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Lymphangiogenic Growth Factor Responsiveness Is Modulated by Postnatal Lymphatic Vessel Maturation

Terhi Karpanen,* Maria Wirzenius,*
Tajja Mäkinen,* Tanja Veikkola,*
Hidde J. Haisma,[†] Marc G. Achen,[‡]
Steven A. Stacker,[‡] Bronislaw Pytowski,[§]
Seppo Ylä-Herttuala,[¶] and Kari Alitalo*

From the Molecular/Cancer Biology Laboratory and Ludwig Institute for Cancer Research, Biomedicum Helsinki and Haartman Institute, University of Helsinki and Helsinki University Central Hospital, Helsinki, Finland; A.I. Virtanen Institute,[¶] University of Kuopio, Kuopio, Finland; the Department of Therapeutic Gene Modulation,[‡] University of Groningen, Groningen, The Netherlands; the Ludwig Institute for Cancer Research,[§] Royal Melbourne Hospital, Victoria, Australia; and Molecular and Cellular Biology,[†] ImClone Systems, New York, New York*

Lymphatic vessel plasticity and stability are of considerable importance when attempting to treat diseases associated with the lymphatic vasculature. Development of lymphatic vessels during embryogenesis is dependent on vascular endothelial growth factor (VEGF)-C but not VEGF-D. Using a recombinant adenovirus encoding a soluble form of their receptor VEGFR-3 (Ad-VEGFR-3-Ig), we studied lymphatic vessel dependency on VEGF-C and VEGF-D induced VEGFR-3 signaling in postnatal and adult mice. Transduction with AdVEGFR-3-Ig led to regression of lymphatic capillaries and medium-sized lymphatic vessels in mice under 2 weeks of age without affecting collecting lymphatic vessels or the blood vasculature. No effect was observed after this period. The lymphatic capillaries of neonatal mice also regressed partially in response to recombinant VEGFR-3-Ig or blocking antibodies against VEGFR-3, but not to adenovirus-encoded VEGFR-2-Ig. Despite sustained inhibitory VEGFR-3-Ig levels, lymphatic vessel regrowth was observed at 4 weeks of age. Interestingly, whereas transgenic expression of VEGF-C in the skin induced lymphatic hyperplasia even during embryogenesis, similar expression of VEGF-D resulted in lymphangiogenesis predominantly after birth. These results indicate considerable plasticity of lymphatic vessels during the early postnatal period but not thereafter, suggesting that anti-lymphangiogenic therapy can be safely applied in adults. (*Am J Pathol* 2006, 169:708–718; DOI: 10.2353/ajpath.2006.051200)

The lymphatic vasculature collects extravasated fluid, macromolecules, and cells of the immune system from the interstitium and after filtration through a series of lymph nodes returns them back to the blood circulation. The lymph vessels also absorb and transport dietary lipids from the intestine.¹ In contrast to blood vessels, lymphatic capillaries start blind ended, have a discontinuous basement membrane, and are not covered by pericytes, whereas collecting lymphatic vessels are surrounded by a smooth muscle cell layer. The lymphatic endothelial cells lack tight interendothelial junctions and are attached to the surrounding extracellular matrix by anchoring filaments, while valves serve to prevent lymph backflow in the absence of a strong propulsive pressure.² Defects of lymphatic vessel function can lead to lymphedema, a condition characterized by swelling of extremities due to fluid accumulation in tissues.³ Lymphatic vessels also represent the primary route of metastatic spread for many types of human cancers,⁴ and they are furthermore involved in the regulation of inflammatory responses in various pathological conditions.^{5,6}

The mechanisms controlling development of the blood vasculature are relatively well characterized, but the molecular mechanisms regulating the growth and function of lymphatic vessels are only starting to be elucidated. Vascular endothelial growth factor receptor (VEGFR)-3 is initially expressed in all endothelial cells of mouse em-

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Current address of T.M.: Department of Molecular Neurobiology, Max-Planck-Institute of Neurobiology, Martinsried, Germany; current address of T.V.: Roche Oy, Espoo, Finland.

Address reprint requests to Kari Alitalo, M.D., Ph.D., Molecular/Cancer Biology Laboratory, Biomedicum Helsinki, P.O.B. 63 (Haartmaninkatu 8), FI-00014 University of Helsinki, Finland. E-mail: kari.alitalo@helsinki.fi.

bryos.⁷ *VEGFR-3*-deficient mice die at embryonic day 9.5 (E9.5) because of a failure in remodeling of the primary vascular plexus.⁸ Later in embryonic development, when the lymphatic vessels start to sprout at approximately E10.5, the expression of *VEGFR-3* decreases in blood vessels and becomes restricted almost exclusively to the lymphatic endothelium.⁷ The two known ligands of *VEGFR-3*, vascular endothelial growth factor (VEGF)-C and VEGF-D, have been shown to induce primarily lymphangiogenesis.^{9,10} VEGF-C is necessary for the initial sprouting and migration of lymphatic endothelial cells from embryonic veins, and mice lacking *VEGF-C* die prenatally,¹¹ whereas VEGF-D is dispensable for embryonic lymphatic development.¹²

We have shown that the ligand-binding domain of *VEGFR-3* fused to the Fc-region of human immunoglobulin γ chain (*VEGFR-3-Ig*) is a potent inhibitor of VEGF-C-induced tumor lymphangiogenesis and lymphatic, but metastasis.^{13,14} To apply inhibition of ligand-induced *VEGFR-3* signaling to prevent lymphatic metastasis in cancer patients, it would be important to know how this affects the normal lymphatic vasculature. Here we show that inhibition of the interaction of *VEGFR-3* with its ligands, by adenovirus-encoded soluble *VEGFR-3-Ig*, recombinant *VEGFR-3-Ig* protein, or by inhibitory *VEGFR-3* antibodies, causes systemic regression of normal lymphatic capillaries and medium-sized lymphatic vessels during the first 2 weeks of postnatal life. After that time, the lymphatic vasculature becomes independent of *VEGFR-3* ligands, and the lymphatic vessels regenerate even in the presence of neutralizing concentrations of *VEGFR-3-Ig*. Furthermore, we demonstrate that whereas transgenic overexpression of VEGF-C induces lymphangiogenesis during embryogenesis, VEGF-D stimulates lymphatic vessel growth predominantly after birth, indicating additional changes related to postnatal lymphatic maturation.

Materials and Methods

Mice and Adenoviruses

The *VEGFR-3^{+LacZ}*, K14-VEGF-C, K14-VEGF-C156S, and K14-VEGF-D mice have been described previously.⁸⁻¹⁰ NMRI *nu/nu* and *nu/+* mice were from Harlan (Horst, The Netherlands). All experiments involving mice were approved by the Provincial State Office of Southern Finland and they were performed in accordance with institutional guidelines. Recombinant adenoviruses encoding *VEGFR-3-Ig* (Ad*VEGFR-3-Ig*), VEGF-C (Ad*VEGF-C*), β -galactosidase (Ad*LacZ*), and *VEGFR-2-Ig* (Ad*VEGFR-2-Ig*) were produced as described.^{13,15}

In Vitro Testing of the Adenoviruses

HepG2 cells were transduced with 100 pfu/cell of Ad*VEGFR-3-Ig* or Ad*LacZ* and metabolically labeled with 100 μ Ci/ml [³⁵S]methionine and [³⁵S]cysteine (Redivue ProMix; Amersham Pharmacia Biotech, Uppsala, Sweden). The labeled fusion protein was precipitated with protein A-Sepharose (Amersham Pharmacia Biotech)

and analyzed by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Alternatively, unlabeled conditioned medium from Ad*VEGFR-3-Ig*-transduced HepG2 cells or, as a control, polyclonal rabbit antibodies against human VEGF-C (antiserum 882),¹⁶ were used to bind metabolically labeled VEGF-C from the conditioned media of HepG2 cells transduced with Ad*VEGF-C* or Ad*LacZ* adenoviruses. The complexes were precipitated, washed, and analyzed by 12.5% SDS-PAGE under reducing conditions.

