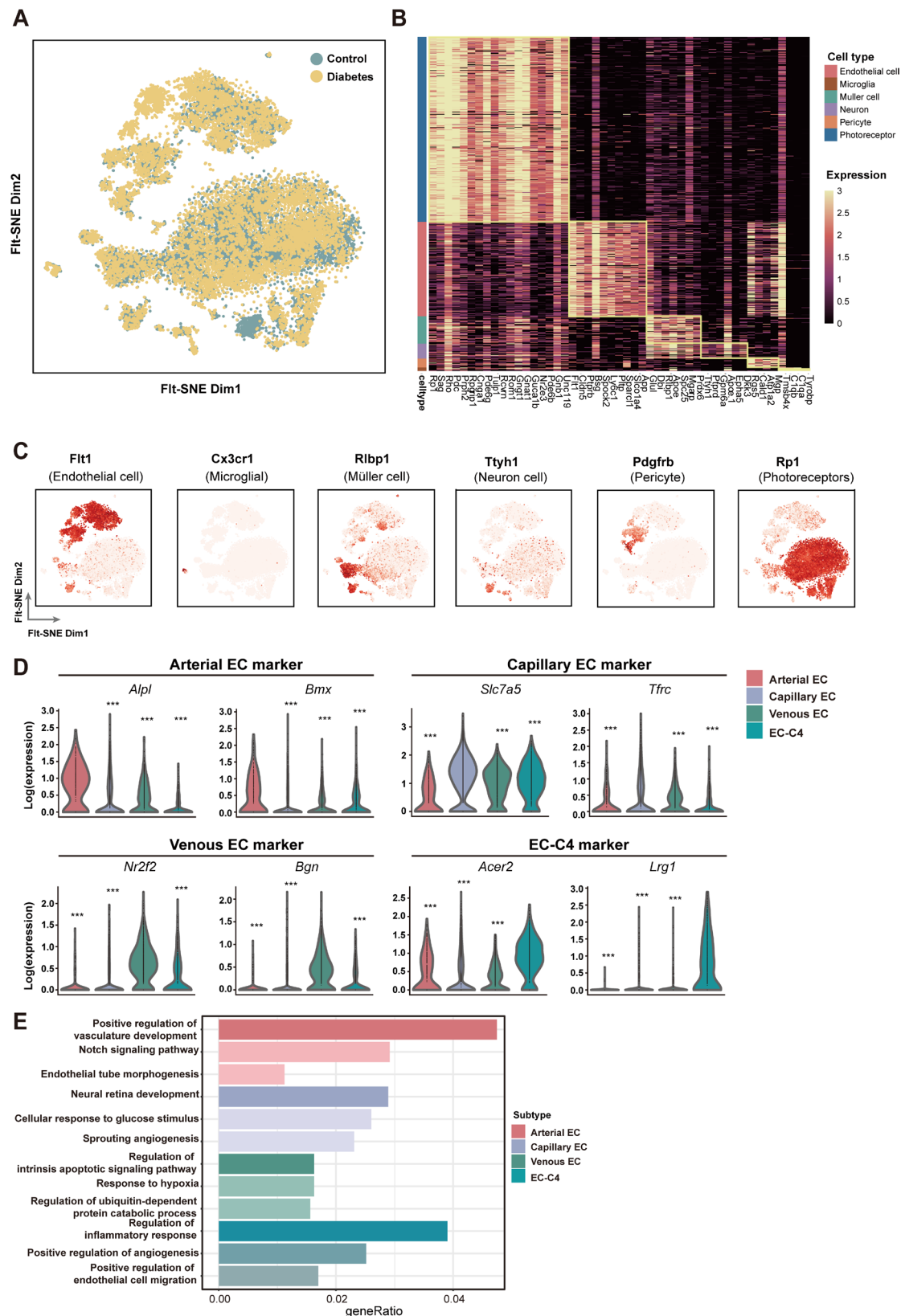
7 RNA was extracted from the cultured cells using TRIzol reagent (Invitrogen, Carlsbad,
8 CA, USA). Two μ g total RNA was reverse-transcribed with SuperScript III and
9 random primers (Cat No.12574035, ThermoFisher Scientific, Grand Island, NY).
10 Real-time PCR was performed using Brilliant II SYBR Green qPCR Master Mix
11 (Stratagene, CA) on an ABI 7900HT Real-Time PCR System (Life Technologies,
12 CA). Results were normalized to those of GAPDH. The primer sequences are
13 presented in Table S11.

