Published in final edited form as:

FEBS J. 2015 April ; 282(8): 1458–1467. doi:10.1111/febs.13234.

Simultaneous *in vivo* imaging of blood and lymphatic vessel growth in Prox1-GFP/Flk1::myr-mCherry mice

Jimmy Zhu, BS^{#1}, Jennifer Dugas-Ford, PhD^{#1}, Michael Chang, BS¹, Patryk Purta, BS¹, Kyu-Yeon Han, PhD¹, Young-Kwon Hong, PhD², Mary E. Dickinson, PhD³, Mark I. Rosenblatt, MD, PhD, MBA¹, Jin-Hong Chang, PhD^{1,*}, and Dimitri T. Azar, MD, MBA¹

¹Department of Ophthalmology and Visual Sciences, Illinois Eye and Ear Infirmary, University of Illinois at Chicago, Chicago, IL, USA

²Department of Surgery, Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

³Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX, USA

These authors contributed equally to this work.

Abstract

The ability to visually observe angiogenesis and lymphangiogenesis simultaneously and repeatedly in living animals could greatly enhance our understanding of the interdependence of these processes. To generate a mouse model that affords such visualization via *in vivo* fluorescence imaging, we bred Prox1-GFP mice with Flk1::myr-mCherry mice to generate Prox1-GFP/Flk1::myr-mCherry mice, in which lymphatic vessels emit green fluorescence and blood vessels emit red fluorescence. Corneal neovascularization was induced in these mice using three injury models: vascular endothelial growth factor (VEGF) pellet implantation, basic fibroblast growth factor (bFGF) pellet implantation, and alkali burn injury. Vessel growth was observed *in vivo* by stereomicroscopy on days 0, 3, 7, and 10 after pellet implantation or alkali injury as well as in flat-mounted corneas via confocal microscopy after the final *in vivo* imaging time point. We observed blood and lymphatic vessel growth in all three models, with the most significant growth occurring from days 0–7. Upon VEGF stimulation, the growth kinetics of blood and lymphatic vessels were similar. Blood vessels exhibited similar growth patterns in VEGF- and bFGF-stimulated corneas. Alkali burn injury induced robust angiogenesis and lymphangiogenesis. In this study, the intrinsic fluorescence of blood and lymphatic endothelial cells in Prox1-GFP/Flk1::myr-mCherry mice permitted simultaneous *in vivo* imaging of angiogenesis and lymphangiogenesis. This imaging ability allowed us to differentiate the processes as well as observe their interdependence and will be valuable in the development of therapies targeting angiogenesis and/or lymphangiogenesis.

*Corresponding author: Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, 1855 W. Taylor Street, Chicago, IL 60612. changr@uic.edu.

AUTHOR CONTRIBUTIONS J.H.C. and D.T.A. planned experiments; J.Z., J.D-F., M.C., P.P. and J.H.C. performed experiments; D.T.A., K.Y.H., J.Z., J.D-F. and M.C. analyzed data; Y.K.H. and M.E.D. contributed reagents/other essential material; J.H.C. and M.I.R. revise manuscript and J.Z., J.D-F., J.H.C. and D.T.A. wrote the paper.

Conflict of interest: The authors have no conflicts of interest to declare.

Keywords

Cornea; angiogenesis; lymphangiogenesis; in vivo imaging; endothelial cells

INTRODUCTION

Blood and lymphatic vessels together maintain homeostasis throughout the body. Specifically, blood vessels bring nutrients, oxygen, signaling molecules (e.g., hormones), and immunologically responsive cells to tissues while removing metabolic waste. The lymphatic system, in equilibrium with blood circulation, removes excess fluid and proteins from blood and tissues and provides immune surveillance against foreign antigens. Angiogenesis (the formation of new blood vessels) has been studied extensively, as it plays a significant role in several pathological conditions including cardiovascular diseases and cancer[1-7]. The study of lymphangiogenesis (the formation of new lymphatic vessels) has not been equally extensive. However, interest in this process is growing due to recent discoveries of the roles of lymphangiogenesis in physiological and pathological processes, particularly metastasis[8-10]. Nevertheless, our understanding of angiogenesis and lymphangiogenesis remains limited because the processes are rarely studied simultaneously.

