In vivo imaging of lymphatic vessels in development, wound healing, inflammation, and tumor metastasis

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Lymphatic vessel growth or lymphangiogenesis occurs during embryonic development and wound healing and plays an important role in tumor metastasis and inflammatory diseases. However, the possibility of noninvasive detection and quantification of lymphangiogenesis has been lacking. Here, we present the Vegfr3EGFPLuc mouse model, where an EGFP-luciferase fusion protein, expressed under the endogenous transcriptional control of the Vegfr3 gene, allows the monitoring of physiological and pathological lymphangiogenesis in vivo. We show tracking of lymphatic vessel development during embryogenesis as well as lymphangiogenesis induced by specific growth factors, during wound healing and in contact hypersensitivity (CHS) - induced inflammation where we also monitor down-regulation of lymphangiogenesis by the glucocorticoid dexamethasone. Importantly, the Vegfr3-reporter allowed us to tracking tumor-induced lymphangiogenesis at the tumor periphery and in lymph nodes in association with the metastatic process. This is the first reporter mouse model for luminescence imaging of lymphangiogenesis. It should provide an important tool for studying the involvement of lymphangiogenesis in pathological processes.

In adults, lymphangiogenesis occurs during the formation of the corpus luteum and during wound healing, and may be abundant in association with pathological processes (1). Thus, lymphangiogenesis typically occurs at sites of inflammation induced by factors produced by macrophages and granulocytes. The process contributes to reduction of tissue edema and activation of immune responses by facilitating fluid drainage and the transport of dendritic cells and macrophages (2–4). The lymphatic system is also a main route of tumor dissemination and metastasis. Tumor invasion to regional lymph nodes is often the first sign of tumor metastasis and a marker of poor prognosis. Stimulation of growth and dilation of preexisting lymphatic vessels by the tumor facilitates tumor spread via the lymphatics (5–7) and the contribution of intratumoral lymphangiogenesis may depend on tumor type (8–12). Moreover, lymphangiogenic growth factors produced by the tumor may induce sinusoidal hyperplasia in the regional lymph node and this process may further contribute to tumor metastasis (13–15). However, the events that lead to dissemination of tumor cells through the lymphatic system are still poorly understood. Elucidation of these steps is crucial for the design of new anti-metastatic therapeutic strategies.

Despite the importance of lymphangiogenesis as a therapeutic target, still there are very few models to trace and study this process in vivo. Several transgenic mouse lines for fluorescent visualization of lymphatic vessels have recently been reported. All these lines are based on gene-targeted BAC transgenic constructs to express either GFP (16), mOrange (17), or Tomato (18) fluorescent proteins under Prox-1 transcriptional control. Positron emission tomography (PET) combined with radio-labeled anti-LYVE-1 antibodies have also been used for in vivo imaging of lymphatics in mice (19). We have generated a reporter mouse line for in vivo imaging of lymphatic vessels in which a dual reporter for fluorescence and luminescence (EGFPLuc) is expressed under the endogenous transcriptional control of the Vegfr3 (vascular endothelial growth factor receptor 3) gene, the first lymphatic marker discovered (20). Although Vegfr3 is also expressed, to some extent, in the tip cells of the newly formed blood capillaries (21–23) and in fenestrated endothelium (24), it is still one of the best markers for all lymphatic endothelial cells. Outside the vascular system Vegfr3 expression has been described in subpopulations of monocytes and macrophages (25) and in embryonic osteoblasts and neural progenitors (26, 27). We have validated the reporter for tracking lymphatic vessels in vivo during development, and lymphangiogenesis after administration of specific growth factors and inhibitors, in wound healing, inflammation and tumor dissemination.

**Results**

**Generation of Vegfr3EGFPLuc Knockin Mice.** To create the reporter allele, an EGFPLuc-encoding sequence, preceded by an IRES (internal ribosome entry site) element, was introduced into the 3′-UTR of the mouse Vegfr3 locus by gene targeting (Fig. 1A). Details of the targeting strategy are described in Fig. S1. The introduction of the IRES-EGFPLuc cassette in the 3′-UTR of the Vegfr3 gene does not significantly alter the expression of Vegfr3 (Fig. S2). The level of the EGFPLuc protein is proportional to gene dose in +/KI and KI/KI embryos, respectively (Fig. S2B).