14 **Transendothelial Electrical Resistance (TEER) Assay**

15 The TEER value was evaluated using a commercially available TEER measurement
16 equipment (EVOM2, World Precision Instruments) equipped with a chopstick
17 electrode pair (STX3, World Precision Instruments). The TEER values of the
18 transwell membranes were measured in triplicate. The TEER values were calculated
19 as follows: $TEER (\Omega/cm^2) = (total\ resistance\ across\ the\ cellular\ monolayer\ on\ the$
20 $membrane\ blank\ resistance\ of\ the\ membrane) / surface\ area\ of\ the\ membrane.$

21

1 Supplemental Figures and Figure Legends



2

1 **Figure S1. Global view of the FACS CD31+ cells isolated from the retina of**
2 **diabetic mice**

3 (A) t-SNE visualization of the FACS CD31+ cells from the retina of diabetic mice.
4 Cells are colored by cell conditions. (B) Heatmap showing average scaled expression
5 levels (color-scaled, row-wise Z scores) of the top differentially expressed genes
6 across the endothelial cell-type clusters. (C) t-SNE visualization of endothelial cell
7 clusters. Color represents z-score-scaled gene expression values. (D) Violin plots
8 showing the scaled expression values of EC markers across EC subtypes. (E) Bar plot
9 showing the enrichment of functional pathways in each endothelial cell subtypes as
10 compared with other EC subtypes. The bars are colored by cell subtypes. The color
11 intensity reflects the $-\log_{10}$ transformed adjusted p -values for the pathways.
12