Treatment of Mice with Adenovirus-Encoded Ligand Traps, Blocking Antibodies, or Recombinant Proteins

One-, four-, or seven-day-old mouse pups were injected intraperitoneally with 5×10^8 pfu/30 to 50 μ l and mice 14 days or older with 1×10^9 pfu/60 to 100 μ l of Ad*VEGFR-3-Ig*, Ad*VEGFR-2-Ig*, Ad*LacZ*, or a corresponding volume of phosphate-buffered saline (PBS). Mice were injected intraperitoneally once a day or every second day with 30 mg/kg or 60 mg/kg of mF4-31C1, a rat monoclonal antibody against mouse *VEGFR-3* that blocks ligand binding¹⁷; 25 mg/kg of recombinant *VEGFR-3-Ig* fusion protein¹⁸; 20 mg/kg AFL4, a rat monoclonal antibody against mouse *VEGFR-3* that blocks ligand binding¹⁹; or control (nonblocking rat monoclonal antibodies against mouse *VEGFR-2*,¹⁹ recombinant *VEGFR-1-Ig* fusion protein¹⁸ or PBS) in a volume of 20 to 100 μ l.

Visualization of Blood and Lymphatic Vessels

Fluorescent whole-mount immunostaining was performed as described previously²⁰ with polyclonal rabbit antibodies against mouse LYVE-1¹¹ and monoclonal rat antibodies against mouse PECAM-1 (BD Pharmingen, San Diego, CA) using Alexa Fluor 594-conjugated goat anti-rabbit and Alexa Fluor 488-conjugated goat anti-rat antibodies (Molecular Probes, Eugene, OR) for detection. Ear tissues were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and analyzed with a LSM510 Meta confocal microscope (Carl Zeiss, Heidelberg, Germany). Other tissues were analyzed with a stereomicroscope (Leica, Wetzlar, Germany).

Paraffin sections from paraformaldehyde-fixed tissues were immunostained with monoclonal rat antibodies against *VEGFR-3*¹⁹ or PECAM-1 (BD Pharmingen) or rabbit antibodies against LYVE-1¹¹ using tyramide signal amplification kit (NEN Life Sciences, Boston, MA). The lymphatic vessels of *VEGFR-3^{+LacZ}* mice were stained with X-gal (Sigma-Aldrich). For visualization of functional lymphatic vessels, fluorescein isothiocyanate-conjugated dextran (2000 kd; Sigma-Aldrich, St. Louis, MO) was injected intradermally into the ear or tail, and the uptake of the dye by lymphatic vessels was analyzed by fluorescence microscopy.

Detection of VEGFR-3-Ig in Serum

The concentration of VEGFR-3-Ig fusion protein in the serum was determined by specific enzyme-linked immunosorbent assay as described.¹⁸ Data are expressed as average \pm SD. To test VEGFR-3-Ig binding properties, serum obtained from AdVEGFR-3-Ig-transduced mice was used to precipitate metabolically labeled VEGF-C from the conditioned medium of VEGF-C-transfected 293T cells.

Pharmacokinetics of mF4-31C1 and VEGFR-3-Ig

Female *nu/nu* mice were injected intraperitoneally with 20 mg/kg of mF4-31C1. Two mice per group were bled at the indicated times after treatment. Plasma concentration of mF4-31C1 was determined using an enzyme-linked immunosorbent assay. Briefly, 96-well Maxi-sorp microtiter plates (Nunc, Roskilde, Denmark) were coated with the extracellular domain of mouse VEGFR-3 fused with secreted human alkaline phosphatase (sR3-AP).¹⁷ Plasma samples, serially diluted in blocking buffer (PBS containing 5% freeze-dried milk), were added to the wells and incubated at room temperature for 1 hour. Bound mF4-31C1 was detected with donkey anti-rat IgG-horseradish peroxidase conjugate (Amersham Pharmacia Biotech) using TMB peroxidase substrate (KPL, Gaithersburg, MD). The change in optical density was measured using a microplate reader (Molecular Devices, Sunnyvale, CA). Pharmacokinetic parameters were calculated by non-compartmental analysis using the WinNonlin program (Pharsight Corporation, Mountain View, CA). To determine the systemic half-life of VEGFR-3-Ig in mice, 10 μ g of recombinant VEGFR-3-Ig protein were injected intravenously into three female C57BL/6 mice in a volume of 200 μ l, and blood samples were collected from the tail vein at the indicated time points. Serum VEGFR-3-Ig concentration was quantified by enzyme-linked immunosorbent assay.¹⁸ Data are expressed as average \pm SD.

Quantitation of Lymphatic Vessels

At E14.5, we quantified the number of X-gal-stained skin lymphatic vessels at 0.3, 0.6, and 0.9 mm distance from the dorsal midline as well as the skin area covered by X-gal-positive lymphatic vessels on the dorsal side of K14-VEGF-D \times *VEGFR-3^{+/LacZ}*, K14-VEGF-C \times *VEGFR-3^{+/LacZ}*, and *VEGFR-3^{+/LacZ}* embryos. At P1, P7, and P14, the area covered by lymphatic vessels in the skin of K14-VEGF-C, K14-VEGF-D, and wild-type littermate mice was quantified from photomicrographs of LYVE-1-stained skin sections (six photomicrographs/mouse) using the Image-Pro Plus program (Media Cybernetics, Silver Spring, MD). Data are expressed as average \pm SD.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For the determination of the relative expression levels of VEGF-C and VEGF-D in the K14-VEGF-C, K14-VEGF-D, and wild-type littermate mice, RNA was extracted from whole embryos at E14.5 and from ventral skin at E17.5 using the RNeasy mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed from 0.5 μ g of total RNA with the QuantiTect reverse transcription kit (Qiagen), and 1/20 of the reverse transcription reaction was used as a template in the quantitative PCR reaction performed with the DyNAmo HS SYBR Green qPCR kit (Finnzymes, Espoo, Finland) using the ABI 7500 SDS real-time PCR instrument (Applied Biosystems, Foster City, CA). The oligonucleotide primers used were the following: 5'-GGCTGGCAACATAACAGAGA-3' and 5'-GTGGCATGCATTGAGTCTTT-3' for human VEGF-C, 5'-CTTGCTGGAACAGAAGACCA-3' and 5'-TACAGACA-CACTCGCAACGA-3' for human VEGF-D, and 5'-ACA-ACCTTGGCATTGTGGAA-3' and 5'-GATGCAGGGATG-ATGTTCTG-3' for mouse GAPDH. Relative expression of human VEGF-C and VEGF-D were normalized to mouse GAPDH expression.

