Lymphangiogenesis and angiogenesis: concurrence and/or dependence? Studies in inbred mouse strains

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ABSTRACT **Genetic background significantly affects angiogenesis in mice. However, lymphangiogenic response to growth factors (GFs) in different strains has not been studied. We report constitutive expression of corneal lymphatics that extends beyond the limits of normal limbal vessels. In untreated corneas, the total number ($P=0.006$), the number above blood vessels ($P=10^{-8}$), and the area of preexisting lymphatics ($P=0.007$) were significantly higher in C57BL/6 than in BALB/c mice. Normal corneas of three other strains, the *nu/nu*, 129E, and Black Swiss mice, showed in most parameters intermediate phenotypes. *FGF-2*$^{-/-}$ mice showed significantly less preexisting lymphatics than control ($P=0.009$), which suggests a role for this GF in lymphatic development. VEGF-A-induced corneal lymphangiogenic response was significantly higher in BALB/c mice ($P=0.03$), but it did not differ significantly in C57BL/6 mice, when compared to PBS-implanted control. FGFR-3 expression was higher in C57BL/6 than BALB/c mice, which suggests GF-receptor heterogeneity as a possible explanation for strain-dependent differences. The heterogeneity of preexisting lymphatic vessels in the limbal area significantly correlated with the extent of corneal lymphangiogenesis (VEGF-A: $r=0.7$, $P=0.01$; FGF-2: $r=0.96$, $P=10^{-5}$) in BALB/c but not in C57BL/6 mice. Removal of conjunctival lymphatics did not affect GF-induced lymphangiogenesis. This work introduces physiological expression of lymphatics without blood vessels, which indicates that angiogenesis and lymphangiogenesis, even though intricately related, may occur independently. Furthermore, we show strain-dependence of normal and GF-induced lymphangiogenesis. These differences may affect disease development in various strains.—Nakao, S., Maruyama, K., Zandi, S., Melhorn, M. I., Taher, M., Noda, K., Nusayr, E., Doetschman, T., Hafezi-Moghadam, A. Lymphangiogenesis and angiogenesis: concurrence and/or dependence? Studies in inbred mouse strains. *FASEB J.* 24, 504–513 (2010). www.fasebj.org**

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Avascularity of the cornea is necessary for its transparency (1). The normal cornea but not the conjunctiva is also devoid of lymphatic vessels (2). Lymphangiogenesis occurs in vascularized (3, 4) and inflamed corneas (5–7). However, the cellular and molecular mechanisms of corneal lymphangiogenesis are not well understood.

Using the corneal micropocket assay, Rohan and co-workers (8–10) revealed differences in angiogenesis in various mouse strains in response to growth factors (GFs). Vascular endothelial growth factor A (VEGF-A)- or fibroblast growth factor 2 (FGF-2)-induced corneal angiogenesis in BALB/c mice is significantly higher than in C57BL/6 mice (8), which suggests a key role for genetic background in angiogenic potential.

The current paradigm regards lymphangiogenesis secondary to angiogenesis (11), which suggests common molecular and cellular origins for both processes. Save for the report by Chang *et al.* (12), which demonstrates that low-dose FGF-2 selectively induces pathological lymphangiogenesis in the cornea, current literature gives no indication of alone-standing lymphatics under physiological conditions. Furthermore, the mechanisms underlying the selective lymphangiogenic growth of low-dose FGFs remain to be elucidated. Both VEGF-A and FGF-2 induce lymphangiogenesis in corneas of C57BL/6 mice (13, 14). Also, VEGF-C induces lymphangiogenesis in various models (15, 16) and angiogenesis under certain conditions (17). However, the lymphangiogenic response to GFs in various strains has not been studied.

Here, we investigate the lymphangiogenic potential of inbred mouse strains after VEGF-A, FGF-2, and VEGF-C corneal implantation. Elucidating the heterogeneity in preexisting and the mechanisms underlying the growth of lymphatics may explain differences in disease development in various mouse models.

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MATERIALS AND METHODS

Animals

All animal experiments were approved by the Animal Care Committee of the Massachusetts Eye and Ear Infirmary. Male 6- to 10-wk-old C57BL/6J (000664; Jackson Laboratory, Bar Harbor, ME, USA), BALB/cN (BALB; Taconic, Hudson, NY, USA), *nu/nu* (088; Charles River Laboratories, Wilmington, MA, USA), 129E (476; Charles River Laboratories), and Black Swiss (BLKSW; Taconic) mice were purchased. The development of *Fgf2*-null mice was described previously (18).

Corneal micropocket assay in mice

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Poly-HEMA pellets (0.3 μl, P3932; Sigma, St. Louis, MO, USA) containing 200 ng VEGF-A (293-VE; R&D Systems, Minneapolis, MN, USA), 100 ng FGF-2 (3139-FB; R&D Systems) or 400 ng VEGF-C (4634; BioVision, Mountain View, CA, USA) were prepared and implanted into the corneas. VEGF-A, FGF-2 or VEGF-C pellets were positioned at ~0.8–1 mm distance to the corneal limbus. After implantation, bacitracin ophthalmic ointment (E. Fougera & Co., Melville, NY, USA) was applied to each eye to prevent infection. Six days after implantation, digital images of the corneal vessels were obtained using OpenLab 2.2.5 software (Improvision Inc., Waltham, MA, USA) with standardized illumination and contrast.

Whole-mount immunofluorescence

Eyes were enucleated and fixed with 4% paraformaldehyde for 30 min at 4°C. For whole-mount preparation, the corneas were microsurgically exposed by removing other portions of the eye. Radial cuts were then made in the cornea. Tissues were washed with PBS 3 times for 5 min and then placed in methanol for 20 min. Tissues were incubated overnight at 4°C with anti-mouse CD31 mAb (5 μg/ml, 550274; BD Pharmingen, San Jose, CA, USA) and anti-mouse LYVE-1 Ab (4 μg/ml, 103-PA50AG; ReliaTech GmbH, Wolfenbüttel, Germany), diluted in PBS containing 10% goat serum and 1% Triton X-100. Tissues were washed 4 times for 20 min in PBS followed by incubation with Alexa Fluor488 goat anti-rat IgG (20 μg/ml, A11006; Invitrogen, Carlsbad, CA, USA) and Alexa Fluor647 goat anti-rabbit IgG (20 μg/ml, A21244; Invitrogen) overnight at 4°C. Corneal flatmounts were prepared on glass slides using a mounting medium (TA-030-FM, Mountant Permafluor; Lab Vision Corporation, Fremont, CA, USA). The flatmounts were examined by fluorescence microscopy, and digital images were recorded using OpenLab 2.2.5 software with standardized illumination and contrast. Angiogenesis and lymphangiogenesis were quantitatively analyzed using Scion Image 4.0.2 software (Scion Corp., Frederick, MD, USA). The number of the lymphatic vessels was obtained by counting. The distance of the tips of the newly grown vessels (angiogenic and lymphatic) from the preexisting limbal vessels and vessel length was measured using OpenLab software.