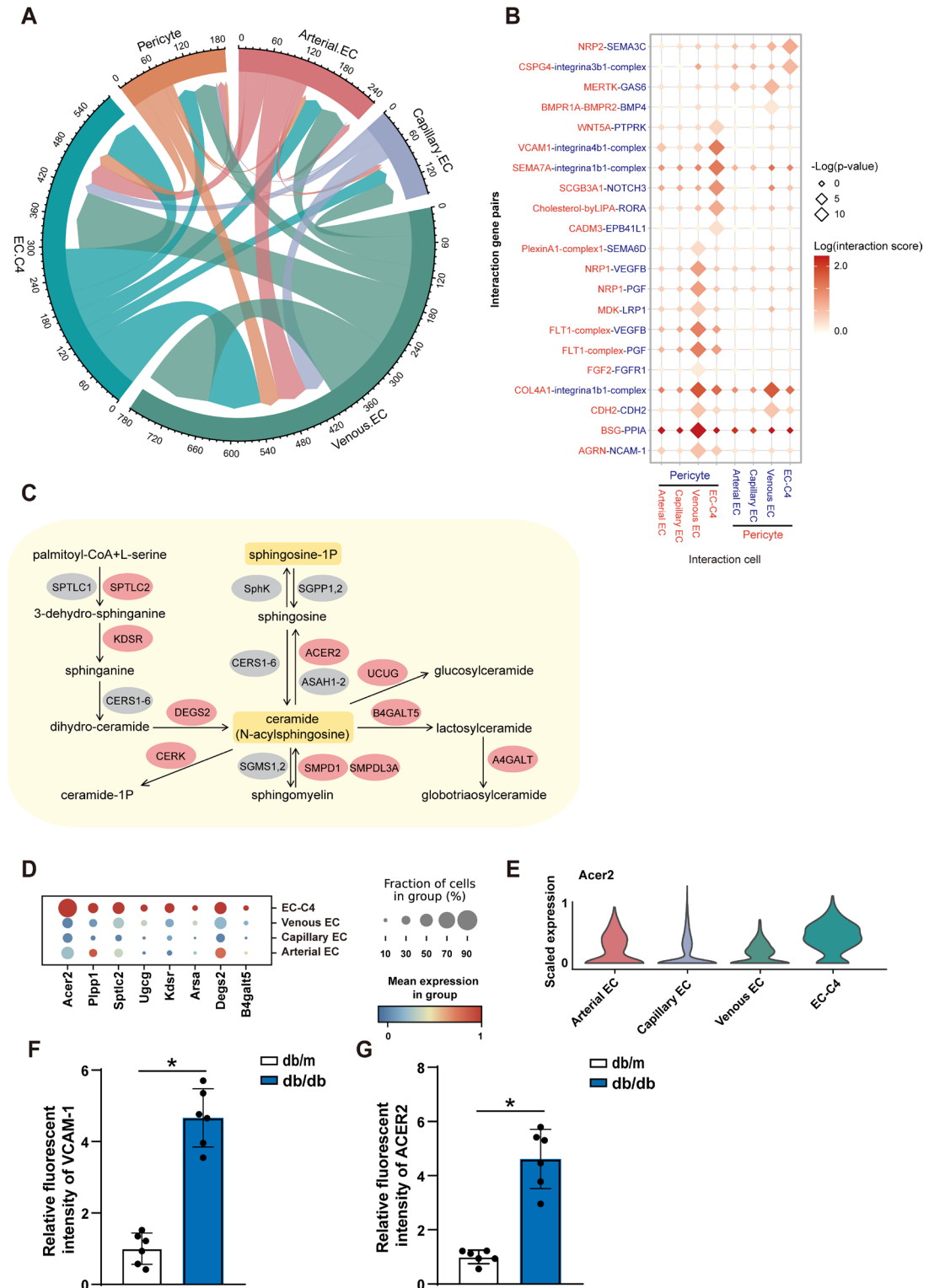


Figure S2. Expression level of sphingolipid metabolism-related genes in endothelial cell subtypes

(A) Chord diagram showing the ligand-receptor pairs between pericytes and

1 endothelial cell subtypes. The arrow width represents the number of ligand-receptor
2 pairs that are significantly enriched in each interacting pair between cell types. (B)
3 Dot plot showing the ligand-receptor interaction scores between pericytes and
4 endothelial cell subtypes. The color intensity reflects the average interaction score
5 within each cluster, and the size of each dot reflects the $-\log_{10}$ transformed p -value.
6 P -values were calculated using the CellPhoneDB method; only interaction pairs with
7 adjusted p -values < 0.05 are shown in the plot. (C) The main metabolism pathway of
8 ceramide and sphingosine-1-phosphate synthesis. (D) Dot plot for the gene expression
9 value. The size of each circle reflects the percentage of cells in a cluster where the
10 gene is detected, and the color intensity reflects the average expression level within
11 the cluster. Only genes with an adjusted p -value < 0.05 are shown in the plot. (E)
12 Violin plots showing the scaled gene expression values in endothelial cell subtypes.
13 The expression level of ACER2 was compared between EC-C4 and other cell type.
14 T-test was applied for statistical analysis. *** $p < 1e-3$. (F) Quantification of VCAM-1
15 expression patterns in cross-sections of mouse eyes from indicated groups. ($n = 6$). (G)
16 Quantification of ACER2 expression patterns in cross-sections of mouse eyes from
17 indicated groups. ($n = 6$). The data represent mean \pm SD. P -values correspond to
18 unpaired two-tailed t -test.