Results

AdVEGFR-3-Ig Establishes Long-Term Inhibition of VEGF-C and -D in Vivo

We used a recombinant adenovirus, AdVEGFR-3-Ig, to inhibit VEGFR-3 signaling in mice of different ages. AdVEGFR-3-Ig-transduced cells secreted a protein of the expected size (Figure 1A), which was able to bind VEGF-C (Figure 1B). Also, the VEGFR-3-Ig fusion protein in the serum of mice transduced intraperitoneally with AdVEGFR-3-Ig 1 week after birth, and analyzed at 2 or 4 weeks of age, was able to bind VEGF-C (Figure 1C). The systemic half-life of VEGFR-3-Ig in C57BL/6 mice was determined to be between 2 to 3 days (Figure 1D).

AdVEGFR-3-Ig Induces Postnatal Lymphatic Vessel Regression

To determine whether the lymphatic vasculature of postnatal mice depends on ligand-induced VEGFR-3 signaling, *VEGFR-3^{+/LacZ}* mice were transduced intraperitoneally with 5×10^8 pfu of AdVEGFR-3-Ig or AdLacZ or a corresponding volume of PBS at days 1, 7, or 14 after birth. An initial lymphatic vessel network is present on day 1 after birth (see Figure 6, I and L).^{18,21} The mice treated with AdVEGFR-3-Ig at the age of 1 or 7 days were almost completely devoid of lymphatic capillaries and medium-sized lymphatic vessels in the intestine, mesenterium, diaphragm, stomach, and heart, when analyzed 1 week after the injection. Only a few thin remnants of lymphatic vessels, likely without a lumen, and some isolated lymphatic endothelial cells remained in these tissues, as determined by whole-mount β -galactosidase staining

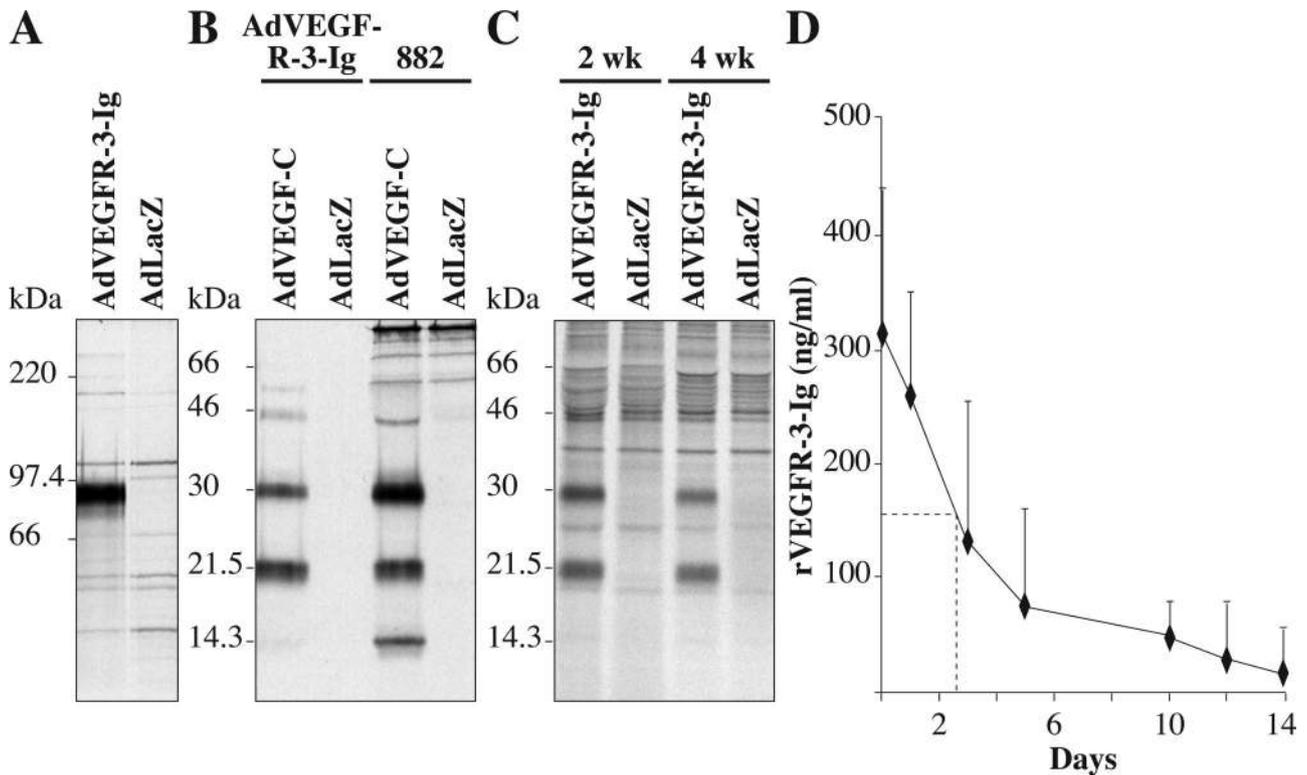


Figure 1. AdVEGFR-3-Ig establishes long-term inhibition of VEGF-C *in vivo*. **A:** AdVEGFR-3-Ig- or AdLacZ-transduced cells were metabolically labeled and the conditioned media were precipitated with protein A Sepharose. **B** and **C:** Metabolically labeled VEGF-C was precipitated with VEGFR-3-Ig produced by AdVEGFR-3-Ig-transduced cells or with the 882 polyclonal VEGF-C antiserum (**B**) or using serum from nude mice transduced intraperitoneally with AdVEGFR-3-Ig or AdLacZ 1 week after birth and analyzed at the age of 2 or 4 weeks (**C**). The bound proteins were analyzed by 7.5% (**A**) or 12% (**B** and **C**) SDS-PAGE under reducing conditions. **D:** Concentration of VEGFR-3-Ig in the circulation of C57BL/6 mice as a function of time after a single intravenous injection of 10 μg of the recombinant protein. Dashed line indicates half of the protein concentration obtained 5 minutes after injection.

(Figure 2, A–H; and data not shown). However, large collecting lymphatics, for example the thoracic duct, were not affected. This may reflect the expression of VEGFR-3 in lymphatic capillaries but not in collecting lymphatic vessels.²² Only occasional thin lymphatic vessels were detected in the skin sections of the AdVEGFR-3-Ig-treated mice by immunohistochemical analysis with antibodies against the lymphatic endothelial markers LYVE-1, VEGFR-3, or podoplanin (Figure 2I and data not shown). On the contrary, lymphatic vessels were abundant in the skin of AdLacZ- and PBS-treated control mice (Figure 2J). However, the blood vessels of the AdVEGFR-3-Ig-treated mice appeared normal in number and size, as analyzed by immunohistochemical staining with antibodies against the pan-endothelial marker platelet endothelial cell adhesion molecule 1 (PECAM-1) (Figure 2, K and L).

