Inhibition of Lymphangiogenesis and Hemangiogenesis in Corneal Inflammation by Subconjunctival Prox1 siRNA Injection in Rats

Chang Rae Rho,1,2 Jun-Sub Choi,2 Minkoo Seo,3 Suk Kyeong Lee,3 and Choun-Ki Joo2,4

1Department of Ophthalmology and Visual Science, Daejeon St. Mary’s Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea
2Catholic Institute for Visual Science, College of Medicine, The Catholic University of Korea, Seoul, Korea
3Department of Medical Lifescience, College of Medicine, The Catholic University of Korea, Seoul, Korea
4Department of Ophthalmology and Visual Science, Seoul St. Mary’s Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea

Correspondence: Choun-Ki Joo, Department of Ophthalmology and Visual Science, Seoul St. Mary’s Hospital, College of Medicine, The Catholic University of Korea, #222 Banpo-daero, Seocho-gu, Seoul 137-701, Republic of Korea; ckjoo@catholic.ac.kr.
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PURPOSE. Prospero homeobox 1 (Prox1) siRNA is a small interfering RNA that is designed to specifically bind Prox1 mRNA. We determined whether Prox1 siRNA inhibits lymphangiogenesis and hemangiogenesis after acute corneal inflammation.

METHODS. Three Prox1 siRNAs were synthesized and investigated for their effects on Prox1 mRNA expression and tube formation in human dermal lymphatic endothelial cells (HDLECs) in vitro. The in vivo effects of Prox1 siRNA were assessed in alkali burn-induced inflammatory corneal neovascularization in rats. Prox1 siRNA was administered via subconjunctival injection. Corneal flat mounts were stained for lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 to show lymphatic vessels. Lymphangiogenesis and hemangiogenesis were analyzed morphometrically using Image J software. Corneal inflammatory cell infiltration was evaluated by immunostaining for F4/80 and CD45. Protein levels of LYVE-1, podoplanin, VEGF receptor 2 (VEGFR2), and VEGFR3 were analyzed by Western blotting.

RESULTS. Prox1 siRNA treatment decreased Prox1 mRNA expression and tube formation in cultured HDLECs. Subconjunctival injection of Prox1 siRNA significantly inhibited alkali burn-induced lymphangiogenesis and hemangiogenesis in the cornea compared with those of scrambled siRNA (negative control). This inhibition was comparable to that induced by bevacizumab (positive control). Prospero homeobox 1 knockdown by Prox1 siRNA also inhibited macrophage and leukocyte infiltration into the cornea. Prox1 siRNA downregulated the expression of all four proteins.

CONCLUSIONS. Prox1 siRNA is a strong inhibitor of inflammatory corneal lymphangiogenesis and hemangiogenesis in vivo. Prox1 siRNA may be useful in preventing immune rejection after penetrating keratoplasty by suppressing lymphangiogenesis.

Keywords: cornea, lymphangiogenesis, Prox1

The healthy cornea is devoid of blood and lymphatic vessels and actively sustains an avascular state.1 However, various inflammatory conditions or diseases can break down this status and cause pathologic corneal hemangiogenesis and lymphangiogenesis, which lead to loss of transparency and decreased visual acuity.2 This pathologic process involves enzymatic degradation and remodeling of the extracellular matrix and capillary endothelial cell migration and proliferation.3 Stimulation by angiogenic factors, including VEGF and FGF, triggers proliferation and migration of vascular endothelial cells into surrounding tissues.4 Although keratoplasty is required for a vascularized cornea, new hemangiogenesis and lymphangiogenesis increase graft rejection following keratoplasty. Postoperative blockade of VEGF using the VEGF trap had a beneficial effect of reducing the ingrowth of blood and lymphatic vessels and improving graft survival after low-, intermediate-, and high-risk keratoplasty.5–7 The relative contribution of blood and lymphatic vessels in rejection has been explored. The results showed that sole blockade of lymphangiogenesis improves graft survival as effectively as preventing both vessel types, which suggests that lymphatic vessels are more influential than blood vessels during corneal graft rejection.1,8

Treatments for corneal neovascularization include topical corticosteroids, photocoagulation, conjunctival resection, β-irradiation, cauteronization, topical ascorbic acid, and cryotherapy.9,10 However, none of these options completely resolves neovascularization, and some increase the risk of cataract or glaucoma.9 Anti-VEGF treatments, which inhibit hemangiogenesis, block lymphangiogenesis and can also delay epithelial and stromal wound healing and cause neurotoxicity.11,12

