Alternatively spliced VEGF receptor-2 is an essential endogenous inhibitor of lymphatic vessel growth

sVegfr-2 is an alternatively spliced isoform of Vegfr2. (a) Representative western blot of mouse cornea with an antibody against the amino (N)-terminus of Vegfr-2 reveals an
immunoreactive band at 75 kDa but not 230 kDa. n = 12. (b) Predicted structure of sVegfr-2 protein and its unique carboxyl (C) terminus amino acid sequence (pink) compared to membrane bound (mb) Vegfr-2. Ig1-7, immunoglobulin-like domains (blue circles); transmembrane domain (orange); TK, tyrosine kinase domain (green-yellow ellipse). (c) Diagram of pre-mRNA exon-intron structure of Vegfr2 (center box - not scaled). Retention of intron 13 (downward dotted arrows) yields a novel mRNA species with a unique 3’-tail (red). Upward dotted arrow showing mbVegfr2. mb, membrane bound; s, soluble. (d) PCR amplification of 393 bp (*) product from a mouse cornea cDNA encompassing the splice site giving rise to sVegfr2 mRNA. Adjacent lane (water) shows (template negative) control. n = 5. bp, base pairs (bottom left). (e) 400 bp amplicon from 3’RACE PCR shows that the third polyadenylation signal sequence is active. n = 5. PA1-3, potential polyadenylation signal sites (bottom right).
Complete mouse sVegfr2 transcript (cDNA) sequence (5'-3') and its cloning from corneal cDNA. (a) 5' and 3' untranslated region (UTR, (lowercase), open-reading frame (ORF) (uppercase). Exon boundaries are indicated by vertical lines ( | ). Novel 3' end of exon 13 shown in red. Red bar ( | ) shows alternative splicing site. Sequences targeted by primers underlined by arrow (forward →) (reverse ←). Blue arrows sVegfr2 primer set for RT-PCR. Orange arrows sVegfr2 primer set used for real-time PCR. Green arrows correspond to primer set used to clone the ORF of sVegfr2. Polyadenylation signal site is highlighted in yellow. (b) RT-PCR was used to clone the open-reading-frame (ORF) of sVegfr2 (* 2.1 kb band; lanes 1 and 2 cDNA from two independent RT reactions). M, marker; lane 3, negative control (H2O).
Characterization of anti-sVegfr-2 antibody (AA21127). (a) ELISA showed that the custom rabbit antibody against the C-terminus of sVegfr-2 (AA21127) specifically detected immobilized immunizing peptide (gmeaslgdriamp; blue bar) compared to an immobilized control peptide (green bar). (b) Representative western blots of supernatants from Chinese Hamster Ovary (CHO) cells that were transfected with empty plasmid (pNull) or plasmids coding for mbVegfr-2 (pmbVegfr-2) or sVegfr-2 (psVegfr-2), and probed with AA21127, rabbit antibody against the amino (N) terminus of Vegfr-2 (T014), and goat antibody against the N-terminus of Vegfr-1 (Santa Cruz, sc-31173), show that T014 recognizes both sVegfr-2 and mbVegfr-2, whereas
AA21127 recognizes only sVegfr-2. AA21127 also does not recognize sVegfr-1, which is constitutively produced by CHO cells.
**Supplementary Figure 4**