The cornea is an excellent model for studying the growth of blood and lymphatic vessels; its location on the surface of the animal makes it easily accessible for experimental manipulation and its inherent avascular nature allows observation of vessel formation. These characteristics have made corneal neovascularization (CNV), which includes both angiogenesis and lymphangiogenesis, a valuable model system for studying the molecular mechanisms of vessel development[11, 12]. CNV can be experimentally induced in mice via several methods including chemical burn, suturing, corneal transplantation, viral infection (e.g., herpes simplex virus-1), or implantation of growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)[13, 14].

Large superficial blood vessels can sometimes be viewed in the cornea using traditional microscopy, depending on the experimental model and strain of mouse. However, the colorless lymphatic vessels underlying blood vessels are invisible to the naked eye. Several markers aid *in vitro* visualization of blood and lymphatic vessels, including fetal liver kinase (Flk)1/VEGF receptor (VEGFR)2, CD31, and platelet endothelial cell adhesion molecule (PECAM) for blood vessels and lymphatic vessel endothelial receptor (LYVE)-1, Prox1, podoplanin, and VEGFR3 for lymphatic vessels[15, 16]. However, use of these markers to observe vessels requires post-mortem staining of tissues from multiple animals at each experimental time point. Furthermore, observation of the sprouting patterns of individual blood and lymphatic vessels in relation to each other and visualization of the overall vasculature in the same animal over time is not possible with immunostaining methods. Given the limitations of the current marker systems and the need to better understand the simultaneous development of blood and lymphatic vessels during CNV, our aim was to generate a line of mice that enables simultaneous *in vivo* observation of blood and lymphatic vessel growth via fluorescence microscopy.

To achieve this aim, we crossed Prox1-GFP mice with Flk1::myr-mCherry mice to generate Prox1-GFP/Flk1::myr-mCherry mice. Prox1-GFP mice express green fluorescent protein (GFP) under the promoter of Prox1, a marker of lymphatic endothelial cells but not blood endothelial cells[17-19], whereas Flk1::myr-mCherry mice express the protein mCherry (a red fluorophore) under the promoter of Flk1, a marker of blood endothelial cells[20]. In the newly created line of mice, we could observe sprout formation, vessel branching, and experimentally induced blood and lymphatic vessel growth via *in vivo* fluorescence microscopy over multiple time points in the same mice.

RESULTS

To demonstrate the usefulness of Prox1-GFP/Flk1::myr-mCherry mice for *in vivo* studies of blood and lymphatic vessel formation in the cornea, we used three separate animal models of CNV: VEGF-pellet implantation, bFGF-pellet implantation, and alkali burn injury. In all three models, we were able to observe and track independently (single color) and in coordination (two-color) the overall blood and lymphatic vessel growth as well as the growth of individual vessels.

Blood and lymphatic vessel growth after VEGF pellet-induced corneal injury

We first observed corneal angiogenesis and lymphangiogenesis after corneas were exposed to high levels of VEGF. By observing the normally avascular cornea of Prox1-GFP/Flk1::myr-mCherry mice before and after implantation of a VEGF pellet, we could track continuous vessel formation and progression over time under a SteREO Lumar microscope. The distribution and morphology of blood (mCherry, red) and lymphatic (GFP, green) vessels could be clearly distinguished before VEGF stimulation (Fig. 1AD), when occasional stumps of lymphatic vessels (arrows in Fig. 1A) and loops of blood vessels (Fig. 1B) were identified shallowly penetrating the same regions of the cornea in both the experimental and control corneas. Extensive blood and lymphatic vessel growth occurred following VEGF pellet implantation (Fig. 1E-G, I-K, M-O).

By day 3 (Fig. 1E-G), all lymphatic stumps had elongated to form vessels invading the cornea and growing towards the pellet. We observed *de novo* budding of lymphatic vessels (arrowheads, Fig. 1E) from the limbus, although it is possible that these vessels were simply continuations of lymphatic stumps too minute to be visualized on day 0. Through day 7 (Fig. 1I-K), lymphatic vessels continued to elongate and branch, and one potentially new vessel (indicated by an asterisk) appeared. Between days 7 and 10 (Fig. 1M-O), we observed very little growth or branching of previously sprouted lymphatic vessels and no sprouting of new vessels. However, we also did not observe any vessel regression.