**Detection of EGFP in Lymphatic Vessels During Development.** VEGFR3 is first detected in blood vessel endothelial cells at 8.5 d of mouse embryonic development (E8.5), thus preceding the differentiation of the lymphatic vasculature (E9.0–E9.5) (28). Upon formation of the lymphatic vascular network from preexisting embryonic blood vessels, Vegfr3 expression is high in the developing lymphatic endothelium and is down-regulated in blood vessels (29). Detection of Vegfr3 expression by direct EGFP fluorescence in whole-body Vegfr3EGFPLuc embryos allows direct tracking of the early stages of mouse lymphatic vessel development. At E11.5, low EGFP expression is detected in embryonic blood vessels (Fig. 1B, red arrows), whereas a strong fluorescence signal is also detected in cells committed to form the primary lymphatic network (Fig. 1B, white arrows). At E12.5, a lymphatic vascular structure expressing high levels of EGFP/Vegfr3 is observed. Simultaneously, EGFP fluorescence decreases in the blood vessels. At E13.5, a vascular lymphatic network expressing high levels of EGFP has developed and EGFP expression is no longer detected in the blood vessels (Fig. 1B). In normal skin, EGFP fluorescence is detected in lymphatic vessels
but not in blood vessels (Fig. 1C, I and II). EGFP fluorescence can be monitored simultaneously with EGFP immunofluorescence detection (Fig. S3A). Costaining with different lymphatic markers shows coexpression of EGFP and Vegfr3 in all endothelial cells of the dermal lymphatic network (Fig. 1D). In the skin of 3-wk-old mice EGFP and VEGFR3 colocalize with the lymphatic marker LYVE-1 in lymphatic capillaries (Fig. 1E and Fig. S3B, respectively). EGFP is not expressed in platelet endothelial cell adhesion molecule (PECAM)1+ blood vessels where only autofluorescence of red blood cells is detected (Fig. 1E, white arrowheads). EGFP fluorescence is also detected in growing blood capillaries of the postnatal retina (Fig. 1C, III and IV) where VEGFR3 regulates angiogenic sprouting (21).

**Whole Body Imaging of Postnatal Vegfr3 Expression.** Vegfr3 is expressed at low levels in adult lymphatic vessels and is up-regulated during lymphangiogenesis (30). Moreover, mouse lymphatic vessels become independent of VEGFR3 signaling for maturation after the first 2 wk of postnatal life, although they still respond to VEGF-C/VEGFR3 signaling (30, 31). Whole-body luciferase activity was monitored in Vegfr3EGFPluc mice from the early postnatal period to adulthood. Luciferase signal strongly decreases between weeks 2–5 of postnatal life (Fig. 2A and B), coinciding with the time during which lymphatic vessels become independent of VEGFR3 signaling and reaches a basal level at around 10 wk of age. A similar decrease is observed when luciferase signal is quantified using a region of interest (ROI) defined in the hind right paw, devoid of hair (Fig. S4A). Quantification of luciferase activity from tissue extracts shows a parallel down-regulation from postnatal weeks 4–14 (Fig. S4B). Western blot analysis confirmed that EGFPluc protein levels decrease in parallel to those of VEGFR3 in different organs, including skin (Fig. S4C). As expected, whole-body luciferase signal in homozygous versus heterozygous mice is proportional to the reporter gene dose (Fig. 2A and Fig. S4A). Altogether, these results show that Vegfr3 expression is developmentally regulated during early postnatal life in mice.

**Imaging of Lymphangiogenesis Induced by Specific Growth Factors.** To test the specificity and sensitivity of the Vegfr3EGFPluc reporter for imaging lymphangiogenesis in adult mice, we injected immune compromised athymic nu/nu (Crl:NU(lico)-Foxn1nu)
mice, homozygous for the Vegfr3<sup>EGFPLuc</sup> allele, intradermally with adenovirus expressing angiogenic or lymphangiogenic growth factors: AdVEGF-B167 (heparin binding VEGF-B) (32), AdVEGF-E (33), or AdVEGF-C (34). Among them, AdVEGF-C has been shown to elicit a strong lymphangiogenic response in the skin (30). Injection of AdVEGF-C results in an eightfold increase of luciferase signal over the basal level in the treated ears, whereas neither AdVEGF-E nor AdVEGF-B167 significantly increased luciferase activity (Fig. 3 A and B). As previously described, an angiogenic response was also observed in AdVEGF-C-treated ears (22), although weaker than the one induced by AdVEGF-E expression (Fig. 3C) indicating that the induction of luciferase signal is not related to angiogenesis. Anti-LYVE-1 immunohistochemistry confirmed an increase in lymphatic vessel density and lumen enlargement in sections of AdVEGF-C-treated ears compared with those treated with either AdVEGF-E or AdVEGF-B167 (Fig. S5).

**Vegfr3 Expression and Lymphangiogenesis in Skin Wound Healing.** Wound healing is a complex process in which different cell types are implicated including fibroblasts, endothelial cells, and circulating inflammatory cells, recruited to the wound area in response to tissue injury (35). A strong correlation between macrophage recruitment and wound healing has been reported previously (36). Moreover, during skin wound healing, angiogenesis and lymphangiogenesis take place at the periphery of the wound, contributing to optimal healing (37, 38). We have used the Vegfr3<sup>EGFPLuc</sup> reporter to image lymphangiogenesis during skin wound healing and to further explore its dependence on inflammation. Punch biopsy wounds were generated on the backs of Vegfr3<sup>EGFPLuc</sup>+/+KI females. An increase in luciferase signal was observed early after wounding, mainly at the periphery of the wound, reaching a peak at day 8 when wound area was 30–40% of the original size (Fig. 4A and B). The increment in luciferase signal was transient, returning to the basal level 23 d after wounding, when reepithelialization is complete (Fig. 4B and C). Accumulation of lymphatic vessels with expanded lumens in the wound granulation tissue was confirmed by LYVE-1 immunohistochemistry (Fig. 3D). The density of VEGFR3<sup>+</sup> lymphatic vessels in the wound granulation tissue was quantified at different healing times (Fig. S6), showing a good correlation with the luminescent signal (Fig. 4B). These results show that lymphangiogenesis is a transient process during wound healing and demonstrate a strong correlation between luciferase induction and lymphatic vessel density in the wound area in our model.

