Netrin-4 Mediates Corneal Hemangiogenesis but Not Lymphangiogenesis in the Mouse-Model of Suture-Induced Neovascularization

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PURPOSE. Netrin-4, a secreted protein, is found in the basement membrane of blood vessels and acts as a key regulator of angiogenesis. Here we investigated the role of Netrin-4 in the mouse-model of suture-induced corneal hem- and lymphangiogenesis.

METHODS. Corneal hem- and lymphangiogenesis were induced in Netrin-4-deficient (Ntn4<sup>−/−</sup>) and wild-type (WT) mice by placing three 11-0 nylon sutures intrastromally. Fourteen days after suturing, the vascularized area was analyzed via corneal flat mount immunohistochemistry. Messenger RNA levels for VEGF-A, VEGF-C, Lyve-1, Netrin-4, Unc5H2, “deleted in colon cancer” receptor, and Neogenin in treated and nontreated mouse corneas, cultured human corneal keratocytes (HCK) and epithelial cells (HCEC-HCET) were analyzed by quantitative PCR.

RESULTS. In wild-type mice, Netrin-4 mRNA expression in the cornea decreased in growing corneal neovascularization after suturing. Correspondingly, Ntn4<sup>−/−</sup> mice showed an increased vascularized area compared to that in WT mice. Expression of VEGF-A mRNA was higher in Ntn4<sup>−/−</sup> versus WT mice. There was no Netrin-4 expression in lymphatic vessels and the area of lymphatic vascularization did not differ between Ntn4<sup>−/−</sup> and WT mice, nor did expression of VEGF-C and Lyve-1 mRNA. Human corneal epithelial cells showed mainly Netrin-4 mRNA expression, which increased after stimulation, while HCK demonstrated Unc5H2 mRNA expression. Expression of VEGF-A, Netrin-4, Unc5H2, and Neogenin mRNA in HCEC and HCK did not differ significantly between the serum-free condition and VEGF-A or Netrin-4 stimulation.

CONCLUSIONS. Absence of Netrin-4 increased corneal hemangiogenesis but not lymphangiogenesis in the mouse-model of suture-induced neovascularization. Netrin-4 acted as an antiangiogenic factor in the cornea, with which the healthy cornea is enriched via its expression by corneal epithelial cells.

Keywords: Netrin-4, lymphangiogenesis, corneal neovascularization

The normal, healthy cornea lacks both blood and lymph vessels, establishing part of the immune and angiogenic privilege of the cornea. Inflammatory corneal disease or surgical manipulation may induce outgrowth of blood and lymph vessels from the limbus into the avascular cornea. This leads to reduced transparency of the cornea, causing visual loss, as well as loss of corneal immune privilege, which is associated with a higher risk of graft rejection after corneal transplantation. Lymphangiogenesis is essential to mediate immune reaction after corneal grafting. Key regulators of inflammation-driven hem- and lymphangiogenesis of the avascular cornea are growth factors of the VEGF family: VEGF-A, -C, and -D.1<sup>-10</sup>,<sup>-15</sup>

The breakdown and formation of extracellular matrix (ECM) plays a central role in vessel growth. Netrins are a family of laminin-related ECM molecules, initially identified as axonal guidance molecules. In mammals, five members of the Netrin family have been identified that act through six putative receptors, including “deleted in colon cancer” (DCC), Neogenin, and members of the Unc5 subfamily. Initial reports on the role of Netrin-4 outside the nervous system demonstrated its essential involvement in regulating mammary and lung morphogenesis, as well as hem- and lymphangiogenesis. Netrin-4 is located in the basement membrane of blood vessels, lending extra support to its potential as a key player in angiogenesis. In vitro, Han et al. showed that Netrin-4 inhibited human umbilical vein endothelial cell (HUVEC) tube formation, viability and proliferation, apoptosis, migration, and invasion in a dose-dependent manner. Netrin-4 has been detected in mouse cornea, with a likely role regulating epithelial cell proliferation or migration. A recent study reported that Netrin-4 suppressed and reversed corneal neovascularization in the alkali burn model. Attempts to identify receptors via which Netrin-4 influences angiogenesis have been sporadic and contradictory. Despite this, it seems that three of the six cognate Netrin-1 receptors, DCC, Neogenin, and Unc5H2, are involved in the interaction between endothelial vessel cells and vascular smooth muscle cells.
In the present study, we analyzed the role of Netrin-4 and its receptors in a mouse-model of suture-induced corneal hemangiogenesis. In contrast to the alkali burn model previously used to study the functional role of Netrin-4 in the cornea, the suture-induced model represents a low-grade inflammation model, displaying not only corneal hemangiogenesis but also lymphangiogenesis. Therefore, this model better represents the involved factors mediating immune reaction after corneal grafting.

