Semaphorin 3F Modulates Corneal Lymphangiogenesis and Promotes Corneal Graft Survival

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Purpose. Corneal vascularization significantly increases the risk for graft rejection after keratoplasty. Semaphorin 3F (Sema3F) is a known modulator of physiologic avascularity in the outer retina. The aim of this study was to investigate whether Sema3F is involved in maintaining corneal avascularity and can reduce the risk for corneal graft rejection.

Methods. Corneal Sema3F expression was investigated using immunohistochemistry and qPCR in human and murine tissue. Pathologic invasion of blood and lymph vessels into corneal tissue was analyzed in the murine corneal suture and high-risk keratoplasty model. The anti-lymphangiogenic effects of Sema3F were further investigated using an in vitro spheroidal sprouting model with supernatant from isolated primary human corneal epithelial cells (hCECs).

Results. Sema3F is constitutively expressed in human and murine corneal epithelium. In the corneal suture model, lymphangiogenesis was significantly suppressed by topical Sema3F treatment ($P = 0.0003$). In the murine high-risk keratoplasty model, pretreatment by topical Sema3F in the inflammation phase significantly promoted subsequent graft survival ($P = 0.0006$). In this model, both lymph- and blood angiogenesis were reduced ($P < 0.05$). In vitro, hCEC supernatant had a direct anti-lymphangiogenic effect on human lymphatic endothelial cells ($P < 0.01$). This effect was completely abolished by addition of anti-Sema3F antibodies.

Conclusions. Sema3F is a novel mediator of corneal avascularity with potent anti-lymphangiogenic properties. Topical treatment with Sema3F eye drops may help to limit corneal vascularization and improve outcomes in high-risk keratoplasty patients.

Keywords: semaphorin, sema3F, cornea, vascularization, angiogenesis, lymphangiogenesis

High-risk keratoplasties are characterized by a prevascularized recipient cornea, which increases the rate of graft rejection.[1] These high-risk vascular beds contain not only visible blood vessels but also clinically invisible lymphatic vessels.[2] It is therefore of significant interest to suppress both blood as well as lymph vessel ingrowth into the normally avascular corneal tissue prior to keratoplasty. One known mediator of physiologic avascularity in the eye is Semaphorin 3F (Sema3F).[3,4] Sema3F is a member of the class 3 family of semaphorins. Class 3 semaphorins are soluble ligands of the neuropilin and plexin receptors and were originally described for their role in axon guidance during neuronal development.[5,6] During axonal path finding, class 3 semaphorins exert an axorepellent effect, steering the axonal growth cone away from the semaphorin source.[7] It was later discovered that the repellent properties of semaphorins are not limited to axons. Through induction of cytoskeletal collapse and retraction of cell protrutions, most semaphorins (including Sema3F) exert antiangiogenic properties.[8–10]

In the eye, various semaphorins have been described as having crucial effects during development.[11,12] One of their main functions is to support correct organization of the neuronal layers in the retina. In the mature eye, however, many semaphorins become downregulated. Sema3F, in contrast, remains constitutively expressed in the photoreceptor and RPE layers of the retina.[3] Importantly, these layers are physiologically avascular. Neither vessels from the retinal vasculature directly above nor from the choroidal vasculature directly below enter this avascular niche of the outer retina.[3,4] Similar to the outer retina, the cornea is a physiologically avascular ocular tissue. We therefore asked, whether Sema3F could play a role in maintaining corneal avascularity. Different from the retina, both blood and lymph vessels have to be considered when investigating corneal avascularity.[13] Corneal invasion by lymphatic vessels in particular was found to play a decisive role in inducing corneal allograft rejection, probably through altered immune response.[14] In avascular, so called normal risk recipients, corneal graft survival rate is approximately 90%.[15,16] This changes dramatically with ingrowth of vessels. Vascularized corneal tissue represents a high-risk situation in which graft survival rates drop below 50% to 0%.[17,18] In this study, we therefore analyzed corneal expression patterns of Sema3F in vitro and in vivo and investigated the role of Sema3F during corneal inflammatory hem- and lymphangiogenesis as well as in a high-risk murine keratoplasty model. Our results demonstrate that Sema3F is physiologically expressed in

both murine and human corneal epithelium and that Sema3F significantly inhibits corneal invasion of lymphatic vessels resulting in significantly improved graft survival following high-risk keratoplasty.

Methods

Mouse Model for Suture Induced Inflammatory Corneal Neovascularization

For this assay female BALB/c mice (6–8 weeks of age) were used. The protocols were in accordance with the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research and institutional guidelines regarding animal experimentation were followed.