Expression of lymphangiogenesis-related factors in the mouse cornea

Total RNA was extracted from 4–6 corneas by Trizol reagent (15596-026; Invitrogen). The RNA (2 μg) sample was transcribed to cDNA with reverse transcription reagents (N808-0234; Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using Step One Plus Real-Time PCR System and mouse FGF-2- or FGFR-3-specific TaqMan Gene Expression Assays for individual mRNAs (100 ng) according to the manufacturer's protocol (Applied Biosystems).

Removal of conjunctiva and cornea lymphangiogenesis

The conjunctiva underneath the limbal vascular plexus was microsurgically removed (width 2–3 mm; height 0.5–1 mm) (see **Fig. 4*D***). Corneal micropocket assay was performed, and bacitracin ophthalmic ointment (E. Fougera & Co.) was applied to each eye to prevent infection. Six days after implantation, digital images of the corneal vessels were obtained using OpenLab software.

Immunohistochemistry

The eyes or Matrigels were harvested and snap-frozen in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA). Sections (10 μm) were prepared, air-dried, and fixed in ice-cold acetone for 10 min. The sections were blocked with 3% nonfat dried milk bovine working solution (M7409; Sigma) and stained with anti-FGF-2 Ab (2 μg/ml, sc-79; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-mouse CD31 mAb (1:50, 550274; BD Pharmingen) and anti-mouse LYVE-1 Ab (1 μg/ml, 103-PA50AG; ReliaTech GmbH), or podoplanin (1 μg/ml, 11–033; AngioBio, Del Mar, CA, USA) and CD11b (1:100, 550282; BD Pharmingen). After an overnight incubation, sections were washed and stained for 20 min with Alexa Fluor488 goat anti-rabbit IgG (10 μg/ml, A11034; Invitrogen), Alexa Fluor488 donkey anti-goat IgG (10 μg/ml, A-11055; Invitrogen), Alexa Fluor647 goat anti-rabbit IgG (10 μg/ml, A21244; Invitrogen), and Alexa Fluor594 goat anti-hamster IgG (10 μg/ml, A21113; Invitrogen). To investigate proliferation of lymphatic endothelial cells, the sections were placed in Target Retrieval Solution, Citrate, pH 6 (S2369A; DakoCytomation, Copenhagen, Denmark) at 97°C for 10 min and allowed to cool for another 10 min at room temperature before staining with anti-Ki67 Ab (150 μg/ml, M7249; DakoCytomation) and anti-mouse LYVE-1 Ab.

Matrigel plug assay

Mice were injected subcutaneously with 0.2 ml Matrigel (356230; BD Biosciences) containing PBS, 1 μg FGF-2 (3139-FB; R&D Systems), or 2 μg VEGF-C (2179-VC; R&D Systems). Ten days after implantation, the Matrigel was harvested, and cryosections (10 μm) were prepared for immunohistochemistry. Digital images of the staining were obtained using OpenLab software. Quantitative analysis of the $CD31^+$, $LYVE-1^+$, $podoplanin^+$ and $CD11b^+$ areas in the Matrigels were performed using Scion Image software.

RESULTS

Comparative analysis of lymphatics in untreated corneas of inbred mouse strains

Corneas of normal C57BL/6, BALB/c, 129E, and Black Swiss mice are clear, avascular, and do not phenotypically differ, save for their pigmentation status (**Fig. 1*A***). As a distinctive feature, corneas of normal *nu/nu* mice (5 of 10 eyes) showed spontaneous blood vessels. To study the preexisting blood and lymphatic vessels in the