**Materials and Methods**

**Animals**

All animal experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the responsible University Animal Care and Use Committees. We purchased C57Bl/6j mice from Charles River (Wilmingon, MA, USA) or Janvier (Cedex, France). Mice that were Ntn4+/− were generated as described on a C57BL/6j-background. Genotype was determined by PCR analysis of genomic DNA prepared from tail or ear samples. Primers used: wild-type (WT) and null allele forward 5' AGCAGCCCTTTAAAAACATCCTGAG 3', WT allele reverse 5'GAAAGCTCAGGGCAAGCTATGTG 3', and null allele reverse 5'CAAATGTGTCAGTTTCATAGCC 3'. Animals were fed regular laboratory chow and water ad libitum. A 12-hour day/night cycle was maintained.

**Mouse Model of Suture-Induced Inflammatory Corneal Neovascularization**

To induce reproducible corneal neovascularization, the protocol was performed as previously described. Mice were deeply anesthetized. The central cornea was marked by a 2-mm trephine, gently placed at the central cornea. Three 110-nm sutures were placed intrastromally with two incursions each. The central cornea was marked by a 2-mm incision. Once enucleated, eyes were rinsed in PBS, fixed in acetone for 8 minutes, and rinsed again in PBS. The sclera was dissected with a circumferential incision parallel to the limbus, followed by removal of the lens and iris. Four radial cuts were made to the cornea, the suture-induced model represents a low-grade inflammation model, displaying not only corneal hemangiogenesis but also lymphangiogenesis. Therefore, this model better represents the involved factors mediating immune reaction after corneal grafting.

**Histology and Immunohistochemistry on Paraffin Sections**

Eyes were fixed in PFA 4% overnight at 4°C or in methacarn (60% methanol, 30% 1,1,1-trichlorethane, 10% acetic acid) overnight at room temperature or routinely processed for paraffin embedding. Sections (5 μm) were used for immunohistochemical analysis. Immunohistochemical staining was performed on deparaffinized sections.

**Cell Culture**

Human corneal keratocytes (HCK)-SV40, and human corneal epithelial cells (HCEC)-SV40, and transformed human corneal epithelial cells (HCET)-SV40, were cultured as previously described. We grew HCECs in Dulbecco’s modified Eagle medium mixed with nutrient mixture F-12 Ham medium, supplemented with 10% fetal calf serum (FCS) and antibiotics. We grew HCET in Dulbecco’s modified Eagle medium mixed with nutrient mixture F-12 Ham medium, supplemented with 5% FCS, 5 μg/mL insulin, 10 ng/mL human epidermal growth factor (hEGF) and antibiotics (Sasaki medium) or keratocytes basal medium ((KBM), CC3104; Lonza, Basel, Switzerland) supplemented with 0.5 mM calcium chloride. hEGF hydrocortisone, insulin, bovine pituitary extract (BPE) and antibiotics

Biologically, Littleton, CO, USA) for 4 days in 1% BSA in PBS with 5% goat serum at 4°C. After PBS wash, corneas after incubating with primary antibody LYVE-1 were incubated in the appropriate secondary antibody, a Cy3-conjugated goat anti-rat IgG (1:300; Jackson ImmunoResearch Laboratories, Inc.) for 1 day at 4°C in the same buffer. After PBS wash, all corneas were mounted flat onto glass slides and subjected to microscopy imaging with a standardized technique to compare vascular density and to analyze areas of hem- and lymphangiogenesis. Images of the flat mounts were captured with a charge-coupled device camera (C4742-95-12ER; Hamamatsu, Hamamatsu City, Japan) attached to a microscope (model MZ FLIII; Leica Microsystems, Bensheim, Germany) and a fluorescence microscope (Axio Imager M2; Zeiss, Göttingen, Germany).