Corticosteroids have been shown to block lymphangiogenesis induced by VEGF-C in the cornea (39) and by tumor cells (40). To investigate the effect of corticosteroids on lymphangiogenesis during skin wound healing, a set of Vegfr3<sup>EGFPLuc</sup> females was treated with dexamethasone (Dex), a synthetic glucocorticoid with a potent anti-inflammatory activity, shown to prevent angiogenesis and lymphangiogenesis associated to chronic inflammation (41) and to block lymphatic vessel regeneration (42). Wound closure was delayed and luciferase activity was significantly reduced in Dex-treated mice (Fig. 4A–C). Quantification of LYVE-1<sup>+</sup> and VEGFR3<sup>+</sup> lymphatic vessel density in the granulation tissue revealed no significant differences at day 8 and only a mild increase at day 14 after wounding in Dex-treated mice compared with control skin (Fig. 4D and Fig. S6). These data show that glucocorticoids block lymphangiogenesis in dermal wound healing and further support the connection between inflammation and lymphangiogenesis.

**Tracking Inflammation-Induced Lymphangiogenesis.** To further investigate the connection between inflammation and lymphangiogenesis, we subjected Vegfr3<sup>EGFPLuc</sup> mice to conventional in vivo inflammation protocols. Complete Freund adjuvant (CFA) is frequently used to stimulate T-cell-mediated immunity, inducing a
strong inflammatory reaction at the injection site with formation of granulomas and hyperplasia and structural changes of lymph nodes (43). Vegfr3EGFPLuc females were treated with i.p. injection of (CFA) (44). A robust and transient increase in luciferase activity was observed in the abdominal area of CFA-treated females (Fig. 5 A and B). Immunohistological analysis of abdominal organs from CFA-treated animals revealed the presence of an inflammatory infiltrate surrounding all abdominal organs (Fig. 5C, black arrowheads), concomitant with a high density of dilated VEGFR3+ lymphatic vessels between the normal and the inflammatory tissue in all organs examined (Fig. 5C, red arrowheads). The majority of inflammatory cells were negative for Vegfr3 expression (Fig. S7A), and, therefore, the induction of luciferase signal reflects the accumulation of Vegfr3-expressing lymphatic vessels in all of the abdominal organs in response to CFA-treatment.

Next, we explored Vegfr3 expression and lymphangiogenesis associated to contact hypersensitivity (CSH), a local inflammatory response induced by exposure to a chemical agent (challenge) to which the individual has been previously sensitized. Vegfr3EGFPLuc +/KI females were prepsensitized 6 d before they were challenged with oxazolone (Oxa). The Oxa challenge induced a two- to threefold increase of luciferase signal over the basal level 2 d after challenge (Fig. 5 D and E), coinciding with the time of maximal inflammation (Fig. S7B). A weak increase of luciferase signal was also observed in the control right ears because of cross-contamination with Oxa during grooming. Immunohistological analysis of the Oxa-treated ears, 2 and 4 d after challenge, revealed a marked and transient expansion of the dermis with severe infiltration of immune cells and lymphatic vessel enlargement (Fig. S7C). A threefold increase of lymphatic vessel density in Oxa-treated ears, compared with control ears, was observed 2 d after the challenge showing a strong correlation with the increase in luciferase signal detected in vivo (Fig. 5F). Dex administration significantly reduced luciferase induction by Oxa challenge (Fig. 5 D and E), as well as inflammation and swelling (Fig. S7 B and C). Histological analysis of Oxa plus Dex-treated ears revealed a decrease in leukocyte infiltration and VEGFR3 immunostaining to levels comparable to those of control ears (Fig. S7C). Dex treatment also prevented the increase in lymphatic vessel density (Fig. 5F). These results further support the association of inflammation and lymphangiogenesis and validate the Vegfr3EGFPLuc model as a reporter of these processes in vivo.

In Vivo Imaging of Tumor-Induced Lymphangiogenesis. Next, we wanted to explore the potential of the Vegfr3EGFPLuc reporter strain for imaging tumor-induced lymphangiogenesis, both at the periphery of the tumor and at the lymph nodes and its correlation with the tumor metastatic potential. Benign pre-metastatic papillomas, promoted by a conventional two-stage skin carcinogenesis protocol (45), are characterized by an accumulation of enlarged lymphatic vessels at the periphery of the tumor (14). We first explored whether the peripheral lymphangiogenic response in dimethylbenzanthracene/12-O-tetradecanoylphorbol-13-acetate (DMBA/TPA)-induced papillomas could be traced by in vivo luminescence detection. Vegfr3EGFPLuc females treated with DMBA/TPA developed papillomas that exhibited a local increase in luciferase emission (Fig. 6A and B). Histological analysis of the papillomas confirmed the accumulation of enlarged LYVE-1+, PECAM1low lymphatic vessels in the periphery of the tumors (Fig. 6 C and D). No significant increase in luciferase signal from other organs, including lymph nodes, was observed in this assay at this stage of tumor progression.