Blood vessels exhibited a different growth pattern. Loops of vessels with no distinct vessel stumps were apparent at the limbal boundary prior to pellet implantation (Fig. 1B). It was unclear whether these vessels elongated or new vessels sprouted upon VEGF stimulation because the growing vessels were exceedingly dense on day 3 (Fig. 1F). Through day 7 (Fig. 1J), blood vessels continued to elongate toward the implanted VEGF pellet. By day 10 (Fig. 1M-O), individual blood vessels became distinct as the overall density of blood vessels decreased.

Images acquired by confocal microscopy after sacrifice of the mice on day 10 (Fig. 1Q-T) confirmed that nearly all lymphatic and blood vessels growing into the cornea after VEGF pellet implantation were visible in the Prox1-GFP/Flk1::myr-mCherry mouse *in vivo*.

Blood and lymphatic vessel growth after bFGF pellet-induced corneal injury

bFGF acts upstream of VEGF signaling and stimulates angiogenesis in the cornea, at least in part, by activating the VEGF/VEGFR system[21, 22]. Although angiogenesis following bFGF stimulation involves a distinct series of molecular events compared to VEGF-stimulated angiogenesis, blood vessels exhibited similar growth patterns in VEGF- and bFGF-stimulated corneas. However, we found that lymphangiogenesis was more robust in the bFGF-induced injury model (Fig. 2A-L) than in the VEGF-induced injury model (Fig. 1A-P). The time courses of lymphatic and blood vessel growth were very similar in VEGF- and bFGF-stimulated conditions: growth was most dramatic between post-implantation days 3 and 7, and after day 7, we observed far less growth.

Blood and lymphatic vessel growth after corneal injury by alkali burn

Alkali burn injury produced robust corneal angiogenesis and lymphangiogenesis (Figs. 3 and 4). Unfortunately, this injury model damages the pupillary sphincter muscle, preventing the pupil from fully constricting and thus exposing the GFP-expressing lens, which makes imaging of consequent vessel growth difficult. However, it was still possible to visualize vessel growth near the limbus at all time points, and as the wounds healed it became possible to image vessel growth closer to the apex of the cornea. Growth appeared by day 3 after injury (Fig. 3A-C, baseline images are not shown but were similar to those in Figs. 1 and 2 for Day 0), with the most robust growth between days 3 and 7 (Fig. 3D-F) and no significant growth or branching between days 7 and 10 (Fig. 3G-I). Growth occurred in 360 degrees around the wound in all cases. Imaging of vessels was possible *in vivo* (Fig. 3) and in flat-mounted corneas (Fig. 4) and revealed smaller-scale patterns of growth, branching, and development. The blood (mcherry) and lymphatic (GFP) vessels in the Prox1-GFP/Flk1::myr-mCherry mouse corneas were co-localized with immunostained PECAM-1 and LYVE-1, respectively (Fig. 5).

DISCUSSION

We generated Prox1-GFP/Flk1::myr-mCherry mice for the purpose of studying the development and progression of blood and lymphatic vessels *in vivo* in the cornea. Simultaneous *in vivo* imaging of angiogenesis and lymphangiogenesis was achieved for the first time through the intrinsic fluorescence of blood and lymphatic endothelial cells in this line of mice. We demonstrated simultaneous visualization of blood and lymphatic vessel growth into the avascular cornea in three models of corneal injury: VEGF pellet implantation, bFGF pellet implantation, and alkali burn injury.

In vivo time-lapse fluorescence imaging revealed blood and lymphatic vessel formation in each model, capturing differences that will allow us to better understand the interdependence of blood and lymphatic vessel development. We found that in all three models the most robust growth of both blood and lymphatic vessels occurred between postoperational days 3

and 7, with very little growth or branching occurring after this period. This suggests that regardless of the cause of injury to the cornea, anti-angiogenic mechanisms within the normally avascular cornea are capable of blocking the progression of both blood and lymphatic vessel growth beyond 1 week. This duration of vessel growth appears to be independent of several factors, including the extent of injury. The least amount of vessel growth occurred in response to VEGF pellet implantation among the injury models applied in this study. Finally, alkali burn injury resulted in 360 degree infiltration of the cornea with blood and lymphatic vessels. Hence, although differences were observed in the numbers of vessels and their growth patterns among the different methods of injury, the time course of vessel growth was the same for all three injury models: dramatic vessel growth between postoperative days 3 and 7 with an abrupt halt at the end of this time period.