**sVegfr-2 but not mbVegfr-2 expressed in wild-type mouse cornea.** (a) Immunolocalization of sVegfr-2 protein (green) using an antibody against its unique C-terminus shows its presence throughout the cornea in newborn mice (P0) and predominantly in the epithelium in adults (P28). sVegfr-2 staining was abrogated by pre-adsorption (Pre-Ad) with immunizing peptide and not observed when anti-sVegfr-2 antibody was replaced with isotype control IgG. Brackets delimit antero-posterior extent of cornea. (b) Corneal flat mount showing uniform distribution of sVegfr-2 throughout the corneal surface (green) with slightly enhanced expression near the limbal arcade (inset box). Limbal vasculature (CD31+) is shown in red. Inset (dotted box) shows higher magnification of corneal-conjunctival interface (dotted blue line) showing expression of sVegfr-
2 in the cornea (CORN), but not in the conjunctiva (CONJ). (c) Cross-sectional image of corneal-conjunctival interface (arrowhead) showing sVegfr-2 immunoreactivity (green) in the corneal epithelium, but not in the conjunctiva. Cell nuclei stained blue by DAPI. (d) Western blot of mouse corneas using either sVegfr-2 (C) or Vegfr-2 (N) antibodies revealed 75 kDa immunoreactive bands present at birth (P0) and declining at P14 and P28. Gapdh was loading control (bottom); n = 12-30. (e) Four hours after suture injury, the mouse cornea exhibited immunoreactivity (green) to an antibody against the C-terminus (C) of sVegfr-2 (bottom right) but not to an antibody against the C-terminus of mbVegfr-2 (top right). Inset [(+) control] displays mbVegfr-2 immunoreactivity in limbal vessel. Cell nuclei stained blue by DAPI. (top). (f) Absence of amplicon corresponding to mbVegfr2 in two independent corneal samples (c 1 and c 2). M, marker; + cont., positive control; Gapdh, loading control; H2O, template negative control. Scale bar: (a,c,e), 50 µm; (b), 500 µm.
**Generation of Vegfr2-flox mice.** (a). Schematic representation of the gene targeting strategy. A loxP site was inserted into the EcoRI site upstream of the first exon of Vegfr2. Another loxP site and PGK-neo cassette flanked by frt sites were inserted into the SmaI site in the first intron of Vegfr2 gene. The originally targeted allele is designated Vegfr2<sup>flox-neo</sup>. PGK-neo cassette in the targeted locus was removed by FLP<sup>e</sup>-mediated recombination. Heterozygous mice carrying the targeted locus without neo are designated Vegfr2<sup>flox</sup> mice here. The generation of Vegfr2<sup>del1</sup> allele
was tested by crossing $Vegfr2^{\text{flox}}$ mice and a Cre deleter strain. Exon 1 is shown as filled box. Probes used for Southern blot analysis are shown as hatched boxes. loxP and frt sites are shown as filled and open boxes, respectively. Primers for PCR genotyping are shown as arrows ($Vegfr$-2-S1, -A, and -S2). H, $\text{HindIII}$; RI, $\text{EcoRI}$; Sl, $\text{SalI}$; Sm, $\text{SmaI}$. (b) Southern blot analysis. (c) PCR genotyping with Vegfr-2-S1, -A, and -S2 primers of mice from crossing $Vegfr2^{\text{flox}}$ mice and a Cre deleter strain.
Supplementary Figure 6