Image analysis was performed with commercial software (OpenLab; Improvision, Inc., Lexington, MA, USA, and ZEN 2012; Zeiss International, Göttingen, Germany). Areas covered by blood and lymph vessels were detected and measured using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Prior to analysis, grayscale images of whole-mount pictures were modified by several filters and vessels were detected by threshold setting to include bright vessels and exclude dark backgrounds. For analysis, the bright artefacts were blackened. Entire corneas were analyzed by two independent observers, blind to treatment status and genotype, to minimize sampling bias. The complete flat-mount area was set to 1, and the vessel-covered areas (determined via threshold setting) were then related to this value.

**Immunohistochemistry and Morphologic Determination of Corneal Whole Flat Mounts**

Once enucleated, eyes were rinsed in PBS, fixed in aceton for 8 minutes, and rinsed again in PBS. The sclera was dissected with a circumferential incision parallel to the limbus, followed by removal of the lens and iris. Four radial cuts were made to allow flattening.

The whole-mount staining protocol was modified from Cellerino et al. Corneas were blocked with 5% BSA in PBS with 10% goat serum and 0.1% Triton X-100 overnight. Then the corneas were incubated with primary antibody against Netrin-4 (rabbit polyclonal, 1:1000) in 1% BSA in PBS with 5% goat serum for 5 days at 4°C. After several PBS washes, corneas were incubated in the appropriate secondary antibody, a Cy3-conjugated goat anti-rabbit IgG (1:400) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) or fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:300; Sigma-Aldrich Corp., St. Louis, MO, USA) for 1 day at 4°C in the same buffer. After PBS wash, corneas were incubated in FITC-conjugated CD31 (1:100; BD Pharmingen, San Jose, CA, USA) for 1 day or in LYVE-1 (1:100; Novus Biological, Littleton, CO, USA) for 4 days in 1% BSA in PBS with 5% goat serum at 4°C. After PBS wash, corneas after incubating with primary antibody LYVE-1 were incubated in the appropriate secondary antibody, a Cy3-conjugated goat anti-rat IgG (1:300; Jackson ImmunoResearch Laboratories, Inc.) for 1 day at 4°C in the same buffer. After PBS wash, all corneas were mounted flat onto glass slides and subjected to microscopy imaging with a standardized technique to compare vascular density and to analyze areas of hem- and lymphangiogenesis. Images of the flat mounts were captured with a charge-coupled device camera (C4742-95-12ER; Hamamatsu, Hamamatsu City, Japan) attached to a microscope (model MZ FLIII; Leica Microsystems, Bensheim, Germany) and a fluorescence microscope (Axio Imager M2; Zeiss, Göttingen, Germany).
(keratinocyte growth medium [KGM]; Lonza). Human corneal keratocytes were grown in KBM (Lonza) supplemented with 0.5 mM calcium chloride, hEGF, hydrocortisone, insulin, BPE, and antibiotics. Cells were grown in a humidified atmosphere containing 5% CO₂ at 37°C. Cultures were serially passaged once a week. Human keratocytes used were from passages 39 through 52, HCEC from passages 50 through 80, and HCET from passages 50 through 80. Detachment of cells for harvesting was performed with accutase (HCK) or trypsin (HCEC and HCET).

For each stimulation, cells were seeded in 8 wells of a 12-well plate and cultured 12 hours serum-free, so that the cells had a confluency of 100% before stimulation. After 12 hours serum-free, HCECs were stimulated with 10% FCS and HCK with supplement, 10% FCS or supplement with 10% FCS for 2 hours. Alternatively, HCEC, HCET (Sasaki medium or KGM) and HCK were stimulated with 1 ng/mL and 50 ng/mL VEGF-A, and with 50 ng/mL and 500 ng/mL Netrin-4. After, stimulating cells were pooled.

RNA Isolation and RT-PCR

Freshly isolated corneas were stored in an RNA stabilization solution (RNeAlater; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Whole corneas were homogenized in lysis buffer (RNeasy Kit; Qiagen, Hilden Germany) and stored at −20°C until RNA isolation. Two or three corneas of two or three mice were pooled for one probe.

