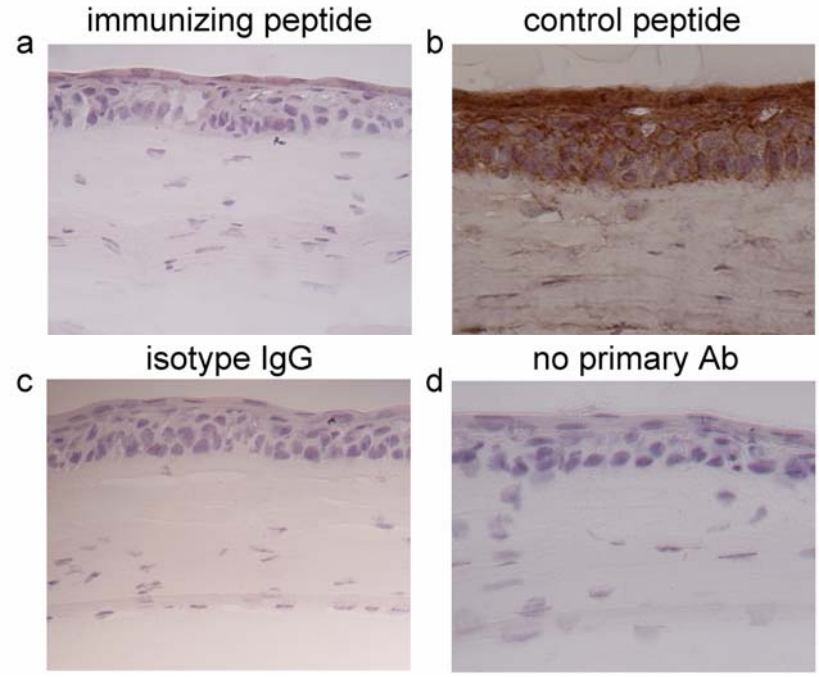
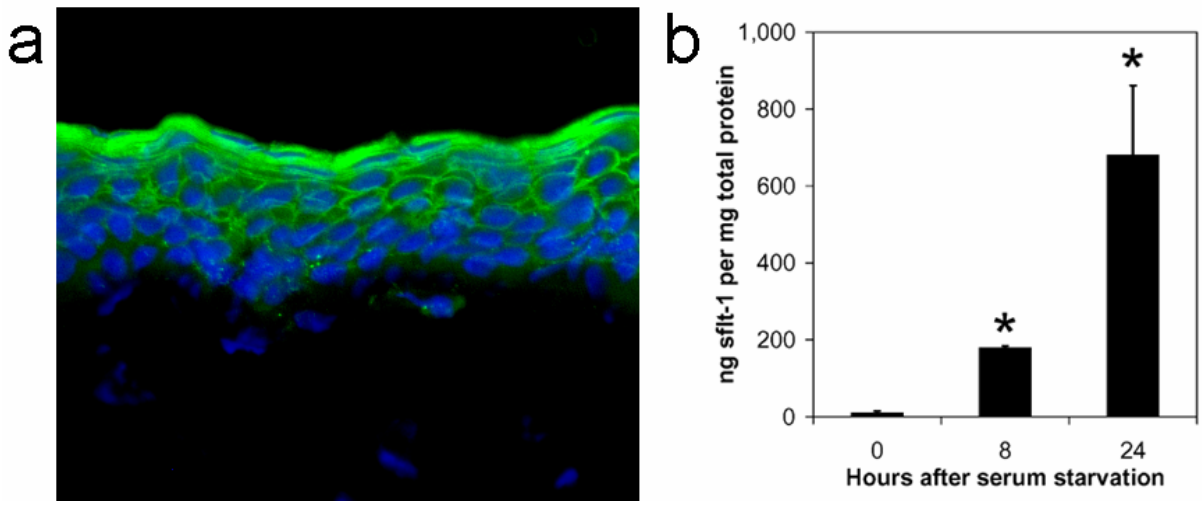
Supplementary Figure S1



