Intravitreal AAV2.COMP-Ang1 prevents neurovascular degeneration in a murine model of diabetic retinopathy

Running Title: AAV2.COMP-Ang1 for diabetic retinopathy

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ABSTRACT

Diabetic retinopathy (DR) is the leading cause of blindness in the working-age population in the United States. The vision-threatening processes of neuroglial and vascular dysfunction in DR occur in concert, driven by hyperglycemia and propelled by a pathway of inflammation, ischemia, vasodegeneration, and breakdown of the blood retinal barrier. Currently, no therapies exist for normalizing the vasculature in DR. Here we show that a single intravitreal dose of adeno-associated virus serotype 2 encoding a more stable, soluble, and potent form of angiopoietin 1 (AAV2.COMP-Ang1) can ameliorate the structural and functional hallmarks of DR in Ins2Akita mice, with sustained effects observed through six months. In early DR, AAV2.COMP-Ang1 restored leukocyte-endothelial interaction, retinal oxygenation, vascular density, vascular marker expression, vessel permeability, retinal thickness, inner retinal cellularity, and retinal neurophysiological response to levels comparable to non-diabetic controls. In late DR, AAV2.COMP-Ang1 enhanced the therapeutic benefit of intravitreally-delivered endothelial colony-forming cells by promoting their integration into the vasculature and thereby stemming further visual decline. AAV2.COMP-Ang1 single-dose gene therapy can prevent neurovascular pathology, support vascular regeneration, and stabilize vision in DR.
INTRODUCTION

Diabetes affects 25.8 million people in the United States and its prevalence is expected to triple in the next 20 years (1). Diabetic retinopathy (DR) is the leading cause of blindness in the working-age population (2). The leading cause of vision loss in DR is diabetic macular edema (DME), a condition in which fluid accumulates underneath the central macula due to a breakdown of the blood retinal barrier (BRB).

Current treatments for DME include laser photocoagulation, intravitreal agents that block vascular endothelial growth factor (VEGF), and/or intravitreal corticosteroids. Such treatments address the downstream consequences but not the vascular endothelial cell loss and ischemia underlying DME (3). Moreover, these therapies improve vision in only a minority of patients (4). Merely 23-33% of patients treated with ranibizumab (5) and 34% of patients treated with aflibercept (6) achieve significant visual gains. Because it creates small burns that can interfere with peripheral vision and overall visual performance, traditional treatment with laser photocoagulation is primarily employed to retard rather than reverse retinal non-perfusion (7). Intravitreal steroids have served as an alternative for patients who have contraindications or are resistant to anti-VEGF agents, but are inferior to VEGF inhibitors in recovering visual acuity and are associated with side effects like cataract and intraocular hypertension (8). Given the suboptimal outcomes, we developed a different approach to DME focusing on the reversal of retinal vascular damage and restoration of normal perfusion.

The underlying pathogenesis of DR is largely due to hyperglycemia (9). Hyperglycemia triggers an inflammatory response leading to leukocyte adhesion, microvascular occlusion, and consequent hypoxia (10,11). Further, hyperglycemia
instigates pericyte loss, compromising endothelial stability and BRB integrity. Eventual
capillary degeneration leads to retinal non-perfusion, exacerbating retinal hypoxia (12).
Consequent pathological VEGF-induced angiogenesis is uncoordinated and results in
immature, leaky vessels with inadequate perfusion, creating a vicious cycle of hypoxia-
driven VEGF secretion and DME (13). Retinal ganglion cell (RGC) loss, neuronal
dysfunction, and changes in vision are also seen in patients with DR, concurrently with
vascular pathology (14). Hence, as a therapeutic goal, vascular stabilization could
promote normal perfusion of metabolically demanding retinal neurons and thereby avert
the sight-threatening sequelae of ischemia and hyperpermeability.

One therapeutic target is angiopoietin 1 (Ang1), a vascular growth factor which
has an abnormally low concentration in the vitreous of patients with DR (15). Ang1, via
binding to the Tie2 endothelial receptor, fosters vessel quiescence & maturation, and
suppresses vascular leakage by preventing VEGF-induced degradation of vascular
endothelial (VE)-cadherin, a transmembrane protein in the adherens junction between
endothelial cells which promotes vascular integrity and decreases vascular permeability
(16,17). Ang1 also promotes the survival of damaged vascular endothelial cells through
the PI3K/Akt cascade (18). Thus, restoration of Ang1 signaling could serve as a possible
solution for preventing endothelial loss, retinal ischemia, and abnormal VEGF expression
in DR (19).

Pharmaceutical development of Ang1 as a viable therapy has been hindered by its
insolubility and aggregation. Ten years ago, a novel, stable, soluble and more potent
version of Ang1, cartilage oligomeric matrix protein Ang1 (COMP-Ang1), was
bioengineered to overcome the limitations of native Ang1 (20). Although the benefits of
COMP-Ang1 have been demonstrated for vasculopathic disorders in animal models of the cardiac (21), nervous (22), and renal (23) systems, this study is the first to test its effectiveness for the prevention and treatment of DR.

We hypothesized that, if introduced early in diabetes, constitutive expression of COMP-Ang1 via adeno-associated virus serotype 2-mediated gene therapy (AAV2.COMP-Ang1), either as a monotherapy or in combination with human-derived endothelial colony-forming cells (ECFCs), could protect retinal neurovascular structure and function in a type 1 diabetic Ins2Akita mouse model of DR. We found that COMP-Ang1 prevents the endothelial loss, capillary dropout, BRB instability, leukocyte dysfunction, neuroretinal attenuation, and visual decline associated with DR. In addition, COMP-Ang1 enhances the therapeutic efficacy of ECFCs in mitigating DR through vascular and visual rehabilitation.

MATERIALS AND METHODS

Mice

This research protocol was approved by the Institutional Animal Care and Use Committee of the University of Utah and conforms to the standards in the ARVO Statement for the Use of Animals in Ophthalmic and Vision research. The diabetic C57BL/6-Ins2^Akita/J (Ins2Akita) and its background wild type (WT) strain, C57BL6/J, were used (24). Mice heterozygous for the Ins2 mutation experience progressive retinal abnormalities 12 weeks after the onset of hyperglycemia and include apoptosis (i.e. endothelial and RGC loss) and functional deficits (increased vascular permeability and decreased neuronal function) (25).
Mice were randomly assigned to one of three experimental groups: AAV2.COMP-Ang1, AAV2.AcGFP (*Aequorea coerulescens* green fluorescent protein), or phosphate-buffered saline (PBS). At 2 months of age (Supplemental Fig. 3), each mouse was anesthetized by intraperitoneal injection of 1.25% tribromoethanol (Sigma-Aldrich, St. Louis, MO) at a dose of 0.025 mL/gram of body weight. Each mouse was treated with either 2 µL of AAV2 solution (2.0 x 10^9 particles) or PBS injected into the vitreous cavity of both eyes with a 33-gauge microsyringe (Hamilton Company, Reno, NV). An additional cohort of mice was treated as described above at 6 months of age. Two weeks later, this cohort received a second intravitreal injection with 1 µL of 1x10^5 ECFCs (Supplemental Fig. 3).