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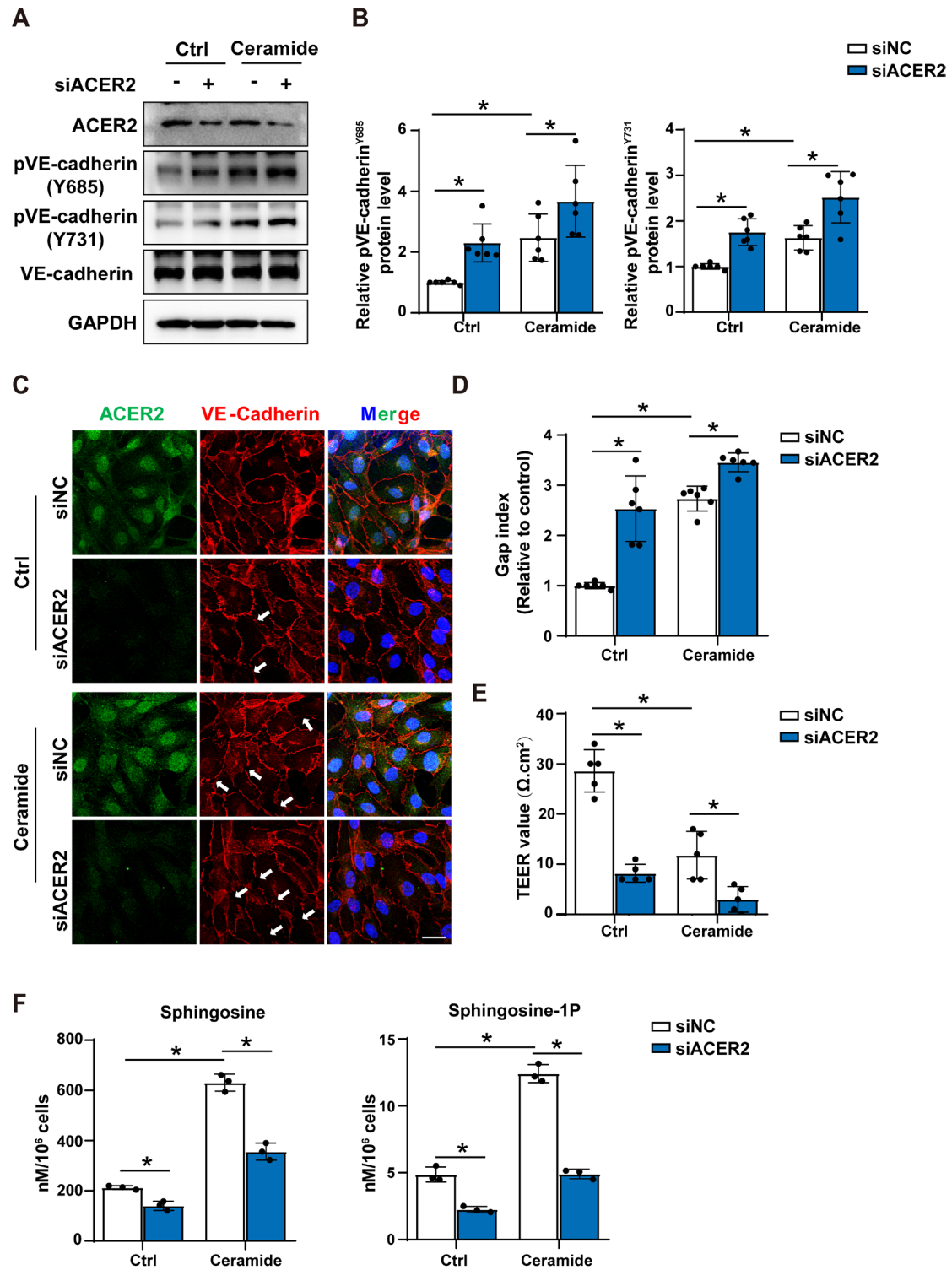


Figure S3. Endothelial ACER2 is essential for endothelial permeability and vascular integrity

(A) Representative western blot analysis of the phosphorylation of VE-cadherin at

1 Y685 and Y731 in HUVECs transfected with indicated siRNA against ACER2 and
2 stimulated with 10μM ceramide for 48h. (B) Quantification of (A). (n = 6). (C)
3 Representative images of HUVEC monolayers stained for VE-cadherin after
4 treatment with indicated siRNA against ACER2 and stimulation with 10μM ceramide
5 for 48h. (Arrow, a typical example of the intercellular gap). (D) Quantification of (C).
6 (n = 6). (E). Changes in transendothelial electrical resistance (TER) in HUVECs
7 transfected with indicated siRNA against ACER2 and stimulated with 10μM ceramide
8 for 48h. (n = 5). (F). Mass spectrometric analysis of sphingosine and
9 sphingosine-1-phosphate (S1P) levels in HUVECs transfected with indicated siRNA
10 against ACER2 and stimulated with 10μM ceramide for 48h (n = 3). Data are
11 represented as mean ± SD. P-values correspond to two-way ANOVA with Tukey's
12 multiple comparisons test in (B), (D), (E) and (F).