Despite the massive regression of the lymphatic vasculature, and in some cases almost complete lack of lymphatic capillaries and medium-sized lymphatic vessels, no signs of lymphedema, chylous fluid accumulation, or growth retardation were observed in the AdVEGFR-3-Ig-transduced mice (data not shown). The average VEGFR-3-Ig level in the sera of the mice that were treated with AdVEGFR-3-Ig at the age of 1 or 7 days was 572 ± 138 ng/ml ($n = 18$) 1 week after injection. Similar regression of the lymphatic vessels occurred in wild-type C57BL/6 littermates when analyzed by immu-

nohistochemical staining of the skin, diaphragm, and intestine (data not shown), which indicates that the regression of lymphatic vessels in the *VEGFR-3^{+LacZ}* mice was not because of *VEGFR-3* haploinsufficiency. In wild-type NMRI mice slightly more lymphatic vessels persisted than in the C57BL/6 mice, indicating variability related to the genetic background (data not shown).

AdVEGFR-3-Ig also induced postnatal regression of the hyperplastic lymphatic vessels in transgenic mice expressing a VEGFR-3-specific mutant form of VEGF-C in the skin under control of the keratin 14 promoter (K14-VEGF-C156S) (Supplemental Figure 1, A–F; see <http://ajp.amjpathol.org>). This indicates that the abnormal, enlarged lymphatic vessels caused by an excessive amount of a VEGFR-3 ligand are dependent on continuous VEGFR-3 signaling during the postnatal period.

In addition to VEGFR-3, the fully processed, mature form of VEGF-C binds to and activates VEGFR-2, which is expressed predominantly in blood vessels and collecting lymphatic vessels.²² The exact role of VEGFR-2 in the lymphatic vasculature is unknown, although there is some indication of its involvement in lymphangiogenesis when stimulated either by VEGF or the mature form of VEGF-C.^{22–24} Treatment of postnatal mice with AdVEGFR-2-Ig did not have any apparent effect on blood or lymphatic vessels in the skin or diaphragm (Supplemental Figure 2, A and B; see <http://ajp.amjpathol.org>; and data not shown). The failure of VEGFR-2-Ig, which was

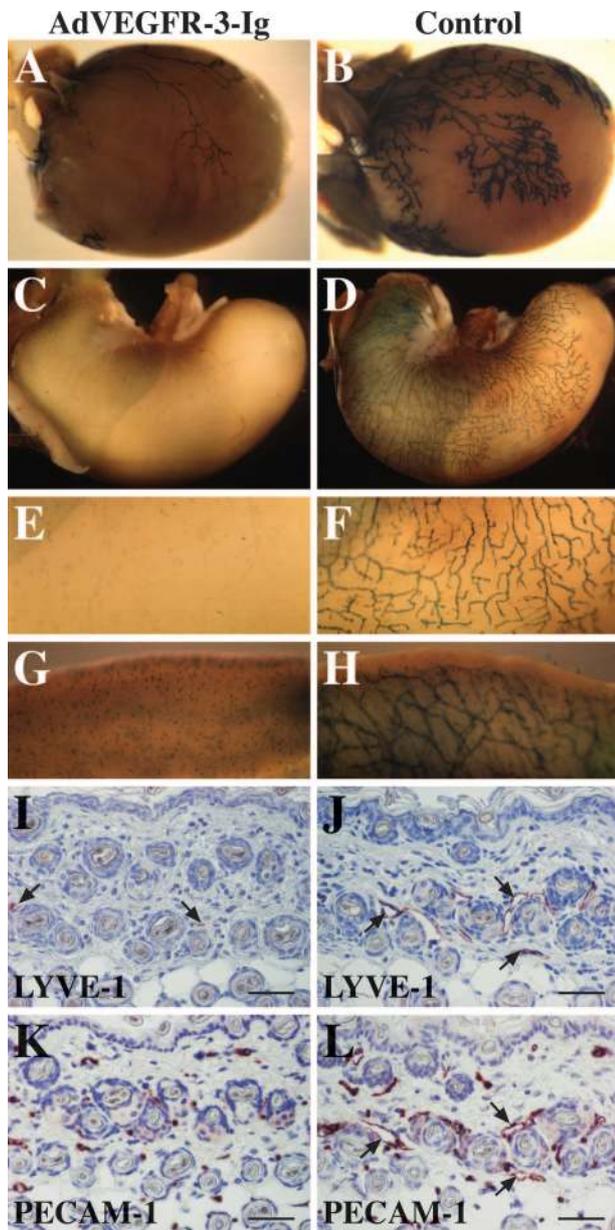


Figure 2. Adenovirally encoded soluble VEGFR-3 causes lymphatic vessel regression in mice under 2 weeks of age but has no effect on the blood vasculature. *VEGFR-3^{+/LacZ}* mice were transduced intraperitoneally with 5×10^8 pfu of AdVEGFR-3-Ig or PBS on day 7 after birth and analyzed 1 week thereafter. The lymphatic vessels were visualized by β -galactosidase staining in the heart (**A** and **B**), stomach (**C–F**), and colon (**G** and **H**). The lymphatic and blood vessels in skin sections were visualized by immunohistochemistry using antibodies against the lymphatic-specific marker LYVE-1 (**I** and **J**, red) or the pan-endothelial marker PECAM-1 (**K** and **L**, red) of AdVEGFR-3-Ig-transduced and control mice, respectively. The vessels with wide lumens indicated with **arrows** in **J** and **L** are lymphatics, which are absent in **I** and **K**. Similar results were observed when the mice were injected at day 1 after birth and analyzed at the age of 7 days. Scale bars = 50 μ m.

produced and circulating in the blood at comparable levels to VEGFR-3-Ig, to affect the vessels of postnatal mice could in part depend on the lower binding affinity of VEGFR-2 for both VEGF-C (kd, 410 pmol/L)¹⁶ and for VEGF (75 to 250 pmol/L)²⁵ as compared to that of VEGFR-3 for VEGF-C (135 pmol/L)¹⁶ or of VEGFR-1 for VEGF (25 pmol/L).²⁵