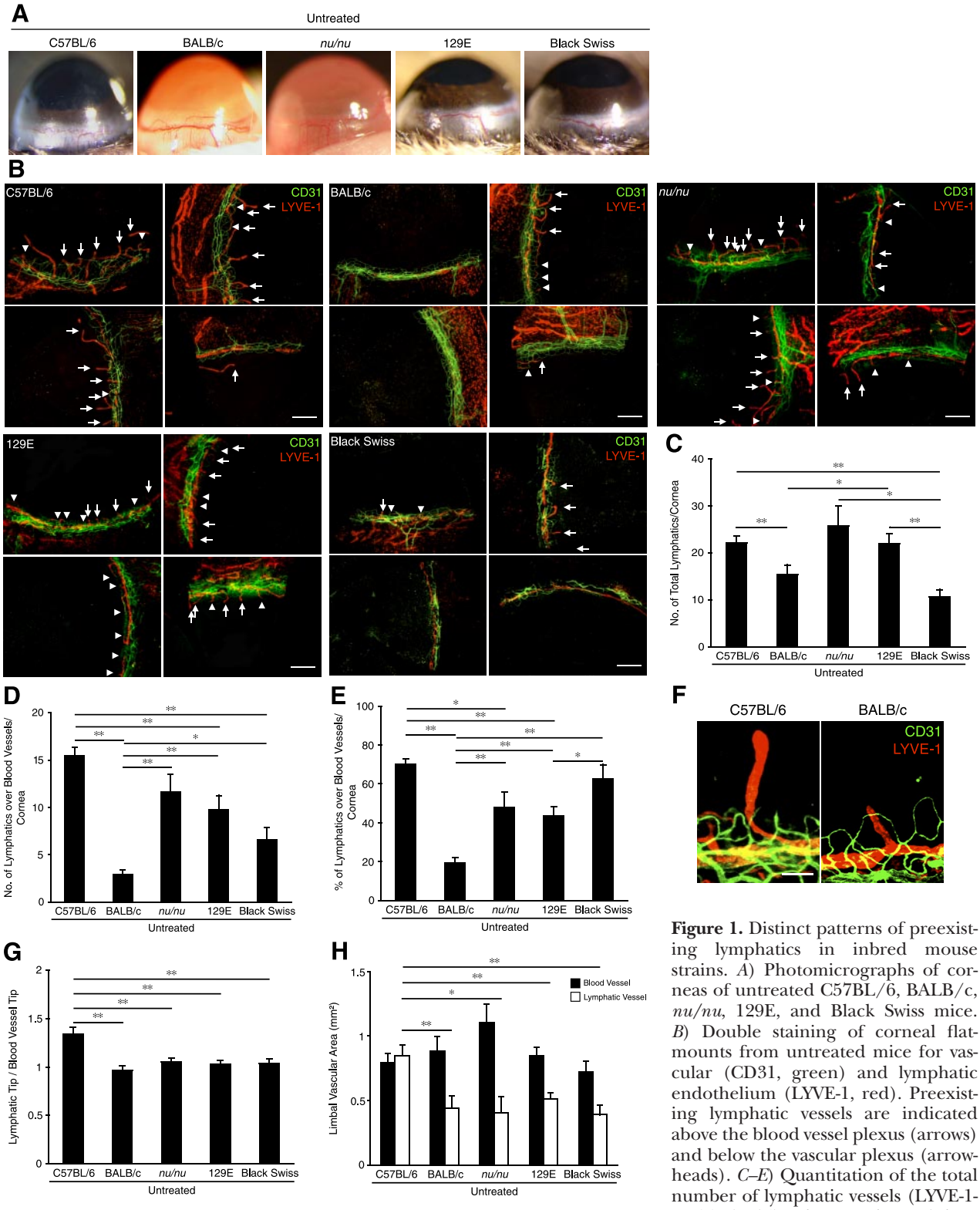

Figure 1. Distinct patterns of preexisting lymphatics in inbred mouse strains. *A*) Photomicrographs of corneas of untreated C57BL/6, BALB/c, *nu/nu*, 129E, and Black Swiss mice. *B*) Double staining of corneal flatmounts from untreated mice for vascular (CD31, green) and lymphatic endothelium (LYVE-1, red). Preexisting lymphatic vessels are indicated above the blood vessel plexus (arrows) and below the vascular plexus (arrowheads). *C–E*) Quantitation of the total number of lymphatic vessels (LYVE-1-positive) (*C*), the number of lymphatic vessels over the vascular plexus per cornea (*D*), and the percentage of lymphatics over blood vessels (*E*) in untreated corneas of C57BL/6 (n=12), BALB/c (n=11), *nu/nu* (n=7), 129E (n=7), and Black Swiss mice (n=8). *F*) Distinct patterns of blood (CD31) and lymphatic vessels (LYVE-1) in untreated corneas of C57BL/6 and BALB/c mice. *G*) Ratio of the distance of lymphatic tips and blood vessel tips from limbal vessels in untreated corneas of C57BL/6 (n=58), BALB/c (n=36), *nu/nu* (n=76), 129E (n=81), and Black Swiss mice (n=49). *H*) Quantitative analysis of blood vessel (black) and lymphatic area (white) in untreated corneas of C57BL/6 (n=11), BALB/c (n=4), *nu/nu* (n=7), 129E (n=7), and Black Swiss mice (n=8). $^*P < 0.05$; $^{**}P < 0.01$. Scale bars = 400 μm (*B*); 100 μm (*F*).

normal murine cornea, we immunostained corneal flatmounts of untreated mice with CD31 and LYVE-1 Abs, respectively. Vascular loops in the vicinity of the limbus preexisted in all strains (Fig. 1*B*). LYVE-1$^+$ lymphatic vessels in untreated corneas showed round tips and followed the limbal curvature along with the vascular plexus (Fig. 1*B*). In BALB/c, 129E, and Black Swiss mice, preexisting lymphatics were together with limbal vessels, whereas in C57BL/6 and *nu/nu* mice, some lymphatics surpassed the limit of limbal vessels and were found individually in the cornea (Fig. 1*B*). The density of lymphatics was significantly higher in C57BL/6 mice (n=12) than BALB/c (n=11, P=0.006), or Black Swiss mice (n=8, P=0.00003) (Fig. 1*C*). In comparison, the lymphatic density was significantly higher in *nu/nu* mice (n=7) than in Black Swiss mice (P=0.01) (Fig. 1*C*). Furthermore, the corneal lymphatic density was significantly higher in 129E (n=7) than BALB/c mice (P=0.03) (Fig. 1*C*). The percentage of lymphatics over blood vascular plexus was highest in C57BL/6 (n=12) and lowest in BALB/c (n=11) among all examined strains ($P=4\times10^{-11}$) (Fig. 1*E*). Also, the ratio of lymphatic/blood vessel length was significantly higher in C57BL/6 (n=58) compared to BALB/c mice (n=36, $P=0.5\times10^{-4}$) (Fig. 1*F, G*). The length ratio in *nu/nu*, 129E, or Black Swiss mice was significantly lower than C57BL/6 but not BALB/c mice (Fig. 1*G*). The number of preexisting lymphatics above the blood vascular plexus was significantly higher in C57BL/6 (n=12), compared to BALB/c (n=11, $P=10^{-8}$), 129E (n=7, P=0.003), or Black Swiss (n=8, P=0.00009), but not *nu/nu* mice (n=7, P=0.1) (Fig. 1*D*). The lymphatic area was significantly larger in C57BL/6 (n=11) compared to BALB/c (n=6, P=0.007), *nu/nu* (n=7, P=0.01), 129E (n=7, P=0.003), or Black Swiss mice (n=8, P=0.0008). However, the blood vascular area did not differ significantly between these strains (n=4–11) (Fig. 1*H*).

FGF-2 and FGFR-3 expression in untreated cornea and corneal lymphatics in *Fgf2*$^{-/-}$ mice

Low-dose FGF-2 selectively induces lymphangiogenesis (12). To investigate whether FGF-2 contributes to the phenotype of the preexisting corneal lymphatics in C57BL/6 and BALB/c mice, we investigated FGF-2 expression in untreated C57BL/6 and BALB/c corneas with real-time PCR and immunohistochemistry. In line with previous reports (19), we found FGF-2 expression in corneas of both strains (Supplemental Fig. 1). Real-time PCR showed similar FGF-2 expression levels in untreated corneas of adult C57BL/6 and BALB/c mice (n=4, P=0.9) (**Fig. 2*C***).

However, in *Fgf2*$^{-/-}$ mice, the number of corneal lymphatics (n=8) was significantly lower than in the WT counterparts (n=12, P=0.009) (Fig. 2*A*). Furthermore, the number of lymphatics over the vascular plexus was significantly higher in WT (n=12) mice, compared to that in *Fgf2*$^{-/-}$ mice (n=8, P=0.047) (Fig. 2*A, B*). To examine the expression level of FGFR-3, which is important for FGF-2-induced lymphangiogenesis (20), we performed real-time PCR. Untreated corneas of C57BL/6 mice showed significantly higher levels of FGFR-3 expression than BALB/c mice (n=3, P=0.01) (Fig. 2*C*).