Another important benefit of this mouse line is the ability to image vessels in a single mouse at multiple time points, which allows for more refined tracking of vascular changes over time. For example, we were able to observe the growth of individual lymphatic vessels following implantation of a VEGF or bFGF pellet. This resulted in the discovery that many new blood vessels sprout from the limbus following either method of stimulation. Using the same mouse at multiple points also reduces the total number of mice required for experiments, along with the associated time and cost.

There are numerous potential uses for Prox1-GFP/Flk1::myr-mCherry mice in the study of physiologies and pathologies. The value of simultaneously observing hemangiogenesis and lymphangiogenesis in living experimental animals is clear. The most obvious use is crossing these mice to create other transgenic models, such as knock-out lines, in order to study, in real-time, how changes in genetic background lead to changes in either stimulated or endogenous angiogenic processes. Differences in vessel sprouting, vessel branching, blood flow, and vessel regression can be studied *in vivo* over multiple time points and under various experimental conditions in any line of mice bred with the Prox1-GFP/Flk1::myr-mCherry line. Considering these advantages, we believe Prox1-GFP/Flk1::myr-mCherry mice will be a valuable tool for future research in angiogenesis and lymphangiogenesis.

MATERIALS AND METHODS

Generation of Prox1-GFP/Flk1::myr-mCherry mice

The Prox1-GFP mouse expresses GFP under the Prox1 promoter. This mouse was generated using a Prox1-GFP bacterial artificial chromosome (BAC) construct that extends from 115 kb upstream to 81 kb downstream of the Prox1 start codon, a region that includes all known Prox1 regulatory elements[18]. In the Tg(Flk1::myr-mCherry) mice, mCherry expression is driven by a blood endothelial-specific portion of the Flk1 promoter, with the myristoylation (myr) tag directing the fluorescent protein to the cell membrane[20]. Prox1-GFP mice were bred with Flk1::myr-mCherry mice to generate Prox1-GFP/Flk1::myr-mCherry mice. Studying the cornea in albino mice was impossible because the lens in Prox1-GFP mice exhibits strong green fluorescence while the pupil contains no melanin to block lens fluorescence. We therefore bred Prox1-GFP mice into a C57BL/6 background prior to breeding with Flk1::myr-mCherry mice. This resulted in a normal, pigmented pupil, which allows *in vivo* viewing of fluorescent blood and lymphatic vessels. Flk1::myr-mCherry mice

were originally acquired in the FVB/NJ background. We bred these mice with the C57BL/6 strain of Prox1-GFP mice to produce a Prox1-GFP/Flk1::myr-mCherry line of brown mice. All animal experiments were performed using protocols approved by the Animal Care Committee of the University of Illinois at Chicago.

Animal care for surgery and imaging

Male or non-pregnant female adult (6–8 weeks old, 5 mice per group) Prox1-GFP/Flk1::myr-mCherry mice were used in all experiments. Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). Once sedated, one drop of 1% proparacaine was administered topically to the eye for local anesthesia. Eyelashes were clipped above and below the eye in preparation for surgical procedures and *in vivo* imaging. For all procedures, eyes were imaged *in vivo* by SteREO Lumar microscopy (see below) on post-treatment days 0, 3, 7, and 10. Eyes were enucleated, dissected, and flat-mounted for confocal microscopy on postoperative day 10 after the final *in vivo* imaging session.

Corneal micropocket assays

VEGF, bFGF (FGF2), and blank (phosphate-buffered saline, PBS) pellets used for implantation were made according to the methods of Tang et al. with modifications [23]. Pellets contained 10% (w/v) sulfuric acid (Sigma-Aldrich, St. Louis, MO), 12% (w/v) poly-hydroxyethylmethacrylate (HEMA; Sigma-Aldrich) dissolved in absolute ethanol, and recombinant human VEGF-A (R&D Systems, Minneapolis, MN), bFGF, or PBS. Pellets were formed by pipetting 0.5 μ l poly-HEMA solution onto UV-exposed parafilm, which yielded 20 pellets containing 150 ng VEGF, 80 ng bFGF, or PBS. Pellets were dried at room temperature before they were stored at -80°C or used. Once the animal was deeply anesthetized and the eye was prepared for surgery (see above), we used a modified von Graefe knife to create a corneal micropocket in the epithelium. The initial incision was made parallel to and approximately 1 mm from the limbus. The knife was inserted into the incision site and advanced laterally, parallel to the limbus, to create a 0.5-mm micropocket within the corneal stroma. The poly-HEMA pellet was carefully inserted into this pocket. Antibiotic erythromycin (Bausch and Lomb, Tampa FL) ointment was applied to the eye following pellet implantation to prevent infection, and Buprenex (Reckitt Benckiser Healthcare, England) was administered immediately after the procedure and twice the following day to alleviate post-surgical pain.