**Virus Vector Construction**

The plasmids pAAV.COMP-Ang1 and pAAV.AcGFP were created by incorporating the COMP-Ang1 cDNA from the pCMV-dhfr2-COMP-Ang1 (donated generously by the Koh Laboratory, KAIST, Daejeon) into pAAV-MCS (Agilent Technologies, Santa Clara, CA), while pAAV.AcGFP was created by the same technique with AcGFP cDNA from the pIRES2-AcGFP1 plasmid (Clontech Laboratories, Mountain View, CA) (Supplemental Fig. 3).

For in vivo assays requiring imaging with the Spectralis HRA+OCT (Heidelberg Engineering), mice were anesthetized by an inhalation of 3% isoflurane/O_2_ mixture in a closed canister at a flow rate of 1.0 Lpm. Pupils were dilated with a 1% tropicamide.

Retinas were harvested and processed for in situ hybridization (ISH) according to standard protocols using procedures to avoid RNAse contamination.
Reverse Transcriptase Polymerase Chain Reaction for COMP-Ang1 mRNA Expression

Primer sequences for COMP-Ang1 (University of Utah Gene Core, Salt Lake City, UT) were:

COMP-Ang1 F 5’-GCTCTGTTTTCCTGCTGTCC 3’
COMP-Ang1 R 5’-GTGATGGAATGTGACGCTTG 3’

Primer sequences for the internal control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were:

GAPDH F 5’-AACCTTGGGATTGTGGAAGGG 3’
GAPDH R 5’-ACCAGTGGATGCAGGGATGAT 3’

Immunoprecipitation and Western Blot

Retinas were harvested and protein lysate samples were immunoprecipitated with anti-FLAG M2 affinity gel (Sigma-Aldrich). Eluted proteins samples were run on 12% SDS-PAGE. Overall protein levels were compared with anti-GAPDH (1:3000, Abcam, Cambridge, MA) and anti-β-actin (1:3000, Abcam). Samples were tested for anti-VEGF-A (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), anti-VE cadherin (1:1000, Abcam), and anti-phospho-Src (PY419, 1 µg/mL, R&D Systems).

Immunofluorescence of Retinal Flat-Mounts for Vascular Markers

Enucleated globes were fixed in 4% paraformaldehyde (PFA) followed by retinal dissection. Specimens were stained with 1:200 alpha-smooth muscle actin (α-SMA) antibody or neuron-glial antigen 2 (NG-2) antibody conjugated with cyanine dye (Cy3) (Sigma-Aldrich) and 5µg/ml AlexaFluor 647 conjugated isolectin GS-IB4 (Invitrogen) in blocking buffer overnight at 4 ºC. After washing, the retina was flat-mounted on a glass
slide. Full retinal field immunofluorescence (IF) images were captured at low
magnification, followed by increasing magnification of each quadrant with scanning laser
confocal microscopy (Olympus America).

Trypsin Digest for Retinal Vascular Architecture

Enucleated globes were fixed in 2% formalin. The retina was detached around the
sub-retinal space. The optic nerve was cut under the disc. The specimen was digested at
37°C in 2.5% trypsin/0.2M TRIS at pH 8.0 for 30-60 minutes. Specimens were
transferred to distilled water and to 0.5% triton X-100 surfactant/distilled water and left at
room temperature for another hour. Lastly, the specimen was moved to 0.1% triton X-
100/distilled water for mounting and dried in a 37°C incubator. Samples were stained
with periodic acid Schiff staining and imaged with light microscopy (Olympus, Center
Valley, PA). Acellular capillaries and pericytes were counted in each of a total of 5 high-
powered fields (HPFs) per retinal quadrant.

Transendothelial Electrical Resistance for BRB Integrity

In vitro measurements of transendothelial electrical resistance (TER) were
performed with an electrical cell–substrate impedance sensing (ECIS) system (Applied
Biophysics, Troy, NY). Human retinal microvascular endothelial cells (HrMVECs) (Cell
Systems, Kirkland, WA) were seeded (50,000 cells/well) onto fibronectin-coated gold
microelectrodes in ECIS culture wells (8W10E+, Applied Biophysics) and incubated
overnight at 37°C in complete medium (EBM-2 + EGM2-MV, Lonza Group, Basel,
Switzerland) until cell resistance reached a plateau. Cells were serum-starved for one
hour until resistance was stabilized (1200 Ω). Each well received one of three
experimental treatments: COMP-Ang1 protein (Enzo Life Sciences, Farmingdale, NY),
VEGF protein (R&D, Minneapolis, MN), or PBS. Monitoring was continued for 21 hours. The data from triplicate wells were averaged and presented as normalized resistance versus time.

**Miles Assay for Retinal Vasopermeability**

Mice were administered Evans blue (EB) (Sigma-Aldrich) at a dosage of 20 mg/kg through tail vein injection. After 4 hours, the vasculature was perfused with PBS. The retinas were next harvested and placed in formamide at 70°C for 18 hours. Samples were centrifuged for 2 hours at 40,000 g in a 0.2 µm filter. EB concentration was detected spectrophotometrically by subtracting absorbance at 620 nm from 740 nm.

**Acridine Orange Fluorography for Leukocyte Transendothelial Migration**

Acridine orange (AO (Acros Organics, Geel, Belgium), 0.10%/PBS) was filtered with a 0.22 µm filter. The solution (0.05mL/min for a total of 1 minute) was injected into the tail vein. Imaging utilized Spectralis HRA+OCT (Heidelberg Engineering, Heidelberg, Germany) with a 488nm argon blue laser with a standard 500nm long-pass filter. Images were acquired from both eyes with a 55-degree lens utilizing the movie mode on the Spectralis HRA+OCT (Heidelberg Engineering).

**Flow Assay for Leukocyte-Endothelial Interaction**

HrMVECs were cultured in parallel-plate fibronectin-coated flow chambers (µ-slide VI 0.4, ibidi USA, Madison, WI) until 80% confluent and exposed to tumor necrosis factor alpha (TNF-α) (10ng/mL) (R&D Systems, Minneapolis, MN) or vehicle control for 3 hours. Human leukocytes were isolated as described previously in accordance with Institutional Review Board guidelines (26) and diluted in warmed ultrasaline (Lonza Group) to 1 x 10^6 cells/ml. Leukocytes were pumped through the
parallel plate flow chambers using a syringe pump (Harvard Apparatus, Holliston, MA) at 1 dynes/cm$^2$ (typical venous shear stress). Differential interference contrast (DIC) images were acquired at a rate of 1/second for 1 minute, and the number of leukocytes adhered to or rolling on the monolayer was quantified as leukocytes/frames/second. Three independent flow wells were averaged to attain the reported values.