Prior to surgery, each animal was deeply anesthetized with an intramuscular injection of Ketanest (8 mg/kg; Pfizer, Berlin, Germany) and Rompun (0.1 mL/kg; Bayer, Leverkusen, Germany). Three 11-0 nylon sutures (Serag Wiessner, Naila, Germany) were placed intrastromally each extending over 120° of corneal circumference. The outer point of entry was near the limbus, and the inner exit point was the corneal center equidistant from limbus to obtain standardized angiogenic responses in three parts of the cornea. Sutures were left in place for the duration of the experiment. The operated mice were randomized. The treatment group ($n = 10$) received recombinant mouse Semaphorin 3F (R&D Systems, Minneapolis, MN, USA) as topical drops (50 μg/drop) three times daily for 14 days while the control group ($n = 9$) received saline eye drops. Eye drops were prepared one day prior to the experiment by resolving Sema3F in sterile PBS with 1% BSA. Eye drops were aliquoted into daily doses and stored at 4°C until use. Eye drop application was blinded. All mice were euthanized after 2 weeks.

Corneal Whole Mounts and Morphologic Assessment of Hemangiogenesis and Lymphangiogenesis

Lymphatic vessels were stained with rabbit anti-mouse LYVE-1 (1:200, AngioBio, San Diego, CA, USA) and blood vessels were stained with an anti-CD-31-FITC (1:100, Acris Antibodies GmbH, Hiddenhausen, Germany) antibody. LYVE-1 was detected with a Cy3-conjugated secondary antibody. Isotype control was assured with a FITC-conjugated normal rat2A IgG for CD31-FITC and with a normal rabbit IgG (both Santa Cruz Biotechnology, Santa Cruz, CA, USA) for LYVE-1. Quantification analysis was performed using multiple digital images that were automatically assembled from the flatmounts using Olympus BX53 fluorescence microscope (Olympus, Hamburg, Germany) and Cell^f Software (Olympus) as described previously.[19] In brief, CD31 positive structures were defined as blood vessels, while Lyve-1–positive structures bigger than 300 pixels were defined as lymphatic vessels. Areas covered by vessels were quantified and normalized to total corneal area.

To quantify corneal macrophages, LYVE-1–positive cell number was obtained using Cell^f software. First, the area of lymphatic vessels was substracted from the overall LYVE-1–positive signal in the cornea. The resulting area represents the area covered by LYVE-1 macrophages. This value was divided by the average area covered by a single LYVE-1–positive cell to calculate the total number of macrophages.

Corneal Transplantation

In order to prepare a high-risk environment for corneal graft rejection, the recipient corneas were pretreated by placing three interrupted 11-0 nylon sutures (Serag Wiessner, Naila, Germany) in corneal stromas of 6- to 8-week-old BALB/c mice. The sutures were left in place for 2 weeks. During that time, the treatment group ($n = 10$) received recombinant mouse Sema3F (R&D Systems) as topical drops (50 μg/drop) three times daily while the control group ($n = 9$) received saline eye drops. After 14 days, Sema3F treatment was stopped, sutures removed and penetrating corneal transplantation performed as previously described.[20] Briefly, donor corneas were excised by marking the central cornea with a 2.0-mm trephine and cut with curved Vannas scissors. Until grafting, corneal tissue was placed in chilled PBS. Recipients were anesthetized as above and the graft bed was prepared by trephining a 1.5-mm site in the central cornea of the right eye and discarding the excised cornea. The donor cornea was applied to the bed and secured in place with eight interrupted 11-0 sutures. Antibiotic ointment (Refobacin; Merck KGaA, Darmstadt, Germany) was placed on the corneal surface and the eyelids were closed with 8-0 suture (Serag Wiessner, Naila, Germany). To study graft survival, tarsorrhaphy and corneal sutures were removed after 7 days. Groups were blinded and grafts were then examined weekly until week 8 after transplantation by slit-lamp microscopy and scored for opacity by two investigators (score 0 = clear cornea, 1 = minimal superficial opacity, 2 = minimal deep [stromal] opacity pupil margin and iris vessels visible], 3 = moderate stromal opacity [only pupil margin visible], 4 = intense stromal opacity (only a portion of pupil margin visible], 5 = maximum stromal opacity, anterior chamber not visible) as described previously.[20] Grafts with opacity scores more than 2 were considered as rejected.

FACS Analyses

In the high-risk keratoplasty model, draining submandibular lymph nodes were harvested at respective time points and cells were isolated. Erythrocytes were lysed using red blood cell lysis buffer and cell suspensions were washed with flow cytometry buffer containing 1% fetal bovine serum (FBS) and 20 μM HEPES. Cell suspensions were stained extracellularly for CD3 (Pe-Cy7; Biolegend, San Diego, CA, USA), CD4 (APC; eBioscience, Waltham, MA, USA), CD8 (APC-Cy7; Biolegend), CD11c (PE; Biolegend), CD11b (Pe-Cy7; Biolegend), CD25 (PE; Biolegend), and CD45 (APC; Biolegend) for 30 minutes on ice. Some samples were then fixed and permabilized using eBioscience Fix/Perm kit and stained for intracellular Foxp3 (FITC, eBioscience). Data were acquired by Canto I (BD Bioscience, San Jose, CA, USA) and analyzed using FlowJo (v.10; Flowjo, Ashland, OR, USA).