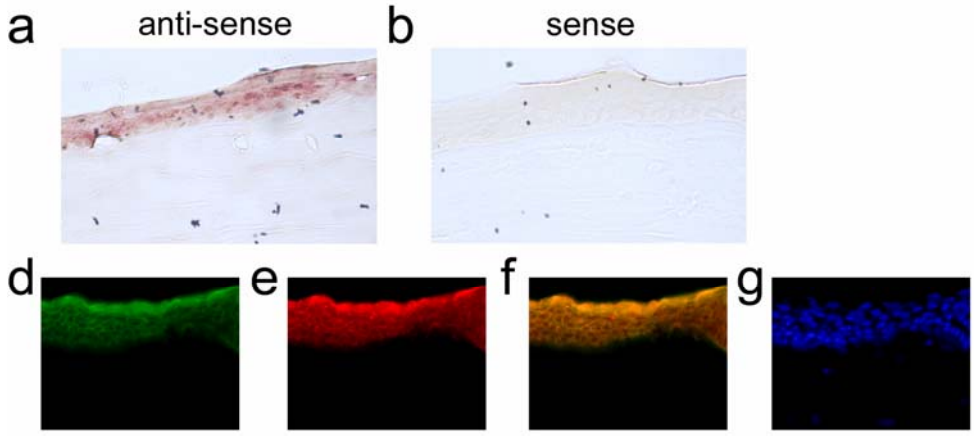
Specificity of sflt-1 immunolocalization. a,b, sflt-1 staining (brown) in mouse cornea was inhibited by the immunizing peptide (**a**) but not by an unrelated, negative-control peptide (**b**). No staining was observed when anti-sflt-1 antibody was replaced with isotype control IgG (**c**) or when it was omitted (**d**). Cell nuclei stain blue.

Supplementary Figure S2



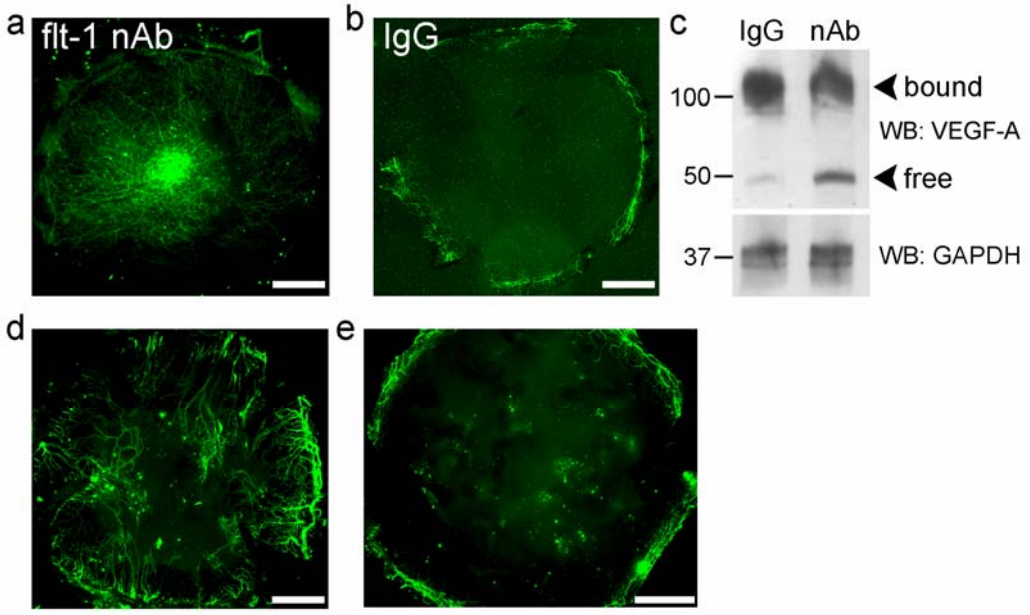
sflt-1 is present extracellularly *in vivo* and *in vitro*. a, The pattern of sflt-1 immunostaining (green) is diffuse and extends beyond cell borders in the superficial layers of mouse corneal epithelium, and appears external to cytoplasm and in intercellular spaces in the deeper layers. Cell nuclei stained blue by DAPI. **b,** ELISA demonstrates that mouse corneal epithelial cells constitutively secrete sflt-1. *$P <$ 0.05 Bonferroni corrected Mann Whitney U test compared to previous time point. n=6. Error bars depict s.e.m.