Melanoma is one of the tumors that metastasize mainly through invasion of the regional lymph nodes (46–49). To explore the capacity of the reporter to track lymphangiogenesis in a highly metastatic tumor context, we used a xenograft assay by s.c. injection of the metastatic mouse melanoma cell line (B16-V5) (50) in Vegfr3EGFPLuc +/KI females, backcrossed into the B6(Cg)-Tyrc-2J genetic background for 5 generations. Luciferase signal was induced in mice during B16-V5 tumor xenograft growth (Fig. 6E). Because of the high melanin content and dark pigmentation of the B16-V5 cells, any light emission from the tumor is expected to be mostly absorbed by the melanin, making it impossible to properly quantify the intratumor lymphangiogenesis (yellow arrowhead in expanded panel of Fig. 6E). Instead, an increase in luciferase signal is detected in the periphery of the tumor (Fig. 6A, white circle) that correlates with the accumulation of enlarged LYVE-1+ lymphatic vessels in the surrounding skin (Fig. S84). The luciferase signal at the periphery of the tumor increased proportionally to the increase in tumor volume (Fig. S8F), reaching a maximum of fourfold over the basal luminescence level when the tumor size was ~1 cmm. Moreover, an increase in luciferase signal parallel to tumor growth was also observed in distant parts of the body, particularly in lymph nodes (Fig. 6F, red arrowheads). Quantification of the luciferase signal from both the proximal (inguinal) and distal (axillary) lymph nodes when the tumor was 1 cmm in size also

![Fig. 5. Imaging of inflammation-induced lymphangiogenesis.](image-url)
Therefore, the driven by the transcriptional control of the isolated metastatic cells proximal to lymphatic vessels (Fig. S9). Maximal luciferase signal, revealing the presence of very few node was analyzed by S-100 immunostaining at the time of detection of lymphatic vessels (LYVE-1+) and blood vessels (strong PECAM1+). In the dual point to each one of the papillomas in females. (51) using the same approach, suggesting a common mechanism of postnatal transcriptional down-regulation for these endothelial receptors. This down-regulation may account for the low expression levels of the EGFPLuc reporter in adult mice. By contrast, in previously described transgenic reporter models in which fluorescent protein expression is driven by Proxl transcriptional control elements, reporter expression is sustained from embryos to adults (16, 17). This may represent an advantage of these models for fluorescence visualization of individual lymphatics in adults. However, the luciferase in our reporter provides an excellent tool for noninvasive whole-body imaging through all stages of development, including adult tissues. Luciferase signal can be detected from a depth of 2 cm in tissues, and sensitivity of luminescence is several orders of magnitude higher than that of fluorescence (52), which makes it optimal for noninvasive detection and quantification of lymphangiogenesis even from internal organs.

We have thoroughly tested the Vegfr3EGFPLuc model as a reporter of lymphangiogenesis in vivo in adult mice. AdVEGF-C-induced lymphangiogenesis results in a strong (eightfold) local increase in luciferase activity in the ear assay, whereas AdVEGF-E, a potent angiogenic growth factor, or AdVEGF-B167 do not increase luciferase signal significantly over that mediated by injection of PBS in the same conditions. We also observed an increase in luciferase signal associated with lymphangiogenesis induced in wound healing and in acute inflammation assays. Because Vegfr3 expression has been reported in inflammatory monocytes and macrophages recruited to the injured tissue (25, 36), the global increase in luciferase signal in these assays may, in principle, result from both an increase in lymphatic vessel density and the recruitment of VEGFR3+ inflammatory cells. However, we have found a tight correlation between the increase in luciferase signal and the increase in lymphatic vessel density during wound healing and in inflammation, with no significant contribution of VEGFR3+ cells from the inflammatory infiltrate. Thus, the luciferase signal reflects mostly the increase of VEGFR3+ lymphatic vessel density.