Total RNA of HCEC, HCET, and HCK was extracted after RNA isolation. Two or three corneas of two or three mice after suture placement to induce corneal hem- and lymphangiogenesis. In wild-type mice, immunohistochemical staining showed a strong expression of Netrin-4 in the basement membrane of the corneal epithelium (Bowman's layer) and endothelium (Descemet membrane) and in the basement membrane of the corneal vessels (Fig. 1, Supplementary Fig. S2). Corneal epithelium showed a strong, unspecific staining in sagittal sections (compare Supplementary Fig. S1). Costaining demonstrated a colocalization of Netrin-4 and CD31, a marker of blood vessel endothelium (Figs. 1A, 2C, Supplementary Fig. S3). In contrast, Netrin-4 was not expressed in lymphatic vessels, shown as absence of colocalization of Netrin-4 and LYVE-1 staining (Figs. 1B, 3C). In mice that were Netrin-4/−, Netrin-4 expression was not detectable (Figs. 1, 2C, 3C).

Corneal Unc5H2 expression was not detectable in sagittal sections of wild-type and Netrin-4/− mice after suture placement.

**Table. Primer Sequences**

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<th>Reverse Sequence (5’ → 3’)</th>
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**Statistical Analysis**

All results are expressed as the mean ± SD or mean normalized expression (MNE) ± SD for real-time RT-PCR results. After normal distribution testing (Shapiro–Wilks) the data were compared via unpaired t-test if the Levenne-test showed equal variance; otherwise, a nonparametric test (Mann-Whitney U) was used. Differences were considered statistically significant when P < 0.05.

**RESULTS**

**Netrin-4 Expression in the Cornea After Suture-Induced Hem- and Lymphangiogenesis**

Corneal Netrin-4 expression was examined in wild-type and Netrin-4/− mice after suture placement to induce corneal hem- and lymphangiogenesis. In wild-type mice, immunohistochemical staining showed a strong expression of Netrin-4 in the basement membrane of the corneal epithelium (Bowman’s layer) and endothelium (Descemet membrane) and in the basement membrane of the corneal vessels (Fig. 1, Supplementary Fig. S2). Corneal epithelium showed a strong, unspecific staining in sagittal sections (compare Supplementary Fig. S1). Costaining demonstrated a colocalization of Netrin-4 and CD31, a marker of blood vessel endothelium (Figs. 1A, 2C, Supplementary Fig. S3). In contrast, Netrin-4 was not expressed in lymphatic vessels, shown as absence of colocalization of Netrin-4 and LYVE-1 staining (Figs. 1B, 3C). In mice that were Netrin-4/−, Netrin-4 expression was not detectable (Figs. 1, 2C, 3C).

Corneal Unc5H2 expression was not detectable in sagittal sections of wild-type and Netrin-4/− mice after suture placement.
versus 0.131

4. The corneal epithelium shows a strong, unspecific staining

0.057, Ntn4

6 compared to that of WT mice (0.137

121.60

compared to untreated wild-type mice (Fig. 4). Expression of VEGF-A mRNA increased significantly after suture placement in both wild-type and Ntn4 knockout (Ntn4−/−) mice (ΔΔCT-value wild-type 2.82 ± 0.0001, Ntn4−/− 4.30 ± 10−3 versus 2.34 ± 10−3 ± 0.34 ± 10−3, P = 0.0011; Fig. 4A). The increase in VEGF-A mRNA expression in the WT mice coincided with a decrease in Ntn4 mRNA expression. Expression of VEGF-C and Lyve-1 mRNA showed no significant difference between suture-treated and untreated mice (ΔΔCT-value VEGF-C wild-type 1.35 ± 0.50 ± 0.20 ± 10−3, Ntn4−/− 1.67 ± 10−3 ± 0.20 ± 10−3 versus 1.40 ± 10−3 ± 0.24

5. Absence of Netrin-4 Increased Suture-Induced Hemangiogenesis, but Not Lymphangiogenesis

To investigate the effect of Netrin-4 depletion on corneal hemangiogenesis, blood and lymph vessel area as indicated by staining against CD31 and Lyve-1, respectively, was measured in corneal flat mounts 14 days after suture placement and normalized to total corneal area. We found a significant increase in vascularized area in Ntn4−/− mice compared to that of WT mice (0.157 ± 0.040 versus 0.204 ± 0.057, P = 0.01, n = 8; Figs. 2A, 2B). In contrast to blood vessel area, lymphatic vascularized area did not differ significantly between WT and Ntn4−/− mice (0.161 ± 0.049 versus 0.131 ± 0.038, P = 0.16, n = 9; Figs. 3A, 3B).