**Immunoperoxidase Staining for Retinal Hypoxia**

Mice received an intraperitoneal injection of the bio-reductive hypoxia marker pimonidazole (Hypoxyprobe, Burlington, MA) at 60 mg/kg (3). Three hours later, retinas were harvested and stained with a hypoxyprobe-1 monoclonal antibody conjugated to fluorescein isothiocyanate (FITC) to detect reduced pimonidazole adducts (Hypoxyprobe) forming in pO$_2$<10 mmHg.

**Optical Coherence Tomography for Retinal Thickness**

Mice were imaged bilaterally with optical coherence tomography (OCT) (Spectralis HRA+OCT, Heidelberg Engineering). Retinal cross-sectional thickness was measured 250µm relative to the optic nerve head, using the en face image as a guide. Measurements were recorded for each retinal quadrant and averaged to attain the reported values.

**Microsphere Fluorescence for Retinal Vasopermeability**

Each mouse received a dosage of 100 µL/20 gram tail vein injections with 100 nm microspheres (Magsphere, Pasadena, CA) conjugated to either the near infrared fluorophore ZW800 (Flare Foundation, Boston, MA) (4) or GFP (Magsphere). Bilateral
imaging by Spectralis HRA+OCT (Heidelberg Engineering) was performed with FA and indocyanine green (ICG) modalities.

**Immunofluorescence for Retinal Thickness and VE-Cadherin**

Enucleated globes were fixed in 4% PFA. The globes were cut in 10 µm sections and stained with anti-VE cadherin antibody (1:200, Abcam) and 4’,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Sections were captured with scanning laser confocal microscopy (Olympus America).

**Immunofluorescence of Retinal Flat-Mounts for RGC Density**

4-month-old mice received intravitreal injections of either AAV2.COMP-Ang1 or PBS as described above. 6 months later, retinas were fixed and dissected as described above for flat mounts. Specimens were labeled with 1:200 pan-Brn3 antibody (Santa Cruz Biotechnology), followed by AlexaFluor 546 conjugated secondary antibody (Invitrogen), and counterstained with DAPI (Sigma-Aldrich). Eight fields were imaged for each retina using the confocal 40X oil objective; these comprised four evenly spaced fields (one per quadrant) adjacent to the optic nerve and four fields (similarly spaced) near the flat mount periphery. Images were counted blind by two separate investigators. Counts were averaged for each retina and compared across control and experimental groups.

**Electroretinography for Retinal Function**

Mice were dark-adapted, anesthetized with ketamine/xylazine (90 mg/10 mg per kg body weight), and placed on a controlled warming plate (TC-1000, CWE Instruments, Ardmore, PA) (5). Electroretinograms (ERGs) were taken between a gold corneal electrode and a stainless-steel scalp electrode with a 0.3- to 500-Hz band-pass filter.
(UTAS E-3000, LKC Technologies, Gaithersburg, MD). The photoflash unit was calibrated to deliver 2.5 cd s/m² at 0 dB flash intensity and scotopic measurements were recorded with flash intensities increasing from 0.0025 to 250 cd s/m². The b-wave amplitudes were determined in scotopic conditions, and the mean values at each stimulus intensity were compared with an unpaired two-tailed t-test.

**Optokinetic Tracking for Visual Acuity**

Optomotor reflex-based spatial frequency threshold tests were conducted in a visuomotor behavior measuring system (OptoMotry, CerebralMechanics, Lethbridge, AB, Canada). Tracking was defined as a reproducible smooth pursuit with a velocity and direction concordant with the stimulus. Trials of each direction and spatial frequency were repeated until the presence or absence of the tracking response could be established unequivocally. Rotation speed (12°/s) and contrast (100%) were kept constant.

**ECFCs**

Fresh human cord blood, donated under full ethical approval by healthy volunteers at the Northern Ireland Blood Transfusion Service (Belfast, United Kingdom), underwent density gradient fractionation for the isolation of mononuclear cells (MNCs) and were selected for ECFCs via resuspension in complete medium (EBM + EGM-2 MV, Lonza Group) supplemented with 10% FBS and seeding onto 24-well culture plates pre-coated with rat tail collagen type 1 (BD Biosciences, Bedford, UK) at a density of 1 x 10⁷ cells/mL. Cells were labeled (Qtracker 655, Invitrogen, Life Technologies, Carlsbad, CA) per manufacturer’s instructions.

**Immunofluorescence of Retinal Flat-Mounts for ECFC Engraftment**

From the mice that had received intravitreal ECFCs, harvested retinas were fixed
and dissected as described above for flat mounts (Supplemental Fig. 3b). Specimens were
stained with 5µg/ml AlexaFluor 647 conjugated isolectin GS-IB4 (Invitrogen) and
mounted on a glass slide as described above. ECFC integration was counted in 4 high-
powered fields in each retinal quadrant.

**Scratch Assay for ECFC Migration**

ECFCs were plated on rat-tail collagen-coated 6 well plates pre-labeled with
traced lines (27). When cells were 90% confluent, a uniform straight scratch was made in
the monolayer using a 200 µl pipette tip. After injury, cells were washed, medium was
changed, and reference photographs were taken within each region marked by the lines
using a phase contrast microscope (Eclipse E400, Nikon, Tokyo, Japan). Wells were
incubated with the experimental treatment (doses of COMP-Ang1 protein from 0-1000
ng/mL) and images were captured at hourly intervals. Endothelial cell migration was
quantified by calculating the proportion of denuded area.

**Matrigel Assay for ECFC Tubulogenesis**

ECFCs were labeled with a fluorescent dye (PKH Cell Linker Kit for General
Cell Membrane Labeling, Sigma-Aldrich) as previously described (28). Next, they were
suspended in growth factor reduced basement membrane matrix (Matrigel, Becton
Dickinson Biosciences, Franklin Lakes, NJ) and 50 µL aliquots were spotted onto a 24-
well plate. Spots were covered with DMEM containing 5% porcine serum and treated
with either control or increasing doses of COMP-Ang1. After 24 hours, wells were
assessed for the presence of tubules. Images were acquired by using a laser confocal
microscope (Nikon).

**Statistical Analysis**
All numerical data were analyzed in Excel (Microsoft, Redmond, WA) and presented as the mean ± standard deviation (stdev). Student’s two-tailed t-test, with α level = 0.05, was used to compare differences between 2 samples. ANOVA test, with p<0.05, followed by Tukey post hoc analysis, was used to compare differences between 4 groups.