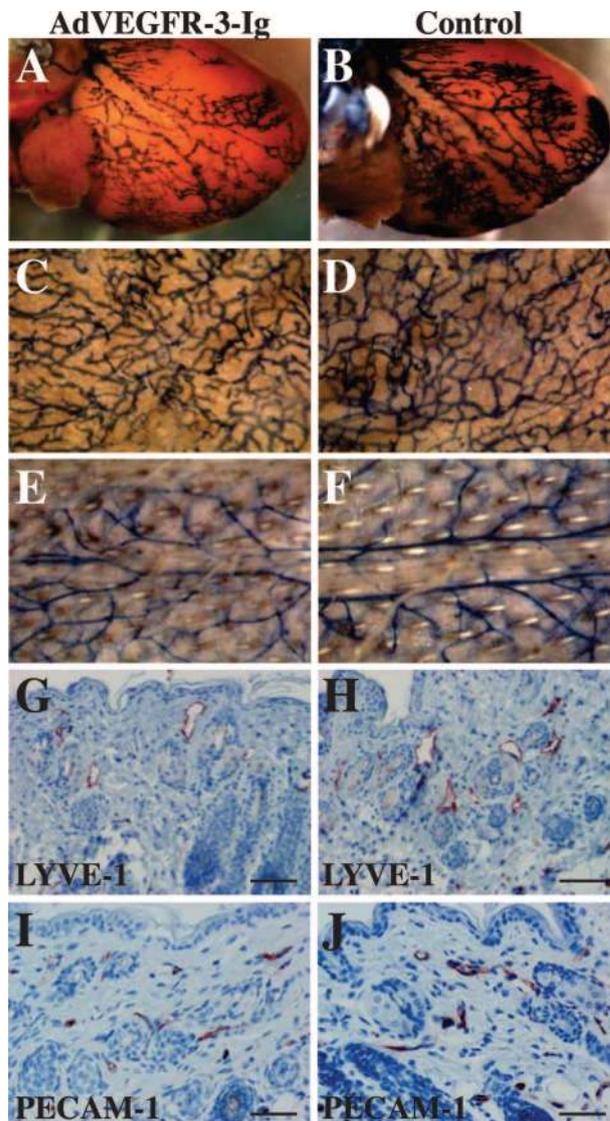


Figure 3. Lymphatic vessels become resistant to inhibition of VEGFR-3 signaling after the first 2 postnatal weeks. β -Galactosidase staining of hearts (**A** and **B**), ears (**C** and **D**), and tail skin (**E** and **F**) and immunohistochemical staining of skin sections using antibodies against LYVE-1 (**G** and **H**, red) and PECAM-1 (**I** and **J**, red) of *VEGFR-3^{+/LacZ}* mice transduced intraperitoneally with AdVEGFR-3-Ig or PBS, respectively, at the age of 2 weeks and analyzed 1 week thereafter. Scale bars = 50 μ m.

Lymphatic Vessels Become Independent of the Ligand-Activated VEGFR-3 Pathway after the First Two Postnatal Weeks

When the *VEGFR-3^{+/LacZ}* mice were transduced with AdVEGFR-3-Ig at the age of 2 weeks or older and the lymphatic vessels were analyzed 1 week after the injection, no regression of the already formed lymphatic vessels or inhibition of further lymphangiogenesis was observed (Figure 3, A–H). The level of VEGFR-3-Ig in the sera of these mice was 562 ± 78 ng/ml ($n = 5$) at the time of analysis, which would neutralize ~ 100 ng/ml of VEGF-C.¹⁸ The VEGFR-3-Ig in the sera of these mice was functional as demonstrated by its ability to precipitate VEGF-C, and no detectable amounts of potentially neu-

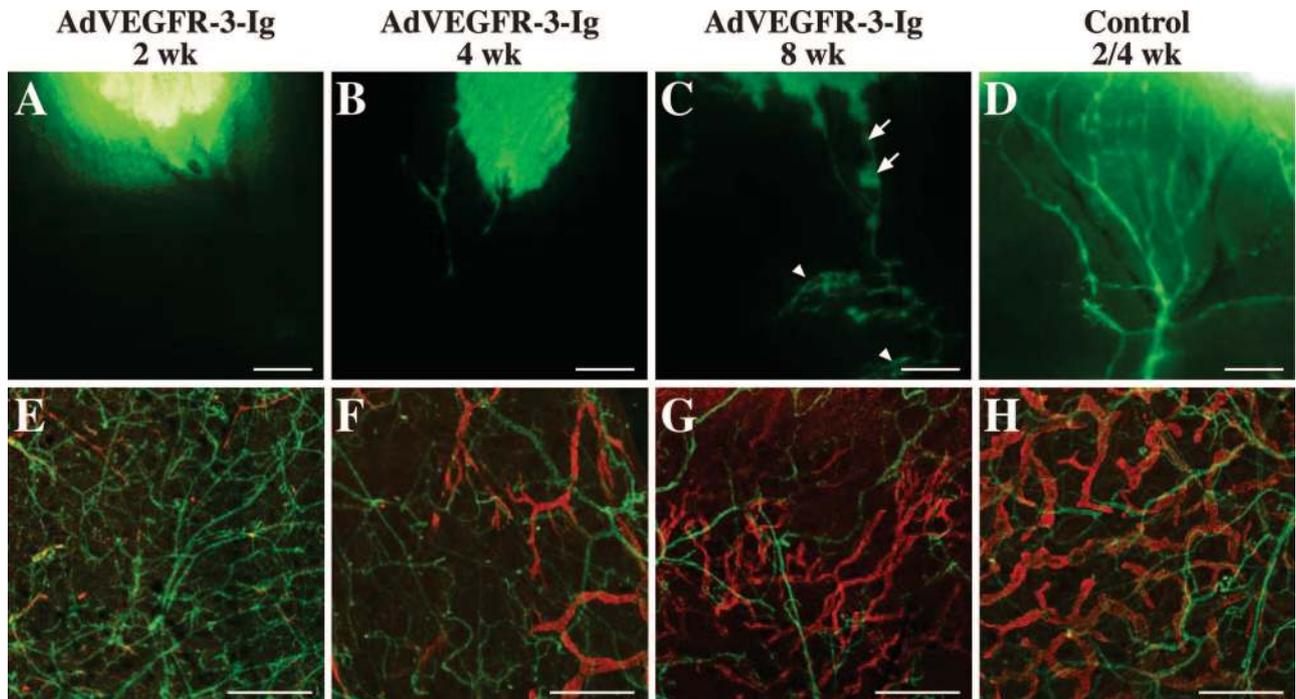


Figure 4. Lymphatic vessels start to regrow 4 weeks after birth despite neutralizing VEGFR-3-Ig levels in the serum. Fluorescent dextran lymphangiography in the ear skin (A–D) as well as whole-mount LYVE-1 (red) and PECAM-1 (green) staining of the ears (E–H) of *nu/nu* mice injected intraperitoneally with AdVEGFR-3-Ig (A–C and E–G) or AdLacZ (D and H) at the age of 1 week and analyzed at the indicated time points. Scale bars: 1 mm (A–D); 500 μ m (E–H).

tralizing antibodies against VEGFR-3-Ig were observed by Western blotting (data not shown).

Lymphatic Vessels Start to Regenerate during the Fourth Postnatal Week Despite Neutralizing Levels of VEGFR-3-Ig in the Serum

To avoid possible immune reactions against the adenovirus or the human protein and to allow longer observation times, the following experiments were done in *nu/nu* mice, which were transduced with AdVEGFR-3-Ig or AdLacZ 4 to 7 days after birth. At the age of 2 weeks, the functional lymphatic vessels in the ear, tail, and hind limb were visualized by fluorescent lymphangiography. In the ears of the AdVEGFR-3-Ig-treated mice, the tracer remained at the site of the injection or, in some cases, was taken up by a single thin lymphatic vessel, suggesting that the ears of the AdVEGFR-3-Ig-treated mice contained no or only few functional lymphatic vessels (Figure 4A). This was further confirmed by whole mount immunofluorescent staining for LYVE-1 (Figure 4E). In the tail of the AdVEGFR-3-Ig-treated mice, most of the small lymphatics were missing, but the fluorescent dextran was still drained through the main collecting lymphatic vessels, which appeared thinner than the ones in the control mice (data not shown). The para-aortic and axillary lymph nodes were smaller than those of the control mice, but the thoracic duct appeared unaffected (data not shown). In the diaphragm, almost complete regression of the lymphatic vessels was observed (data not shown).