Decrease in Unc5H2 mRNA Expression After Suture-Induced Hem- and Lymphangiogenesis

Levels of Unc5H2, DCC, and Neogenin mRNA expression were measured in WT and Ntn4−/− mice 14 days after suture placement (Fig. 5). Expression of Unc5H2 mRNA decreased after suture placement in wild-type (ΔΔCT-value 26.21 ± 10−3 ± 0.75 ± 10−3 ± 1.34 ± 10−3 versus 26.37 ± 10−3 ± 3.95 ± 10−3 and significantly in Ntn4−/− mice (ΔΔCT-value 33.33 ± 10−3 ± 2.22 ± 10−3 ± 1.54 ± 10−3 versus 4.83 ± 10−3 ± 1.22 ± 10−3, P = 0.03; Fig. 5A). In contrast to Unc5H2, DCC, and Neogenin mRNA expression did not differ significantly between treated and untreated WT or Ntn4−/− mice (ΔΔCT-value DCC wild-type 1.96 ± 10−3 ± 1.54 ± 10−3 versus 1.75 ± 10−3 ± 1.38 ± 10−3, Ntn4−/− 0.80 ± 10−3 ± 0.48 ± 10−3 versus 1.14 ± 10−3 ± 1.16

5. Decrease in Netrin-4 mRNA Expression and Increase in VEGF-A mRNA Expression After Suture-Induced Hem- and Lymphangiogenesis

Netrin-4 mRNA expression was analyzed in untreated and suture-treated WT mice after 14 days. A significant decrease in Netrin-4 mRNA expression was measured in suture-treated compared to untreated wild-type mice (ΔΔCT-value wild-type 121.60 ± 10−3 ± 40.84 ± 10−3 versus 53.05 ± 10−3 ± 12.41 ± 10−3, P < 0.001; Fig. 4B). Messenger RNA levels of VEGF-A, VEGF-C, and Lyve-1 were measured in suture-treated and untreated WT and Ntn4−/− mice (Fig. 4). Expression of VEGF-A mRNA increased significantly after suture placement in both wild-type and Ntn4−/− mice (ΔΔCT-value wild-type 2.82 ± 0.0001, Ntn4−/− 4.30 ± 10−3 versus 2.34 ± 10−3 ± 0.34 ± 10−3, P = 0.0011; Fig. 4A). The increase in VEGF-A mRNA expression in the WT mice coincided with a decrease in Ntn4 mRNA expression. Expression of VEGF-C and Lyve-1 mRNA showed no significant difference between suture-treated and untreated mice (ΔΔCT-value VEGF-C wild-type 1.35 ± 0.50 ± 0.20 ± 10−3, Ntn4−/− 1.67 ± 10−3 ± 0.20 ± 10−3 versus 1.40 ± 10−3 ± 0.24

5. Lack of Netrin-4 Increased VEGF-A mRNA Expression, but Had no Influence on VEGF-C and Lyve-1 mRNA Expression

Mice that were Ntn4−/− showed significantly higher VEGF-A mRNA expression 14 days after suture placement compared to wild-type mice (ΔΔCT-value wild-type 6.12 ± 10−3 ± 0.35 ± 10−3 versus Ntn4−/− 2.34 ± 10−3 ± 0.35 ± 10−3, P = 0.004; Fig. 4A). There was no significant difference in VEGF-C and Lyve-1 mRNA expression between suture-treated WT and Ntn4−/− mice (ΔΔCT-value VEGF-C wild-type 0.90 ± 10−3 ± 0.20 ± 10−3 versus Ntn4−/− 1.40 ± 10−3 ± 0.24 ± 10−3, Lyve-1 wild-type 2.38 ± 10−3 ± 1.08 ± 10−3 versus Ntn4−/− 2.18 ± 10−3 ± 0.83 ± 10−3).

Netrin-4 mRNA Is Mainly Expressed in Corneal Epithelial Cells, Whereas Unc5H2 mRNA Is Expressed in Corneal Keratocytes