Supplementary Figure S3



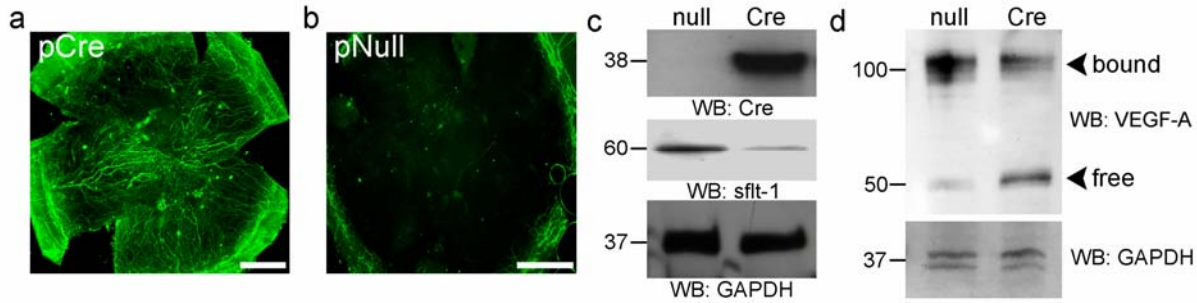
VEGF-A produced by the cornea colocalizes with sflt-1. a,b, VEGF-A mRNA was detected by in situ hybridization in mouse cornea. Antisense RNA probes showed red reactivity in the epithelium. Sense RNA probes showed negligible reactivity. **d-g**, Immunoreactivities of sflt-1 (green, **d**) and VEGF-A (red, **e**) colocalize in the mouse cornea (merge of green and red channels appears yellow, **f**). **g**, Cell nuclei stained blue by DAPI.

Supplementary Figure S4



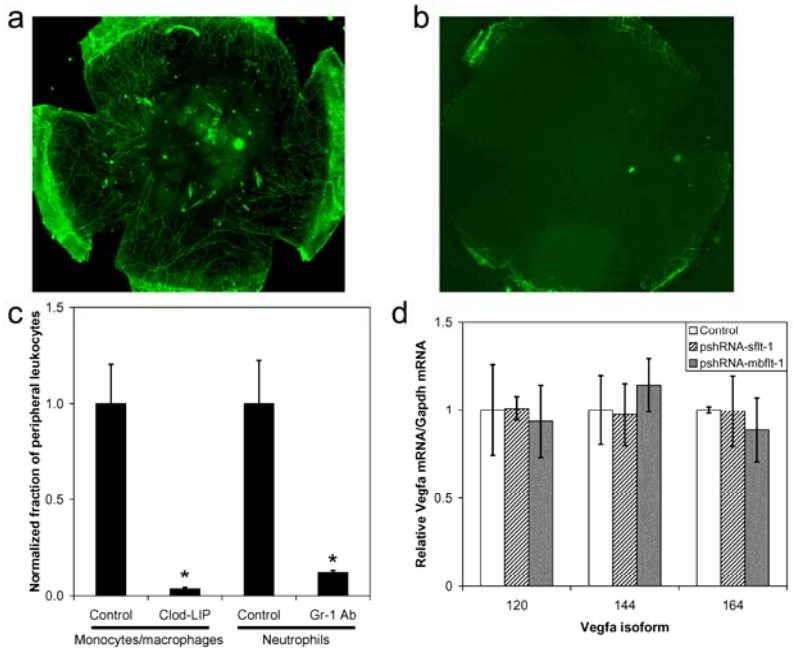
Neutralizing flt-1 protein abolishes corneal avascularity. a,b, Representative flat mounts show that corneal injection of anti-flt-1 neutralizing antibody (nAb) but not isotype control IgG injection elicits invasion of $CD31^+$ (green) $LYVE\text{-}1^-$ blood vessels into cornea of mice 14 days after injection. n=14. **c**, Representative non-reducing western blot at 1 hour after injection of nAb but not control IgG shows a shift in VEGF-A from bound to free form. n=6. **d,e**, Representative flat mounts show that subconjunctival injection (10 μg) of neutralizing anti-flt-1 antibody (**d**) but not isotype control IgG (**e**) elicits corneal vascularization in mice 10 days after injection. n=10. Scale bars, 900 μm.