GF-induced corneal lymphangiogenesis in inbred mouse strains

To study possible strain dependence of GF-induced lymphatic response, we implanted VEGF-A, FGF-2, VEGF-C, or control pellets in corneas of C57BL/6 and BALB/c mice. Corneas implanted with control pellets showed no angiogenesis. In contrast, VEGF-A, FGF-2, and VEGF-C induced significant corneal angiogenesis in either strain (**Fig. 3*A, B***). In line with previous reports (8), both VEGF-A and FGF-2 but not VEGF-C elicited significantly greater angiogenic responses in BALB/c than in C57BL/6 mice (n=10–11, P=0.01) (Fig. 3*C*). Examination of the other 3 strains—*nu/nu*, 129E, and Black Swiss—showed differing amounts of preexisting lymphatics, as well as GF-induced lymphangiogenesis (Supplemental Fig. 2). FGF-2 and VEGF-C caused significant lymphangiogenesis in C57BL/6 (n=3–13, $P_{\text{FGF-2}}$=0.02, $P_{\text{VEGF-C}}$=0.04) as well as BALB/c (n=4–11, $P_{\text{FGF-2}}$=0.002, $P_{\text{VEGF-C}}$=0.0002) mice, whereas VEGF-A significantly increased lymphatic area in BALB/c (P=0.03) but not C57BL/6 (P=0.4) mice (Fig. 3*D*). Furthermore, VEGF-A, FGF-2, and VEGF-C increased the number of lymphatic tips in BALB/c (n=4–11, $P_{\text{VEGF-}}$=0.02, $P_{\text{FGF-2}}$=0.01, $P_{\text{VEGF-C}}$=0.01) and C57BL/6 mice (n=3–13, $P_{\text{VEGF-A}}=0.7\times10^{-3}$, $P_{\text{FGF-2}}$=0.009, $P_{\text{VEGF-C}}$=0.01) compared to PBS-implanted controls (Fig. 3*E*). However, the lymphatic area in either strain did not differ (n=3–11, $P_{\text{VEGF-A}}$=0.8, $P_{\text{FGF-2}}$=0.7, $P_{\text{VEGF-C}}$=0.5) (Fig. 3*D*). Also, no significant difference was found in the number of lymphatic tips in the GF-implanted corneas between C57BL/6 and BALB/c mice (n=3–13, $P_{\text{VEGF-A}}$=0.7, $P_{\text{FGF-2}}$=0.5, $P_{\text{VEGF-C}}$=0.4) (Fig. 3*E*).

Effect of preexisting conjunctival lymphatics on corneal lymphangiogenesis

In eyes that had preexisting conjunctival lymphatics at the pellet implantation sites, as confirmed by immunohistochemistry, we evaluated GF-induced lymphangiogenesis (**Fig. 4*A, B***). The conjunctiva of some C57BL/6 and BALB/c mice were devoid of LYVE-1$^+$ lymphatic tube structures (not, however, LYVE-1$^+$ cells) (Fig. 4*A*). Of the conjunctival sites of GF-implanted eyes, 73.1 and 62.5% showed apparent lymphatic tube structures in C57BL/6 and BALB/c mice, respectively (Fig. 4*A, B*). To investigate whether preexistence of conjunctival lymphatics affects GF-induced corneal lymphangiogenesis, we implanted VEGF-A in BALB/c corneas with and without LYVE-1$^+$ lymphatic tube structures and quantified the lymphangiogenic response. BALB/c mice with preexisting conjunctival lymphatics showed significantly more VEGF-A-induced corneal lymphangiogenesis than animals without (n=4–7, $P=0.7\times10^{-4}$) (Fig. 4*C*). In contrast, corneal angiogenesis was not affected by existence or absence of conjunctival lymphatics