Gene silencing by small interfering RNA (siRNA) allows effective control over posttranscriptional gene regulation. Small interfering RNA interferes with the expression of specific genes via a complementary nucleotide sequence; binding to mRNAs induces cleavage of the mRNA. Prospero homeobox 1 (Prox1) is a homeobox transcription factor essential for the develop-
ment and maintenance of lymphatic vasculature. Prospero homeobox 1 knockout in embryonic mice results in a loss of lymphangiogenesis without affecting hemangiogenesis. The specific effects of Prox1 siRNA on corneal lymphangiogenesis have not yet been explored. In this study, we evaluated the effects of subconjunctivally administered Prox1 siRNA in a rat model of alkali burn-induced inflammatory corneal neovascularization.

METHODS

Materials

Three Prox1 siRNAs were synthesized by Genolution Pharmaceutical, Inc. (Seoul, Korea). The sequences are listed in the Table. Bevacizumab (Avastin, Roche, Basel, Switzerland) was used as a positive control, and scrambled siRNA (Genolution Pharmaceutical, Inc.) was used as a negative control for the anti-lymphangiogenic effects. Liposomes (BPM-LPS, Bioplymed, Seoul, Korea) were used to stabilize the Prox1 and scrambled siRNAs.

Cell Culture

Human dermal lymphatic endothelial cells (HDLECs) (C-12217, Promocell GmbH, Heidelberg, Germany) were grown on attachment factor-coated plates in M199 medium supplemented with 20% fetal bovine serum, 3 ng/mL basic FGF (Sigma-Aldrich Corp., St. Louis, MO, USA), and 10 U/mL heparin (Sigma-Aldrich Corp.). Human dermal lymphatic endothelial cells were used at passages 4 to 6. Human dermal lymphatic endothelial cells (1 × 10⁴) were inoculated on Matrigel-coated plates in serum-free M199 supplemented with 1% (vol/vol) penicillin-streptomycin. All cells were treated with VEGF (20 ng/mL, Sigma-Aldrich Corp.). Three experimental groups were treated with the respective Prox1 siRNA (10 μM), whereas control cells were treated with scrambled siRNA (10 μM). Formation of tube-like structures was observed under a phase-contrast microscope and quantified by counting the number of tubes formed in three randomly chosen fields using ImageJ v.1.47 image analysis software (National Institutes of Health, Bethesda, MD, USA).

Animals

Eight-week-old male Sprague–Dawley rats (weight, 250–300 g) were used. The experimental, positive-control, and negative-control groups each comprised 15 animals per procedure. The animals were treated according to the regulations of the Ethics Committee of the Catholic University of Korea, Seoul, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All efforts were made to minimize suffering and the number of animals used.

Induction of Corneal Neovascularization (CNV)

Corneal neovascularization was induced by alkali injury using a method described previously with minor modifications. In brief, animals were anesthetized with an intraperitoneal injection of zolazepam (30 mg/kg) and Xylazine (10 mg/kg), supplemented with topical anesthesia (0.5% proparacaine hydrochloride, Alcaine eye drops, Alcon, Inc., Fort Worth, TX, USA). Under a surgical microscope, circular filter paper (3.0-mm diameter), soaked with 4 μL of 0.5 M sodium hydroxide, was placed in the central cornea of the right eye for 30 seconds. The alkali-burned area and conjunctival sac were rinsed with 0.9% saline (10 mL) for 1 minute. The dose-dependent response was examined using subconjunctivally administered Prox1 siRNA (Prox1-1) at 1, 5, 10, and 20 μM. Lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 staining showed dose-dependent inhibition of lymphatic vessels, with 10 μM as the lowest dose showing the most effective inhibition of lymphangiogenesis. This dose was used in subsequent experiments (Supplementary Fig. S1).