Alkaline burn

A 1.5-mm diameter circle was cut from Whatman #3 filter paper by a trephine and soaked in 2 μ L of 1 N sodium hydroxide solution. The filter paper was placed over the center of the cornea of the left eye for 60 seconds. It was then carefully removed, and the wound surface was immediately flushed with saline and treated with antibiotic erythromycin ointment (Bausch and Lomb, Tampa, FL). Buprenex (Reckitt Benckiser Healthcare, England) was administered after surgery and twice the following day to alleviate pain.

SteREO Lumar microscopy

Mice were prepared for surgery as described above. Following proparacaine application and eyelash clipping, the eye was briefly rinsed and the corner blotted dry. The deeply sedated mouse was immobilized for microscopy using a jaw lock. The camera (Zeiss SteREO Lumar V12, Jena, Germany) was focused onto the cornea at 30× magnification. The exposure time was 200 ms for GFP detection and 3100 ms for mCherry detection. Z-stack images were taken at approximately 100-μm intervals from the base of the cornea to its apex, and the images were flattened. Imaging was performed from directly above and on the lateral side of the cornea where the pellets were implanted. Our preliminary data showed an improved mCherry images can be visualized via Zeiss Axiozoom V16 microscope (Jena, Germany, data not shown).

Wholemount Immunohistochemistry

Alkaline burn-treated mouse eyes were enucleated, and whole corneas were cut with four radial incisions from the peripheral rim. The corneas were fixed in 4% paraformaldehyde for 4 h at room temperature. The eye was washed once with phosphate-buffered saline (PBS) for 3 min and then washed with 1% Triton X 100 in PBS for 30 min. Blocking was then performed using 5 % normal donkey serum in PBS for 1 h. The primary (anti-LYVE-1 or PECAM-1) antibodies were applied for 8 h (or overnight) at 4°C. After washing, the secondary antibody was incubated for 4 h. The corneas were washed three times with PBS and flattened on a glass slide for viewing with a confocal microscope (Zeiss 710 confocal microscope, Jena, Germany). The antibodies used were rabbit anti-LYVE-1 (1:50, Abcam, Cat# ab14917, Cambridge, MA), rat anti-mouse PECAM-1 (1:50, CD31; MEC 13.3, cat# 553370, BD Pharmingen, San Jose, CA), and Alexa-conjugated 647 conjugated donkey anti-rabbit or -rat antibody (1:100; Jackson ImmunoResearch, West Grove, PA).

Confocal microscopy

Eyes were enucleated and directly fixed in 4% paraformaldehyde overnight. After four washes with PBS, corneas were dissected, flat-mounted onto glass slides with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA), and covered with a coverslip. Flat-mounted corneas were imaged using a Zeiss LSM 710 confocal microscope at 10× magnification. Z-stacks and 5×5 grid images were recorded.

ACKNOWLEDGMENTS

This study was supported by grants for the National Institutes of Health EY10101 (D.T.A.), R01EY018594 (M.I.R.), EY023691, EY021886, I01 BX002386 (J.H.C), EY01792, UL1TR000050, and an unrestricted grant from Research to Prevent Blindness, New York, NY.

Abbreviations

bFGF	basic fibroblast growth factor
CNV	corneal neovascularization
Flk	fetal liver kinase

GFP	green fluorescent protein
HEMA	hydroxyethylmethacrylate
LYVE-1	lymphatic vessel endothelial receptor 1
PBS	phosphate-buffered saline
PECAM	platelet endothelial cell adhesion molecule
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