(a) LeCre / Vegfr2^{+/+} vs. LeCre / VegFr2^{loxP/loxP}

(b) Cd31, Lyve-1, and MERGE

(c) Lyve-1, Meca-32, and MERGE

(d) LeCre / Vegfr2^{+/+} vs. LeCre / Vegfr2^{loxP/loxP}

(e) LeCre / Vegfr2^{loxP/loxP} (sutured)
$\text{LeCre/Vegfr2}^{\text{loxP/loxP}}$ mouse cornea lacks sVegfr-2 and has lymphatic but not blood vessels. (a) Immunofluorescence reveals absence of sVegfr-2 in the cornea of $\text{LeCre/Vegfr2}^{\text{loxP/loxP}}$ mice compared to littermate control. Cell nuclei stained with DAPI. (b) Representative flat mount of the cornea of a $\text{LeCre/Vegfr2}^{\text{loxP/loxP}}$ mouse shows that all vessels expressing the pan-endothelial cell marker CD31 (red) also express Lyve-1 (green), a lymphatic endothelial cell marker. The merged image shows areas of colocalization of CD31 and Lyve-1 in the same vessels. The absence of blood vessels is confirmed by the lack of CD31$^+$ Lyve-1$^-$ vessels. (c) Representative corneal flat mounts (top panels) show invasion of Lyve-1$^+$ (green) lymphatic but not MECA-32$^+$ (red) blood vessels into the cornea of a $\text{LeCre/Vegfr2}^{\text{loxP/loxP}}$ mouse. (d) Corneal flat mounts of $\text{LeCre/Vegfr2}^{\text{loxP/loxP}}$ and littermate control mice showing normal MECA-32$^+$ limbal blood vessels that do not invade the cornea. (e) Corneal flat mount and microphotograph of corneal neovascularization (MECA-32$^+$ blood vessels) of the $\text{LeCre/Vegfr2}^{\text{loxP/loxP}}$ 10 days after suture placement. Scale bar: (a) 50 µm; (b-e), 500 µm.
Postnatal loss of Vegf-c in mouse cornea. (a) Vegf-c was immunolocalized (red) in the newborn wild-type mouse corneal epithelium (middle right). Immunofluorescence was not observed when anti-Vegf-c antibody was replaced with isotype control IgG (top right). Vegf-c expression was not detected in the adult cornea (bottom right). Cell nuclei stained blue by DAPI (left). Scale bar, 50 µm. Brackets delimit antero-posterior extent of cornea. (b) Representative
western blot of wild-type mouse cornea detected Vegf-c species that migrated at relative molecular masses of 25, 43, and 58 kDa in the newborn (left lane) but not adult (right lane) cornea. Gapdh was loading control (bottom), n = 12. (c) RT-PCR shows amplicon corresponding to Vegfc in the neonate cornea; Gapdh, loading control; H2O, template negative control, water.
sVegfr-2 blocked lymphangiogenesis induced by mouse Vegf-c and human VEGF-D, but not by mouse Vegf-d. (a) Corneal area occupied by lymphatic vessels (Lyve-1+) induced by pmVegf-c and pVEGF-D injection in wild-type mouse corneas was reduced by psVegfr-2 compared to pNull; however, psVegfr-2 did not inhibit pVegf-d induced lymphangiogenesis. NS,
not significant; *, $P < 0.05$; Significance by Mann Whitney U test. n = 4. Error bars depict s.e.m.

(b) Representative corneal flat mounts showing decreased Lyve-1$^+$ lymphatic vessels (green) in corneas co-transfected in vivo with pmVegf-c/pS Vegfr-2 and phVegf-d/pS Vegfr-2 compared to pmVegf-c/pNull and pVEGF-D/pNull, respectively. No change in Lyve-1$^+$ lymphatic vessel density was seen between pVegf-d/pS Vegfr-2 and pVegf-d/pNull. Scale bar 500µm.
**Vegf-d is not expressed in the neonatal cornea.** (a) Vegf-d was immunolocalized (green) in the mouse lung, but not in the newborn (P0) wild-type mouse cornea. Immunofluorescence was not observed when anti-Vegf-d antibody was replaced with isotype control IgG. Cell nuclei stained blue by DAPI. Scale bar, 50 µm. (b) Representative western blot showing the absence of Vegf-d in P0 wild-type mouse cornea. Recombinant Vegf-d (rVegf-d) was used as positive control. Gapdh was loading control (bottom), n = 8.
Suture injury induced sVegfr-2 expression in corneal epithelium and not in macrophages.

(a) sVegfr2 mRNA detected by in situ hybridization in the wild-type mouse corneal epithelium (SUT (−); top row) and increased staining intensity 4 hours following suture placement (SUT (+); bottom row) (left). Antisense probes (AS) show purple-blue reactivity. Sense RNA probes (S) show negligible reactivity. Sutured corneas exhibited substantially greater immunolocalization (green) of sVegfr-2 protein compared to uninjured corneas (right panels). (b) Immunolocalization of sVegfr-2 protein (red) to the corneal epithelium of sutured corneas, but
not in infiltrating macrophages (F4/80<sup>+</sup> cells, green). Epi, epithelium; Str, stroma \textbf{(a,b)}. Cell nuclei stained blue by DAPI. Scale bars, 50 µm.
sVegfr2 expression is widespread. Northern blotting using poly(A) positive RNA from various mouse organs with a riboprobe targeting the region coding to the extracellular domain of Vegfr-2 (blue) revealed two distinct bands at 6 kb and 4 kb corresponding to mbVegfr2 and sVegfr2. Riboprobe targeting the unique 3’UTR of sVegfr2 (red) detects a single band at 4 kb corresponding to sVegfr2.
**Supplementary Figure 12**

**mbVegfr-2 is not expressed in skin epithelium or hair follicles and Vegf-c is expressed in newborn, but not adult mouse skin.** (a) mbVegfr-2 was immunolocalized (green) in the skin vasculature (arrows), but not in the epithelial cells or hair follicles, using an antibody targeted to the C-terminus of mbVegfr-2. Superficial keratin autofluorescence is observed. (b) Vegf-c was immunolocalized (red) in the newborn wild-type mouse skin (left) but not detected in the adult mouse skin (middle). Immunofluorescence was not observed when anti-Vegf-c antibody was replaced with isotype control IgG (right). Scale bar, 50µm. (a,b) Cell nuclei stained blue by DAPI.
Characterization of anti-human sVEGFR-2 antibody (AA21129) and sVEGFR2 sequence.  