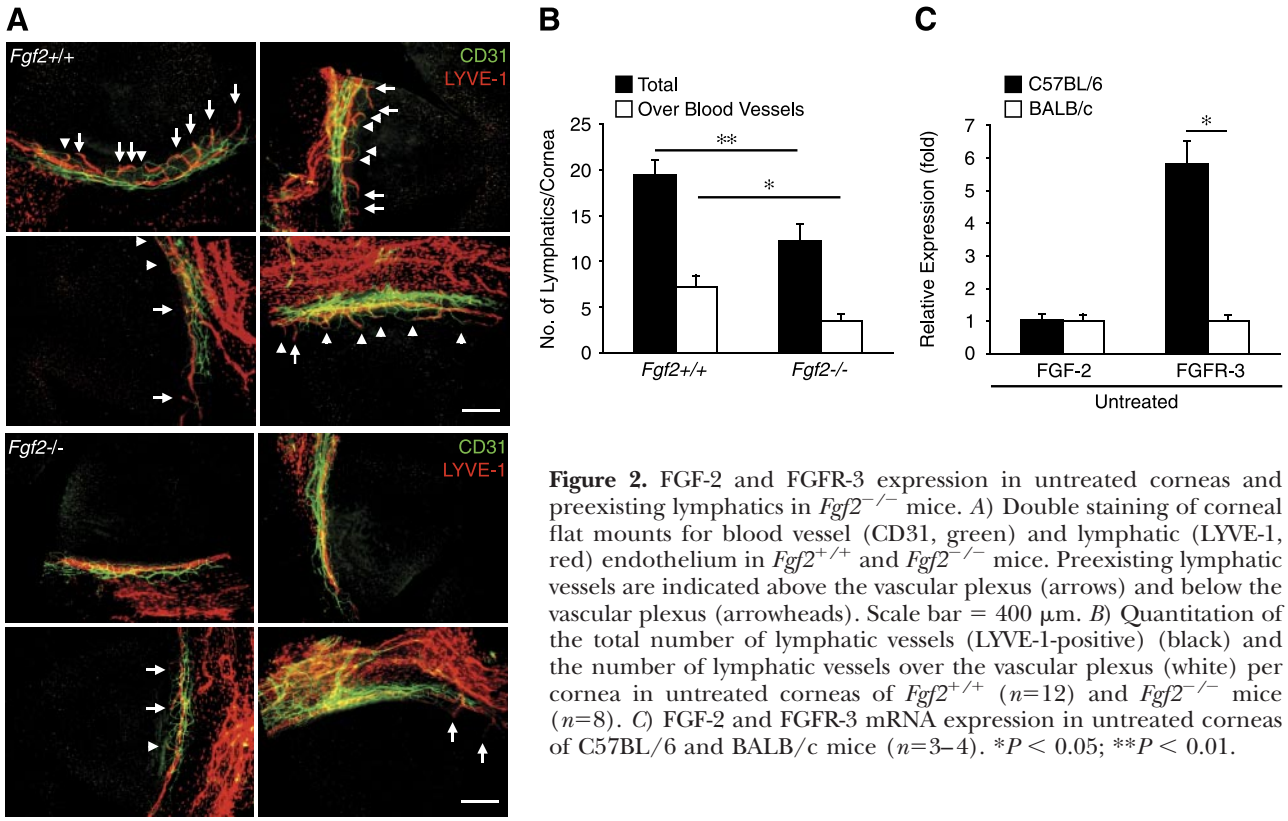

Figure 2. FGF-2 and FGFR-3 expression in untreated corneas and preexisting lymphatics in *Fgf2*$^{-/-}$ mice. *A*) Double staining of corneal flat mounts for blood vessel (CD31, green) and lymphatic (LYVE-1, red) endothelium in *Fgf2*$^{+/+}$ and *Fgf2*$^{-/-}$ mice. Preexisting lymphatic vessels are indicated above the vascular plexus (arrows) and below the vascular plexus (arrowheads). Scale bar = 400 μm. *B*) Quantitation of the total number of lymphatic vessels (LYVE-1-positive) (black) and the number of lymphatic vessels over the vascular plexus (white) per cornea in untreated corneas of *Fgf2*$^{+/+}$ (n=12) and *Fgf2*$^{-/-}$ mice (n=8). *C*) FGF-2 and FGFR-3 mRNA expression in untreated corneas of C57BL/6 and BALB/c mice (n=3–4). *$P < 0.05$; **$P < 0.01$.

(n=4–7, P=0.18) (Fig. 4*C*). Next, we studied GF-induced corneal lymphangiogenesis in C57BL/6 and BALB/c mice. In VEGF-A-implanted C57BL/6 mice, the lymphangiogenic area in eyes with conjunctival lymphatics (n=7) was significantly greater than in eyes without conjunctival lymphatics (n=2, P=0.04). Similarly, in FGF-2-implanted BALB/c mice, the lymphangiogenic area in eyes with conjunctival lymphatics (n=8) was significantly greater than in eyes without (n=2, P=0.02). However, in FGF-2-implanted C57BL/6 mice, the lymphangiogenic area with (n=4) or without conjunctival lymphatics did not differ significantly (n=2, P=0.2).

We hypothesized that preexisting conjunctival lymphatics may be necessary for GF-induced corneal lymphangiogenesis. To address this, we microsurgically removed the conjunctiva without affecting the limbus and implanted VEGF-A into the corneas. Surprisingly, VEGF-A implantation caused lymphangiogenesis as well as angiogenesis in corneas of mice after conjunctival removal (Fig. 4*E, F*). In the control animals that were PBS implanted after conjunctival removal, lymphangiogenesis was stronger than without the removal (n=4 and 5; P=0.04) (Fig. 4*F, G*). Hyperplasia of lymphatics around the area where conjunctiva was excised occurred on d 6 (Fig. 4*F*). Removal of the conjunctiva significantly enhanced VEGF-A-induced corneal angiogenesis (n=7 and 5; P=0.009) but not lymphangiogenesis (n=7 and 5; P=0.3) (Fig. 4*F, H*). These data suggest that the conjunctiva, including its LYVE-1$^+$ lymphatics and cells, may be not necessary for corneal lymphangiogenesis.

The preexisting lymphatics along the limbal vascular plexus are heterogeneously distributed (Fig. 4*A*). VEGF-A- as well as FGF-2-induced corneal lymphangiogenesis correlated with the occurrence of preexisting lymphatics in the limbus in BALB/c ($R_{\text{VEGF-A}}$=0.74, $P_{\text{VEGF-A}}$=0.01, $R_{\text{FGF-2}}$=0.96, $P_{\text{FGF-2}}$=1.0×10^{-5}) but not C57BL/6 mice ($R_{\text{VEGF-A}}$=0.02, $P_{\text{VEGF-A}}$=0.9, $R_{\text{FGF-2}}$=0.32, $P_{\text{FGF-2}}$=0.5) (Fig. 4*I, J*).

Lymphangiogenesis in Matrigel plug assay of C57BL/6 and BALB/c mice

To examine whether the strain-dependent differences in GF-induced lymphangiogenesis are also found outside of the eye, we used the *in vivo* model of subcutaneous Matrigel implantation (21). FGF-2-, VEGF-C-, or PBS-containing Matrigel was implanted in C57BL/6 and BALB/c mice and 10 d later immunostained with CD31 and LYVE-1 Abs. In both strains, LYVE-1$^+$ tube structures as well as CD31$^+$ vessels were visible (**Fig. 5*A***). Both LYVE-1$^+$ tube structures and CD31$^+$ vessels were significantly less in C57BL/6 than in BALB/c mice in FGF-2-containing Matrigel (n=4, P_{CD31}=0.008, $P_{\text{LYVE-1}}$=0.02) (Fig. 5*B, C*). However, no difference was found in LYVE-1$^+$ and CD31$^+$ areas between these strains in PBS- or VEGF-C-containing Matrigels (n=4, PBS: P_{CD31}=0.4, $P_{\text{LYVE-1}}$=0.5; VEGF-C: P_{CD31}=0.5, $P_{\text{LYVE-1}}$=0.6) (Fig. 5*B, C*). Furthermore, because LYVE-1 is also expressed in macrophages (6), we stained the corneas for podoplanin, another lymphatic marker. The podoplanin$^+$ area in BALB/c mice was significantly larger