Cell Culture

After obtaining informed consent, primary human corneal epithelial cells (hCEC) were isolated form human donor corneas from the Eye Bank of the Eye Center, Freiburg Medical Center and cultured in MEM/Earls (10% fetal calf serum, 1% L-glutamin, und 1% penicillin/streptomycin). For immunohistochemistry, cells were cultured on coverslips with fibronectin coating. Supernatant was collected from confluent cells and stored at −80°C. Human lymphatic endothelial cells (hLEC) were purchased from Science Cell (Berlin, Germany) and cultured in EBM Medium with growth supplements.

Spheroid Sprouting Assay

HLECs from passage 1 to 4 were used for spheroid sprouting experiments. The preparation of endothelial cell spheroids was performed as described previously.[21] Briefly, cells were harvested from subconfluent monolayers by trypsinization and suspended in medium containing 10% FBS and 0.25% (wt/vol) carboxymethylcellulose (Sigma, Taufkirchen, Germany). Five hundred

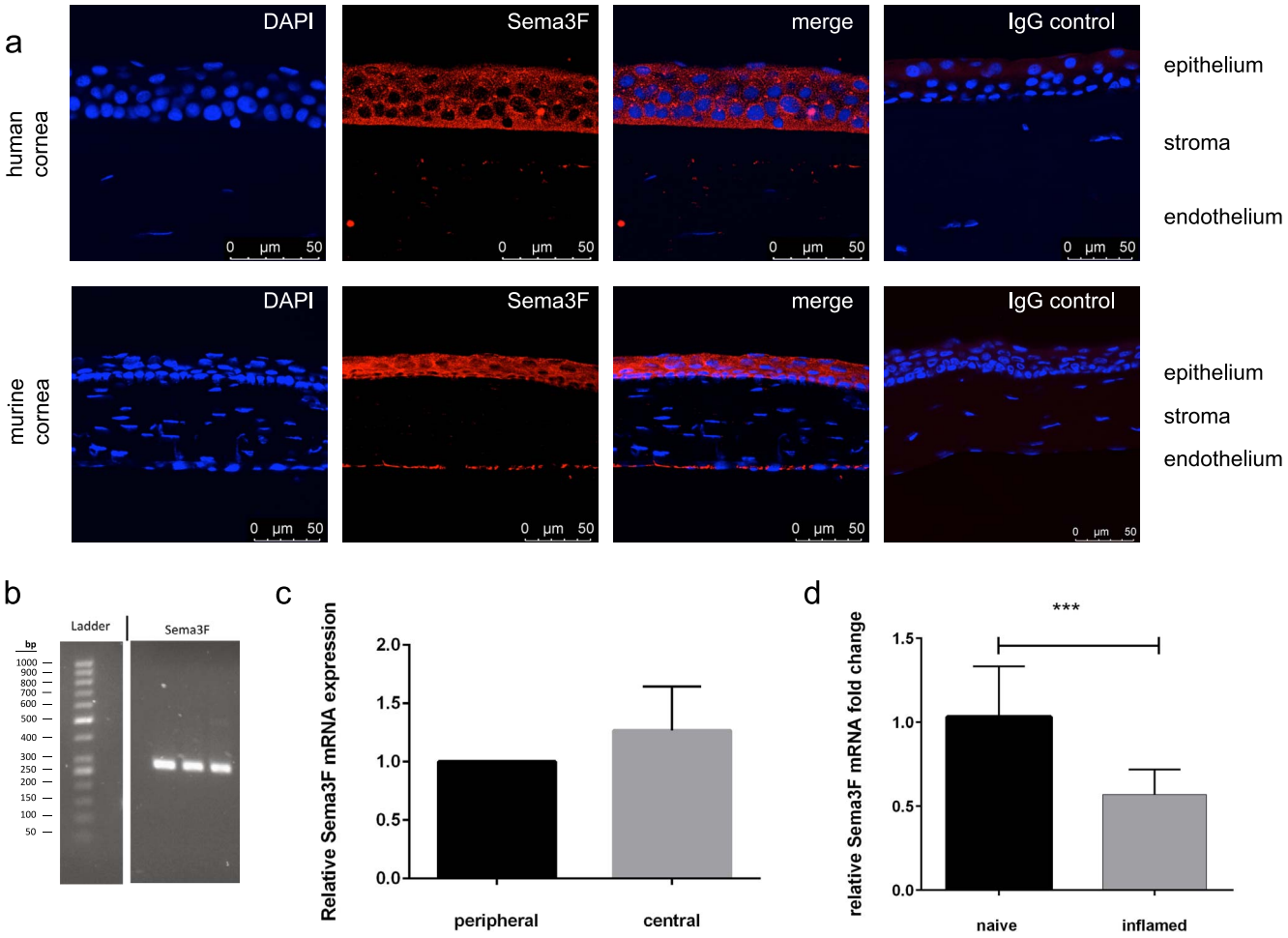

Figure 1. Corneal expression of Sema3F. (**a**) Sema3F is constitutively expressed with high intensity in both human (*top*) and murine corneal epithelium (*bottom*). IgG controls stained negative providing evidence for specificity of the obtained Sema3F signal. (**b**) PCR analysis confirming corneal Sema3F expression. The gel shows mRNA expression from three independent experiments. (**c**) Sema3F is equally expressed in central and peripheral corneal tissue in murine and human tissue. (**d**) Corneal Sema3F expression is downregulated in suture induced inflammation. Real-time PCR on the expression of Sema3F in naïve versus inflamed corneas. Sema3F expression is about twofold downregulated under inflammatory conditions; $P = 0.0008$, $n = 3$.