RESULTS

Intravitreal AAV2 Gene Therapy is Safe for the Mouse Retina

The long-term safety of intraocular AAV-mediated gene therapy has been validated by numerous animal and human studies in which vector inoculation was well-tolerated and unaccompanied by any structural or functional defects (29). We recently reported that the intraocular injection of exogenous AAV2 constructs did not impact retinal thickness, influence electroretinographic (ERG) responses, or increase the risk of retinochoroidal apoptosis (30). Concordant with prior results, the present study found no significant differences (p=0.3 at -30 dB, 0.6 at 0 db, and 0.6 at 20 db) in scotopic and photopic b-wave amplitudes on ERG between WT mice treated with intravitreal AAV2.COMP-Ang1, AAV2.AcGFP, or PBS (Supplemental Fig. 1). Furthermore, optokinetic tracking (OKT) response of no injection WT control mice did not change appreciably compared to PBS-treated mice (p=0.5) over the course of 5 weeks (Supplemental Fig. 1B).

Intravitreal AAV2.COMP-Ang1 Expresses COMP-Ang1 in the Mouse Retina

AAV2 localization and transfection in the retina was confirmed by in vivo and ex vivo detection of the fluorescent gene product of the sham viral control by confocal
microscopy; further, COMP-Ang1 mRNA and protein expression was verified by ex vivo immunoassay.

AcGFP fluorescence was initially observed at 1 week post-injection and persisted through to the 4 months post-injection endpoint (Supplemental Fig. 2A). As anticipated based on previous experience with intravitreal AAV2 (31), AcGFP signal was visualized in all retinal quadrants at the level of the ganglion cell-inner plexiform layer (GC-IPL) (Supplemental Fig. 2B and C).

COMP-Ang1 production in the mouse retina was demonstrated via semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) (Supplemental Fig. 2D), in situ hybridization (ISH) (Supplemental Fig. 2F-H), and immunoblotting (Supplemental Fig. 2E) at the 4 months post-injection endpoint. In parallel with AcGFP fluorescence, ISH revealed increased amounts of COMP-Ang1 mRNA in the inner retina, predominantly in the GC-IPL (Supplemental Fig. 2F-H).

**COMP-Ang1 Prevents Breakdown of Vascular Structure**

The principal morphologic features of DR are pericyte, endothelial cell, and capillary dropout (32). Concordantly, retinal vessel density was discernably reduced in immunofluorescence (IF) and trypsin digest images of Ins2Akita control (PBS and AAV2.AcGFP) versus WT retinas (Fig. 1A-C). Endothelial cell (p<0.01, Fig. 1D) and pericyte (p<0.01, Fig. 1E) coverage were significantly decreased in the diabetic controls compared to WT, while capillary acellularity was increased (p<0.01, Fig. 1F).

IF and trypsin digest images of AAV2.COMP-Ang1 exhibit an improvement in vessel density compared to diabetic controls (Fig. 1A-C), accompanied by a significant drop in acellular capillary density (p<0.01 vs. AAV2.AcGFP, p<0.01 vs. PBS, and
p<0.01 vs. WT, Fig. 1F). AAV2.COMP-Ang1 significantly rescued endothelial cell loss relative to controls (WT = 23.2%, AAV2.COMP-Ang1 = 20.5%, PBS = 15.5%, AAV2.AcGFP = 15.3%, p<0.01 Fig. 1D) but not pericyte coverage (WT = 6.5%, AAV2.COMP-Ang1 = 3.8%, PBS = 4.3%, AAV2.AcGFP = 3.9%, p=0.9, Fig. 1E)

Previous reports have demonstrated that the rescue of pericytes, the major source of endogenous Ang1 in the capillary unit (33), is not necessary for Ang1 rescue of function as long as Ang1 is available in adequate quantities (34). Our data suggest that in lieu of pericytes, AAV2.COMP-Ang1 can provide sufficient Ang1 endothelial trophic signaling to prevent capillary dropout (Fig. 1F).

**COMP-Ang1 Promotes BRB Integrity**

Ischemia from capillary dropout in DR stimulates VEGF production and consequent vascular hyperpermeability (3). Accordingly, the transendothelial electrical resistance (TER) of HrMVECs decreased after treatment with VEGF compared to PBS in vitro (p<0.01, Fig. 2A), while EB extravasation was elevated in PBS and AAV2.AcGFP treated diabetic controls (3.8 and 3.1 fold respectively, Fig. 3A) compared to WT mice (p<0.01). Microsphere leakage was similarly elevated in diabetic controls (Fig. 3B)

COMP-Ang1 significantly increased the TER (p<0.01, Fig. 2A) of HrMVECs and decreased EB extravasation (p<0.01, Fig. 3A) and microsphere (Fig. 3B) leakage in diabetic mice.

These results indicate that COMP-Ang1 restores the barrier function of the retinal vasculature in DR. To explore the molecular underpinnings of this finding, we investigated the influence of COMP-Ang1 on VEGF-A, the proto-oncogene non-receptor tyrosine kinase Src, and the intercellular junction adhesion molecule VE-cadherin.
VEGF-A is known to induce vessel leakage in DR through Src-mediated downregulation of VE-cadherin, while Ang1 is known to upregulate VE-cadherin (16,35).

COMP-Ang1 decreased Src phosphorylation (Fig. 2B) and increased VE-cadherin expression in HrMVECs (Fig. 2C). Both Ins2Akita and WT retinas treated with AAV2.COMP-Ang1 had reduced levels of VEGF-A (Fig. 2D, Supplemental Fig. 4) and increased levels of VE-cadherin (Fig. 2E, Supplemental Fig. 4).

Ang1 acts on the PI3K/Akt cascade to prevent the apoptosis of damaged vascular endothelial cells (18), considering the reduction in capillary acellularity and endothelial cell loss with COMP-Ang1, we explored Akt phosphorylation as a possible mechanism for COMP-Ang1-mediated survival of endothelial cells. COMP-Ang1 increased Akt phosphorylation at the serine 473 residue in both ECFCs and human umbilical vein endothelial cells (HUVECs) (Fig. 2F).

**COMP-Ang1 Reduces Leukocyte-Endothelial Adhesion and Leukostasis**

DR is characterized by a chronic, subclinical inflammatory response that is thought to play a critical role in its pathogenesis (36). The less deformable and more activated leukocytes in DR are conjectured to contribute to retinal nonperfusion and capillary dropout through increased attachment to endothelial cells and entrapment within the capillaries (37).

Leukocyte adhesion to the vascular wall is mediated, in part, by TNF-α (38). Correspondingly, the endothelial monolayer of HrMVECs exposed to TNF-α experienced an abnormally high rate of leukocyte adherence. Treatment with COMP-Ang1 protein decreased the number of adherent leukocytes per minute by 80% (p<0.01, Fig. 2G).
On AO leukocyte fluorography (39), leukocyte rolling was significantly elevated in Ins2Akita control (9.8 cells/min) versus WT retinas (3 cells/min, p<0.01, quantitative image Fig. 3C, representative image Fig. 3D with white arrows pointing to leukocyte aggregations at the bifurcation). AAV2.COMP-Ang1 was able to reduce this rate to below the disease-free baseline (2.8 cells/min, p<0.01, Fig. 3C, Supplemental Videos 1-4).

These results indicate that the improvement in vascular parameters by COMP-Ang1 may have an anti-inflammatory component.