(a) ELISA showed that the custom rabbit antibody against the C-terminus of human sVEGFR-2 (AA21129) specifically detected immobilized immunizing peptide (gretildhcæavgmp; blue) compared to an immobilized control peptide (green). (b) The complete sequence of human sVEGFR2 open-reading frame. Exon boundaries are indicated by vertical lines ( | ). Novel 3' end of exon 13 shown in red.
Supplementary Methods

Generation of Vegfr2-flox mice. The targeting vector for a conditional allele for the Vegfr2 gene was constructed as follows. A 4.8 kb genomic fragment between HindIII and SmaI from the Vegfr2 locus harboring the first exon was subcloned and a single loxP site was introduced into EcoRI site with a disruption of the original site. A 5.7 kb genomic fragment between SmaI and SalI from the Vegfr2 locus was subcloned into the vector mentioned above, followed by the insertion of a construct harboring another loxP site and PGK-neo cassette flanked by frt sites. PGK-HSV-tk cassette was added to the targeting vector at the end. The vector was linearized with NotI and electroporated into R1 ES cells. Correctly targeted ES cell lines (Vegfr2-flox-neo allele) were verified by Southern blot analysis. Chimeric mice were generated by morula aggregation, and males were crossed with ICR (Harland Sprague Dawley) random outbred females. After successful germline transmission of the targeted allele, the PGK-neo cassette flanked by frt sites was removed to establish Vegfr2-flox allele by crossing with an FLPe deleter strain. Deletion was verified by Southern blotting and PCR analysis. To test the deletion of the exon 1 from the Vegfr-2 gene (Vegfr2-del1 allele), Vegfr2-flox heterozygous mice were crossed with a Cre deleter strain (a gift from A. Nagy, Samuel Lunenfeld Research Institute, Toronto, Canada). Deletion was verified by Southern blotting and PCR analysis. We also confirmed that the embryonic phenotype of Vegfr2\textsuperscript{del1/del1} mice was identical to that of Vegfr2\textsuperscript{−/−} mice (data not shown). PCR analysis for genotyping Vegfr2 mutant mice was performed with tail DNA at an annealing temperature of 65 °C with the following primers: Vegfr-2-S1, 5′-TGGAGAGCAAGGCGCTGCTAGC-3′; Vegfr-2-A, 5′-CTTTCCACTCCTGCTACCTAG-3′; and Vegfr-2-S2, 5′-AATTTGGGTGCCATAGCCAATC-3′. The wild-type, flox, del1 alleles gave 322-bp, 439-bp, and 218-bp bands, respectively.

Corneal micropocket angiogenesis assay. VEGF-C pellets containing 160 ng of recombinant human VEGF-C (R&D Systems, 2179-VC-025/CF) was placed into a mouse micropocket as
previously described. Corneas were isolated, pellets were removed and corneal VEGF-C was quantified at 2 and 4 days after pellet placement.

**sVegfr-2 overexpression vector construction.** The cloned coding sequence of sVegfr2 was extracted from agarose gel, sequenced and inserted into a pcDNA3.1 (Invitrogen) overexpression vector (psVegfr-2) according to manufacturer instructions.

**sVegfr-2 enforced expression.** Human embryonic kidney (HEK) 293 cells, Chinese hamster ovary (CHO) cells and mouse corneal epithelial (KEPI) cells were cultured in DMEM (Invitrogen) containing 10% FBS, penicillin G (100 units/ml), streptomycin sulfate (0.1 mg/ml) (all from Sigma Aldrich) at 37 °C, 10% CO2 and 90% room air. Upon attaining 80% confluence the cells were transfected (Lipofectamine 2000, Invitrogen) with psVegfr-2 or an empty pcDNA3.1 plasmid (pNull) in serum-free media. Supernatant fractions from the media were collected 24 h following serum starvation and analyzed for protein content. Similarly, intrastromal corneal injections (20 μg) of psVegfr-2 or pNull were performed *in vivo* for enforced expression studies.