We also show that our model is suitable for imaging tumor-induced lymphangiogenesis. Papillomas generated by the conventional DMBA/TPA skin carcinogenesis exhibit accumulation of lymphatic vessels in the peripheral skin that can be detected by an increase in luciferase signal in our model. Moreover, s.c. injection of the highly metastatic B16-V5 mouse melanoma cell line results in induction of luciferase signal not only at the tumor periphery but also, and very interestingly, at the proximal and distal lymph nodes. The increase of luciferase signal correlates with an expansion of the vascular network, increase in lymph node volume and follicular hyperplasia. Regional lymph node metastasis represents the first step in the dissemination for a variety of human malignant tumors, including melanoma, and invasion of sentinel lymph nodes by tumor cells is associated to bad tumor prognosis (53). Several groups have reported that tumor-induced lymphangiogenesis at sentinel lymph nodes, precedes and contributes to tumor metastasis (14, 54). In our model, the increase in luciferase signal at the proximal lymph node also seems to precede lymph node metastasis since at the time of maximal luciferase induction it is hard to find metastatic cells in the lymph node. Interestingly, we also detected an increase in luciferase signal at distant lymph nodes, in agreement with previous observations that tumor

![Fig. 6. Tumor lymphangiogenesis. (A) Skin papillomas (arrowheads) induced by administration of DMBA/TPA on the dorsal skin of Vegfr3EGFPLuc−/− females. (B) Corresponding whole-body luciferase emission (arrowheads point to each one of the papillomas in A) (C and D) Immunohistochemical detection of lymphatic vessels (LYVE-1) and blood vessels (strong PECAM1+) surrounding the papillomas in consecutive sections of the same tumor. Vessels are indicated by arrowheads. (E) Whole-body dorsal images of luciferase in a Vegfr3EGFPLuc female injected s.c. with B16-V5 cells, at different times after injection. Arrowheads: position of the injection and of the resulting tumor. Note that the dark pigmentation of the B16-V5 melanoma cells blocks luciferase emission from the tumor (yellow arrowhead in the expanded panel). The white lines surround the signal from the periphery of the tumor. (F) Whole-body lateral images of luciferase activity in a Vegfr3EGFPLuc female injected with B16-V5 cells. Lymph node luciferase signal is indicated by red arrowheads.

showed a fourfold increase over the basal level in both cases (Fig. S8G). Histological analysis of the inguinal lymph node shows that the increase in luciferase emission correlates with an increase in lymph node volume, follicular hyperplasia, and expansion of a vascular network of LYVE-1-reactive vessels (Fig. S8 H–E). The presence of metastatic cells in the inguinal lymph node was analyzed by S-100 immunostaining at the time of maximum luciferase signal, revealing the presence of very few isolated metastatic cells proximal to lymphatic vessels (Fig. S9). Therefore, the Vegfr3EGFPLuc mouse model is suitable for imaging not only tumor-induced lymphangiogenesis at the periphery of the tumor but also the responses of the sentinel and distant lymph nodes associated with the metastatic process.

**Discussion**

We describe a knockin mouse model for in vivo imaging of lymphatics and lymphangiogenesis based on the expression of the dual fluorescence and luminescence reporter (EGFPLuc) driven by the transcriptional control of the Vegfr3 gene. The reporter was targeted to the 3′-UTR of Vegfr3. No phenotype has been detected in Vegfr3EGFPLucKI/KI mice. Individual lymphatics can be visualized at the cellular level by direct EGFP fluorescence and by EGFPLuc immunodetection both in embryos and during the first weeks after birth in our model; however, EGFP is harder to detect in adult mice. Quantification of whole-body luciferase signal, as well as luciferase activity in tissue extracts, shows that Vegfr3 transcriptional activity is strongly down-regulated during postnatal development. We also show that EGFPLuc protein down-regulation parallels that of VEGFR3. A similar level of down-regulation has been described for Vegfr2 (51) using the same approach, suggesting a common mechanism of postnatal transcriptional down-regulation for these endothelial receptors. This down-regulation may account for the low expression levels of the EGFPLuc reporter in adult mice.
produced VEGF-C induces lymph node lymphangiogenesis and promotion of metastasis beyond the sentinel lymph nodes (14). Moreover, our model also detects a generalized or “systemic” increase in luciferase signal outside of the lymph nodes in response to tumor growth that may reflect a tumor-induced systemic lymphangiogenic or inflammatory response with increased VEGFR3 expression levels. This response needs further investigation and indicates that tumors may induce a systemic lymphangiogenic response that prepares and facilitates their metastatic spread to distant organs, thus supporting classical Paget’s hypothesis of “seed and soil” for tumor dissemination and metastasis. This is a unique description of noninvasive optical imaging of tumor-induced lymphangiogenesis and lymph node metastasis in mice. Our model may provide a unique tool for analyzing and predicting metastatic potential and for developing antimitastatic therapies.

Materials and Methods

The Vegfr3Gfp/p reporter allele was maintained in a mixed CD-1;129sv; C57Bl/6J genetic background selecting albino mice for breeding, or backcrossed to albino B6(Cg-Tyr<sup>-/-</sup>)J mice for five generations. All experiments with mice were performed in accordance with protocols revised and approved by the Institutional Ethics Committee. A detailed description of the materials and methods is available in SI Materials and Methods.