Lymphatic vessels of *nu/nu* mice transduced with AdVEGFR-3-Ig at the age of 4 days had started to regenerate when the mice were 4 weeks of age despite the presence of

480 \pm 202 ng/ml ($n = 5$) of functional VEGFR-3-Ig in their sera (Figure 4, B and F, and Figure 1C). In the ear fluorescent dextran was taken up by a few thin lymphatic vessels (Figure 4B). The regeneration of the lymphatic vessels was confirmed in whole-mount immunohistochemistry of the ears, mesenterium, and diaphragm as well as by staining of skin sections (Figure 4F and data not shown). In the ear skin and in the diaphragm, lymphatic regeneration seemed to occur by enlargement and sprouting of the remnants of the regressed lymphatic vessels (Figure 4F and data not shown).

At the age of 8 weeks, the lymphatic vasculature in the skin, diaphragm, and mesenterium had widely regenerated. However, both the function (Figure 4C) and the organization (Figure 4G) of the regenerated lymphatic vessels appeared abnormal when compared to skin lymphatic vasculature in control mice of the same age (data not shown; Figure 4H). In these mice, fluorescent dextran was taken up by fewer lymphatic vessels and more slowly than in the control mice, and the lymphatic vessels appeared leaky (Figure 4C, arrows). Furthermore, dextran backflow occurred from the precollecting lymphatic vessels to the smaller capillaries, suggesting defective valves in the regenerated lymphatic vasculature (Figure 4C, arrowheads). The blood vessels of the AdVEGFR-3-Ig-treated mice were not affected at any age (Figure 4, E–H).

Effects of VEGFR-3-Blocking Antibodies and Recombinant VEGFR-3-Ig

To exclude any contribution by responses against adenoviruses, we investigated if the lymphatic vessel regres-

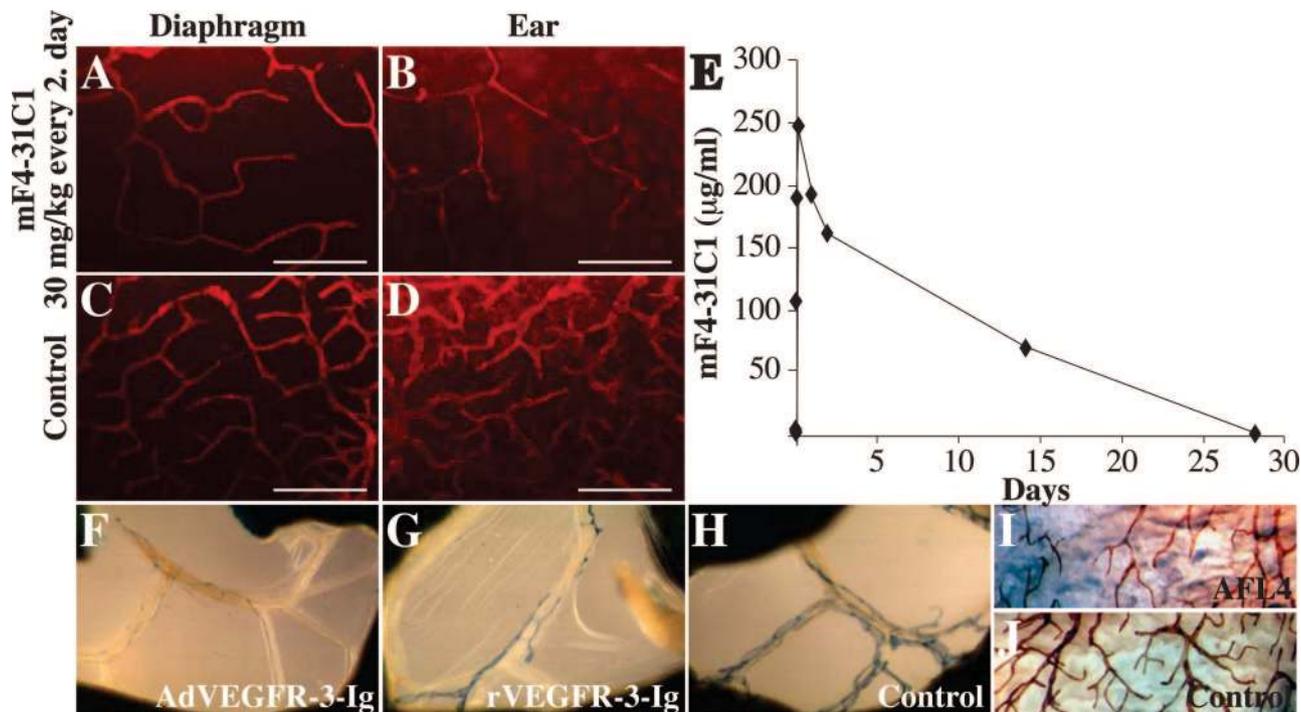


Figure 5. Intra-peritoneally injected blocking antibodies against VEGFR-3 or recombinant VEGFR-3-Ig induce a partial regression of lymphatic vessels. **A–D:** *nu/nu* mice were injected intra-peritoneally with mF4-31C1 blocking antibodies against VEGFR-3 at a dose of 30 mg/kg (**A** and **B**) or with PBS (**C** and **D**) every 2nd day starting at day 4 after birth. Eight days later the diaphragm (**A** and **C**) and the ears (**B** and **D**) were analyzed by fluorescent whole-mount staining with LYVE-1 antibodies (red). Similar results were obtained when a dose of 60 mg/kg every day was used. **E:** Pharmacokinetics of mF4-31C1 in *nu/nu* mice. **F–H:** *VEGFR-3^{+/LacZ}* mice were either transduced with AdVEGFR-3-Ig at a single dose of 5×10^8 pfu (**F**) or injected intra-peritoneally with recombinant VEGFR-3-Ig proteins (**G**) or control proteins (**H**) at a dose of 25 mg/kg once a day starting 1 day after birth and the mesenteric lymphatic vessels were stained with X-gal at the age of 7 days. **I** and **J:** *nu/nu* mice were injected intra-peritoneally with AFL4 blocking antibodies against VEGFR-3 (**I**) or with control antibodies (**J**) at a dose of 20 mg/kg once a day starting 1 day after birth and the lymphatic vessels in the stomach were stained in whole mount with VEGFR-3 antibodies at the age of 7 days. Scale bars = 500 µm.

sion could be reproduced by antibodies blocking VEGFR-3 signaling. The mF4-31C1 rat antibodies that block mouse VEGFR-3¹⁷ or, as a control, nonblocking rat antibodies against mouse VEGFR-2¹⁹ or PBS were injected intra-peritoneally into *nu/nu* mice starting on day 4 after birth. Eight days thereafter the lymphatic vessels were analyzed by immunohistochemistry in diverse organs. Partial regression of lymphatic vessels was detected in the ear skin and diaphragm at a dose of 30 mg/kg injected every second day (Figure 5, A–D). Increasing the dose to 30 mg/kg or even to 60 mg/kg daily did not result in a more complete lymphatic vessel regression (data not shown), suggesting that 30 mg/kg every second day is a saturating dose. The systemic half-life of mF4-31C1 was found to be rather long, more than 5 days in *nu/nu* mice (Figure 5E). Partial regression of the lymphatics could also be observed in the mesenterium, stomach, and diaphragm of *VEGFR-3^{+/LacZ}* mice injected intra-peritoneally with a daily dose of 25 mg/kg of recombinant VEGFR-3-Ig protein (Figure 5G and data not shown) or of *nu/nu* mice injected daily with 20 mg/kg of AFL4 rat antibodies against mouse VEGFR-3 (Figure 5I and data not shown).