cells were seeded per hanging drop to assemble into a single spheroid within 24 hours at 37°C and 5% CO_2. After 24 hours, spheroids were harvested and used for sprouting analysis in a type I collagen matrix. Per group, 30 to 40 endothelial cell spheroids were seeded into 0.5 mL of collagen solution in nonadherent 24-well plates with a final concentration of rat type I collagen of 1.5 mg/mL. Freshly prepared gels were transferred rapidly into a humidified incubator (37°C, 5% CO_2) and after the gels had solidified, 0.1 mL serum-free media or 0.1 mL hCEC supernatant was added per well ± VEGF-C (9199-VC-025; RnD, Wiesbaden, Germany) ± anti-Sema3F antibody (rabbit, C135015; LSBio, Eching, Germany). Spheroid gels were incubated for 24 hours in a humidified incubator. Images were acquired using ProgRes CF (Jenoptik, Jena, Germany). A minimum of 15 spheroids were imaged per well and sprouting was quantified using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry

Cryofixed enucleated murine eyes and cryofixed human corneas were used. Sections were fixed in methanol at −20°C for 10 minutes and permeabilized using 2% Triton X-100 in PBS for 2 minutes. After washing in PBS, slides were blocked with 5% goat or donkey serum in PBS and stained overnight at 4°C in PBS with 0.3% Triton X-100, 1% goat or donkey serum and anti-Sema3F primary antibody (rabbit, Ab135880, 1:250; abcam, Berlin, Germany). Controls were stained with isotype control antibody (rabbit, 31235, 1:100; Thermo Fisher Scientific, Dreieich, Germany). Alexa Fluor 488 anti-rabbit was used as secondary antibody (goat, 111-547-003, 1:500; Jackson, Ely, Cambridgeshire, UK) and slides were mounted with DAPI/Moviol (475904; Calbiochem, Darmstadt, Germany). Images were acquired using a Leica Confocal TCS SP8-DMi8 microscope (Leica, Wetzlar, Germany).

RNA Isolation and PCR

Total RNA was extracted from human corneas or fresh murine corneas using RNeasy kit (Qiagen, Hilden, Germany) and reverse-transcribed with SuperScript III Reverse Transcriptase (Life Technologies, Darmstadt, Germany). PCR was performed on a Chromo4 PTC-200 (Biorad, Munich, Germany). PCR products were run on agarose gels for quality control. Primer sequences: mSema3f: 5′-CCG CCT CAC TGT CGC TG-3′ (Forward); 5′-GTA GGG ATC GCG GGC AAG GC-3′ (Reverse); hSema3f : 5′-CAG CCG ACG GGC GCT ATG AG-3′ (Forward); hSemaF: 5′-CAG TCA GCA CAG GCA GCC CC-3′ (Reverse)