13

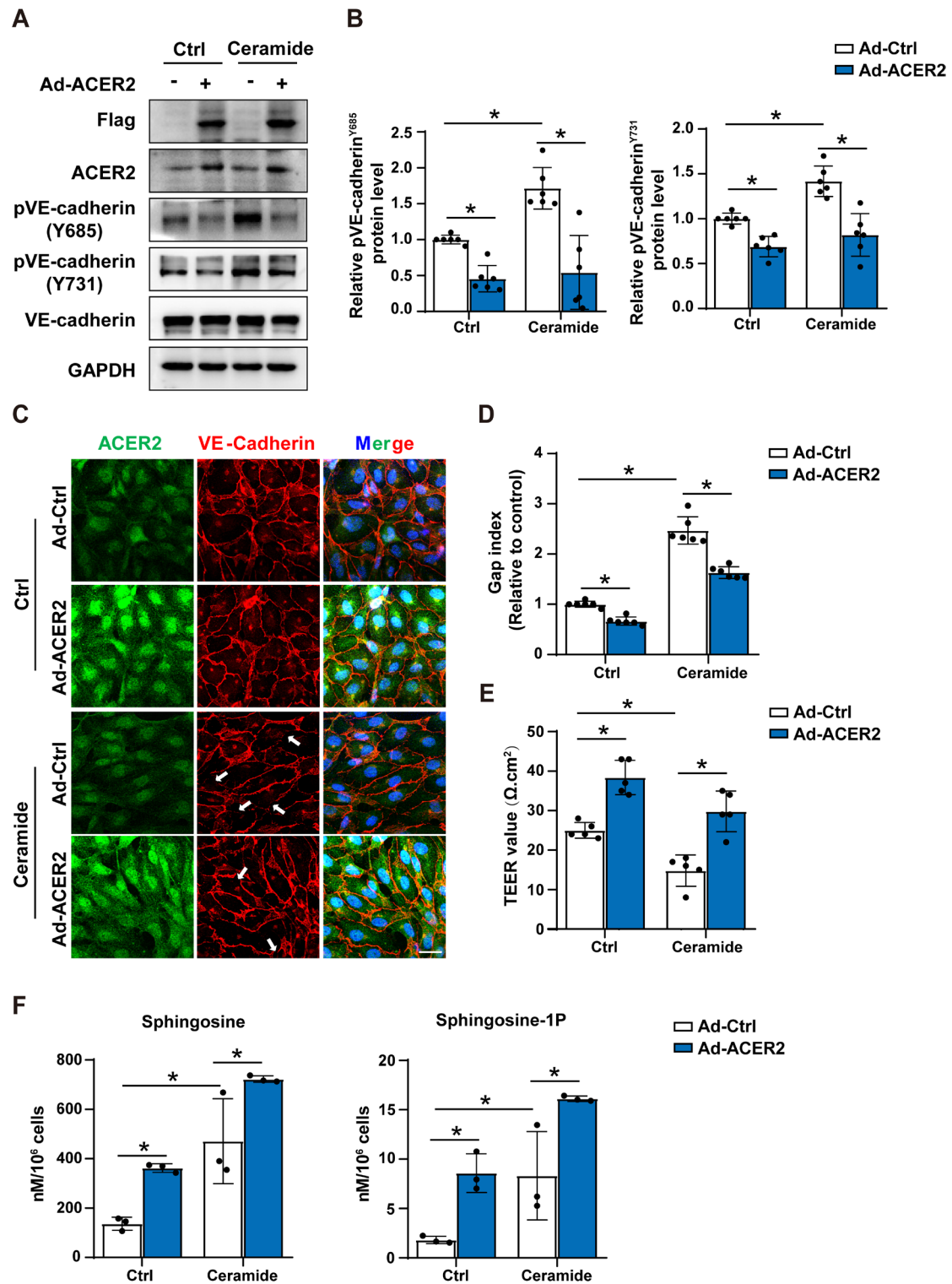


Figure S4. Endothelial ACER2 is essential for endothelial permeability and vascular integrity

(A) Representative western blot analysis of the phosphorylation of VE-cadherin at

1 Y685 and Y731 in HUVECs infected with indicated adenovirus and stimulated with
2 10μM ceramide for 48h. (B) Quantification of (A). (*n* = 6). (C) Representative images
3 of HUVEC monolayers stained for VE-cadherin after treatment with indicated
4 adenovirus and stimulation with 10μM ceramide for 48h. (Arrow, a typical example
5 of the intercellular gap). (D) Quantification of (C). (*n* = 6). (E) Changes in
6 transendothelial electrical resistance (TER) in HUVECs infected with indicated
7 adenovirus and stimulated with 10μM ceramide for 48h. (*n* = 5). (F). Mass
8 spectrometric analysis of sphingosine and sphingosine-1-phosphate (S1P) levels in
9 HUVECs infected with indicated adenovirus and stimulated with 10μM ceramide for
10 48h. (*n* = 3). Data are represented as mean ± SD. *P*-values correspond to two-way
11 ANOVA with Tukey's multiple comparisons test in (B), (D), (E) and (F).