**Corneal Transplantation.** Following anesthesia, donor (C57Bl/6J, 6 to 8 weeks of age) corneal grafts were excised from a central 2-mm corneal button using trephine and Vannas scissors (Inami, Japan). The corneal buttons were kept in cold sterile PBS until the time of transplantation. The recipient corneal graft beds were prepared by similarly removing 1.5-mm corneal button, with trephine and Vannas scissors (Inami, Japan). The transplant recipients were Balb/C mice between 6 and 8 weeks of age. The donor corneas were held in place by 10-12 interrupted 11-0 sutures (Mani, Japan). Antibiotic ointment (Poly-Bac, Akorn) were applied to the corneal surface for 8 days after surgery and sutures were removed 7 days after the procedure. Recombinant Vegfr-2/Fc (10 μg, R&D Systems), IgG/Fc (10 μg, Jackson Immunoresearch) or sVegfr-2 (10 μg) were injected in the corneal bed immediately before transplantation. Post-operative evaluations were performed under a biomicroscope on a weekly basis by 2 independent
examiners. Mice with post surgical complications (i.e. – synechiae, hyphema, cataract, collapsed anterior chamber) were excluded from the study. Transplant survival was determined by an established semi-quantitative method \(^5\) based on the clinical appearance of the grafted corneas: corneas with an opacity score >2 (Moderate stromal opacity, where pupil is visible and iris obscured) lasting for over 8 weeks were considered rejection.

**Corneal angiogenesis assay.** Corneas were transfected \textit{in vivo} \(^6,7\) by intrastromal injections of naked plasmids (4 \(\mu\)g) coding for Vegf-a (Addgene plasmid 10909), Vegf-c (gift of K. Miyazono, University of Tokyo, Japan), Vegf-d (Open Biosystems, 3028644) and VEGF-D (TrueClone, Origene, SC122680). Plasmids coding for sVegfr-2 (psVegfr-2) or pNull (empty plasmid-fellow eye) and Vegfr-2/Fc or sVegfr-2 (5 \(\mu\)g) were co-administered into the cornea at day 0 and day 7. At day 14, mice were euthanatized and angiogenesis was quantified by corneal flat mounts as previously described\(^7\).

**Generation of sVEGFR-2 specific antibodies.** Peptide synthesis was performed from C-terminus to N-terminus using Fmoc chemistry and a solid support resin. Synthesized peptides were purified and examined by MALDI-TOF mass spectrometry and reversed phase HPLC. Purified (GMEASLGDRİAMP, mouse and GRETILDHCAEAVGMP, human) peptides include an N-terminal cysteine for directional conjugation to the carrier protein keyhole-limpet hemocyanin (KLH). Peptide immunogens conjugated to KLH were used to generate immune responses in rabbits (pathogen-free, barrier-raised New Zealand White Rabbits). Immunizations and sera collections were performed using a 79-day immunization protocol, then the sera was purified using proprietary peptide affinity chromatography techniques (ECM Biosciences).

**Enzyme Linked Immunosorbent Assay (ELISA).** Microtiter plates (96-well; Pierce) were incubated with 10 ng/well purified peptides (21127) or control peptide for 2 h at RT. After two washing steps with PBS to remove unbound peptide, plates were blocked with 3% BSA in PBS. Next, purified rabbit antibody targeted against the C-terminus sequence of sVegfr-2 (AA21127)
was added at various dilutions for 1 h. Plates were washed twice with 0.1% Tween 20 in TBS, then incubated for 30 min with donkey anti-rabbit Ig coupled to horseradish peroxidase (1:10,000 in TBS; Jackson Immunoresearch). After washing with 0.1% Tween 20 in TBS, 100 µl of the colorimetric HRP substrate solution (OPD; Pierce) was added to each well. After 5 min, the reaction was stopped with 100 µl of 1 M H₂SO₄, and the plates were read at 650 nm in a microplate reader. ELISAs were also used according to the manufacturer’s instructions to quantify sVegfr-2 (Quantikine, R&D Systems), mouse Vegf-c (E0145m, USCNlife), and human VEGF-C (Quantikine, R&D Systems). Measurements were normalized to total protein (Bio-Rad).