Supplementary Figure S5



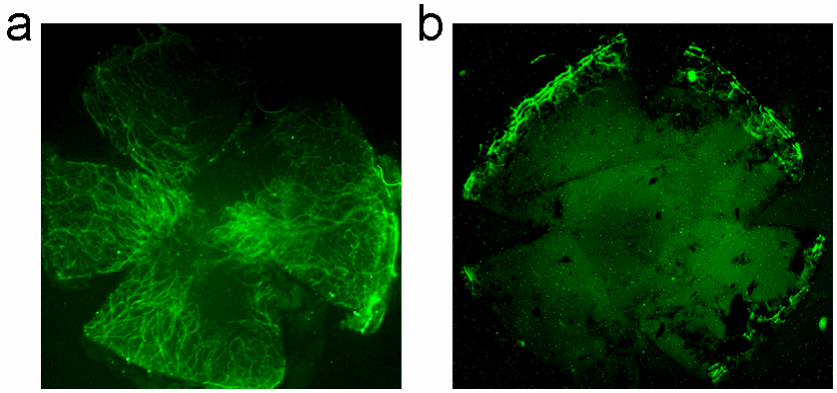
Neutralizing flt-1 gene expression abolishes corneal avascularity. a,b, Representative flat mounts at 14 days after injection of pCre but not pNull induces corneal vascularization in *flt-1*$^{loxP/loxP}$ mice. n=10. **c**, Representative reducing western blots show that in *flt-1*$^{loxP/loxP}$ mouse corneas, at 2 days after pCre injection, Cre is expressed and sflt-1 expression is reduced compared to pNull injection. n=6. **d**, Representative non-reducing western blot shows a shift in VEGF-A from bound to free form in eyes injected with pCre compared to pNull. n=6. Scale bars, 900 μm.

Supplementary Figure S6



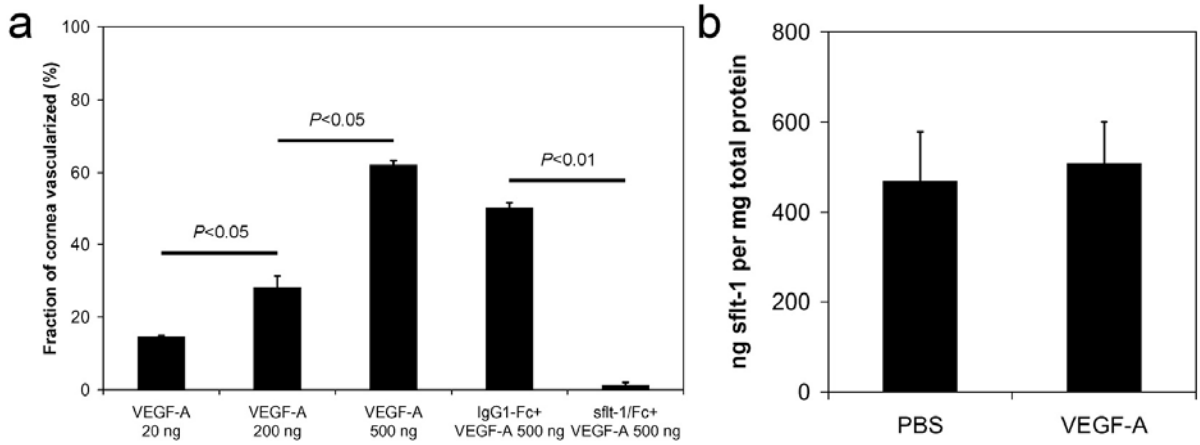
Corneal vascularization induced by sflt-1 shRNA not due to inflammation. a,b, Representative flat mounts demonstrate that pshRNA-sflt-1 induced corneal vascularization 10 days after injection in mice systemically depleted of monocytes/macrophages and neutrophils by injection of clodronate liposomes and anti-Gr-1 antibody for 3 consecutive days (**a**) and not by monocyte/macrophage and neutrophil depletion alone (**b**). n=5. **c,** Flow cytometry reveals the monocyte/macrophage and neutrophil fractions of peripheral blood leukocytes, normalized to control levels, were markedly suppressed by clodronate liposomes and anti-Gr-1 antibody injection compared to controls (PBS-liposomes and non-immune rat IgG, respectively) 3 days after initial injection. n=6, *P<0.05 compared to controls. **d**, pshRNA-sflt-1 did not elevate VEGF-A mRNA levels compared to pshRNA-mbflt-1 or control uninjected corneas. Individual VEGF-A isoform levels measured by real time RT-PCR 2 days after injection were divided by GAPDH levels and normalized to control levels. No pairwise differences were statistically significant by Bonferroni corrected Mann Whitney U test. n=12. Error bars depict s.e.m.