Animal Treatment

Immediately following the alkali injury, rats were randomly assigned to one of the three groups (15 rats per group). Rats received Prox1 siRNA (20 μL), bevacizumab (20 μL, 25 mg/mL), or scrambled siRNA (20 μL) by subconjunctival injection.

| TABLE. Three Prox1 siRNAs Were Synthesized and Assessed in Terms of Their Effects on Prox1 mRNA Expression |
| Name | Sequence (5’ to 3’) |
| Prox1-1 | GAGUGGACAUUGAGAGAAUU |
| Prox1-2 | ACAAAGGCAGCAGCAAAGCUU |
| Prox1-3 | GAUGGUAACCUGUCAGAAUU |

Tube Formation Assay

The inhibitory function of Prox1 siRNAs on the lymphangiogenesis of HDLECs was determined in tube formation experiments using the MILLIPORE In Vitro Angiogenesis Kit (MILLIPORE, Billerica, MA, USA), according to the manufacturer’s protocol. Ninety-six-well plates were coated with cold liquid ECMatrix (70 μL/well) and incubated at 37°C in a humidified 5% CO₂ incubator for 1 hour to promote solidification. Human dermal lymphatic endothelial cells (5 × 10⁴) were seeded into 96-well plates with polymericized ECMatrix and incubated with conditioned media at 37°C for 4 to 6 hours. Three experimental groups were treated with the respective Prox1 siRNA (10 μM), whereas control cells were treated with scrambled siRNA (10 μM). Formation of tube-like structures was observed under a phase-contrast microscope and quantified by counting the number of tubes formed in three randomly chosen fields using ImageJ v.1.47 image analysis software (National Institutes of Health, Bethesda, MD, USA).
Ofloxacin ophthalmic ointment (Ocuflox, Samil, Seoul, Korea) was administered postoperatively.

**Corneal Neovascularization Assay**

Corneal neovascularization was observed 7 days after alkali burning, and photographs were taken using a digital camera (NEX5, SONY, Tokyo, Japan) with identical focus and exposure through the eyepiece of a NIKON SMZ-645 (Nikon Corp. Instruments Co., Tokyo, Japan) dissecting microscope at 20× magnification (15 corneas per group). Each photograph was analyzed at the same magnification using ImageJ software. The innermost new vessel in the cornea was outlined, and the area between the border and the limbus was defined as the vascularized area. The degree of neovascularization was determined by dividing the vascularized area by the total corneal area (measured in pixels). Photographs were analyzed in random order by two double-blinded investigators to minimize observer bias.

**Corneal Flat Mounts and Morphologic Analysis of Lymphangiogenesis**

One week after the alkali burn treatment, rats were euthanized, and their eyes were collected. Enucleated eyes were fixed in 10% formalin. Five corneas in each group were used for flat mounts. Fixed tissues were stained overnight at 4°C with rabbit anti-mouse LYVE-1 antibody (1:500; Abcam, Eugene, OR, USA). Tissues were then washed three times, and LYVE-1 was detected using a Texas Red-conjugated secondary goat anti-rabbit antibody (1:100; Abcam). After three additional washes in PBS, all corneas were transferred to Superfrost slides (Menzel-Gläser, Braunschweig, Germany), covered with Dako mounting medium (Hamburg, Germany), and stored at 4°C in the dark. Stained whole mounts were viewed under a fluorescence microscope (Axio Observer Z1, Carl Zeiss GmbH, Jena, Germany), and digital pictures were taken with a 14-bit monochrome charge coupled device camera (Axiocam HR, AxioVision, Carl Zeiss). The area covered by lymphatic vessels was determined using the ImageJ software.

**Immunostaining**

Five formalin-fixed corneas in each group were embedded in paraffin and sectioned at 4 μm for routine histologic processing. To observe inflammatory cells and angiogenesis in detail, sections were stained using an anti-CD45 antibody (leukocyte marker, dilution 1:100, BD Biosciences, Franklin Lakes, NJ, USA), anti-F4/80 antibody (macrophage marker, dilution 1:200, Abcam), and anti-phalloidin antibody (to detect F-actin; dilution 1:500, Abcam) for 16 hours at 4°C. After three washes with PBS for 15 minutes, antibody-stained sections were stained using a Texas Red–conjugated secondary antibody (Abcam) for 1 hour, washed three times with PBS, and counterstained. Stained sections were examined by fluorescence microscopy (Axio Imager 2, Carl Zeiss GmBH) at 100× magnification.