REFERENCES

1. Azar DT. Corneal angiogenic privilege: angiogenic and antiangiogenic factors in corneal avascularity, vasculogenesis, and wound healing (an American Ophthalmological Society thesis). *Trans Am Ophthalmol Soc.* 2006; 104:264–302. [PubMed: 17471348]
2. Chang JH, Gabison EE, Kato T, Azar DT. Corneal neovascularization. *Curr Opin Ophthalmol.* 2001; 12:242–9. [PubMed: 11507336]
3. Tshionyi M, Shay E, Lunde E, Lin A, Han KY, Jain S, Chang JH, Azar DT. Hemangiogenesis and lymphangiogenesis in corneal pathology. *Cornea.* 2012; 31:74–80. [PubMed: 22030600]
4. Folkman J. Tumor angiogenesis: therapeutic implications. *The New England journal of medicine.* 1971; 285:1182–6. [PubMed: 4938153]
5. Ware JA, Simons M. Angiogenesis in ischemic heart disease. *Nat Med.* 1997; 3:158–64. [PubMed: 9018233]
6. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature.* 2000; 407:249–57. [PubMed: 11001068]
7. Ellenberg D, Azar DT, Hallak JA, Tobaigy F, Han KY, Jain S, Zhou Z, Chang JH. Novel aspects of corneal angiogenic and lymphangiogenic privilege. *Prog Retin Eye Res.* 2010; 29:208–48. [PubMed: 20100589]
8. Cao Y. Opinion: emerging mechanisms of tumour lymphangiogenesis and lymphatic metastasis. *Nature reviews Cancer.* 2005; 5:735–43.
9. Tammela T, Zarkada G, Wallgard E, Murtomaki A, Suchting S, Wirzenius M, Waltari M, Hellstrom M, Schomber T, Peltonen R, Freitas C, Duarte A, Isoniemi H, Laakkonen P, Christofori G, Yla-Herttuala S, Shibuya M, Pytowski B, Eichmann A, Betsholtz C, Alitalo K. Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. *Nature.* 2008; 454:656–60. [PubMed: 18594512]
10. Stacker SA, Achen MG, Jussila L, Baldwin ME, Alitalo K. Lymphangiogenesis and cancer metastasis. *Nature reviews Cancer.* 2002; 2:573–83.
11. Kenyon BM, Voest EE, Chen CC, Flynn E, Folkman J, D'Amato RJ. A model of angiogenesis in the mouse cornea. *Invest Ophthalmol Vis Sci.* 1996; 37:1625–32. [PubMed: 8675406]
12. Cao R, Lim S, Ji H, Zhang Y, Yang Y, Honek J, Hedlund EM, Cao Y. Mouse corneal lymphangiogenesis model. *Nature protocols.* 2011; 6:817–26.
13. Wuest TR, Carr DJ. VEGF-A expression by HSV-1-infected cells drives corneal lymphangiogenesis. *J Exp Med.* 2010; 207:101–15. [PubMed: 20026662]
14. Ling S, Lin H, Liang L, Xu J, Xu C, Zhao W, Liu Z. Development of new lymphatic vessels in alkali-burned corneas. *Acta Ophthalmol.* 2009; 87:315–22. [PubMed: 18811642]
15. Podgrabska S, Braun P, Velasco P, Kloos B, Pepper MS, Skobe M. Molecular characterization of lymphatic endothelial cells. *Proc Natl Acad Sci U S A.* 2002; 99:16069–74. [PubMed: 12446836]
16. Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. *Cell.* 1999; 98:769–78. [PubMed: 10499794]

17. Hagerling R, Pollmann C, Kremer L, Andresen V, Kiefer F. Intravital two-photon microscopy of lymphatic vessel development and function using a transgenic Prox1 promoter-directed mOrange2 reporter mouse. *Biochemical Society transactions*. 2011; 39:1674–81. [PubMed: 22103506]
18. Choi I, Chung HK, Ramu S, Lee HN, Kim KE, Lee S, Yoo J, Choi D, Lee YS, Aguilar B, Hong YK. Visualization of lymphatic vessels by Prox1-promoter directed GFP reporter in a bacterial artificial chromosome-based transgenic mouse. *Blood*. 2011; 117:362–5. [PubMed: 20962325]
19. Truong TN, Li H, Hong YK, Chen L. Novel characterization and live imaging of Schlemm's canal expressing Prox-1. *PloS one*. 2014; 9:e98245. [PubMed: 24827370]
20. Poche RA, Larina IV, Scott ML, Saik JE, West JL, Dickinson ME. The Flk1-myr::mCherry mouse as a useful reporter to characterize multiple aspects of ocular blood vessel development and disease. *Dev Dyn*. 2009; 238:2318–26. [PubMed: 19253403]
21. Onguchi T, Han KY, Chang JH, Azar DT. Membrane type-1 matrix metalloproteinase potentiates basic fibroblast growth factor-induced corneal neovascularization. *Am J Pathol*. 2009; 174:1564–71. [PubMed: 19264910]
22. Han KY, Fahd DC, Tshionyi M, Allemann N, Jain S, Chang JH, Azar DT. MT1-MMP modulates bFGF-induced VEGF-A expression in corneal fibroblasts. *Protein and peptide letters*. 2012; 19:1334–9. [PubMed: 22670674]
23. Tang Z, Zhang F, Li Y, Arjunan P, Kumar A, Lee C, Li X. A mouse model of the cornea pocket assay for angiogenesis study. *Journal of visualized experiments : JoVE*. 2011