Supplementary Figure S7



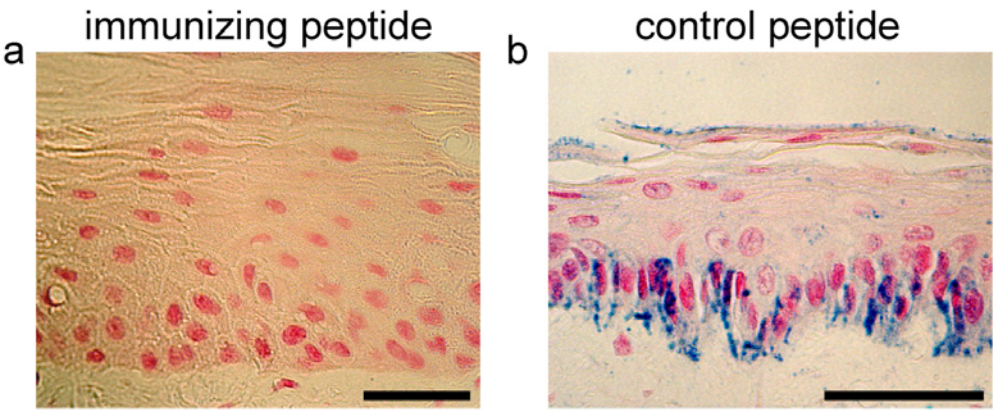
Corneal vascularization induced by sflt-1 shRNA is specifically due to mRNA knockdown. a, Representative flat mount shows that $p_2$shRNA-sflt-1, targeted against a different sequence than by pshRNA-sflt-1, also induced corneal vascularization. n=10. **b**, Representative flat mount shows that psflt-1*, a plasmid coding for a sflt-1 translationally silent mutant refractory to pshRNA-sflt-1, preserved corneal avascularity in eyes treated with pshRNA-sflt-1. n=10.

Supplementary Figure S8



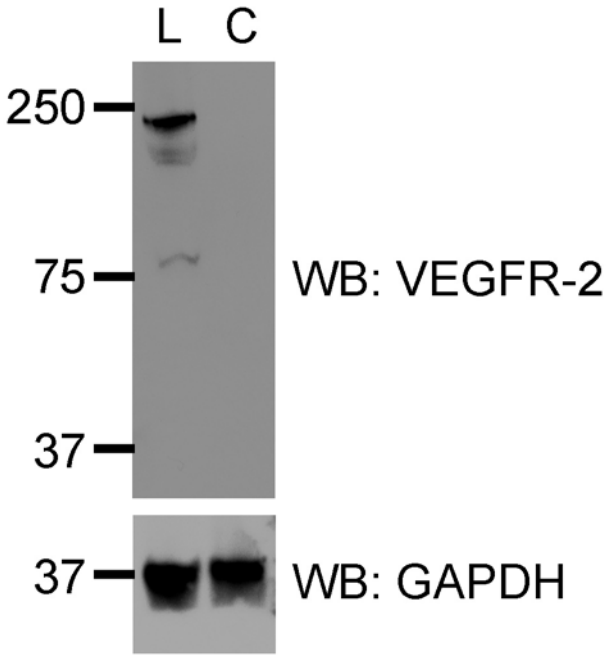
Exogenous VEGF-A induces corneal vascularization by overwhelming endogenous sflt-1. a, Recombinant mouse VEGF-A_{164} injection induces CV in a dose-dependent manner and is blocked by co-administration (5 μg) of recombinant sflt-1/Fc but not isotype control IgG1-Fc. Area of CV 10 days after injection measured by morphometric analysis of $CD31^+$ $LYVE\text{-}1^-$ blood vessel coverage of cornea. Statistically significant differences are displayed. n=6. **b,** ELISA of corneal sflt-1 levels 3 days after injection were not significant different between PBS- and VEGF-A-injected eyes. Significance by Bonferroni corrected Mann Whitney U test. n=8. Error bars depict s.e.m.