12

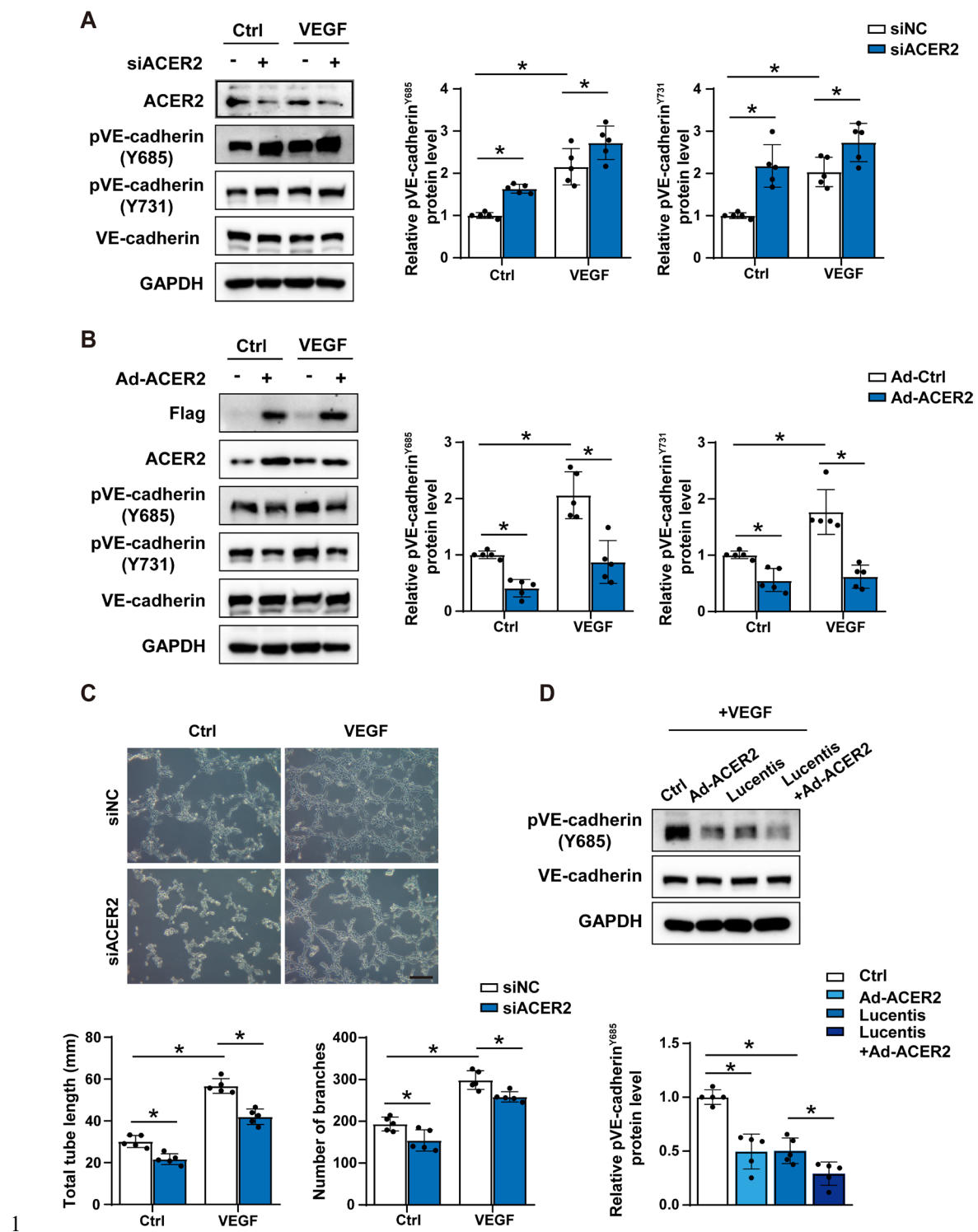


Figure S5. Endothelial ACER2 suppresses VEGF-downstream signaling

(A) Representative western blot analysis of the phosphorylation of VE-cadherin at Y685 and Y731 in HRECs transfected with indicated siRNA against ACER2 and stimulated with 25ng/mL VEGF for 10min. (*n* = 5). (B) Representative western blot