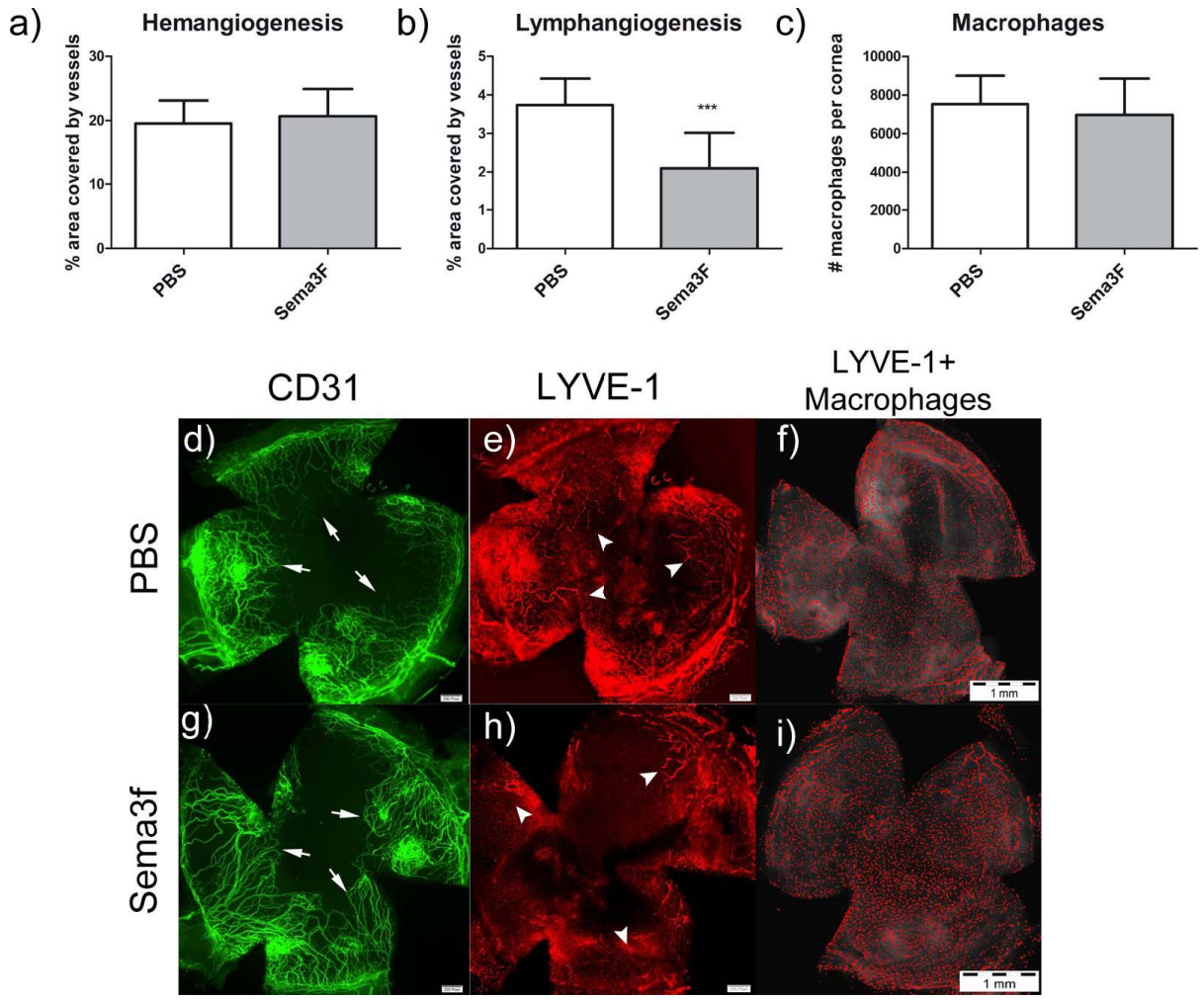

FIGURE 2. Specific inhibition of corneal lymphangiogenesis by Sema3F in the corneal suture model. Quantification (**a–c**) and representative images (**d–i**) of Sema3F effects in the corneal suture model. Sema3F topical eye drops significantly reduce inflammatory corneal lymph- (**b**) but not hemangiogenesis (**a**). LYVE-1+ macrophage recruitment was not affected by Sema3F treatment (**c, f, i**). Exemplary corneal flat mounts show comparable ingrowth of CD31+ blood vessels (**d, g**) but suppressed ingrowth of LYVE-1+ lymphatic vessels with Sema3F treatment (**e, h**). *Arrows* point to blood vessel tips in (**d**, **g**) and lymphatic vessel tips in (**e**, **h**). *P* value for lymphangiogenesis: $P = 0.0003$; $n = 10$ per group.

MTT Assay for Cell Proliferation

HLEC from passage 3 to 5 were used for this assay. Cells were seeded in a 96-well plate with 0.1 mL endothelial medium containing growth supplements and incubated for 24 hours at 37°C and 5% CO_2. Cells were then starved for 24 hours in endothelial basal medium with 6% FBS and no growth supplements. Afterward, cells were stimulated with supernatant from HCEC or control media and incubated for 72 hours. After the media was removed, 0.1 mL of phenol red-free endothelial basal media containing 10% of 12 mM MTT (#M6494; Invitrogen Thermo Fisher Scientific, Dreieich, Germany) was added to each well and cells were incubated for 4 hours at 37°C and 5% CO_2. For analysis, 0.085 mL media was removed from each well and 0.05 mL dimethyl sulfoxide was added. After incubation for 10 minutes the optical density was measured at 540 nm using a Tecan Spark microplate reader (Tecan, Grödig, Austria).

Statistics

Statistical analyses were performed using GraphPad Prism (v6.0; GraphPad Software, Inc., San Diego, CA, USA). The results of the spheroid-sprouting assays and tube formation assays were analyzed using ANOVA with Tukey multiple comparison test. Gene expression and corneal angiogenesis readings were compared using unpaired *t*-test. *P* values in Kaplan-Meier plots were calculated using Log-rank (Mantel-Cox) Test.

RESULTS

In order to investigate corneal expression of Sema3F, we performed immunohistochemistry on human and mouse tissue (Fig. 1A). We found strong Sema3F expression in the corneal epithelium and very limited expression in the stroma. Note that these samples are from healthy corneas, thus providing evidence for Sema3F being physiologically expressed in the cornea under normal resting conditions in adult eyes. IgG controls stained negative in all samples. In order to validate the immunohistochemistry results, we analyzed corneal Sema3F expression using PCR (Fig. 1B). The results confirmed strong corneal Sema3F expression under physiologic conditions. There was no gradient in physiologic Sema3F expression between central and peripheral cornea (Fig. 1C). Under suture-induced inflammatory conditions, however, Sema3F expression was found to be significantly downregulated (Fig. 1D).