Supplementary Figure S9



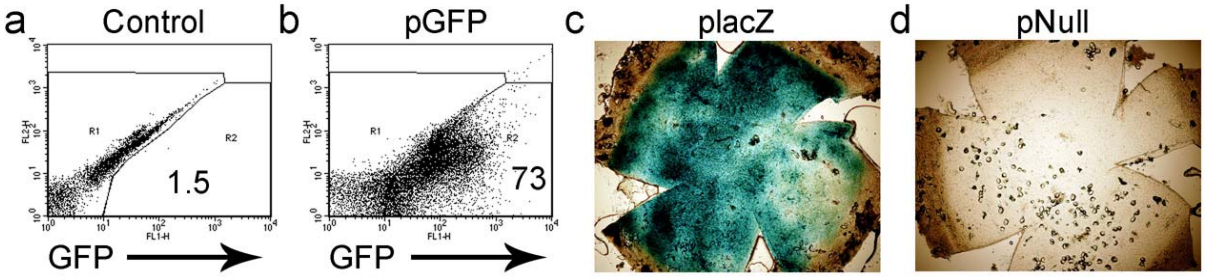
Manatee cornea contains mbflt-1. mbflt-1 staining (blue) in the manatee cornea was inhibited by the immunizing peptide (**a**) but not by an unrelated, negative-control peptide (**b**). Nuclei stained red. Scale bars, 200 μm.

Supplementary Figure S10



Normal cornea does not express VEGFR-2. Representative reducing Western blot shows VEGFR-2 in mouse lung homogenates (L) but not mouse cornea lysates (C). Equal loading was confirmed by detection of GAPDH. n=10.

Supplementary Figure S11



Naked plasmids transfect mouse corneas *in vivo*. (**a,b**) Representative flow cytometry data reveal that greater than 70% of corneal cells express GFP 1 day after injection of pGFP (**b**) compared to pNull (**a**). (**c,d**) Xgal staining of flat mounts confirms that approximately 75% of corneal cells express β-galactosidase (blue) 1 day after injection of placZ (**c**) compared to pNull (**d**). n=6.

Supplementary Methods

Animals. A/J, C57Bl/6J, *corn1*, *Ifng*$^{-/-}$, *ROSA26R* (*Gt(ROSA)26Sor*^{tm1Sor}/J) lacZ reporter (The Jackson Laboratory), *Vegfb*$^{-/-}$ (The Jackson Laboratory; gift of D.A. Greenberg, Buck Institute for Age Research), and Balb/c mice (Harlan Laboratories) were used. *Ifnar1*$^{-/-}$ (gift of H.W. Virgin, Washington University) *Pax6*$^{+/-}$, *Pax6*$^{+/+}$, and *Plgf*$^{-/-}$ mice have been previously described[1-3]. *flt-1*$^{loxP/loxP}$ mice, generated and characterized by Genentech, will be described elsewhere. Dolphin, dugong, elephant, human, manatee, and whale eyes were collected in accordance with applicable regulations. Experiments were approved by institutional review boards and conformed to the Association for Research in Vision and Ophthalmology Statement on Animal Research.

Hypoxia. Hypoxia was induced by placing C57Bl/6J mice into 8% O_2 PEGASS chambers (Columbus Instruments) for 24 hours.

Vectors. siRNA expression cassettes (SECs) were developed by *in vitro* amplification by PCR. Multiple sequences were screened to identify the best targets for mbflt-1 (AAACAACCACAAAAUACAACA) and sflt-1 (#1: AAUGAUUGUACCACACAAAGU; #2: UCUCGGAUCUCCAAAUUUA), which were ligated into the pSEC Neo vector. Plasmids were prepared (Plasmid Mini Prep kit, Eppendorf) and sequenced to confirm the in-frame sequence of the inserts. psflt-1* was generated by site directed mutagenesis (Stratagene) of 2278-AATGATTGTACCACACAAAGT in psflt-1 (gift of V.L. Bautch, University of North Carolina) to AACGACTGCACAACTCAGAGC.

Immunohistochemistry. Deparaffinized sections were incubated with serum-free protein block (Dako or Biogenex). Endogenous peroxidase and alkaline phosphatase were quenched with H_2O_2 and levamisole (Vector Laboratories). Immunolocalization was performed with rabbit antibody against the unique carboxyl-terminus of sflt-1 (1:1000; ref. 4), rabbit antibody against the unique carboxyl-terminus of mbflt-1 (1:1000; clone C-17, Santa Cruz

Biotechnology), goat antibody against mouse VEGF-A (1:200; R&D Systems), goat antibody against human vascular cell adhesion molecule-1 (1:100; Santa Cruz Biotechnology), rabbit antibody against Cre recombinase (1:5,000; EMD/Novagen) using biotin-streptavidin–horseradish peroxidase, alkaline phosphatase, or immunofluorescent methods using FITC- and PE-conjugated secondary antibodies (Vector Laboratories). Counterstain was obtained with Mayer's hematoxylin (Lillie's Modified, Dako), Nuclear FastRed (Vector Laboratories), or DAPI (1:25,000; Molecular Probes). Specificity was assessed by staining with control isotype non-immune IgG, omitting primary antibody, or pre-adsorbing the primary antibody with a ten-fold molar excess of the immunizing peptide.