1 analysis of the phosphorylation of VE-cadherin at Y685 and Y731 in HRECs infected
2 with indicated adenovirus and stimulated with 25ng/mL VEGF for 10min. ($n = 5$). (C)
3 Representative bright field images of HRECs showing that knockdown of ACER2
4 abolished VEGF-induced tube formation. HRECs were transfected with control
5 siRNA or ACER2 siRNA and treated with 25ng/ml of VEGF (scale bars: 500 μ m)
6 (upper). Quantification of total tube length and number of branches (lower). ($n = 5$).
7 (D) Representative western blot analysis of the phosphorylation of VE-cadherin at
8 Y685 in HRECs infected with indicated adenovirus or Lucentis and stimulated with
9 25ng/mL VEGF for 10min. ($n = 5$).

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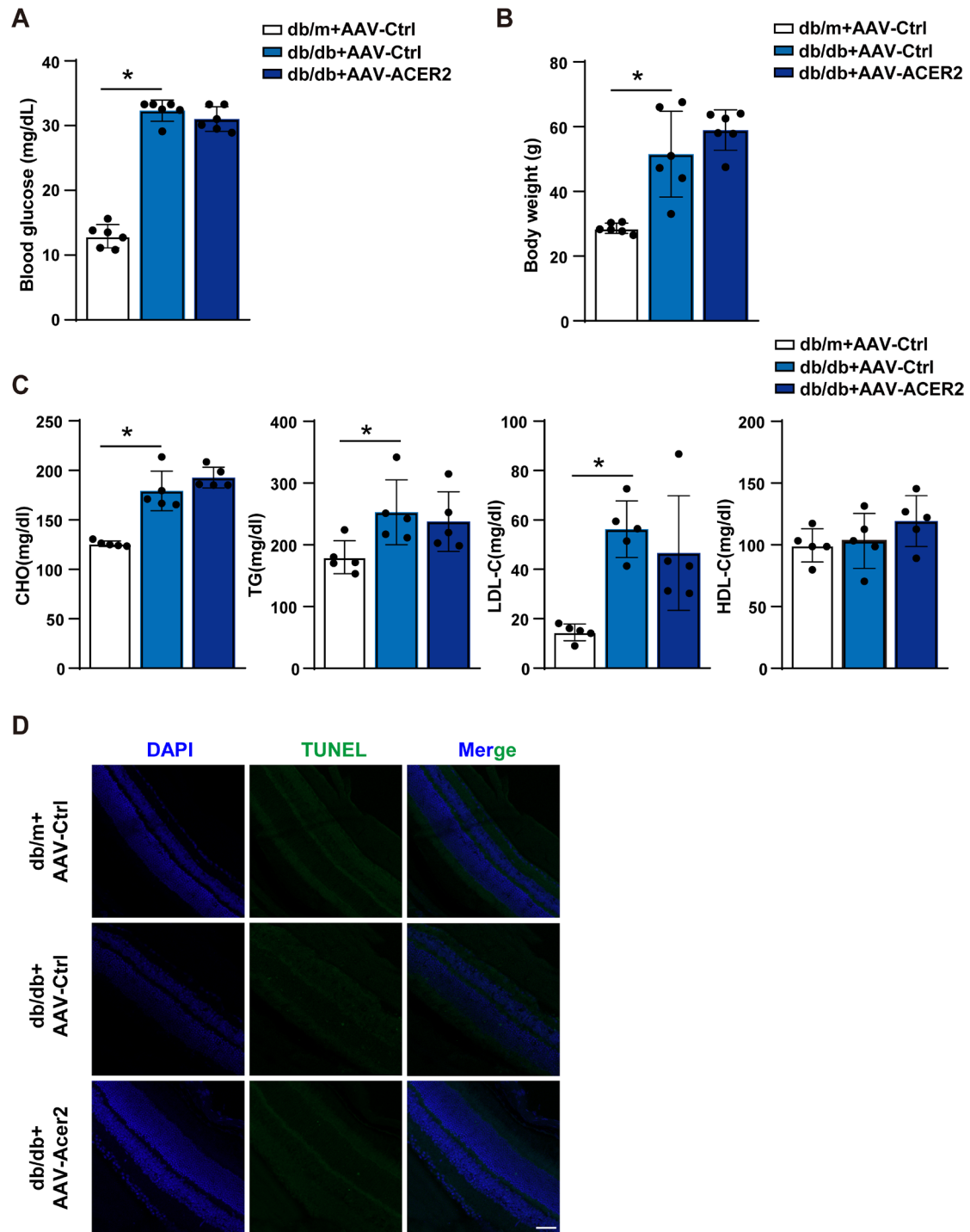