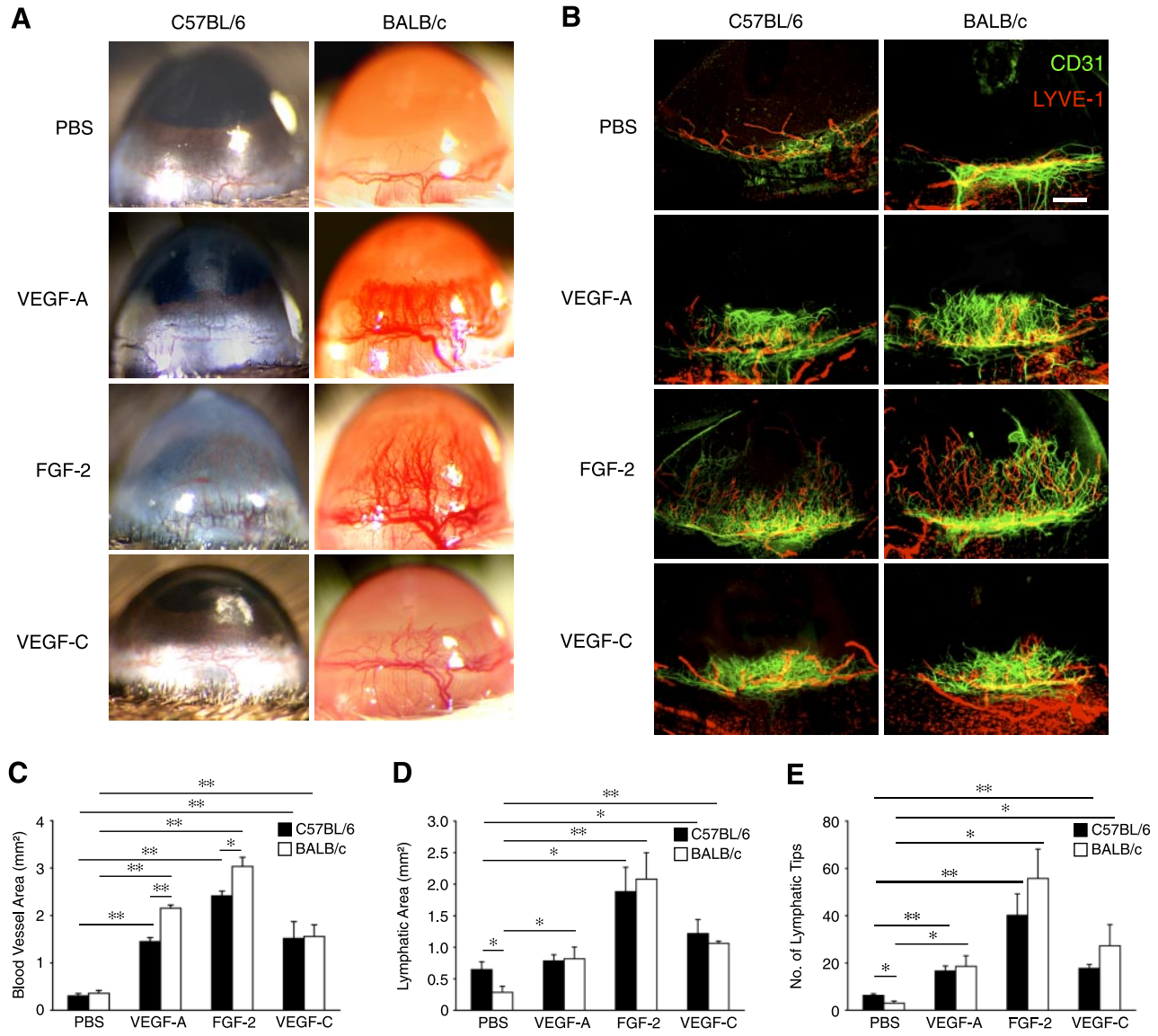

Figure 3. VEGF-A, FGF-2 and VEGF-C-induced corneal angiogenesis and lymphangiogenesis in C57BL/6 and BALB/c mice. *A*) Pellets containing VEGF-A (200 ng), FGF-2 (100 ng), VEGF-C (400 ng), or vehicle control (PBS) were implanted into corneas of C57BL/6 and BALB/c mice. Corneal angiogenesis and lymphangiogenesis were examined 6 d after implantation. *B*) Double staining of corneal flat mounts for angiogenic (CD31, green) and lymphangiogenic (LYVE-1, red) endothelium. Scale bar = 400 μm. *C, D*) Quantitative analysis of angiogenesis (*C*) and lymphangiogenesis (*D*) in PBS-, VEGF-A-, FGF-2-, or VEGF-C-implanted corneas on d 6 (n=5–11). *E*) Quantitation of the number of lymphatic tips in corneas of VEGF-A-, FGF-2-, VEGF-C-, or vehicle control-implanted eyes (n=3–13). *$P < 0.05$; **$P < 0.01$.

than C57BL/6 mice in the FGF-2-containing Matrigel (n=4, P=0.047). However, in PBS- or VEGF-C-containing Matrigel, no difference was found between these strains (n=4, P_{PBS}=0.5, $P_{VEGF\text{-}C}$=0.4) (Fig. 5*D*, *E*). To investigate differences in inflammatory cell infiltration between these strains, we next quantified $CD11b^+$ cells in GF-containing Matrigels. FGF-2-containing Matrigels showed significantly higher numbers of $CD11b^+$ cells in BALB/c than in C57BL/6 mice (P=0.02), while VEGF-C-containing (P=0.3) or PBS Matrigels (P=0.8) did not differ (n=4 each group) (Fig. 5*D*, *F*). To examine whether the lymphangiogenesis is due to lymphatic endothelial cell proliferation in the Matrigel, immunohistochemistry for Ki67 and LYVE-1 was performed. The number of $Ki67^+$ cells in FGF-2-containing Matrigel was significantly higher than control in BALB/c (n=4, P=0.01) but not C57BL/6 mice (n=4, P=0.2) (Fig. 5*G*, *H*). These results indicate a higher sensitivity for FGF-2- but not VEGF-C-induced lymphangiogenesis and angiogenesis in BALB/c than C57BL/6 mice.

DISCUSSION

Normal cornea is devoid of lymphatics (2). Furthermore, lymphatic growth presumably follows angiogenesis (22). Physiologically preexisting lymphatics in untreated corneas are round tipped and distinct from the sprouting lymphatics. Untreated C57BL/6 corneas showed significantly more preexisting lymphatics over