**Western Blot Analysis of Corneal Proteins**

The protein levels of LYVE-1, podoplanin, VEGF receptor 2 (VEGFR2), and VEGFR3 1 week after the alkali burn treatment were evaluated by Western blotting. Three groups were included to compare across groups and experimental conditions: normal cornea without any treatment, normal cornea with bevacizumab injection (negative control for bevacizumab), and alkali burn without treatment. Five corneas were pooled from each group. Briefly, excised corneas were homogenized in 100 mL lysis buffer (Proprep Protein...
Extraction Solution, INTRON Biotechnology, Sungnam, Korea) using a Precellys 24 bead–based homogenizer (Bertin Technologies, Villeurbanne, France). Proteins (15 μg protein per sample) were electrophoresed on a 10% SDS-polyacrylamide gel. Proteins were electroblotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), which were blocked using 3% BSA solution. The membranes were incubated using antibodies against rat LYVE-1, podoplanin, VEGFR2, or VEGFR3. After washing with Tris-buffered saline containing 0.05% Tween (TBST), the blots were incubated with the respective secondary peroxidase-labeled antibody for 1 hour at room temperature, washed four times with TBST, and processed for chemiluminescent detection of immunoreactive proteins using a peroxidase substrate (Lumigen PS-3, Lumigen, Southfield, MI, USA).

**Statistical Analysis**

The Kruskal–Wallis test was performed for multiple comparisons among groups, and SPSS 17.0 (SPSS, Chicago, IL, USA) was used for all analyses. A value of \(P < 0.05\) was considered statistically significant.

**RESULTS**

**Prospero Homeobox 1 siRNA Inhibits Prox1 Expression in HDLECs**

We first investigated the effect of Prox1 siRNA on HDLECs by performing real-time PCR to compare Prox1 mRNA expression. As demonstrated in Figure 1, all Prox1 siRNAs significantly suppressed VEGF-induced Prox1 mRNA expression in HDLECs (\(P < 0.05\)). Prospero homeobox 1-1 was the most effective for inhibiting Prox1 mRNA expression.

**Prox1-1 siRNA Suppresses HDLEC Tube Formation**

We confirmed the anti-lymphangiogenic effect of Prox1 siRNA by performing an in vitro tube formation assay. When the extent of tube formation was assessed, a rich network of tubular structures in HDLECs was observed in the scrambled siRNA groups. Prospero homeobox 1 siRNA treatment significantly impaired the tube forming activity of HDLECs compared with the scramble control (Fig. 2). Quantitative analysis demonstrated that Prox1-1 exerted the greatest inhibitory effect on tube formation (approximately 67%). Based on real-time PCR and tube formation experiments, Prox1-1 was used in subsequent in vivo experiments.

**Prospero Homeobox 1-1 Suppresses Alkali Burn–Induced Corneal Hemangiogenesis and Lymphangiogenesis**

Next, we investigated the effect of subconjunctivally injected Prox1-1 on inflammation-triggered growth of blood and lymphatic vessels. On day 7 after alkali injury, Prox1-1–treated corneas displayed significantly less neovascularization than that of scrambled siRNA-treated corneas; the Prox1-1 effects were similar to those of bevacizumab (vascularized area of the cornea: scrambled siRNA, 47.9 ± 13.4%; Prox1-1, 26.6 ± 8.9%; bevacizumab, 20.5 ± 9.8%) (\(P = 0.005\); Fig. 3). Lymphatic vessel endothelial hyaluronan receptor-1–stained corneal flat mounts exhibited inhibited lymphangiogenesis in Prox1-1 and bevacizumab-treated corneas compared with those treated with scrambled siRNA (relative area to the negative control: scrambled siRNA, 100.0%; Prox1-1, 43.0 ± 14.4%; bevacizumab, 51.3 ± 19.2%) (\(P = 0.19\); Fig. 4). Phalloidin immunostaining identified more new vessel walls in scrambled siRNA–treated corneas than in those treated with Prox1-1 or bevacizumab (Supplementary Fig. S2).