**COMP-Ang1 Reduces Hypoxia**

Leukostasis has been proposed as a mechanism of capillary non-perfusion and retinal hypoxia (40). Since hypoxia is a potent inducer of VEGF-A, we further assessed the relationship between COMP-Ang1 and hypoxia. Pimonidazole staining was increased in the diabetic control mice relative to WT mice whereas it was reduced nearly to baseline levels in AAV2.COMP-Ang1 mice (Fig. 3E).

Collectively, our outcomes demonstrate a pathway for COMP-Ang1-mediated retinal vascular functional stabilization. The inhibition of leukocyte adhesion and stimulation of Akt phosphorylation lead to the preservation of perfusion and normalization of tissue oxygenation, whereas the inhibition of VEGF-A and Src phosphorylation lead to the preservation of VE-cadherin and normalization of permeability.

**COMP-Ang1 Prevents Retinal Neuronal Dysfunction**

DR causes neural degeneration of the inner retina (14). Consistent with this, the retinas on optical coherence tomography (OCT) and IF images from Ins2Akita control mice (185 µm) were qualitatively and quantitatively thinner than WT mice (210 µm,
p<0.01, Fig. 4A-D). In parallel, GC-IPL cell density was also decreased in Ins2Akita control (68 nuclei/300 μm length) versus WT (45 nuclei/300 μm length) retinas (p<0.001, Fig. 4B-D), a 34% loss of cells.

AAV2.COMP-Ang1 preserved retinal thickness (WT = 210 μm; AAV2.COMP-Ang1 = 205 μm, AAV2.AcGFP = 181 μm, PBS = 185 μm; p<0.01; Fig 4A,D) and prevented loss of cells in the GC-IPL (65 nuclei/300 μm length, p=0.03) in Ins2Akita mice (Fig. 4B-D).

Further characterization of GC-IPL cells with the RGC-specific marker, Brn3, revealed no difference in RGC counts within the central retinas of PBS versus COMP-Ang1 treated Ins2Akita mice (p=0.7, Fig. 4E). However, peripheral retinas showed a 17% loss of ganglion cells (Fig. 4F). Although this difference was not statistically significant (p = 0.07), the trend shows an effect size similar to that reported for RGC loss in the peripheral retina of diabetic versus WT mice (41), suggesting that a larger sample size would provide sufficient power to confirm an effect.

In sum, these data suggest that AAV2.COMP-Ang1 is beneficial in preventing diabetes-induced GC-IPL atrophy and peripheral RGC cell loss, but may also target or recruit non-RGC cell types within the inner retina for neuroprotection.

**COMP-Ang1 Prevents Visual Dysfunction**

Patients with DR manifest with visual deficits early in the disease, and animals exhibit changes in visual acuity and contrast sensitivity through impaired visual tracking behavior and delayed retinal electrical responses (42). In line with this, ERG and OKT responses were within normal limits for WT mice but abnormally depressed in diabetic control mice (at -40 dB, WT = 127, PBS = 57, AAV2.AcGFP = 73 μvolts, p<0.01; at 4
months post-injection, WT = 0.388, PBS = 0.184, AAV2.AcGFP = 0.174 cycles/degree, p<0.01; Fig. 5A-C).

AAV2.COMP-Ang1 treatment diminished the dampening in scotopic b-wave amplitudes (185 µvolts, p<0.01, Fig. 5A and B) caused by anomalous photoreceptor-bipolar communication in DR (43). Likewise, Ins2Akita mice treated with AAV2.COMP-Ang1 were able to avert the deterioration of OKT (0.312 cycles/degree; p<0.01; Fig. 5C).

Together, spatial resolution and ERG data show that AAV2.COMP-Ang1 can preserve retinal neurophysiological function.

**COMP-Ang1 Enhances ECFC Treatment Effect**

The recellularization and resultant refunctioalization of acellular capillaries could theoretically halt the nonperfusion at the root of DR pathophysiology, but in diabetes the endogenous reparative cells responsible for this task have a decreased ability to associate with existing vascular networks (44). The exogenous delivery of human-derived ECFCs, as evidenced by their utility in oxygen-induced retinopathy (45), may be able to compensate for this deficiency but have not yet been explored in the context of DR. We know that ECFCs express high levels of the Ang1 receptor, Tie2 (46), and that Ang1 promotes the differentiation of stem cells into vasculogenic cells for vessel engraftment and reformation. Therefore, we tested the regenerative potential of dual therapy with COMP-Ang1 and ECFCs.

COMP-Ang1 demonstrated a dose-dependent increase in 6-hour migration (2.5-fold increase over control at 10 ng/mL, p<0.01, Fig. 6A) and 24-hour tubulogenesis (4.3 fold over control p<0.01, Fig. 6B) of ECFCs in vitro, as assessed by scratch migration
assay and matrigel tube formation assay, respectively. In vivo, aged 26-week-old
Ins2Akita mice treated with AAV2.COMP-Ang1 had increased 72-hour ECFC vessel
integration on confocal microscopy (Fig. 6C, Supplemental Videos 5-6) and 2-month
visual response with OKT (AAV2.COMP-Ang1 = 0.307, AAV2.AcGFP = 0.251, PBS =
0.263 cycles/degree; p<0.01; Fig. 6D).

Our results show that COMP-Ang1 boosts the capacity of ECFCs to rebuild
vessels and counteract vision loss in DR.

DISCUSSION

This study established the salutary effects of COMP-Ang1 in ameliorating pivotal
pathogenic events in the trajectory toward DME through neurovascular normalization.
Structural and functional indices of neurovascular restoration to a state more consistent
with a homeostatic disease-free phenotype by COMP-Ang1 included endothelial and
capillary density; vessel permeability; VEGF-A, phospho-Src, VE-cadherin, and
phospho-Akt levels; leukocyte-endothelial interaction and retinal hypoxia; neuroretinal
thickness, GC-IPL cellularity, and peripheral RGC density; and most importantly, vision.
Furthermore, COMP-Ang1 augmented the effectiveness of ECFCs in regenerating
vessels and stabilizing vision in advanced DR. Based on these results, our working
model for further study centers on the premise of COMP-Ang1 directly or indirectly
suppressing inflammation and modulating the actions of VEGF, Src, VE-cadherin, and
Akt at the molecular level, thereby influencing downstream processes of perfusion and
BRB reinforcement vital for neurovascular stability.
Our study corroborates and extends work which showed that AAV-mediated Ang1 gene therapy could suppress vascular leakage in stroke (47) and intravitreal recombinant COMP-Ang1 protein could suppress vascular leakage in choroidal neovascularization (48). Our results advance the field by 1) demonstrating that sustainable delivery of COMP-Ang1 to the retina can be achieved with a single intravitreal injection, 2) testing the therapeutic effects of COMP-Ang1 in an animal model of DR, and 3) introducing the potential for COMP-Ang1 to be used in conjunction with ECFCs for the treatment of advanced DR.