The above shown data has led us to the hypothesis that corneal epithelial cells express primarily Netrin-4 whereas keratocytes primarily express VEGF-A. To investigate this role of corneal epithelial cells and keratocytes in maintenance of theangiogenic environment, mRNA expression of VEGF-A, Netrin-4, Unc5H2, Neogenin, and DCC was analyzed in HCEC and HCK. To calm down proliferative activity in the cell cultures the cells were kept under serum-free conditions for 24 hours prior to experimentation. To investigate whether the keratocytes or corneal epithelial cells can basically differentially express VEGF-A, Netrin-4, or the designated Netrin-4
receptors we used FCS as a strong although unstimulated stimulator. We compared the mRNA production of these targets between serum-free versus stimulated for 2 hours (HCEC with 10% FCS and HCK with supplement, 10% FCS or supplement with 10% FCS; Fig. 6). Both cell lines showed low VEGF-A mRNA expression in serum-free conditions (Fig. 6A) that increased significantly after stimulation (ΔΔCT-value HCK 4.6 × 10^{-3} ± 2.1 × 10^{-3} versus 16.0 × 10^{-3} ± 9.6 × 10^{-3}, P =

**FIGURE 2.** Netrin-4 and blood vessels. Corneal flat mounts (A) and magnification at the limbal border (C) of WT and Ntn4−/− mice 14 days after suture placement. Endothelium of vessels is visualized by CD31 staining (green) and Netrin-4 by Netrin-4 staining (red). Immunohistochemical costaining showed a strong expression of Netrin-4 colocalized with endothelial vessel staining. (B) Quantification of vascularized area compared to total corneal area in WT and Ntn4−/− mice (mean ± SD). There was a significant increase in the vascularized area after suturing in Ntn4−/− mice compared to WT mice (P = 0.01).

**FIGURE 3.** Netrin-4 and lymphatic vessels. Corneal flat mounts (A) and magnification at the limbal border (C) of WT and Ntn4−/− mice 14 days after suture placement. Lymphatic vessels are visualized by Lyve-1 staining (red) and Netrin-4 by Netrin-4 staining (green). Costaining showed no colocalization of Netrin-4 and Lyve-1. (B) Quantification of lymphatic vascularized area compared to total corneal area in WT and Ntn4−/− mice (mean ± SD). No significant difference in the lymphatic vascularized area was found between Ntn4−/− and WT mice (P = 0.16).
Netrin-4 Mediates Corneal Hemangiogenesis

FIGURE 4. Differential regulation of VEGF-A, Netrin-4, VEGF-C, and Lyve-1 mRNA expression. mRNA levels of (A) VEGF-A, (B) Netrin-4, (C) VEGF-C, and (D) Lyve-1 in suture-treated (black bar with white dots) and untreated (black bars) WT and suture treated (white bar with black dots) and untreated (white bars) Ntn4−/− mice after 14 days. (A) Expression of VEGF-A mRNA in treated and untreated WT and Ntn4−/− mice. Expression of VEGF-A mRNA increased significantly after suture placement in WT and Ntn4−/− mice (P < 0.0001, P = 0.0011). Mice that were Ntn4−/− showed significantly higher VEGF-A mRNA expression after suturing compared to WT mice (P = 0.0004). (B) Comparison of Netrin-4 mRNA expression in suture-treated and nontreated WT mice. A significant decrease in Netrin-4 mRNA expression can be observed in suture-treated compared to untreated WT mice (P < 0.001). No expression of Netrin-4 mRNA was detected in Ntn4−/− mice. Expression of VEGF-C mRNA in WT and Ntn4−/− mice. In contrast to VEGF-A, VEGF-C mRNA expression did not differ between WT and Ntn4−/− mice. (D) Expression of Lyve-1 mRNA in WT and Ntn4−/− mice. In comparison to VEGF-C, Lyve-1 mRNA expression showed no significant difference between WT and Ntn4−/− mice.

No Effect of VEGF-A and Netrin-4 Stimulation on VEGF-A, Netrin-4, Unc5H2, and Neogenin mRNA Expression of Corneal Epithelial Cells and Corneal Keratocytes

To investigate the effect of Netrin-4 and VEGF-A on corneal epithelial cells and keratocytes, the mRNA expression of VEGF-A, Netrin-4, Unc5H2, and Neogenin was measured in HCEC, HCET, and HCK after stimulation with VEGF-A (1 ng/mL or 50 ng/mL) or with Netrin-4 (50 ng/mL or 500 ng/mL) for 2 hours (Fig. 7, see Supplementary Fig. S4). Expression of VEGF-A, Netrin-4, Unc5H2, and Neogenin mRNA did not differ significantly in HCEC, HCET, and HCK between serum-free and VEGF-A or Netrin-4 stimulated cells.