**Inflammatory Cell Infiltration Was Decreased by Subconjunctivally Injected Prox1-1**

To investigate the effect the subconjunctivally injected Prox1-1 on corneal inflammation, we immunostained corneas for CD45 and F4/80 to detect leukocyte and macrophage infiltration, respectively, after alkali burns. CD45 staining showed significantly fewer leukocytes in Prox1-1–treated corneas compared with those treated with scrambled siRNA (relative area to the negative control: scrambled siRNA, 100.0%; Prox1-1, 43.0 ± 14.4%; bevacizumab, 51.3 ± 19.2%) (\(P = 0.19\); Fig. 4). Phalloidin immunostaining identified more new vessel walls in scrambled siRNA–treated corneas than in those treated with Prox1-1 or bevacizumab (Supplementary Fig. S2).
treated corneas compared with those treated with scrambled siRNA (scrambled siRNA, 17.6 ± 4.2 cells per field; Prox1-1, 9.0 ± 3.4 cells per field; bevacizumab, 8.2 ± 3.1 cells per field) (P = 0.025; Fig. 6).

Prox1-1 Decreases LYVE-1, Podoplanin, VEGFR2, and VEGFR3 Expression in the Alkali Burn Cornea Model

We investigated the mechanism by which Prox1-1 inhibits corneal lymphangiogenesis and hemangiogenesis after alkali burns by performing Western blotting for LYVE-1, podoplanin, VEGFR2, and VEGFR3. Low LYVE-1 expression was found in normal rat cornea with or without bevacizumab administration, whereas its expression increased considerably after alkali burns in the scrambled siRNA group. LYVE-1 expression was lower in the Prox1-1 and bevacizumab treatment groups than in the scrambled siRNA group. Identical protein expression patterns were found for podoplanin, VEGFR2, and VEGFR3 (Fig. 7).

DISCUSSION

In this study, we demonstrated that Prox1 siRNA could inhibit alkali burn-induced corneal lymphangiogenesis. The mean area of corneal lymphangiogenesis was significantly smaller in Prox1 siRNA-treated eyes than in those treated with scrambled siRNA; this pharmacologic effect was comparable to that of bevacizumab. Moreover, Prox1 siRNA inhibited corneal hemangiogenesis compared with the negative control, which was unexpected. The neovascularized area was 55% of that in negative control eyes. Prox1 siRNA treatment decreased HDLEC transcription of Prox1, which is known to be important in lymphangiogenesis, and reduced HDLEC tube formation. The in vivo and in vitro results demonstrate that blocking Prox1 reduces corneal lymphangiogenesis.

Previous studies of ocular angiogenesis focused mostly on hemangiogenesis. This focus likely resulted from the fact that new vessels filled with red blood cells are more easily identified than transparent lymphatic vessels and may relate to the recent introduction of anti-VEGF antibodies, which have proven effective in inhibiting new blood vessels in the retina.18 Research on lymphatic vessels has been limited because no appropriate markers for transparent lymphatic vessels have been identified. However, neovascularization in the cornea is more strongly dependent on immune processes compared with that in the retina or choroid. Lymphatic vessels serve as the afferent limb of the immune response; studies of graft rejection after penetrating keratoplasty have shown that vascular lymphatic grafts are less likely to be rejected than those with new lymphatic vessels.8 Thus, we aimed to develop an effective means of inhibiting the growth of afferent lymphatic vessels.

Existing methods to suppress the growth of lymphatic vessels include corticosteroid and anti-VEGF antibodies, including bevacizumab and ranibizumab.12,19–21 An earlier study demonstrated that corticosteroids are strong inhibitors of inflammatory lymphangiogenesis. The main mechanism was proposed to be suppression of macrophage infiltration and proinflammatory cytokine expression and direct inhibition of proliferation of lymphatic endothelial cells.19 However, ste-
Steroids as therapy for eye diseases remain controversial because of their adverse effects, including glaucoma and decreased wound healing. Therefore, steroids are not used to treat inflammatory angiogenesis accompanied by an epithelial defect. Early corneal epithelial healing is important because the corneal epithelium expresses a soluble VEGF-A receptor. The soluble VEGF-A receptor functions as a decoy receptor for secreted VEGF and inactivates membrane-bound VEGF-A receptor 1 and 2 by heterodimerization. Potential side effects of anti-VEGF treatment include delayed epithelial and stromal wound healing, as well as neurotoxic effects.