In situ hybridization. In situ hybridization was carried out on formaldehyde fixed cryosections as previously described[5]. Digoxigenin (DIG)-labeled sense and anti-sense riboprobes were transcribed from mouse sflt-1 and VEGF-A cDNAs using the DIG RNA-labeling kit (Boehringer-Mannheim). The sflt-1 probe corresponded to the divergence site of sflt-1 as previously reported[6]. DIG-labeled probes were hybridized, slides were washed under high-stringency conditions, incubated with alkaline phosphatase-conjugated anti-DIG antibody (1:2000; Boehringer-Mannheim), and stained with NBT/BCIP (Boehringer-Mannheim).

Topical eye drops. Cell permeable enzymatically active Cre recombinase[7,8] (NLS-Cre) or NLS-β-galactosidase (gift of D.J. Forbes, University of California at San Diego; ref. [9]), both containing a 6-His tag and an SV40 derived nuclear localization signal, were dissolved in PBS (0.6 mg/ml) and dropped on to the surface of the cornea at the rate of 1 μl/min for 5 min.

Rescue experiments. Recombinant sflt-1/Fc or control isotype IgG_1-Fc were injected (5 μg /2 μl) into the corneas of fellow eyes of *corn1* (at 2 and 3 weeks of age) and *Pax6*$^{+/-}$ (at 6 and 7 weeks of age) mice and morphometric measurements of vascularized area on corneal flat

mounts were performed, as previously reported[10,11], at 4 (*corn1*) and 8 (*Pax6*$^{+/-}$) weeks of age.

Protein expression. Enzyme-linked immunosorbent assays (ELISAs) were used according to the manufacturer's instructions to quantify sflt-1 (Quantikine, R&D Systems) and free VEGF-A (RELIDA, RELIATech GmbH). Measurements were normalized to total protein (Bio-Rad). As previously described[12], immunoblotting was performed with rabbit antibody against the amino-terminus of flt-1 (1:1,000; Angiobio), rabbit antibodies against the unique carboxyl-terminus of sflt-1 (1:100; ref. [13] or 1:1,000; ref. 4), goat antibody against mouse VEGF-A (1:1,000; R&D Systems), rabbit antibody against mouse VEGFR-2 (1:1,000; clone T014; ref. [14]), or rabbit antibody against Cre recombinase (1:10,000), and loading was assessed with rabbit antibody against human GAPDH (1:2,000; Abcam). Mouse cornea lysates were immunoprecipitated with goat antibody against mouse VEGF-A (2 μg/ml, R&D Systems) immobilized to protein G-agarose, subjected to SDS-PAGE, and immunoblotted with biotinylated goat antibody against the amino-terminus of mouse flt-1 (1:1,000, R&D Systems).

Gene expression. Total mouse cornea RNA was prepared (RNAqueous, Ambion) and cDNA was synthesized by reverse transcription (TaqMan, Applied Biosystems) and analyzed by real-time quantitative polymerase chain reaction (ABI 7000, Applied Biosystems). The primers for sflt-1 were: forward 5′-AGGTGAGCACTGCGGCA-3′, reverse 5′-ATGAGTCCTTTAATGTTTGAC-3′. The primers for VEGF-A were described in ref. [15]. FAM (6-carboxyfluorescein)-labeled probes (Maxim Biotech) were used as target hybridization probes. *sflt-1* and *Vegfa* expression were quantified and normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) or 18S rRNA levels by polymerase

chain reaction with reverse transcription (RT–PCR) using TaqMan gene expression assays (Applied Biosystems).