Figure 1. VEGF pellet implantation induces blood and lymphatic vessel formation

In vivo observation of angiogenesis and lymphangiogenesis in a Prox1-GFP/Flk1::myr-mCherry mouse over 10 days following 150ng VEGF pellet implantation (left 3 columns) or control PBS pellet implantation (far right column). (**A-P**) SteREO Lumar microscopy images of GFP-expressing lymphatic vessels (green), mCherry-expressing blood vessels (red) and overlays (right two columns): (**A-D**) prior to implantation, (**E-H**) 3 days post-implantation, (**I-L**) 7 days post-implantation, and (**M-P**) 10 days post-implantation. (**Q-T**) Confocal imaging of the same corneas on day 10. Scale bar: 500 μ m in (**A-P**) and 200 μ m in (**Q-T**). **Arrows** (**A**): regularly spaced lymphatic vessels penetrating the cornea in the uninjured eye; **arrowheads** (**E**): new lymphatic vessels budding from the cornea 3 days after VEGF pellet implantation; **asterisk** (**I**): one potential new lymphatic vessel budding from the cornea on day 7 after the initial phase; **circle** (**T**): outline of the implanted PBS control pellet.

Figure 2. bFGF pellet implantation induces blood and lymphatic vessel formation
In vivo observation of angiogenesis and lymphangiogenesis in a single Prox1-GFP/
 Flk1::myr-mCherry mouse over 10 days following 80ng bFGF pellet implantation (**A-L**).
 SteREO Lumar microscopy images of GFP-expressing lymphatic vessels (green, left
 column), mCherry-expressing blood vessels (red, middle columns) and overlaid images
 (right column): (**A-C**) prior to implantation, (**D-F**) 3 days post-implantation, (**G-I**) 7 days
 post-implantation, and (**J-L**) 10 days post-implantation. Scale bar: 500 μ m.

Figure 3. Alkali burn results in widespread blood and lymphatic vessel formation

In vivo observation of blood and lymphatic vessel growth in response to alkali burn injury (60-s application of 1N NaOH) in the same Prox1-GFP/Flk1::myr-mCherry mouse. SteREO Lumar microscopy images of GFP-expressing lymphatic vessels (green), mCherry-expressing blood vessels (red), and overlaid images (right) in the same cornea: (A-C) 3 days, (D-F) 7 days, and (G-I) 10 days after injury. Lens epithelial cells intensely expressed Prox1-GFP, and the pupil could not fully constrict after alkali burn injury due to muscle damage. This caused the intense green fluorescence seen in the center of the eye in these images. Scale bar: 500 μ m.

Figure 4. Confocal microscopic observation of blood and lymphatic vessel growth in response to alkali burn injury

Vessel growth was induced by alkali burn (60-s application of 1N NaOH) in different Prox1-GFP/Flk1::myr-mCherry mice following sacrifice at the indicated time points after injury. Confocal images showing GFP-expressing lymphatic vessels (green), mCherry-expressing blood vessels (red), and overlaid images (right) in the cornea: (A-C) prior to injury, (D-F) 3 days, (G-I) 7 days, and (J-L) 10 days after injury. Scale bar: 100 μ m.

Figure 5. Wholemount corneal immunostaining of blood and lymphatic vessels after alkali burn injury

Vessel growth was induced by alkali burn (60-s application of 1N NaOH) in Prox1-GFP/Flk1::myr-mCherry mice. Corneas were immunostained with anti-LYVE-1 antibody (C; blue) or anti-PECAM-1 antibody (G, blue). Immunostaining and the intrinsic expression of GFP by lymphatic vessels (A, E; green) and mCherry-expressing blood vessels (B, F; red) were visualized. Overlaid images (D, H; merged, right) are also shown. Scale bar: 50 μ m.