Figure S6. Endothelial ACER2 protects against diabetes induced vascular leakage and retinal neurovascular disease.

db/m and db/db mice were infected with AAV9-Ctrl or AAV9-ACER2 delivered by intravitreal injection. (A-B) Blood glucose and body weight in the three groups. ($n =$

1 6). (C) Plasma levels of total cholesterol (CHO), triglycerides (TG), low-density
2 lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) in
3 the indicated groups. ($n = 5$). (D) Representative TUNEL assay images of the eye
4 sections from the three groups. ($n = 5$). The data represent mean \pm SD. P -values
5 correspond to one-way ANOVA with Tukey's multiple comparisons test in (A), (B)
6 and (C).

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1 **Table S1.** Basic information on single-cell data processed in the analysis.

2 **Table S2.** Differentially expressed genes detected in each of the six retina cell types.

3 **Table S3.** The difference in the proportion of EC subtypes between diabetic and

4 control mice. Chip-square test with B-H adjusted was applied for statistical analysis.

5 **Table S4.** Differentially expressed genes detected in each of the four EC subtypes.

6 **Table S5.** Gene ontology terms-enriched in each of the four EC subtypes.

7 Corresponding to Figure S1D.

8 **Table S6.** Ligand-receptor pairs illustrating cell-cell communication among pericyte

9 and EC subtypes.

10 **Table S7.** Differentially expressed genes detected between EC-C4 and the other EC

11 subtypes.

12 **Table S8.** Gene ontology terms-enriched in EC-C4 as compared to other EC subtypes.

13 Corresponding to Figure 2B.

14 **Table S9.** Expression levels of specific markers across EC subtypes. Expression

15 levels were compared using a t-test and adjusted with the Benjamini–Hochberg

16 method. Corresponding to Figure S2D-E.

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1 **Table S10. Clinical information about the patients.**

2

Stage of DR	NDM	NPDR-NDME	NPDR-DME	PDR-A	P
N	25	25	25	25	NA
Male:Female	10:15	11:14	10:15	10:15	0.95
Age, years	69.28±9.60	61.12±11.80	56.36±14.21	53.64±12.95	0.13
Diabetes duration, years	0	13.55±8.77	9.45±7.43	14.42±7.23	0.07
HbA1c, %	5.76±0.64	7.94±1.54	7.38±1.64	8.30±1.78	0.19
HbA1c, mmol/mol	39.45±7.03	63.49±17.08	57.12±7.88	67.26±19.45	0.19
Fasting blood glucose mmol/L	5.89±1.84	9.97±3.16	9.11±2.54	11.42±7.20	0.28
Total cholesterol, mmol/L	4.64±0.92	4.64±1.07	4.67±0.85	5.78±1.74	0.00
Triglycerides, mmol/L	1.30±0.47	2.01±1.15	2.09±0.97	3.44±3.65	0.08
Data are mean ± standard deviation. <i>P</i> values were calculated using ANOVA and the χ^2 test for continuous and categorical variables, respectively.					

3

4 **Table S11. Primer sequences for real time PCR**

Gene	Forward primer	Reverse primer
ACER2	5'- TGGTGCAGGACAACACTACAC -3'	5'- GCAGATGGGCGGTAAAATGAA -3'
ICAM-1	5'-GTGATGCTCAGGTATCCATCCA-3'	5'-CACAGTTCTCAAAGCACAGCG-3'
VCAM-	5'-AGTTGGGGATTTCGGTTGTTCT-3'	5'-CCCCTCATTCCTTACCACCC-3'
TNF α	5'-CGTCGTAGCAAACCACCAAG-3'	5'-GGCAGAGAGGAGGTTGACTT-3'
GAPDH	5'- GAAGGTGAAGGTCGGAGTC -3'	5'- GAAGATGGTGATGGGATTTC -3'

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