Flow cytometry. Suspensions of cells isolated from mouse cornea by incubation with collagenase D (20 U/ml; Roche Diagnostics) and keratanase (5 U/ml; Sigma-Aldrich) treatment were incubated in Fc block (0.5 mg/ml; BD Pharmingen) for 15 min on ice. GFP expression was quantified using Alexa Fluor 488-conjugated rabbit antibody against GFP (1:500; Molecular Probes). Cells were stained after fixation with 4% paraformaldehyde and permeabilization with 1% Triton X-100 (Sigma Aldrich) and were subjected to FACS analysis (FACSCalibur, BD Biosciences). Monocytes/macrophages ($CD11b^+$ $CD115^+F4/80^+$) and neutrophils ($CD11b^+F4/80^-$ $Gr\text{-}1^+$), in cells isolated from cardiac blood after erythrocyte hemolysis with lysis buffer (eBioscience), were gated by FITC-conjugated rat antibody against mouse CD11b (1:100; eBioscience), RPE-Cy5-conjugated rat antibody against mouse F4/80 (1:10; Serotec), PE-conjugated rat antibody against mouse CD115 (1:1,000; eBioscience), and Alexa Fluor 647-conjugated rat antibody against mouse Gr-1 (1:100; eBioscience).

Cell culture. Cultured mouse corneal epithelial cells (gift of J.Y. Niederkorn, UT Southwestern Medical Center) were maintained in EMEM supplemented with 10% FCS, 1% glutamine, and antibiotics at 37 °C under 5% CO_2. Secreted sflt-1 levels in the supernatant were measured by ELISA (R&D Systems) at 0, 8 and 24 h after serum starvation.

Statistics. Differences in incidence of CV and mean levels of protein, mRNA, and CV area were compared with two-tailed Fisher's exact test and Mann Whitney U test with Bonferroni correction for multiple comparisons, respectively. P values < 0.05 were considered significant. Data are presented as mean±s.e.m.

Supplementary Notes

1. Muller, U. et al. Functional role of type I and type II interferons in antiviral defense. *Science* **264**, 1918-21 (1994).
2. Quinn, J. C., West, J. D. & Hill, R. E. Multiple functions for *Pax6* in mouse eye and nasal development. *Genes Dev* **10**, 435-46 (1996).
3. Carmeliet, P. et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med* **7**, 575-83 (2001).
4. Orecchia, A. et al. Vascular endothelial growth factor receptor-1 is deposited in the extracellular matrix by endothelial cells and is a ligand for the α5β1 integrin. *J Cell Sci* **116**, 3479-89 (2003).
5. Witta, J. et al. Nigrostriatal innervation is preserved in Nurr1-null mice, although dopaminergic neuron precursors are arrested from terminal differentiation. *Brain Res Mol Brain Res* **84**, 67-78 (2000).
6. He, Y. et al. Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. *Mol Endocrinol* **13**, 537-45 (1999).
7. Lin, Q., Jo, D., Gebre-Amlak, K. D. & Ruley, H. E. Enhanced cell-permeant Cre protein for site-specific recombination in cultured cells. *BMC Biotechnol* **4**, 25 (2004).
8. Jo, D. et al. Epigenetic regulation of gene structure and function with a cell-permeable Cre recombinase. *Nat Biotechnol* **19**, 929-33 (2001).
9. Harel, A. et al. Importin β negatively regulates nuclear membrane fusion and nuclear pore complex assembly. *Mol Biol Cell* **14**, 4387-96 (2003).
10. Ambati, B. K. et al. Sustained inhibition of corneal neovascularization by genetic ablation of CCR5. *Invest Ophthalmol Vis Sci* **44**, 590-3 (2003).

11. Ambati, B. K., Joussen, A. M., Kuziel, W. A., Adamis, A. P. & Ambati, J. Inhibition of corneal neovascularization by genetic ablation of CCR2. *Cornea* **22**, 465-7 (2003).

12. Nozaki, M. et al. Loss of SPARC-mediated VEGFR-1 suppression after injury reveals a novel antiangiogenic activity of VEGF-A. *J Clin Invest* **116**, 422-9 (2006).

13. Hiratsuka, S., Minowa, O., Kuno, J., Noda, T. & Shibuya, M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc Natl Acad Sci U S A* **95**, 9349-54 (1998).

14. Feng, D. et al. Ultrastructural localization of the vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) receptor-2 (FLK-1, KDR) in normal mouse kidney and in the hyperpermeable vessels induced by VPF/VEGF-expressing tumors and adenoviral vectors. *J Histochem Cytochem* **48**, 545-56 (2000).

15. Zhang, L. et al. Different effects of glucose starvation on expression and stability of VEGF mRNA isoforms in murine ovarian cancer cells. *Biochem Biophys Res Commun* **292**, 860-8 (2002).