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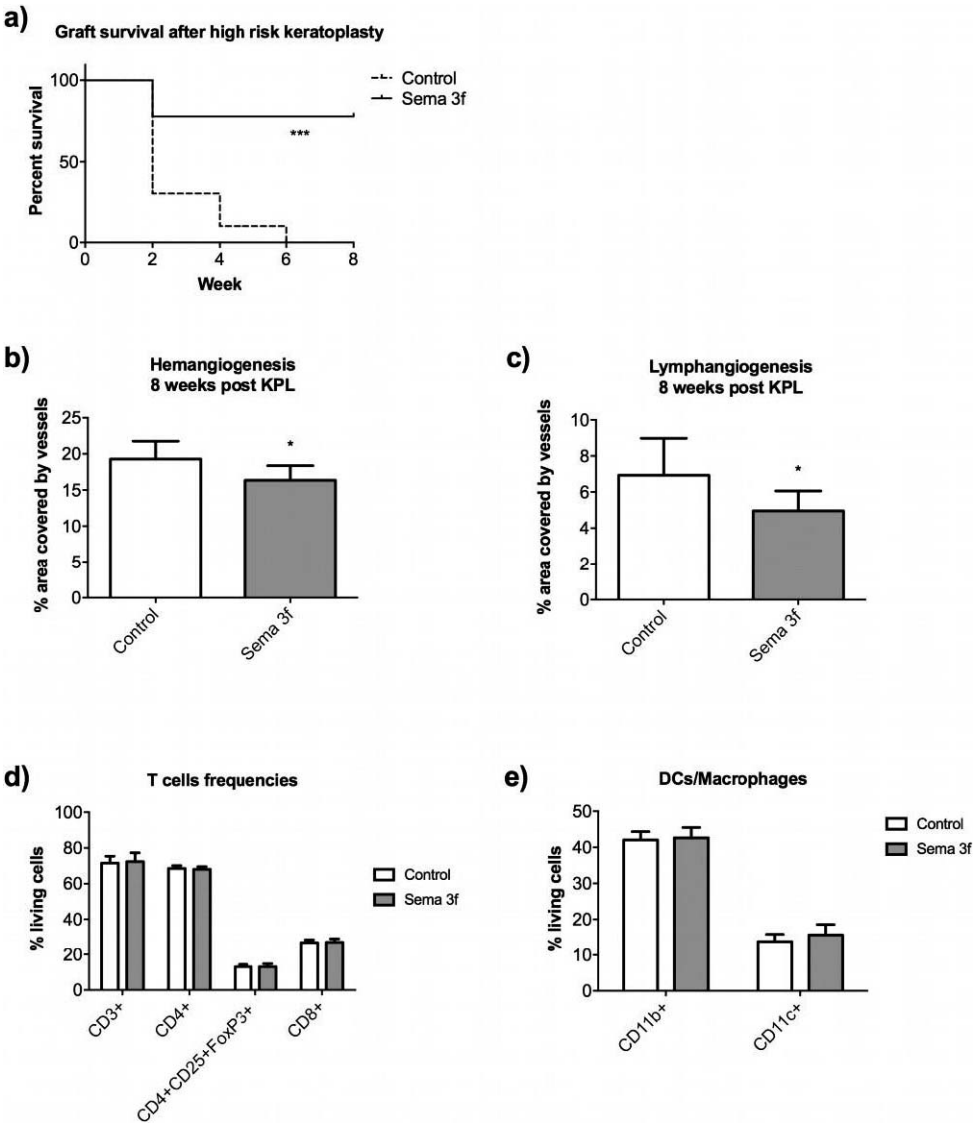

FIGURE 3. Sema3F eye drops improve graft survival after high-risk keratoplasty through long lasting anti-angiogenic effects. (**a**) Graft survival in a murine high-risk keratoplasty model was significantly improved with topical Sema3F eye drops ($P = 0.0006$; $n = 10$). (**b, c**) Quantification of CD31+ blood vessels and LYVE-1+ lymphatic vessels 8 weeks after keratoplasty revealed a long-lasting inhibitory effect of Sema3F on corneal hem- and lymphangiogenesis ($P < 0.05$; $n = 10$). (**d, e**) Number of T cells and macrophages in the lymph nodes was not altered by Sema3F treatment 8 weeks after keratoplasty ($P > 0.05$; $n = 10$).

In order to investigate whether corneal Sema3F expression plays a functional role in maintaining corneal avascularity in vivo, we performed functional experiments using the corneal suture model. We found that Sema3F applied as topical eye drops specifically inhibited the outgrowth of corneal lymphatic but not blood vessels after corneal suture injury (Fig. 2). As it was shown that LYVE-1+ macrophages are involved in recruitment and formation of corneal lymphatic vessels,[22] we quantified this immune cell population in the cornea by immunohistochemistry. Interestingly, recruitment of macrophages, which has in other models been reported to be modulated by class 3 semaphorins,[23] remained unchanged after 14 days of suture induced inflammation.