DISCUSSION

Originally identified as an axon-guidance molecule, Netrin-4 has also been shown to regulate angiogenesis and lymphangiogenesis.12,21,24–26,29 In this study, we demonstrate that Netrin-4 plays an antiangiogenic role in the pathophysiology of corneal hemangiogenesis, but not corneal lymphangiogenesis, using Ntn4−/− mice and a suture-induced inflammatory mouse model.

As shown by our own data and in accordance with that previously published, Netrin-4 is located in the basement membrane of the corneal endothelium (Descemet), the corneal...
epithelium as well as in the basement membrane of corneal blood vessels. The latter observation led to the hypothesis that Netrin-4 might play a role in angiogenesis. In our study, the Netrin-4 strongly colocalized to the basement membrane of the endothelium of blood vessels, but not to lymphatic vessels.

In situ hybridization and microarray analyses have previously shown that vascular endothelial cells, in particular, are a source for Netrin-4. Supportive of a role for Netrin-4 in angiogenesis, in vitro findings by Han et al. demonstrated its concentration-dependent effect on HUVEC tube formation, viability and proliferation, apoptosis, migration, and invasion. A complementary study using human microvascular endothelial cells has shown that Netrin-4 stimulates proliferation, migration, and tube formation at low concentrations, but is inhibitory at higher doses.

An in vivo study reported that Netrin-4 suppressed and reversed corneal neovascularization in the alkali burn model. This is in accordance with our result that the disruption of Netrin-4 expression in mice led to an increase in vascularized area in the suture-induced corneal mouse model. Correspondingly, a lack of Netrin-4 in mice resulted in a significant increase in VEGF-A mRNA expression during suture-induced neovascularization. This again supports data from Han et al. who demonstrated, using the alkali-burn model, that application of Netrin-4 can restore the disrupted balance between VEGF and pigment epithelium-derived factor as an antiangiogenic effect. In their study, VEGF was downregulated after Netrin-4 treatment. Complementary to this, we found increased levels of VEGF-A expression in the absence of Netrin-4. Additionally, our results show a significant decrease in Netrin-4 mRNA expression in wild-type mice after suture placement, comparable to observations made by Han et al. who found a complete absence of Netrin-4 7 days after alkali burn. Taken together, data from Han et al. and our study support the hypothesis that Netrin-4 acts as an antiangiogenic factor and seems to establish an antiangiogenic environment in the cornea under physiologic conditions. Under the pathologic conditions of the suture-induced corneal mouse model, and especially in compared to wild-type mice, the antiangiogenic environment is not maintained. The antiangiogenic influence of Netrin-4 was also investigated by Lejmi et al. in vivo, using three different mouse models. This group suggested that Netrin-4 may act through interaction with basement membrane components, such as laminin, to regulate both endothelial and vascular smooth muscle cell adhesion and migration.

In contrast to its effect on hemangiogenesis, we did not observe an influence of Netrin-4 on corneal lymphangiogenesis. Larrieu-Lahargue et al. showed expression of Netrin-4 in embryonic and adult lymphatic vessels. In our study, we found no colocalization of Netrin-4 with Lyve-1, a known marker for lymphatic vessels. Moreover, the lymphatic vascularized area did not differ significantly between WT and mice, and VEGF-C and Lyve-1 mRNA expression in corneas after suture placement did not differ between WT and mice. This is in contrast to the study by Larrieu-Lahargue et al. which demonstrated an influence of Netrin-4 on induction of lymphangiogenesis in several standard lymphangiogenic models. In their study, overexpression of Netrin-4 in mouse skin increased lymphangiogenesis, probably driven by overexpression of VEGF-C, and induced tumor
lymphangiogenesis. Current data do not support that lack of Netrin-4 has an effect on pathologic corneal lymphangiogenesis. An explanation for that might be that the differentiated cornea has to be kept free of blood and lymphatic vessels. For that purpose, an anti-hemangiogenic environment by enrichment of Netrin-4 in the basement membrane of blood vessels and an anti-lymphangiogenic environment by absence of Netrin-4 in lymphatic vessels are established. In this way the cornea differs from other tissues that express Netrin-4 especially in lymphatic vessels to permit fast lymphatic vessel ingrowth (e.g., in case of injury). Since corneal lymphocytes do not seem to express Netrin-4 especially in lymphatic vessels to permit fast lymphatic vessel ingrowth (e.g., in case of injury). Since corneal lymphocytes do not seem to express Netrin-4, corneal lymphangiogenesis can also develop without Netrin-4, probably because the main stimulus of corneal lymphangiogenesis is mediated by infiltrating macrophages. Netrin-4 independent. In contrast, VEGF-A, especially the isoform 165, plays a critical role for hemangiogenesis, but is dispensable for lymphangiogenesis. Efforts to identify Netrin-4 receptors to date, although few and contradictory, suggest that three Netrin-1 receptors: DCC, Neogenin, and Unc5H2, are involved in the interaction between endothelial vessel cells and vascular smooth muscle cells (VSMC). Notably, Lejmi et al. showed that VSMC express DCC, Neogenin, and Unc5H2 receptors, but silencing all three receptors did not completely inhibit adhesion to Netrin-4. At the mRNA level, we found the expression of all three putative Netrin-4 receptors in the native mouse cornea and of Neogenin and Unc5H2 in corneal cell lines (HCEC, HCET, and HCK). Their expression levels, however, were all relatively low. Among the three candidates, we found that only Unc5H2 mRNA expression was observed in HCK after stimulation, while the Unc5H2 mRNA expression in HCEC remained stable after stimulation (P = 0.016). (D) Neogenin mRNA expression was low in both cell lines (HCEC and HCK) and did not differ after stimulation of the cells.