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Detection of EGFP Fluorescence in Ear Skin and Retina. Ears were dissected and opened in two halves using forceps so that the cartilage remains attached to one of them. The cartilage-free skin was placed on top of ice-cold PBS with the exposed surface facing down. Direct imaging of native EGFP was achieved by mounting the skin between two circular coverslips in PBS in an autofluor chamber. Images were acquired as above using a 20x HCX PL APO CS 0.7 N.A. water immersion objective. For detection of EGFP fluorescence in retina, eyeballs were fixed in 4% PFA for 2 h at 4 degrees in a rotary wheel and washed five times with ice-cold PBS. Retinas were dissected in PBS under a microscope as detailed elsewhere (1) and stained with DAPI (2 μg/mL) in PBS-0.05% Tween for 10 min at RT, washed two times 5 min each in PBS and mounted in Pro-Long Gold Antifade (Molecular Probes). Image acquisition was as described above, using a 40x HCX PL APO 1.25 N.A. oil immersion objective.

Immunofluorescence. The ear skin without cartilage was placed on top of 4% PFA with the exposed surface facing down and fixed for 2 h at RT. Then, it was washed once in PBS for 15 min and in 0.3% Triton X-100 in PBS at RT for 10 min. Blocking was done with 3% skim milk, 0.3% Triton X-100 in PBS ON at 4°C. Primary antibodies were diluted in blocking solution and hybridized ON at 4°C. After washing with 0.3% Triton X-100 in PBS for 2–3 h with several changes, secondary antibodies were diluted in 1% skim milk, 0.3% Triton X-100 in PBS and hybridized at RT for 2 h followed by several washes in 0.3% Triton X-100 in PBS. Skin was mounted in Pro-Long Gold Antifade (Molecular Probes). Primary antibodies were: rabbit anti-EGFP (Invitrogen; A-11122; dilution, 1:50); goat anti-VEGFR3 (R&D Systems; AF743; dilution, 1:100); rabbit anti-PECAM1 (Abcam; ab28364; dilution, 1:50); and rabbit anti-LYVE-1 (Abcam; ab14917; dilution, 1:100). Secondary antibodies were donkey anti-rabbit Alexa Fluor 647; donkey anti-goat Alexa Fluor 555; and chicken anti-rabbit Alexa Fluor 488 (all from Invitrogen and diluted 1:400).

Imaging of Luciferase Activity. An IVIS Spectrum Imaging System (Caliper Life Sciences) was used for in vivo luciferase imaging. Mice were anesthetized with isoflurane (2%) and injected intraperitoneally with 150 mg/kg of luciferin (Caliper Life Sciences) diluted 15 μg/mL in PBS. Sequential images were obtained after luciferin injection every 15 s (maximum light emission, ~20–30 min after luciferin injection). Living Image software (Caliper Life Sciences) was used for data analysis of photons emitted from the specific ROIs defined accordingly for each particular assay. Luciferase activity is in photons per second per square centimeter per steradian (p/s/ cm⁻² sr⁻¹).

Detection of Luciferase of Vegfr3EGFPLuc mice During Postnatal Development. Dorsal and ventral whole-body bioluminescent images were collected from albino heterozygous or homozygous females (n = 7) at different ages with an IVIS imaging system as described above. The ROI was the body of the animal at each time point.

Detection of Luciferase Activity in Tissue Extracts. Mouse tissues were removed and homogenized in three volumes of PBS containing a protease inhibitor mixture (Roche Applied Science) and lysed with passive lysis buffer (Promega). After centrifugation at 9,000 × g for 10 min at 4°C, the supernatant was collected. Luciferase activity was assessed using a luminometer equipped with the Luciferase Assay System (Promega). Protein concentration was estimated by Bradford reagent (Sigma-Aldrich).

Western Blot. Whole tissue extracts were obtained using RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS], containing protein inhibitors. Total protein extracts (100 μg) were loaded on 7.5% or 10% SDS/PAGE gels. The primary antibodies used included: rat anti-mouse VEGFR3 (AFL4) (1:50) (BD Pharmingen), anti-EGFP (1:50) (Chemicon), and mouse monoclonal α-α-tubulin (1:10,000) (Sigma-Aldrich). Alexa 680-coupled (Invitrogen) secondary antibodies were used. The gel was scanned on an Odyssey Infrared Imaging System (LI-COR Biosciences) using the 700-nm channel.

Adenoviral VEGF Expression. Immune compromised athymic nu/nu [Crl:NU(Ico)-Foxn1nu] mice were crossed with Vegfr3EGFPLuc mice to generate double homozygous animals. Injection of recombinant adenoviruses are described elsewhere (2). Recombinant adenoviruses (3.5 × 10⁹ pfu) in PBS (final volume, 50 μL) were injected s.c. in the ears of Vegfr3EGFPLuc; Crl:NU(Ico)-Foxn1nu mice. At least four mice were used in each study group. In vivo bioluminescence emission was monitored at various time points with an IVIS Spectrum as described. At each time point, a circular ROI (18-pixel diameter) including the region of maximum signal was used for quantifying bioluminescence emission in both the control and treated ears. Fourteen days after injection, mice were killed, and the ears fixed in neutral buffered formalin before paraffin embedding and processing.