In addition, our data raise a number of intriguing questions about the mechanism of action for COMP-Ang1 in DR. Intriguingly, we found that both diabetic and non-diabetic mice experienced decreases in VEGF production due to COMP-Ang1 exposure, inviting inquiry about the nature of this relationship and how it is intertwined with oxygen supply and macrophage secretion (48). Equally fascinating, and consistent with evidence of Ang1 neuroprotection in the central nervous system (49), was our discovery that COMP-Ang1 seems to preserve peripheral RGC density. Ins2Akita mice reportedly lose RGCs in the peripheral, but not central retina within three months of developing diabetes (41). We speculate this geographic predilection for both RGC apoptosis and COMP-Ang1 rescue may be tied to the concept of increased vulnerability to hypoxia with distance from the central artery, along with our finding that COMP-Ang1 reduces hypoxia. Moreover, since RGCs accounted for only 50-55% of the GC-IPL cell density conserved by COMP-Ang1 in our study, an exploration into other neuroretinal cell types (e.g. cholinergic amacrine cells (50)) as targets for COMP-Ang1 rescue is merited.
We have shown here that COMP-Ang1 is a safe and effective replacement for endogenous Ang1, which can adequately compensate for deficient Ang1 secretion by pericytes. Our results thus far indicate that COMP-Ang1 suppresses the pathognomonic features of non-proliferative DR and, in contrast to existing therapies, decreases the non-perfusion and ischemia critical to the genesis of proliferative DR. This latter finding, along with the long-term duration of action for a single intravitreal injection of AAV2.COMP-Ang1 relative to anti-VEGF agents, hold immense promise for fulfilling an unmet need in the management of DR if COMP-Ang1 can be successfully translated from the bench to the bedside. Future research will focus on investigating the mechanism of action by which COMP-Ang1 safeguards the retina and the application of COMP-Ang1 to human models of DR.
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GUARANTOR

Balamurali K. Ambati, MD, PhD, MBA, is the guarantor of this work and, as such, had full access to all the data in the study. He takes responsibility for the integrity of the data and the accuracy of the data analysis.

AUTHOR CONTRIBUTIONS

J.M.C., R.R.R., L.S.C, H.U., X.Z., C.O., R.M., P.R.O., S.N., M.F., W.B.M. performed animal studies; J.M.C., R.R.R., H.U., K.W. performed cell culture studies; J.M.C., R.R.R., P.R.O., W.B.M., P.B., D.K. performed visual functional studies; J.M.C., K.W., C.C.G., D.Y.L. performed transendothelial electrical resistance studies; H.U., G.Y.K.,
G.G. developed the plasmids and viral vectors; SKD and SKM were responsible for in
situ hybridization. J.M.C., R.R.R., L.S.C., B.K.A. prepared and wrote the manuscript.

COMPETING INTERESTS
None

DATA AND MATERIALS AVAILABILITY
Not Applicable
FIGURE LEGENDS

Figure 1. AAV2.COMP-Ang1 mitigates diabetic retinal capillary dropout

(A) Representative retinal flatmounts prepared from six month-old mice and stained for isolectin (endothelial cell marker, green) and α-SMA (smooth muscle marker, red).

(B) Magnified view of retina stained with isolectin and NG2 (pericyte marker). Ins2Akita mice experienced pericyte and endothelial dropout; the latter was prevented with a single intravitreal dose of AAV2.COMP-Ang1. (C) Trypsin digest featuring retinas representative of each group. Black arrowheads denote acellular capillaries. (D) Quantification using ImageJ of endothelial coverage and (E) pericyte coverage. (F) Acellular capillaries were manually counted and averaged over an area 1mm². Eight eyes were used in each analysis, data are mean ± stdev. *p<0.01, ANOVA. Post hoc comparisons with a Tukey test to compare means of each group. Scale bars = 600 µm (A), 100 µm (B), 200 µm (C).

Figure 2. COMP-Ang1 increases endothelial integrity

(A) Representative graph of electrical cell substrate impedance sensing (ECIS) of human retinal microvascular endothelial cells (HrMVECs) with COMP-Ang1 (100 ng/mL), VEGF (50 ng/mL), or control (PBS) added to the media. COMP-Ang1 increased resistance of HrMVECs (n=3). Increases in endothelial resistance were correlated with deceased Src phosphorylation (B) and increased VE-cadherin (C) in HrMVECs as demonstrated by Western blot (n=3). Western blot from Ins2Akita mouse retinas demonstrating decreased VEGF-A (D) and increased VE-cadherin (E) in mice treated with AAV2.COMP-Ang1. COMP-Ang1 increased Akt phosphorylation at the serine 473
residue in both ECFCs and HUVECs. (G) COMP-Ang1 reduced TNF-α induced leukocyte rolling in cultured HrMVECs (n=6 per group). *p<0.01, ANOVA

**Figure 3.** COMP-Ang1 enhances vascular barrier function and reduces retinal hypoxia in the diabetic retina. (A) Evans Blue extravasation from the retina of Ins2Akita mouse was increased compared to control; treatment with AAV2.COMPsAng1 returned vascular hyperpermeability to control levels. Eyes from eight mice were used in each analysis; data are mean ± stdev. *p<0.01 compared to WT, *p=0.02 compared to AAV2.AcGFP. (B) Fluorescein angiography (FA) did reveal any leakage in diabetic mice; however, utilizing GFP or the NIR fluorophore ZW800 conjugated to aminated latex microspheres (GFP-ms, or ZW800–ms; 100 nm in diameter) in vivo leakage was captured using the FA or ICG imaging modality on Spectralis, respectively. Note that background GFP fluorescence of the AAV2.AcGFP treated diabetic mice masked signal from the GFP-microspheres. (C) Diabetes induced leukocyte rolling in the retinal vasculature, as captured by acridine orange leukocyte fluorography AOLF. (D) Representative image of AOLF with white arrowheads pointing to adherent and rolling leukocytes. COMP-Ang1 prevents leukostasis and inflammation in this model of diabetic retinopathy; see also Supplemental Video 1. (E) Representative retinas (four mice per group) from mice treated with hypoxyprobe (pimonidazole). COMP-Ang1 reduced hypoxia in diabetic mouse retinas. Scale bars: 600 µm (E), *p<0.01, ANOVA. Post hoc comparisons with a Tukey test to compare means of each group.