**Figure 6.** Differential regulation of VEGF-A, Netrin-4, Unc5H2, and Neogenin mRNA expression in HCEC, HCK. HCEC and HCK were serum-starved with medium for 12 hours. Quantitative PCR was performed to study (A) VEGF-A, (B) Netrin-4, (C) Unc5H2, and (D) Neogenin mRNA expression in HCEC after stimulation for 2 hours with FCS 10% and HCK after stimulation with supplement, FCS 10%, or supplement with FCS 10% for 2 hours. (A) Expression of VEGF-A mRNA in serum-free cell lines, HCEC and HCK, was low. Expression of VEGF-A mRNA increased significantly in both cell lines after stimulation (P = 0.014, P = 0.009). Human cell keratocytes showed significantly higher VEGF-A mRNA expression after stimulation than HCEC (P = 0.014). (B) Comparison of Netrin-4 mRNA expression between HCEC and HCK demonstrated significantly higher Netrin-4 mRNA expression in HCEC compared to HCK serum-free and after stimulation (P = 0.047, P = 0.027). Netrin-4 mRNA expression increased after stimulation in HCEC, but not significantly. In contrast, Netrin-4 mRNA expression was not influenced by stimulation in HCK. (C) Expression of Unc5H2 mRNA was significantly higher in HCK than in HCEC serum-free and after stimulation (P = 0.009, P = 0.014). A significant increase of Unc5H2 mRNA expression was observed in HCK after stimulation, while the Unc5H2 mRNA expression in HCEC remained stable after stimulation (P = 0.016). (D) Neogenin mRNA expression was low in both cell lines (HCEC and HCK) and did not differ after stimulation of the cells.
basement membrane. Given that Netrin-4 is a secreted protein, it is possible that corneal epithelial cells are responsible for secretion of Netrin-4 to establish the non-angiogenic environment of the cornea.

Lejmi et al. observed that Netrin-4 specifically is overexpressed in VEGF-stimulated endothelial cells in vitro and in vivo. The group suggested that Netrin-4 could act as a negative feedback regulator of pathologic angiogenesis at the endothelial cell level. As described above, corneal epithelial cells also express Netrin-4. In this study, however, stimulating corneal epithelial cells or corneal keratocytes with either VEGF-A or Netrin-4 at different concentrations did not influence the expression of VEGF-A, Netrin-4, Unc5H2, or Neogenin mRNA. Thus, these cells do not show an autocrine regulation of Netrin-4 expression by either Netrin-4 or VEGF-A. Therefore, it can be assumed that corneal epithelial cells indeed establish an antiangiogenic environment in the cornea via expression of Netrin-4 to enrich the basement membrane.

In summary, this study demonstrated that the absence of Netrin-4 increased hemangiogenesis in the mouse-model of suture-induced neovascularization, but had no effect on lymphangiogenesis. Netrin-4 acted as an antiangiogenic factor in the cornea. The lack of Netrin-4 resulted in an augmentation of angiogenesis-driving VEGF-A mRNA expression. The receptor Unc5H2 seemed to be the only one of the putative Netrin-4 receptors involved in these processes.

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References

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