**Immunohistochemistry.** Human donor corneas were obtained from the Utah Lions Eye Bank. Tissues were procured and distributed in compliance with the tenets of the Declaration of Helsinki. Research with human corneas was approved by the University of Utah School of Medicine Institutional Review Board. Deparaffinized sections were incubated with 10% normal goat serum (Vector Laboratories). Endogenous peroxidase and alkaline phosphatase were quenched with 3%H₂O₂ and levamisole (Vector Laboratories), respectively. Immunolocalization was performed with rabbit antibodies against the unique C-terminus of mouse sVegfr-2 (1:500, AA21127) and human sVegfr-2 (1:500, AA21129), rabbit antibody against the C-terminus of mbVegfr-2 (1:100, clone ab2349, Abcam), rabbit antibody against Lyve-1 (1:200, clone ab14917, Abcam), rabbit antibody against Prox1 (1:500, clone 102-PA32S, Angiobio) and rabbit antibody against Vegf-c (1:100, clone sc-25783, Santa Cruz Biotechnology), rat antibody against F4/80 (1:400, Serotec). Biotin-streptavidin-horseradish peroxidase (Vector Laboratories), alkaline-phosphatase (Invitrogen), or immunofluorescence (Alexa Fluor 488 and 594, Invitrogen) methods were used. Counterstain was obtained with hematoxylin (Vector Laboratories) or DAPI (1:25,000, Molecular Probes). Substitution of isotype non-immune IgG for the primary antibody, pre-adsorption of the primary antibody with a ten-fold molar excess of
the immunizing peptide, and omission of the primary antibody were used to assess specificity of staining. Images were visualized under light or fluorescent confocal microscopy (Leica SP-5) and analyzed with ImageJ (NIH). Fluorescent images were thresholded equivalently and simultaneously.

**Corneal flat mounts and skin whole mounts.** Following euthanasia the corneas and skin from the abdomen were isolated, washed in PBS and fixed in 4% paraformaldehyde for 1 h and acetone for 20 min at RT. They were then washed in 0.1% Tween20 in PBS and blocked on 3% BSA in PBS for 48 h. Incubation with rabbit anti-mouse Lyve-1 antibody (Abcam; 1:333) and rat anti-mouse CD31 antibody (BD Biosciences; 1:50) or rat-anti-mouse MECA-32 antibody (BD Biosciences; 1:10) or goat anti-mouse Lyve-1 antibody (R&D, 1:100) and rabbit anti-mouse Prox-1 (Angiobio, 1:500) or rabbit anti-sVegfr-2 (AA21127, 1:200) were performed for 48 h at 4 °C. The tissues were again washed in 0.1% Tween20 in PBS and incubated with Alexa Fluor 488 (goat anti-rabbit; 1:200) and Alexa Fluor594 (goat anti-rat; 1:200) or Alexa Fluor488 (donkey anti-goat; 1:200) (Invitrogen) and Cy3 conjugated donkey anti-rabbit (Jackson ImmunoResearch; 1:400) for 24 h. Tissue mounts were visualized under fluorescent microscopy (Leica SP-5) and analyzed with ImageJ (NIH).

**PCR.** RNA was isolated from mouse cornea and human umbilical vein endothelial cells (HUVECs) using RNAqueous (Ambion) kit according to manufacturer instructions. Reverse Transcriptase PCR was carried out with Taqman (Applied Biosystems) per manufacturer instructions. Amplification of \( sVegfr2 \) fragment encompassing the splicing site (Exon13-Intron13 junction) and the complete ORF was performed with the following primers: 5’-CGAGGAGAGAGGGTCATCTC-3’ (forward) / 5’-CAGGGATGCCTCCCATACC-3’ (reverse) and 5’-GCTCTGTGCCCAGCGCGAGGTGCAGGAT-3’ (forward) / 5’-TGCTCTGCTTCCAGGAGTGTGCCAGCCT-3’ (reverse), respectively. Amplification of the ORF of \( sVegfr2 \) was performed with the following primers: 5’-CTCAACTGTCCCTGCGCTG-3’ (forward) / 5’-CGAGGAGAGAGGGTCATCTC-3’ (reverse).
Amplification of loading control Gapdh was performed with the following primers: 5’-AACTTTGTGAAGCTCATTTCCTGGTAT-3’ (forward) / 5’-CCTTGCGTGGCTGGGTGGT-3’ (reverse). mbVegfr2 primers were proprietary from Maxim Biotech. Positive control was mbVegfr2 cDNA (Maxim Biotech). Vegfc amplification was performed with primers: 5’-GTCTGTGTCCAGCGTAGATG-3’ (forward)/ 5’GCTGGCAGAAGACGTCTAAT-3’(reverse).