**Figure 4.** AAV2.COMP-Ang1 prevents diabetes-induced GC-IPL degeneration

(A) Representative figures from optical coherence tomography (OCT) measuring retinal thickness. The red line, generated by OCT software, indicates the retinal surface and...
Bruch’s membrane. Scale bars = 200 µm. (B) Cross sections of six month-old retinas from WT, or Ins2Akita mice treated with PBS, AAV2.AcGFP, or AAV2.COMP-Ang1 stained with DAPI. (C) View of the GC-IPL from mice stained for VE-cadherin (red) or nuclei (DAPI, blue) demonstrating increased VE-cadherin and nuclear staining. Scale bars 30 µm (right). (D) Quantification of retinal thickness from OCT showing that AAV2.COMP-Ang1 prevented diabetes-induced retinal thinning as measured in vivo (*p<0.01 vs. both WT and AAV2.AcGFP). AAV2.COMP-Ang1 prevented diabetes-induced inner retinal layer loss (*p=0.03 ANOVA, with post hoc Tukey test) as measured by nuclei counted in the GC-IPL in retinal cross sections. (E) Representative retinal flat mounts stained with BRN3 (red; a marker for retinal ganglion cells) and isolectin (green; marker for vessels) and DAPI (blue). Qualitatively, fewer peripheral RGCs are observed in PBS-treated Akita retina. (F) Ins2Akita mice showed no difference in central RGCs with either treatment but there was a trend towards reduced peripheral RGCs in PBS-treated mice vs. COMP-Ang1 treated mice (17% fewer ganglion cells in PBS group (p = 0.07)). At least six eyes from each group were tested, data are mean ± stdev. *p<0.01, ANOVA. Post hoc comparisons with a Tukey test to compare means of each group. p, peripheral; c, central.

Figure 5. AAV2.COMP-Ang1 prevents diabetes-induced neural dysfunction

(A) Representative example of ERG response from all groups of mice. Electrical retinal response was elicited and the amplitude of b-wave during scotopic conditions at -3.62 log (Cd s/m²) (-40dB), -2.62 log (Cd s/m²)(-30 dB), -1.62 log (Cd s/m²) (-20 dB), intensity was recorded. (B) Decreased amplitudes were recorded in Ins2Akita mice treated with PBS or AAV2.AcGFP compared to WT mice and AAV2.COMP-Ang1 prevented the
decrease in amplitude. *p<0.01, ANOVA, #p<0.01 compared to AAv2.AcGFP.
Assessing visual acuity was accomplished by testing optomotor tracking response of
Ins2Akita mice treated with AAV2.COMP-Ang1 or control compared to WT. (C)
Ins2Akita mice exhibited decreased optokinetic tracking response (units = cycles/degree).
AAV2.COMP-Ang1 prevented the decrease in visual response; at least six mice from
each group were tested, data are mean ± stdev. *p<0.01, ANOVA. Post-hoc comparisons
with a Tukey test to compare means of each group.

Figure 6. AAV2.COMP-Ang1 enhances ECFC engraftment into the diabetic retina
and prevents further visual decline.

(A) Endothelial colony-forming cells (ECFCs) were plated on collagen-coated wells and
assayed for migration potential under increasing doses of COMP-Ang1. (B) Additionally,
3D tube formation was tested in matrigel. COMP-Ang1 increased migration and tube
formation in a dose dependent manner with maximal effects exerted at 500 ng/mL. Qdot-655
labeled ECFCs were injected intravitreally into aged diabetic mice (6 months, arrow
in D) after the mice had been treated with COMP-Ang1 or control. Three days later
retinas were harvested and stained for blood vessels (isolectin 546) and flatmounted for
confocal analysis. COMP-Ang1 increased ECFC integration into the diabetic retinal
vasculature (see also Supplemental Video 2). (D) Mice treated with COMP-Ang1 or
control plus ECFCs were analyzed for visual tracking ability. COMP-Ang1 plus ECFCs
prevented further declines in spatial frequency threshold. *p<0.001 in vitro experiments
were performed in triplicate on three different ECFC clones (total of nine experiments
per condition). In vivo experiments were performed on five mice per group (ten eyes).
Supplemental Figure 1. AAV2 intravitreal injection does not cause damage in WT retina. (A) WT mice were given intravitreal injections of PBS or AAV2 expressing either AcGFP or COMP-Ang1 and assessed for retinal function via ERG. Compared to PBS injected retinas, there was no difference in b-wave amplitude between groups. (B) These same mice were also tested for visual tracking response over 5 weeks. Time 0 represents the week before injection. There was no difference between groups.

Supplemental Figure 2. Intravitreal injection of AAV2 successfully infects the inner retina. (A) AAV2.AcGFP expression was followed with in vivo confocal ophthalmoscopy (Heidelberg Spectralis) as early as 1 week after injection. Expression remained evident beyond 4 months post-injection. (B) Ex vivo retinal flat mount demonstrating AAV2.AcGFP expression in all four retinal quadrants. (C) Retinal cross-sections demonstrating AAV2.AcGFP expression primarily located in GCL-IPL layer of the retina. (D) Semi-quantitative RT-PCR demonstrated that COMP-Ang1 was expressed only in the eyes of mice injected with AAV2.COMP-Ang1. Flag protein was also found only in mice treated with the AAV2.COMP-Ang1 (E). To localize COMP-Ang1 transcript within the retina, in situ hybridization was utilized. (F) Expression of Ang1 was found in the retinas of mice treated with AAV2. AcGFP. (G) Mice treated with AAV2.COMP-Ang1 showed a demonstrable increase in Ang1 (detecting both native Ang1 and COMP-Ang1 transgene) primarily located in the GCL-IPL. (H) Negative control with Ang1 sense probe.
Supplemental Figure 3. Schematic of treatment paradigm and plasmid design. (A) Ins2Akita mice were treated at 2 months of age with intravitreal injection of either PBS or AAV2 expressing either AcGFP (control) or COMP-Ang1 (treatment). Retinas were harvested at 6 months of age (4 months after injection). (B) In a second cohort of mice, mice were initially injected at the delayed age of 6 months, followed two weeks later by ECFC injection and six weeks later by retinal harvest. (C,D) Plasmid design for COMP-Ang1 and AcGFP.

Supplemental Figure 4. Effects of AAV2.COMP-Ang1 on WT mice. Similar to Ins2Akita mice, intravitreal injection of AAV2.COMP-Ang1 increased VE-Cadherin and decreased VEGF in the retinas of WT mice when compared to AAV2.AcGFP.
Figure 1. AAV2.COMP-Ang1 mitigates diabetic retinal capillary dropout

(A) Representative retinal flatmounts prepared from six month-old mice and stained for isolectin (endothelial cell marker, green) and α-SMA (smooth muscle marker, red). 
(B) Magnified view of retina stained with isolectin and NG2 (pericyte marker). Ins2Akita mice experienced pericyte and endothelial dropout; the latter was prevented with a single intravitreal dose of AAV2.COMP-Ang1. 
(C) Trypsin digest featuring retinas representative of each group. Black arrowheads denote acellular capillaries. 
(D) Quantification using ImageJ of endothelial coverage and (E) pericyte coverage. 
(F) Acellular capillaries were manually counted and averaged over an area 1mm². Eight eyes were used in each analysis, data are mean ± stdev. *p<0.01, ANOVA. Post hoc comparisons with a Tukey test to compare means of each group. Scale bars = 600 µm (A), 100 µm (B), 200 µm (C).
Figure 2. COMP-Ang1 increases endothelial integrity