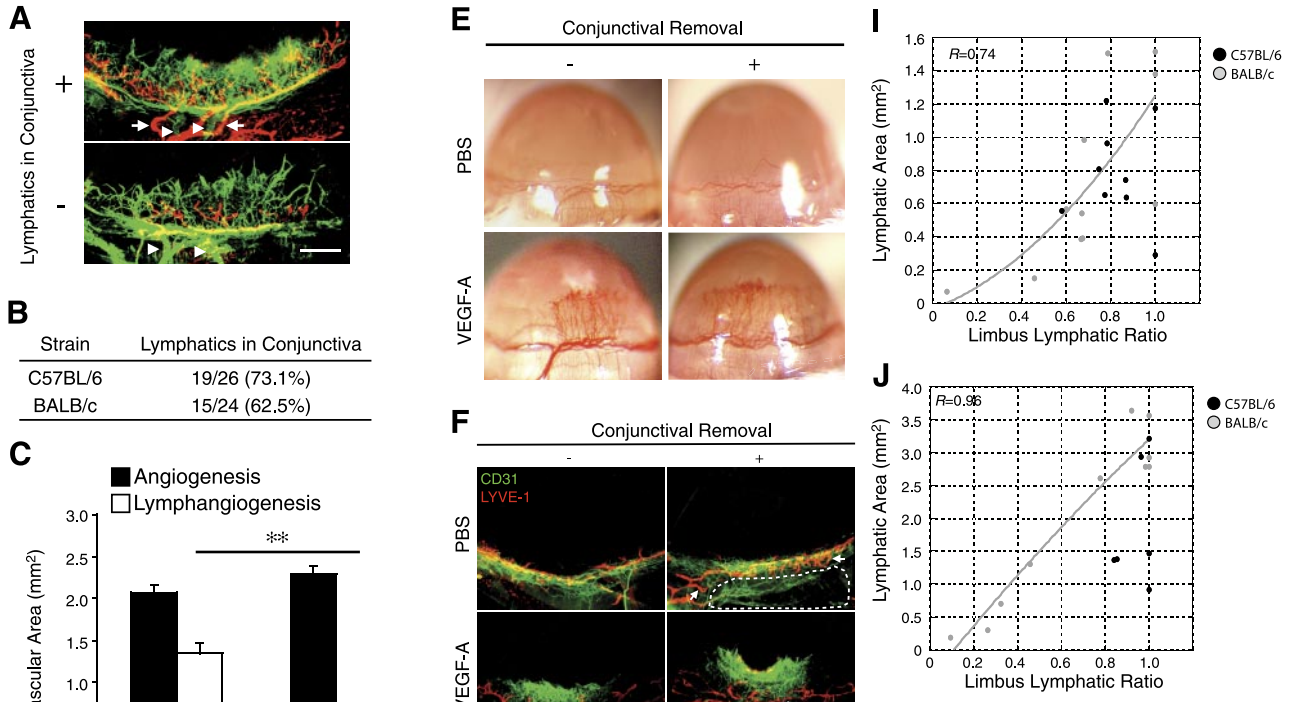

Figure 4. Corneal lymphangiogenesis and conjunctival lymphatic vessels. Corneal flatmounts with lymphangiogenesis were examined on d 6 after implantation and divided into two groups, with or without preexisting lymphatic vessels in the conjunctiva. *A*) Double staining of corneal flatmounts for angiogenesis (CD31, green) and lymphangiogenesis (LYVE-1, red) with or without lymphatic tube structures in VEGF-A-implanted BALB/c mice. Blood vessels (arrowheads) and lymphatic vessels (arrows) in the conjunctiva are indicated. *B*) Frequency of the corneas with LYVE-1$^+$ lymphatic tube structures in the pellet-implanted site of C57BL/6 and BALB/c mice. *C*) Quantitative analysis of lymphangiogenesis (white) and angiogenesis (black) in VEGF-A-implanted BALB/c corneas on d 6 (n=4 and 7). *D*) Photographs and immunohistochemistry of CD31 and LYVE-1 with or without conjunctival removal. *E, F*) Corneal angiogenesis and lymphangiogenesis were examined 6 d after PBS or VEGF-A implantation with or without conjunctival removal. Arrows indicate lymphatic hyperplasia around the excised area. *G, H*) Quantitative analysis of lymphangiogenesis (*G*) and angiogenesis (*H*) in PBS- or VEGF-A-implanted corneas on d 6 with or without conjunctival removal (n=4–7). *I, J*) Correlation between lymphangiogenic area (d 6) and limbus lymphatic ratio in VEGF-A (*I*) or FGF-2 implantation (*J*) (n=6–11). *$P < 0.05$; **$P < 0.01$. Scale bars = 400 μm.

the blood vessels than BALB/c corneas. The phenotype of normal corneas of three other strains, the *nu/nu*, 129E, and Black Swiss mice, differed from C57BL/6 and BALB/c mice. Although, the mechanisms underlying constitutive lymphatic growth in the cornea are not well understood, the difference in preexisting lymphatics between the various strains indicates strain-dependent genetic differences.

In humans, corneal lymphangiogenesis correlates with corneal transplant rejection (23). Preexisting corneal lymphatics may be heterogenic in patients, causing differences in lymphangiogenesis and ultimately transplant rejection. Elucidating the molecular mechanisms, underlying the heterogeneity of the preexisting lymphatics would thus provide useful insights in corneal lymphangiogenesis and potentially help reduce the