VEGF-D Transgene-Induced Lymphatic Hyperplasia Occurs after Birth

We next analyzed the relative contributions of VEGF-C and VEGF-D to embryonic lymphangiogenesis. VEGF-C

is necessary for the initial development of the lymphatic vasculature,¹¹ whereas lymphatic development occurs normally in *VEGF-D*-deficient mice.¹² In K14-VEGF-C and K14-VEGF-C156S mice, the first signs of lymphatic hyperplasia are detected at E13.5, and at E14.5 the cutaneous lymphatic vessels of these embryos are clearly hyperplastic.²² To investigate the ability of VEGF-D to stimulate lymphatic hyperplasia during embryogenesis, we crossed K14-VEGF-D, and as a control, K14-VEGF-C mice with *VEGFR-3^{+/LacZ}* mice. Interestingly, no signs of lymphatic hyperplasia were observed in the K14-VEGF-D × *VEGFR-3^{+/LacZ}* mice when compared to control *VEGFR-3^{+/LacZ}* littermates at E14.5, the age when the cutaneous lymphatic vessels of K14-VEGF-C × *VEGFR-3^{+/LacZ}* mice were already hyperplastic (Figure 6, A–F). At E14.5, the fold increase in the number of lymphatic vessels at 0.6 mm distance from the dorsal midline as compared to the *VEGFR-3^{+/LacZ}* littermates ($n = 3$) was 0.79 ± 0.27 in K14-VEGF-D × *VEGFR-3^{+/LacZ}* embryos ($n = 7$, $P > 0.5$), and 2.15 ± 0.67 in K14-VEGF-C × *VEGFR-3^{+/LacZ}* embryos ($n = 6$, $P < 0.01$). The fold increase in the lymphatic vessel area on the dorsal side as compared to the *VEGFR-3^{+/LacZ}* littermates ($n = 7$) was 1.33 ± 0.17 in K14-VEGF-D × *VEGFR-3^{+/LacZ}* embryos ($n = 7$, $P > 0.02$), and 2.31 ± 0.17 in K14-VEGF-C × *VEGFR-3^{+/LacZ}* embryos ($n = 2$; $P < 0.01$). At this stage the K14-VEGF-D mice expressed the transgene at even higher levels than the K14-VEGF-C mice as deter-

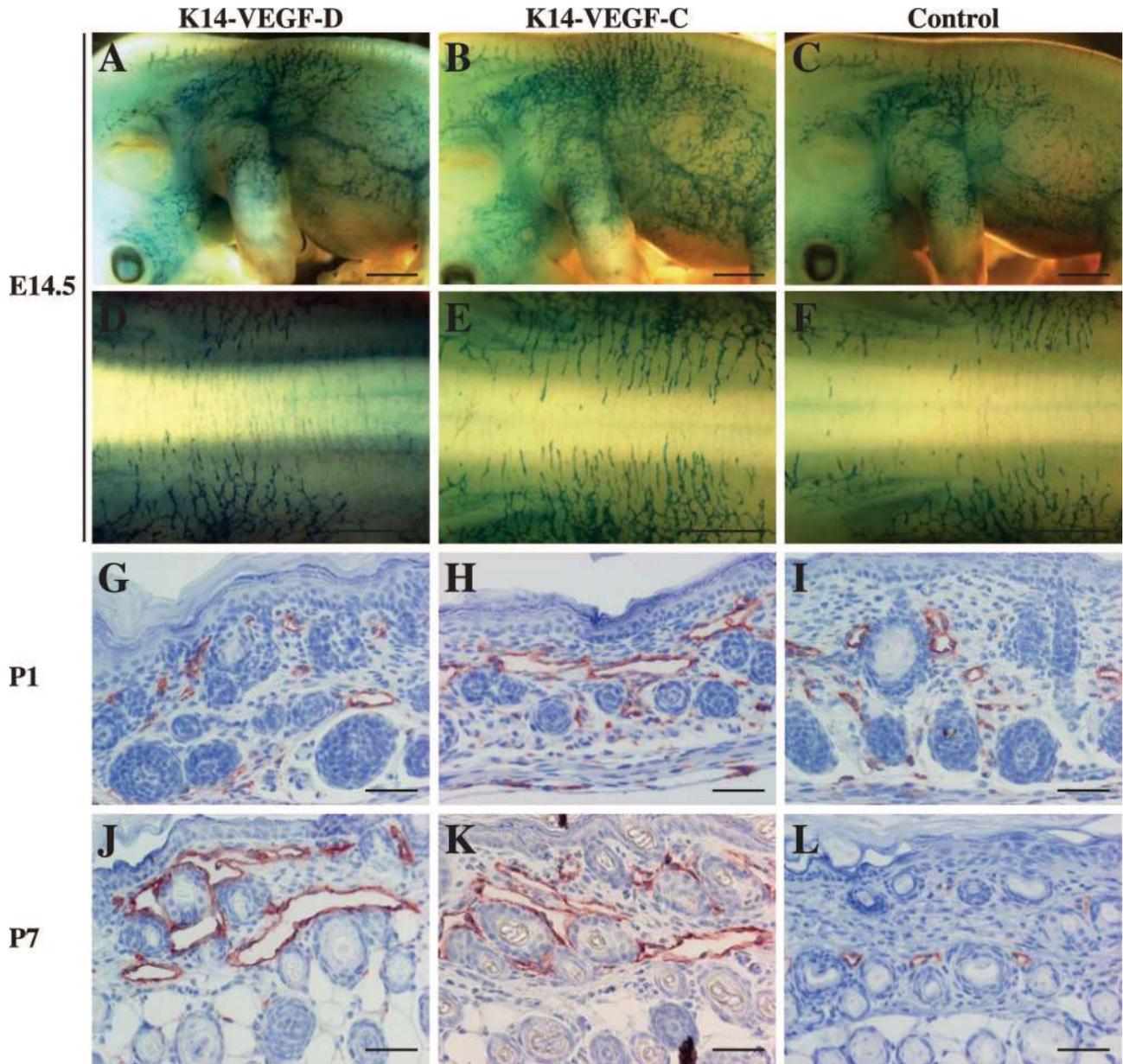


Figure 6. VEGF-D promotes lymphangiogenesis predominantly after birth. **A–F:** Whole-mount β -galactosidase staining of K14-VEGF-D \times *VEGFR-3^{+LacZ}*, K14-VEGF-C \times *VEGFR-3^{+LacZ}*, and control *VEGFR-3^{+LacZ}* mice at E14.5. **G–L:** Immunohistochemical staining for LYVE-1 (red) in the skin sections of K14-VEGF-D and K14-VEGF-C mice as well as in their wild-type littermates at days 1 (**G–I**) and 7 (**J–L**) after birth. Note the progression of lymphatic hyperplasia between P1 and P7 in the K14-VEGF-D skin (**G** and **J**). Scale bars: 1 mm (**A–F**); 50 μ m (**G–L**).

mined by quantitative RT-PCR (data not shown), confirming that the lack of the lymphatic phenotype in the K14-VEGF-D embryos at E14.5 is not because of delay in transgene expression. The first signs of hyperplasia in the cutaneous lymphatic vessels of K14-VEGF-D mice were detected on day 1 after birth (Figure 6, G–I), and at the age of 7 days the lymphatic vessels of K14-VEGF-D mice were indistinguishable from those of K14-VEGF-C mice (Figure 6, J–L). At P7, the fold increase of the lymphatic area as compared to the one in wild-type littermates ($n = 9$) was 7.08 ± 0.17 in K14-VEGF-D mice ($n = 6$, $P < 0.001$) and 6.60 ± 0.59 in K14-VEGF-C mice ($n = 4$, $P < 0.001$). These results suggest that postnatal lymphatic

maturation involves the acquisition of sensitivity to VEGF-D-induced growth signals.