**3’-RACE PCR.** 3’RACE ready cDNA was generated from total RNA using the following primer: 5’-AGAGAATTCACCGGATCCTACCCGGGTTTTTTTTTTTTTTTTTTT-3’. Three potential polyadenylation signal sequences at positions 2360-5 (PolyA1), 3165-70 (PolyA2) and 3956-61 (PolyA3) within intron 13 of Vegfr2 were predicted by PolyA SMV 2.1 software.

Primers were designed to encompass each of the three potential sites yielding an approximate 400 bp PCR product. Forward primers were as follow: 5’-

TGGTACAAGCTTGGTCTCACAGGCAACAT-3’ (PolyA1), 5’-

GCCACACTCATGCCTGTACTCCTCTGG-3’ (PolyA2), 5’-

ACTGCAGTTGGGTGATTTTCAGGAGCAC-3’ (PolyA3). Reverse primer was 5’-

GAGAATTCACCGGATCCTAC-3’.

**DNA sequencing.** PCR products were cloned into TOPO TA vector (Invitrogen) and DNA sequencing was performed by the University of Kentucky Advanced Genetic Technologies Center using multi-color fluorescence based DNA sequencer (ABI 3730xl).

**In situ hybridization.** In situ hybridization was performed on cryosections as previously described. Digoxigenin (DIG)-labeled sense and anti-sense riboprobes were transcribed from the mouse sVegfr2 cDNA using the DIG-RNA-labeling kit (Boehringer-Mannheim). The sVegfr-2 probe targeted a 412 bp fragment (encompassing residues 3603 to 4015) at the unique 3’ end of sVegfr2 mRNA. DIG-labeled probes were hybridized, washed and incubated with alkaline
phosphatase-conjugated anti-DIG antibody (1:2000; Boehringer-Manheim) and stained with BM purple (Roche).

**Northern Blotting.** For mRNA detection, commercially prepared membrane from Ambion containing 2 µg of polyA+ RNA isolated from various mouse organs and fractionated by agarose gel electrophoresis prior to transfer to the membrane were used. Membrane was hybridized in NorthernMax hybridization solution (Ambion) with radiolabeled probes targeted at the unique tail of *sVegfr2* transcript (412 bp, encompassing residues 3603 to 4015 of *sVegfr2*) or a common region present in *mbVegfr2* and *sVegfr2* (822bp, encompassing residues 94-916 of *mbVegfr2* and *sVegfr2*) at 42 °C for 12 h, followed by two post-hybridization washes with 2x SSC, 0.1% SDS and two additional washes with 0.2X SSC, 0.1% SDS, all for 30 min each at 42 °C. The blots were then exposed to a Typhoon phosphoimager screen for 3, then 7 d for image development. The radiolabeled probes were prepared with Prime-it labeling kit (Stratagene) using random primers, high specific activity a-32P-dCTP (6,000 Ci/mmole) and 25 ng of isolated PCR fragments for *sVegfr2* according to the manufacturer’s instructions.

**Statistics.** Mean percentage areas occupied by Lyve-1⁺ (lymphatic vessels) or CD31⁺/Lyve-1⁻ (blood vessels) were calculated in skin whole mounts, and, in corneal flat mounts, were calculated by evaluating all vessels interior to the innermost circumferential limbal vessel, using ImageJ software. The number of Prox1⁺ nuclei within Lyve-1⁺ skin lymphatic vessels were counted in 12 random fields and expressed as LEC density per 100 µm. Mann Whitney U test with Bonferroni correction was used for statistical comparison of multiple variables. Comparison of corneal transplant survival was performed by Kaplan-Meier Survival. The null hypothesis was rejected at *P < 0.05.*
Supplementary Reference List