To analyze if Sema3F-mediated blockage of lymphangiogenesis can prevent breakdown of the immune privilege of the cornea, we used a murine high-risk keratoplasty model. In this model, corneal grafts are placed in prevascularized, highly inflamed recipient corneas.[24] In eyes treated with topical Sema3F eye drops during the prevascularization phase, we found graft rejection in about 30% of eyes through week 8. Control-treated eyes, in contrast, showed graft rejection in about 70% already by week 2 ($P = 0.0006$; Fig. 3a). Interestingly, the Sema3F effect on corneal angiogenesis was sustained long after the acute inflammatory phase and cessation of topical treatment (Fig. 3b). Eight weeks after corneal transplantation (i.e., 8 weeks after cessation of topical Sema3F treatment), the area covered by blood and lymphatic vessels was still significantly reduced in Sema3F-treated corneas versus controls. Different from the corneal suture model, both blood and lymph angiogenesis were reduced by Sema3F in this model (Figs. 3b, 3c). The number of T cells and macrophages in the draining lymph nodes, however, was not altered by Sema3F (Figs. 3d, 3e).

Both in vivo models revealed a potent anti-lymphangiogenic effect of Sema3F. In order to mechanistically analyze whether Sema3F had a direct or indirect effect on lymph angiogenesis we performed additional in vitro experiments. For that purpose, we isolated hCECs. Corneal epithelial morphology