Discussion

This study demonstrates that the lymphatic vasculature of postnatal, but not of adult mice is dependent on ligand-stimulated VEGFR-3 signals. Inhibition of this signaling results in regression of lymphatic capillaries and medium-sized lymphatic vessels during the first 2 weeks after birth. However, the lymphatic vasculature starts to regenerate independently of VEGF-C and VEGF-D by the age

of 4 weeks. Surprisingly, we also found that the postnatal, but not embryonic, lymphatic vessels are sensitive to the induction of hyperplasia by human VEGF-D.

Mice heterozygous for *VEGF-C* deficiency,¹¹ mice harboring an inactivating mutation in the *VEGFR-3* gene, the Chy mice,²⁶ as well as mice expressing VEGFR-3-Ig under a keratin promoter in the skin¹⁸ are born essentially without lymphatic capillaries and medium-sized lymphatic vessels, whereas their large collecting lymphatic vessels appear normal. However, in these mice, lymphatic vascular regeneration occurs in the visceral organs but not in the skin starting at 2 weeks of age. Postnatal regeneration of lymphatic capillaries in most tissues, including the skin, has also been observed in mice deficient of neuropilin-2,²⁷ which acts as a co-receptor for VEGF-C.²⁶ It is as yet not known from where the lymphatic regeneration occurs in these various mice. The large lymphatic vessels in the neuropilin-2-deficient mice and the lymphatic remnants in the AdVEGFR-3-Ig-treated mice could serve as origins of lymphatic sprouting. In contrast, the lymphatic vessels are almost completely absent from the skin of the heterozygous *VEGF-C* gene targeted mice, Chy mice, and K14-VEGFR-3-Ig mice.

Our current results indicate that the VEGF-C/VEGFR-3 pathway is absolutely required for the maintenance of lymphatic vasculature during the first few weeks after birth. Later on the survival of lymphatic vessels becomes independent of VEGFR-3 ligands and their signals, indicating that a process of lymphatic capillary maturation occurs in postnatal mice at ~2 weeks of age. After that, the regrowth of lymphatic vessels occurs without VEGFR-3 ligands, indicating that a VEGF-C- and VEGF-D-independent mechanism that induces lymphatic growth must exist in mice older than 2 to 3 weeks. Although the VEGF-C/VEGFR-3 pathway is not needed after this stage for the growth of the lymphatic vessels, it is apparently functional, because lymphangiogenesis can be stimulated by VEGF-C, when delivered for example via an adenovirus.^{15,22} Possible postnatal factors responsible for the VEGF-C/VEGF-D/VEGFR-3-independent pathway include VEGF,^{23,24} HGF,²⁸ and the angiopoietins,^{20,29,30} which have all been shown to stimulate lymphangiogenesis.

Previous studies have indicated that isolated lymphatic endothelial cells can survive and proliferate to some extent without VEGFR-3 ligand stimulation on fibronectin but not on uncoated tissue culture plates.³¹ This suggests that the extracellular matrix provides important growth-promoting signals for lymphatic vessels. Furthermore, it was shown that integrin $\beta 1$ can directly interact with VEGFR-3 and modulate its phosphorylation, thus affecting lymphatic endothelial cell migration.^{32,33} Integrin $\alpha 9\beta 1$ -deficient mice die 6 to 12 days after birth because of chylothorax caused by a failure of lymphatic vessels, which indicates a crucial function for integrin $\alpha 9\beta 1$ in lymphatic development.³⁴ Indeed, integrin $\alpha 9\beta 1$ was shown to directly bind VEGF-C and VEGF-D and to promote endothelial cell adhesion and migration.³⁵ The molecular mechanism of postnatal lymphatic maturation and of the VEGFR-3 ligand-independent form of lymphangiogenesis could involve such stabilizing signaling by the extracellular matrix, possibly mediated by integrins. How-

ever, it remains to be elucidated why the matrix signals would become activated only a few weeks after birth and why they cannot compensate for the lack of ligand-stimulated VEGFR-3 signaling during embryogenesis and the immediate postnatal period. The fact that VEGF-D is not able to induce lymphangiogenesis during embryogenesis suggests that VEGF-D-induced VEGFR-3 activation might depend on such additional maturation signals for lymphatic endothelial cells.

VEGF gene deletion or daily administration of VEGFR-1-Ig in postnatal mice has been shown to result in endothelial cell apoptosis, leading to severely reduced numbers of blood vessels and increased postnatal lethality.³⁶ The susceptibility of the blood vascular endothelial cells to VEGF depletion is lost, or at least greatly decreased, after the 4th postnatal week, suggesting a process of blood vessel maturation that results in VEGF-independent survival of blood vascular endothelial cells.^{36,37} In the adult, VEGF seems to be required mainly for active angiogenic processes, such as corpus luteum development and wound healing, and to be responsible for angiogenesis in several pathological conditions, such as tumor growth and arthritis.²⁵ VEGF has also been implicated for the survival of fenestrated blood capillaries.³⁸ Coverage of the blood vessels by pericytes starts early, during late embryonic development, indicating that it cannot be the only mechanism of vessel maturation. Because the ablation of the *VEGF* gene during the first postnatal week leads to death in 38% of the pups,³⁶ it would be interesting to know if the blood vessels of the surviving mice can regenerate independently of VEGF in a similar manner to the regeneration of lymphatic vessels without VEGF-C and VEGF-D.

Spread through lymphatic vessels into regional lymph nodes is commonly the first step in the dissemination of human cancer. A correlation between VEGF-C expression by the primary tumor and metastasis to the sentinel lymph nodes has been reported for several types of human cancers.³⁹ Overexpression of VEGF-C and VEGF-D in experimental tumors has been shown to induce tumor lymphangiogenesis and to promote tumor metastasis.^{13,40-42} Furthermore, VEGF-C/D-induced tumor lymphangiogenesis and lymphatic metastasis can be inhibited by blocking the interaction of VEGFR-3 with its ligands.^{13,14,41} It is not known if active lymphangiogenesis occurs in human tumors or if overexpression of VEGF-C by the tumor cells promotes lymphatic metastasis by some more subtle means, for example by facilitating interaction of the tumor cells with the lymphatic endothelium or by priming the lymph nodes for metastatic tumor cells. In any case, our study suggests that the blocking of VEGF-C and VEGF-D should be a safe method to inhibit tumor metastasis because normal lymphatic vessels are not affected by such treatment in adults.

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