In contrast to these anti-lymphangiogenic agents, siRNA is a genetic therapy that acts exclusively on the target gene. The first objective of the present study was to investigate the inhibitory potential of Prox1 siRNA on lymphatic vessels. Prospero homeobox 1 is a key player in lymphatic vasculature formation, acting as a master switch to reprogram blood vascular endothelium toward a lymphatic endothelial fate. Prospero homeobox 1 induces VEGFR3 expression, which is critical in lymphangiogenesis, because it binds to the pro-lymphangiogenic growth factor VEGF-C. For this reason, we chose to target Prox1 using Prox1 siRNA to investigate its role in experimental corneal lymphangiogenesis. Our results indicate that Prox1 knockdown significantly suppressed lymphangiogenesis by 62% in vivo. Inhibition of lymphangiogenesis was further confirmed by the protein assays for LYVE-1, podoplanin, and VEGFR3.

The in vivo experiments demonstrated decreased cellular infiltration on Prox1 siRNA treatment. Recently, Maruyama et al. reported the role of podoplanin in lymphangiogenesis and macrophage functions. They demonstrated that podoplanin neutralization by anti-mouse podoplanin antibody inhibited lymphatic growth and macrophage infiltration. The results of our study and those of Maruyama et al. suggest that lymphatic markers such as Prox1 or podoplanin are promising targets to suppress lymphangiogenesis and inflammation.

The possible linkage mechanism for the anti-inflammatory action of Prox1 siRNA could be angiopoietin-2 (Ang-2). A recent study found Ang-2 to be a target of Prox1. The effect of Ang-2 on inflammatory cell infiltration was investigated by inserting Ang-2-soaked polyvinyl alcohol sponges into mice. The study demonstrated that Ang-2–treated sponges promoted neutrophil and macrophage recruitment compared with PBS-treated sponges. Therefore, we hypothesize that Prox1 siRNA treatment decreases inflammatory cell infiltration via suppressing Ang-2. In addition, Ang-2 is an important modulator of angiogenesis. As shown in Figure 3, corneas treated with Prox1 siRNA were more transparent and contained fewer new vessels than those of control alkali-burned corneas. Several investigations suggested that Ang-2 promotes neovascularization in conjunction with VEGF. In contrast, other reports demonstrated that Ang-2 can promote angiogenesis in the absence of VEGF.
blockade inhibited hemangiogenesis induced by fibroblast growth factor implantation. Yuen et al. demonstrated that corneal lymphangiogenesis was almost abolished and hemangiogenesis was significantly suppressed in Ang-2 knockout mice. Therefore, suppression of Prox1 expression by Prox1 siRNA may have inhibited the expression of Ang-2 in inflammatory alkali-burned corneas, leading to reduced hemangiogenesis.

Figure 6. Prospero homeobox 1-1 siRNA inhibits F4/80(+) leukocyte infiltration after corneal alkali burns. Immunostaining analysis for F4/80 in the corneal stroma in the scrambled siRNA- (A–C), Prox1-1- (D–F), and bevacizumab-treated eyes (H–J). Quantification of infiltrating macrophages per field (J) confirmed these results; scrambled siRNA (17.6 ± 4.2), Prox1-1 (9.0 ± 3.4), and bevacizumab (8.2 ± 3.1). F4/80(+) leukocyte infiltration in Prox1-1- and bevacizumab-treated corneas did not differ significantly. Scale bars: 100 μm.

Figure 7. Effect of Prox1-1 siRNA on LYVE-1, podoplanin, VEGFR2, and VEGFR3 expression after corneal alkali burns. Western blotting demonstrated that LYVE-1, podoplanin, VEGFR2, and VEGFR3 were expressed at low levels in normal rat cornea. A significant increase in LYVE-1, podoplanin, VEGFR2, and VEGFR3 was observed after alkali burns in the scrambled siRNA group. All four proteins were downregulated in the Prox1-1 and bevacizumab groups compared with the scrambled siRNA group.
Lymphangiogenesis and Hemangiogenesis Inhibition

In our study, subconjunctival Prox1 siRNA treatment did not induce any significant side effects, including weight loss, infection, or decreased physical activity. Blocking of VEGFR-3 signaling by a VEGFR-3/immunoglobulin significantly suppressed migration of corneal antigen presenting cells to the corresponding lymph node and decreased corneal transplant rejection. Our results suggest that Prox1 siRNA could be used to improve graft survival following penetrating keratoplasty by suppressing the growth of lymphatic vessels and breaking the adaptive immune arc. Further investigations will determine whether inhibition of lymphangiogenesis by Prox1 siRNA actually promotes graft survival.

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