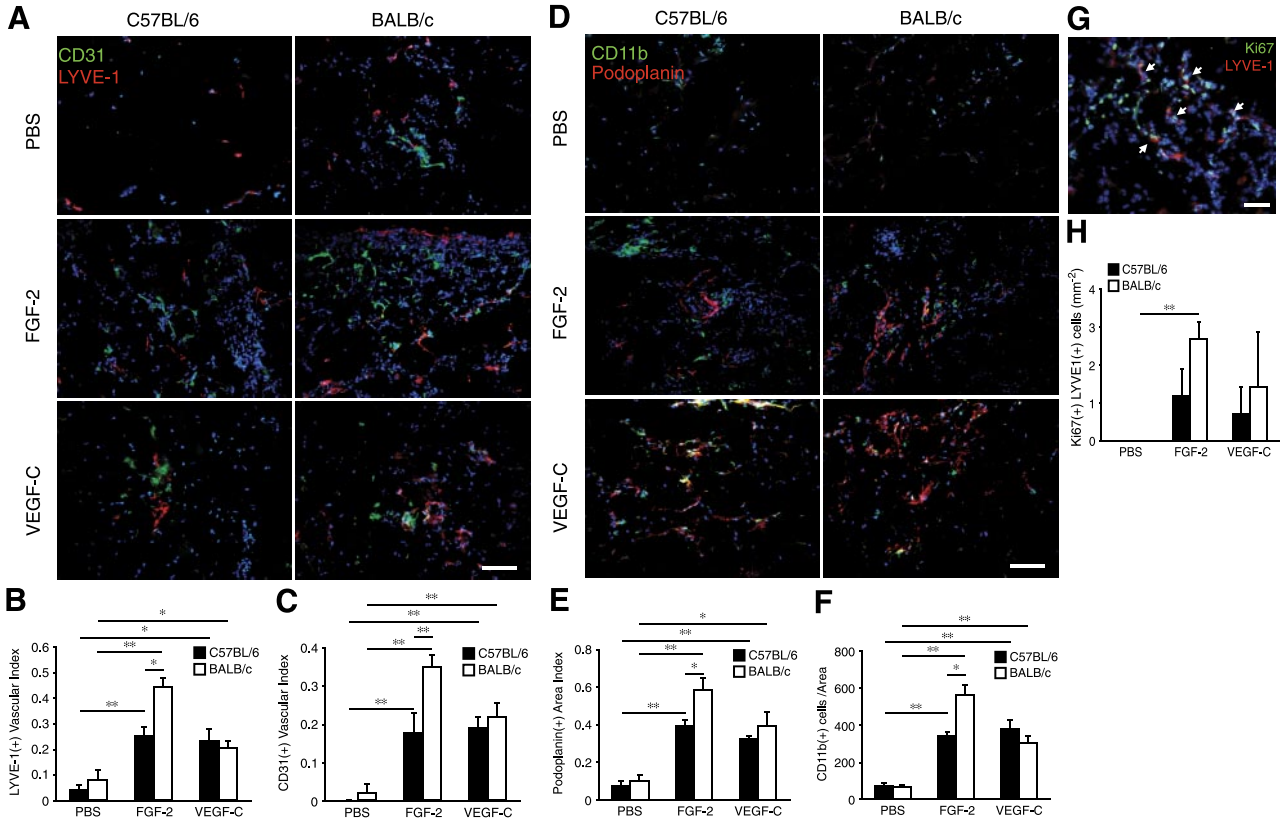

Figure 5. GF-induced angiogenesis and lymphangiogenesis in Matrigel plug assay. *A*) Double staining for angiogenic (CD31, green) and lymphatic endothelium (LYVE-1, red) of PBS-, FGF-2-, or VEGF-C-containing Matrigel in C57BL/6 and BALB/c mice, 10 d after subcutaneous injection of Matrigel. *B, C*) Quantitative analysis of LYVE-1$^+$ area (red, *B*) and CD31$^+$ area (green, *C*) in Matrigel (n=4). *D*) Double staining for lymphatic endothelium (podoplanin, red) and macrophages (CD11b, green) of Matrigel in C57BL/6 and BALB/c mice (d 10). *E, F*) Quantitative analysis of podoplanin$^+$ area (red, *E*) and CD11b$^+$ area (green, *F*) in Matrigel (n=4). *G*) Double staining for lymphatic endothelium (LYVE-1, red) and proliferating marker (Ki67, green) of FGF-2-containing Matrigel in BALB/c mice (d 10). *H*) Quantitative analysis of number of LYVE1$^+$ Ki67$^+$ cells in Matrigel (n=4). *$P < 0.05$; **$P < 0.01$. Scale bars = 100 μm (*A, D*); 50 μm (*G*).

rejection rate in patients. Compared to BALB/c mice, C57BL/6 mice show more vigorous corneal transplant rejection (24). The transplant rejection data together with our finding that C57BL/6 mice have more preexisting lymphatics suggest that the immune privilege in the cornea might be regulated by preexisting lymphatics.

In our experiments, conjunctival blood vessels (arteries and veins) were present in all eyes at the site of angiogenic factor implantation (top side or 12 o'clock). Mouse conjunctiva also contains LYVE-1$^+$ tubelike structures as well as LYVE-1$^+$ cells (25). LYVE-1$^+$ tubelike structures were not always present at 12 o'clock in the conjunctiva. Since normal blood vessels were present in all cases—but lymphatics, only in some cases—the development of lymphatic vessels in the conjunctiva appears to be independent of the blood vessel development. Furthermore, whether LYVE-1$^+$ cells contribute to the lymphatic tube formation or whether they are designated to remain single cells in GF-induced lymphangiogenesis remains unknown.

In eyes with preexisting lymphatics in the conjunctiva, VEGF-A-induced corneal lymphangiogenesis was significantly greater compared with eyes without preexisting conjunctival lymphatics. Interestingly, LYVE-1$^+$ single cells were observed both in conjunctivas with and without preexisting tubelike lymphatics. Although the function of preexisting conjunctival lymphatics during corneal lymphangiogenesis is not understood, our data suggest that these conjunctival lymphatics affect GF-induced corneal lymphangiogenesis. However, these conjunctival lymphatics seem not to be necessary for lymphangiogenesis, as GF-induced lymphangiogenesis also occurs without conjunctival lymphatics. The contribution of tubelike conjunctival lymphatics might be more important than that of LYVE-1$^+$ single cells. Our data suggest that corneal angiogenesis and lymphangiogenesis are independent of conjungtival lymphatics, as in partial conjunctival removal other blood vessels of the conjunctiva might support vascular growth, for instance as a supplier of leukocytes.

The fact that low-dose FGF-2 (12.5 ng) induces lymphangiogenesis without angiogenesis (12) motivated us to investigate whether endogenous FGF-2 contributes to the lymphatic phenotype in C57BL/6 mice. *Fgf2*$^{-/-}$ mice show not only fewer lymphatics but also fewer lymphatic tips that extend beyond the vascular plexus. Intriguingly, FGF-2 expression level did not

differ between C57BL/6 and BALB/c mice, which suggests that differences in FGF-2 expression during development may underlie the lymphatic phenotype that we find in adult mice.

In this study, VEGF-A, VEGF-C, and FGF-2, known angiogenic and lymphangiogenic factors, induced lymphangiogenesis in the cornea (13, 14, 16, 26). These data indicate that GF-induced lymphangiogenic response, defined as amount of new growth per preexisting vessels, is greater in BALB/c than C57BL/6 mice. The fact that both the angiogenic and the lymphangiogenic responses are higher in BALB/c than in C57BL/6 mice suggests strain-dependent common molecular or cellular mechanisms underlying these two phenomena.

Leukocytes, especially macrophages, have a large regulatory effect on corneal angiogenesis and lymphangiogenesis (6, 7, 26–28). $CD11b^+$ cells significantly contribute to lymphangiogenesis (6). More $CD11b^+$ cells were found in FGF-2-containing Matrigel in BALB/c mice compared to C57BL/6 mice. However, lymphangiogenesis or $CD11b^+$ cell infiltration did not differ in VEGF-C-containing Matrigel in these 2 strains. The contribution of immune cells to the strain- or GF-dependent differences in lymphangiogenesis remains to be investigated. FGFR-3, a FGF-2 receptor, is expressed on the lymphatic endothelium (20, 29). Our data show higher FGFR-3 expression in C57BL/6 than BALB/c mice; however, FGF-2 levels do not differ. GF or GF-receptor heterogeneity might contribute to strain-dependent differences in physiological or induced lymphangiogenesis. VEGF-A implantation at 200 ng induces corneal angiogenesis, but not lymphangiogenesis (sprouting but no change in the total surface of the lymphatic). This suggests a higher threshold for VEGF-A-induced lymphangiogenesis than angiogenesis.

Lymphangiogenesis generally accompanies angiogenesis, which may have led to the paradigm that lymphatic growth is a delayed consequence of angiogenesis. Low-dose FGF-2 selectively induces pathological lymphangiogenesis (12). The current study provides evidence for physiological existence of lymphatics in the absence of blood vessels. Moreover, BALB/c shows higher responsiveness to GF-induced lymphangiogenesis compared to C57BL/6 mice, while VEGF-C-induced lymphangiogenesis does not differ in these strains, which suggests differential contribution of the genetic background to various GF-induced lymphangiogenesis. The GF-induced lymphangiogenesis depends highly on preexisting limbal but not conjunctival lymphatics. These findings may help understand pathomechanisms of diseases, where lymphatic growth plays a central role. FJ

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