Wound-Healing Assays. Full-thickness punch biopsy wounds (8-mm diameter) were created on the backs of albino, 12–15-wk-old, Vegfr3EGFPLuc heterozygous females (n = 5), after shaving the area. Wound area was calculated at selected time points after wounding as percentage of the area at day 0 [(V × H) on day n/(V × H) on day 0 × 100%]. V and H are vertical and horizontal diameters, respectively, measured with a caliper. Bioluminescent images were taken as described above. The area of the wound at day 0 was defined as the ROI. For Dex treatment, Vegfr3EGFPLuc heterozygous female (n = 7) were i.p. injected with Dex (Sigma-Aldrich; D1756; 5 mg/kg) daily starting 1 d before wounding and for the length of the assay. Control mice were intraperitoneally injected with PBS.

Inflammation Induced with Complete Freund Adjuvant. Albino 16–18-wk-old Vegfr3EGFPLuc heterozygous females were injected i.p. twice, with an interval of 15 d, with 200 μL of CFA (Difco Laboratories) emulsified 1:1 in PBS as described (3). Control females were injected with PBS. Luciferase emission was measured as described above. The ROI was defined as the abdominal region of each animal. Tissues of treated animals were taken 4 wk after treatment and processed for immunohistochemical analysis with anti-VEGFR3 antibodies.
Induction of Acute CHS. Homozygous, 10–15 wk old Vegfr3\textsuperscript{EGFPLuc} KI/KI females (n = 5) were presensitized by topical application of 50 μL of 2% Oxa (Sigma Aldrich; E0753) in acetone/corn oil, 4:1 vol/vol, to the shaved abdomen and challenged six days later (day 0 in the assay), by topical application of 10 μL of a 1% Oxa solution to the left ear. The right ear was treated with vehicle (acetone/corn oil, 4:1 vol/vol) alone as described (4). For the Oxa + Dex treatment, Dex was administered to another group of Vegfr3\textsuperscript{EGFPLuc} KI/KI females (n = 7) by i.p. injection as described above, starting 1 d before the Oxa challenge. Control mice were intraperitoneally injected with saline. Ear thickness was measured with a caliper at different days after challenge. Bioluminescence images were collected on the day of sensitization and daily for 6 d after Oxa challenge defining as the ROI the treated and control ear of each animal respectively. Left and right ear biopsies (n = 4 mice from each group and time point) were collected 2 and 4 d after Oxa challenge for histology. Lymphatic vessel density was determined by counting the number of VEGFR3\textsuperscript{+} lymphatic vessels per field 20× (3 animals per group at each time point) using a Olympus BX41 microscope with an objective PlanN/20×/0.40/∞/0.17/FN22 and ocular lens WHN10×/22.

Chemical Carcinogenesis Assay. Tumors were initiated on the shaved back skin of 7-d-old Vegfr3\textsuperscript{EGFPLuc} homozygous females with DMBA, followed by promotion with TPA as described (5). Bioluminescence images of the back of the animals were taken every other week and skin papillomas were processed for immunohistological analysis with anti-LYVE1 and anti-PECAM1 antibodies.

B16-V5 Melanoma Xenograft Assay. Mouse B16-V5 melanoma cells were cultured in DMEM (Invitrogen) with 10% (vol/vol) FBS. A total of 5 × 10\textsuperscript{5} cells in 0.1 mL of DMEM (without serum) from subconfluent cultures were s.c. injected in the back of 6 heterozygous, 12-wk-old, Vegfr3\textsuperscript{EGFPLuc} females. Growth of tumors was measured every 2 d by determination of the two orthogonal external diameters using a caliper. Tumor volume was calculated using the formula (a × b\textsuperscript{2} × 0.52), where a and b are the longest and shortest tumor diameters, respectively. Dorsal and lateral whole-body bioluminescence images were taken twice per week, and luciferase activity was analyzed in the peritumoral area and in the lymph nodes as described above. The ROIs for the tumor and lymph nodes were defined at the final time point as a circle surrounding the luminescent area around the tumor or at the position of the lymph node and applying the same ROI to previous time point images.

Immunohistochemistry. The following antibodies were used: rat anti-VEGFR3 (AFL4; BD Pharmingen; dilution, 1:10); rabbit-anti-LYVE1 (Abcam; 768540; dilution, 1:250); rabbit-anti-PECAM1 (Abcam; 805662; diluted 1:75); and rabbit-anti-S-100 (Dako; Z0311; dilution, 1:1,000).

Fig. S1. Gene-targeting strategy and recombination analysis. (A) Schematic drawing of the Vegfr3 gene. Coding exons (black boxes) and 3′-UTR (gray box) are represented. (B) Schematic representation of the targeting vector, homologous recombinant allele (Vegfr3\textsuperscript{neoEGFPLuc}), and Flpe-mediated recombination to eliminate the selection cassette (PGK-neo) flanked by frt sites (black triangles). The IRES-EGFPLuc cassette is integrated 149-nt downstream of the TGA stop codon. (C and D) ES cell clones screening by Southern blot. A total of 60 clones were analyzed, and 18 (30%) were true homologous recombinants. The result of the Southern screening is shown for one of the recombinant ES cell clones. (E) Germ-line chimeras were crossed with transgenic Tg.CAGG-Flpe females (1) to delete the PGK-neo cassette. The excision was analyzed in the progeny by PCR with primers shown in B.