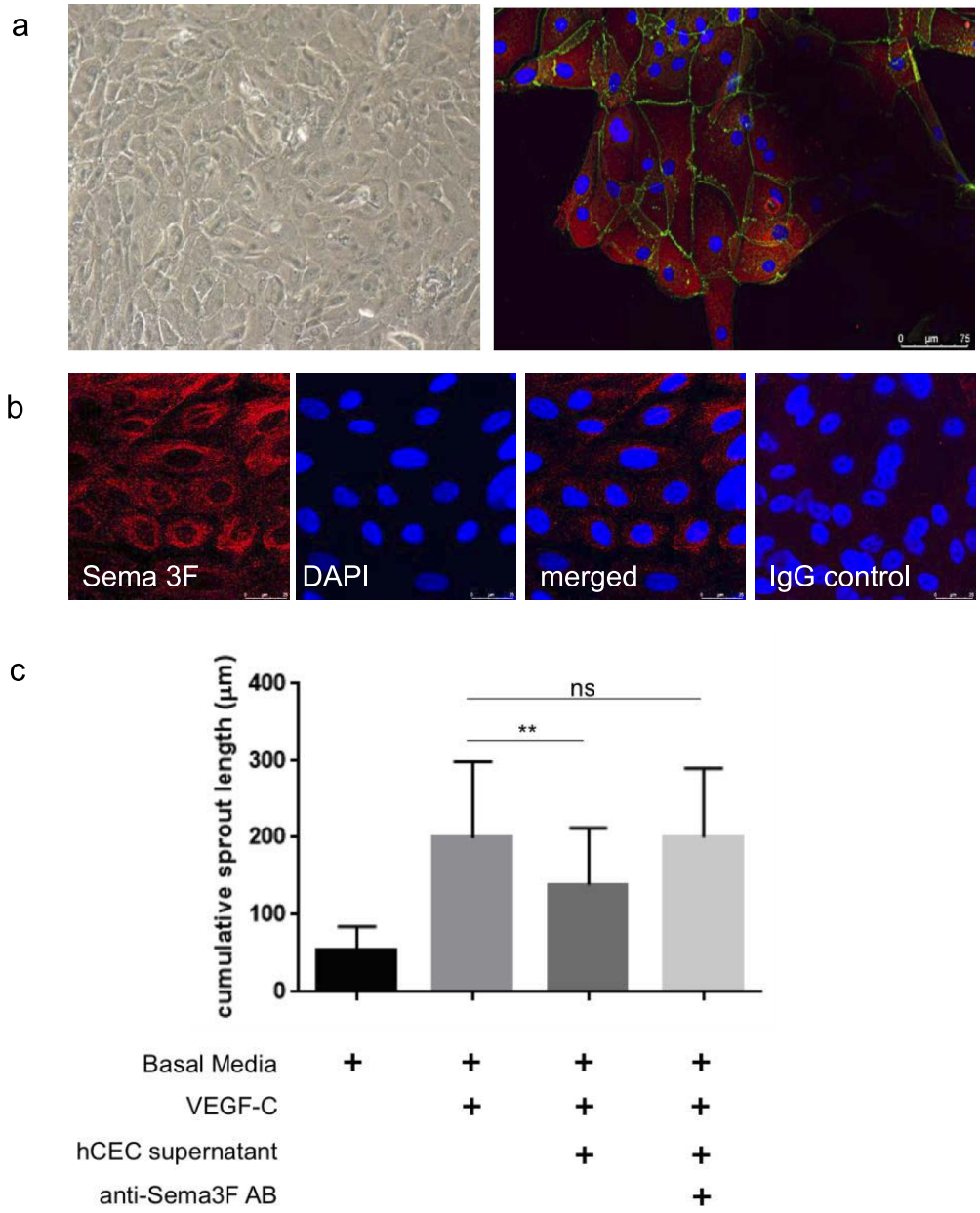

Figure 4. Effect of primary hCECs on lymphangiogenesis in vitro. (**a**) Isolated hCECs exhibit typical epithelial morphology in vitro and express corneal epithelial markers pancytokeratin (*red*) and E-cadherin (*green*). (**b**) Isolated hCECs express Sema3F, confirming the in vivo results. (**c**) Cumulative sprout length of lymphatic endothelial cell spheroids. Stimulation with VEGF-C induces lymphangiogenesis. Addition of hCEC supernatant significantly inhibits VEGF-C–induced lymphangiogenesis ($P < 0.01$; ANOVA corrected for multiple testing). Addition of an anti-Sema3F antibody completely abolishes the anti-angiogenic effect of hCEC supernatant.

and expression of epithelial markers were confirmed (Fig. 4A). In addition, we confirmed that our isolated hCECs express Sema3F in culture (Fig. 4B). Using a functional in vitro sprouting experiment, we evaluated the effect of supernatant from cultured hCECs on lymph angiogenesis (Fig. 4C). Spheroids formed by lymphatic endothelial cells extend tube-like structures resembling primitive lymph vessels when exposed to VEGF-C. This lymph-angiogenic response was significantly inhibited by hCEC supernatant ($P < 0.01$). Addition of a neutralizing antibody against Sema3F completely reversed the lymphangioinhibitory effect of hCEC supernatant, providing evidence that Sema3F mediates the anti-lymphangiogenic effect of hCEC supernatant. In additional experiments, we confirmed that supernatant from hCEC did not induce LEC apoptosis (Supplementary Fig. S1) and that the Sema3F receptor NRP-2 was expressed on LECs (Supplementary Fig. S2).

Discussion

In order to maintain corneal function, the corneal angiogenic privilege must be maintained and pathologic hemangiogenesis and lymphangiogenesis must be suppressed.[25,26] Known mediators of corneal avascularity are sVEGFR2, sVEGFR3, and membrane-bound VEGFR1 and 3. All these factors are expressed in the corneal epithelium.[27–29] In this study, we have identified Sema3F as a novel and potent mediator of corneal avascularity that is constitutively expressed in corneal epithelium.

Human Sema3F is a secreted precursor protein of 100 kDa and is processed into a 95 kDa, a 65 kDa and some smaller isoforms by furin and other endoproteolytic proprotein-convertases.[30,31] It is well established that Sema3F has vasorepellent properties against blood vessels.[3,4] In this study, we have established a novel role for Sema3F in preventing primarily lymphatic vessels from entering the corneal stroma. Furthermore, Sema3F-treated animals showed a significantly improved graft survival. In contrast, no change in the CD4+CD25+FoxP3+ regulatory T cell population in the draining lymph nodes was detectable as observed in other immune modulatory approaches.[32,33] This indicates that blocking lymphangiogenesis by topcical application of Sema3F prior to graft transplantation has no systemic effects on immune cells but hampers the long-term immunoangiogenic response against the allogeneic graft. A full analysis of alloimune response mechanisms is beyond the scope of this study. Anti-lymphangiogenic properties of Sema3F have, however, been described previously for tumor metastasis and dermal lymphatic network formation,[34–36] but not in the context of ocular surface disease or organ transplantation. Interestingly, our results demonstrate direct anti-lymphangiogenic effects of Sema3F. Recruitment of monocytic cells, which has been reported to be modulated by semaphorins,[23,37] was not altered in our experimental setup. This was confirmed by our in vitro experiments demonstrating potent anti-lymphangiogenic effects of conditioned media from primary hCECs in the absence of immunomodulatory cells. The fact that the anti-lymphangiogenic effect induced by hCEC conditioned media was fully reversed by adding a Sema3F antibody provides evidence for the specificity of Sema3F as a hCEC-derived anti-lymphangiogenic factor.

The magnitude of effects observed for topical Sema3F treatment, especially in the high-risk keratoplasty model, is remarkable and may be of clinical significance for patients undergoing penetrating keratoplasty. Especially the fact that early Sema3F treatment in the inflammatory stage of the disease induced a significantly improved graft survival long after Sema3F treatment was stopped points toward useful clinical implications. For example, in patients with corneal chemical burn injuries, topical Sema3F treatment could be administered in the early posttraumatic phase to limit corneal vascularization and improve outcomes after subsequent penetrating high-risk keratoplasty.

In conclusion, this study identified Sema3F as a novel and potent regulator of the corneal lymphangiogenic privilege and as a promising candidate for future clinical use, for example in the form of a truncated Sema3F protein. The fact that lymphangiogenesis was predominantly affected by Sema3F may also have implications for other tissue or organ transplantation settings where continued blood supply is necessary but anti-lymphangiogenic effects might be desirable to suppress the afferent arm of the immune reflex arc to ensure graft survival.

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