(A) Representative graph of electrical cell substrate impedance sensing (ECIS) of human retinal microvascular endothelial cells (HrMVECs) with COMP-Ang1 (100 ng/mL), VEGF (50 ng/mL), or control (PBS) added to the media. COMP-Ang1 increased resistance of HrMVECs (n=3). Increases in endothelial resistance were correlated with decreased Src phosphorylation (B) and increased VE-cadherin (C) in HrMVECs as demonstrated by Western blot (n=3). Western blot from Ins2Akita mouse retinas demonstrating decreased VEGF-A (D) and increased VE-cadherin (E) in mice treated with AAV2.COMP-Ang1. COMP-Ang1 increased Akt phosphorylation at the serine 473 residue in both ECFCs and HUVECs. (G) COMP-Ang1 reduced TNF-α induced leukocyte rolling in cultured HrMVECs (n=6 per group). *p<0.01, ANOVA
Figure 3. COMP-Ang1 enhances vascular barrier function and reduces retinal hypoxia in the diabetic retina. (A) Evans Blue extravasation from the retina of Ins2Akita mouse was increased compared to control; treatment with AAV2.COMP-Ang1 returned vascular hyperpermeability to control levels. Eyes from eight mice were used in each analysis; data are mean ± stdev. *p<0.01 compared to WT, *p=0.02 compared to AAV2.AcGFP. (B) Fluorescein angiography (FA) did reveal any leakage in diabetic mice; however, utilizing GFP or the NIR fluorophore ZW800 conjugated to aminated latex microspheres (GFP-ms, or ZW800–ms; 100 nm in diameter) in vivo leakage was captured using the FA or ICG imaging modality on Spectralis, respectively. Note that background GFP fluorescence of the AAV2.AcGFP treated diabetic mice masked signal from the GFP-microspheres. (C) Diabetes induced leukocyte rolling in the retinal vasculature, as captured by acridine orange leukocyte fluorography AOLF. (D) Representative image of AOLF with white arrowheads pointing to adherent and rolling leukocytes. COMP-Ang1 prevents leukostasis and inflammation in this model of diabetic retinopathy; see also Supplemental Video 1. (E) Representative retinas (four mice per group) from mice treated with hypoxprobes (pimonidazole). COMP-Ang1 reduced hypoxia in diabetic mouse retinas. Scale bars: 600 µm (E), *p<0.01, ANOVA. Post hoc comparisons with a Tukey test to compare means of each group.
Figure 4. AAV2.COMP-Ang1 prevents diabetes-induced GC-IPL degeneration

(A) Representative figures from optical coherence tomography (OCT) measuring retinal thickness. The red line, generated by OCT software, indicates the retinal surface and Bruch’s membrane. Scale bars = 200 µm. (B) Cross sections of six month-old retinas from WT, or Ins2Akita mice treated with PBS, AAV2.AcGFP, or AAV2.COMP-Ang1 stained with DAPI. (C) View of the GC-IPL from mice stained for VE-cadherin (red) or nuclei (DAPI, blue) demonstrating increased VE-cadherin and nuclear staining. Scale bars 30 µm (right). (D) Quantification of retinal thickness from OCT showing that AAV2.COMP-Ang1 prevented diabetes-induced retinal thinning as measured in vivo (*p<0.01 vs. both WT and AAV2.AcGFP). AAV2.COMP-Ang1 prevented diabetes-induced inner retinal layer loss (*p=0.03 ANOVA, with post hoc Tukey test) as measured by nuclei counted in the GC-IPL in retinal cross sections. (E) Representative retinal flat mounts stained with BRN3 (red; a marker for retinal ganglion cells) and isolectin (green; marker for vessels) and DAPI (blue). Qualitatively, fewer peripheral RGCs are observed in PBS-treated Akita retina. (F) Ins2Akita mice showed no difference in central RGCs with either treatment but there was a trend towards reduced peripheral RGCs in PBS-treated mice vs. COMP-Ang1 treated mice (17% fewer ganglion cells in PBS group (p = 0.07)). At least six eyes from each group were tested, data are mean ± stdev. *p<0.01, ANOVA. Post hoc comparisons with a Tukey test to compare means of each group. p, peripheral; c, central.

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Figure 5. AAV2.COMP-Ang1 prevents diabetes-induced neural dysfunction

(A) Representative example of ERG response from all groups of mice. Electrical retinal response was elicited and the amplitude of b-wave during scotopic conditions at -3.62 log (Cd s/m²) (-40dB), -2.62 log (Cd s/m²) (-30 dB), -1.62 log (Cd s/m²) (-20 dB), intensity was recorded. (B) Decreased amplitudes were recorded in Ins2Akita mice treated with PBS or AAV2.AcGFP compared to WT mice and AAV2.COMP-Ang1 prevented the decrease in amplitude. *p<0.01, ANOVA, #p<0.01 compared to AAV2.AcGFP. Assessing visual acuity was accomplished by testing optomotor tracking response of Ins2Akita mice treated with AAV2.COM-P-Ang1 or control compared to WT. (C) Ins2Akita mice exhibited decreased optokinetic tracking response (units = cycles/degree). AAV2.COMP-Ang1 prevented the decrease in visual response; at least six mice from each group were tested, data are mean ± stdev. *p<0.01, ANOVA. Post-hoc comparisons with a Tukey test to compare means of each group.
Figure 6. AAV2.COMP-Ang1 enhances ECFC engraftment into the diabetic retina and prevents further visual decline.

(A) Endothelial colony-forming cells (ECFCs) were plated on collagen-coated wells and assayed for migration potential under increasing doses of COMP-Ang1. (B) Additionally, 3D tube formation was tested in matrigel. COMP-Ang1 increased migration and tube formation in a dose dependent manner with maximal effects exerted at 500 ng/mL. Qdot-655 labeled ECFCs were injected intravitreally into aged diabetic mice (6 months, arrow in D) after the mice had been treated with COMP-Ang1 or control. Three days later retinas were harvested and stained for blood vessels (isolectin 546) and flatmounted for confocal analysis. COMP-Ang1 increased ECFC integration into the diabetic retinal vasculature (see also Supplemental Video 2). (D) Mice treated with COMP-Ang1 or control plus ECFCs were analyzed for visual tracking ability. COMP-Ang1 plus ECFCs prevented further declines in spatial frequency threshold. *p<0.001 in vitro experiments were performed in triplicate on three different ECFC clones (total of nine experiments per condition). In vivo experiments were performed on five mice per group (ten eyes). Scale bars (C): 600 µm (top), 150 µm (middle), and 90 µm (bottom). *p<0.01, ANOVA. Post hoc comparisons with a Tukey test to compare means of each group.

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Supplemental Figure 4. Effects of AAV2.COMP-Ang1 on WT mice. Similar to Ins2Akita mice, intravitreal injection of AAV2.COMP-Ang1 increased VE-Cadherin and decreased VEGF in the retinas of WT mice when compared to AAV2.AcGFP.

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