1. Rodríguez CI, et al. (2000) High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat Genet 25:139–140.

Fig. S2. Analysis of Vegfr3 expression in Vegfr3\textsuperscript{EGFPLuc} embryos. (A) Northern blot of whole E13.5 embryo extracts of the indicated genotypes with a probe homologous to the 3′-UTR region of the Vegfr3 gene. The size of the mRNA for the WT (Vegfr3) and knockin (Vegfr3\textsuperscript{EGFPLuc}) alleles is indicated. GAPDH mRNA levels in the samples are also shown as a loading control. (B) Western Blot for VEGFR3 and EGFP in E13.5 embryos. α-Tubulin immunoblot is included as a loading control.
**Fig. S3.** (A) Colocalization of EGFP by fluorescence and immunofluorescence. EGFP immunofluorescence (EGFP IF) and direct EGFP fluorescence (nEGFP) detection in whole-mount ear skin of KI/KI mice. EGFP fluorescence can still be detected after processing the samples for immunofluorescence as described in SI Materials and Methods. (B) Immunofluorescence of VEGFR3 and LYVE-1 in skin of 3-wk-old mice.

**Fig. S4.** Postnatal regulation of Vegfr3 and EGFPLuc expression. (A) Quantification of luciferase signal from +/-KI and KI/KI mice at different weeks after birth. The ROI used for quantification is an area within the hind left paw devoid of hair. (B) Quantification of luciferase activity in tissue extracts from KI/KI Mice at 2 and 14 wk of age. (C) Western blot analysis of VEGFR3 and EGFPLuc in tissue extracts from KI/KI mice. Tubulin was used as a loading control.

**Fig. S5.** Histological analysis of recombinant AdVEGF-induced lymphangiogenesis. Immunostaining of sections from AdVEGF-C-, AdVEGF-E-, AdVEGF-B167-, and PBS-treated ears with anti-LYVE-1 and anti-PECAM1 antibodies, 14 d after adenoviral vector injection.
Fig. 56. Quantification of lymphatic vessel density in wound-healing assays. Quantification of VEGFR3+ vessels in the wound area at days 8, 14, and 24 after wounding in animals treated with vehicle (Vhc) or with Dex. The density of lymphatic vessels in vehicle-treated animals increases at day 8 but decreases with healing to reach normal levels, whereas in Dex-treated animals, there is not a significant increase at any of the analyzed time points compared with control. The density of lymphatic vessels is represented as mean ± SD. ANOVA: day 8 Vhc vs. Dex, ***P < 0.001; day 8 Vhc vs. control, ***P < 0.001; day 14 Vhc vs. Dex, **P < 0.05; day 14 Vhc vs. control ***P < 0.001.

Fig. 57. CFA- and CHS-induced inflammation. (A) VEGFR3 immunohistochemistry of the inflammatory infiltrate induced by CFA injection. VEGFR3 immunostaining analysis of the peritoneal inflammation induced by injection of CFA in the abdominal cavity. Red arrowheads indicate VEGFR3+ inflammatory cells and black arrowheads indicate dilated lymphatic vessels strongly positive for VEGFR3. (B) Quantification of Oxa-induced inflammation monitored by ear swelling (thickness) at different days after challenge. (C) LYVE-1 immunostaining of vehicle (Vhc)-, Oxa-, and Oxa plus Dex-treated ears 2 d after challenge. Black arrowheads indicate dilated lymphatic vessels.
Fig. S8. Peripheral and lymph node lymphatic response induced by s.c. injection of the B16-V5 melanoma cell line. (A) LYVE-1 immunohistochemistry of the skin lymphatic vessels (red arrowheads) surrounding the tumor. The border between tumor (T) and skin (S) is highlighted in red. (B and C) LYVE-1 immunohistochemistry of the inguinal lymph node of a Vegfr3EGFP::Luc female injected with B16-V5 cells (B) versus that of a nontreated female as a control (C), 15 d after injection (tumor size close to 1 cm³). Note the expansion of the inguinal lymph node. (D and E) Amplified areas (black squares) from b and c, respectively. (F) Quantification of luciferase activity at the periphery of the tumor and tumor volume in B16-V5-injected mice at different times after injection. The basal level of luciferase activity is the luminescence emission monitored from the injected area immediately before injection. Data are represented as means + SD (n = 3 mice). (G) Relative luciferase activity from the proximal (inguinal) and distal (axillar) lymph nodes at the time in which the tumor size was ∼1 cm³ (n = 3).

Fig. S9. Detection of B16-V5 metastasis in the inguinal lymph node. S100 immunohistochemistry of the inguinal lymph node of a mouse injected s.c. with B16-V5 melanoma cells. The lymph node was collected at the final time point of the assay when the tumor was close to 1 cm³ in size. Red arrowhead indicates one S100⁺ metastatic cell in this section. Yellow arrowheads indicate macrophages containing dark pigmented melanin.