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Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins

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Lymphatic vessels are essential for immune surveillance, tissue fluid homeostasis and fat absorption. Defects in lymphatic vessel formation or function cause lymphedema. Here we show that the vascular endothelial growth factor C (VEGF-C) is required for the initial steps in lymphatic development. In *Vegfc*$^{-/-}$ mice, endothelial cells commit to the lymphatic lineage but do not sprout to form lymph vessels. Sprouting was rescued by VEGF-C and VEGF-D but not by VEGF, indicating VEGF receptor 3 specificity. The lack of lymphatic vessels resulted in prenatal death due to fluid accumulation in tissues, and *Vegfc*$^{+/-}$ mice developed cutaneous lymphatic hypoplasia and lymphedema. Our results indicate that VEGF-C is the paracrine factor essential for lymphangiogenesis, and show that both *Vegfc* alleles are required for normal lymphatic development.

The cardiovascular circulatory system consists of a treelike hierarchy of vessels formed from a primitive vascular network[1]. The lymphatic system comprises a separate vascular system that also permeates most organs of the body. The lymphatic vessels drain extracellular fluid and dendritic cell–captured antigens through the secondary lymphoid organs, where lymphocyte activation occurs. The sequential formation of lymph nodes and Peyer's patches requires lymphotoxin $\alpha_1\beta_2$ signaling through the lymphotoxin β-receptor[2–4]. However, the lymphatic vasculature seems to be intact in lymphotoxin β-receptor–deficient mice, indicating that the development of secondary lymphoid organs and the lymphatic vasculature are regulated by a distinct set of genes[5].

The development of the lymphatic vessels in embryos starts when a subset of endothelial cells in the cardinal vein commit to the lymphatic lineage and sprout to form the primary lymph sacs[6–8]. In mice, the lymphatic vasculature starts to develop at embryonic day 10.5 (E10.5), when the cardiovascular system is already functioning. After the formation of the initial lymph sacs, the peripheral lymphatics are generated by centrifugal sprouting. In mice deficient in the *Prox1* homeobox transcription factor gene, the endothelial cells in the cardinal vein fail to induce lymphatic markers such as VEGF receptor 3 (VEGFR-3) or lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), and do not commit to the lymphatic lineage[9,10]. Accordingly, ectopic expression of Prox-1 in cultured primary blood vascular endothelial cells induces several lymphatic endothelial cell–specific genes and downregulates blood vessel–specific genes[11,12].

The gene encoding the receptor tyrosine kinase VEGFR-3 is one of the genes upregulated by Prox-1 in the lymphatic endothelial cells[10,11]. VEGFR-3 is required for remodeling of the blood vascular network at midgestation, but becomes downregulated in the blood vessel endothelia after the emergence of the lymphatic vessels[13,14]. VEGFR-3 activation by its ligands VEGF-C and VEGF-D leads to proliferation, migration and survival of cultured human adult microvascular lymphatic endothelial cells[15]. VEGFR-3 activation also induces lymphangiogenesis in adult tissues[16,17]. Furthermore, missense mutations in the gene encoding VEGFR-3 lead to insufficient signaling through this receptor and hypoplasia of the cutaneous lymphatic network in a subset of families suffering from congenital human lymphedema (Milroy disease; Online Mendelian Inheritance in Man, 153100)[18].

The two known VEGFR-3 ligands, VEGF-C and VEGF-D, belong to the larger VEGF family of growth factors that also includes VEGF, placenta growth factor and VEGF-B. In addition to activating VEGFR-3, VEGF-C and VEGF-D also activate VEGFR-2, which is expressed in both blood and lymphatic vessel endothelia[19–21]. Proteolytic cleavage is an important regulator of the receptor binding and thus the biological activity of VEGF-C and VEGF-D[19,22]. Partially processed forms of VEGF-C and VEGF-D activate VEGFR-3, whereas the fully processed short forms are also potent stimulators of VEGFR-2.

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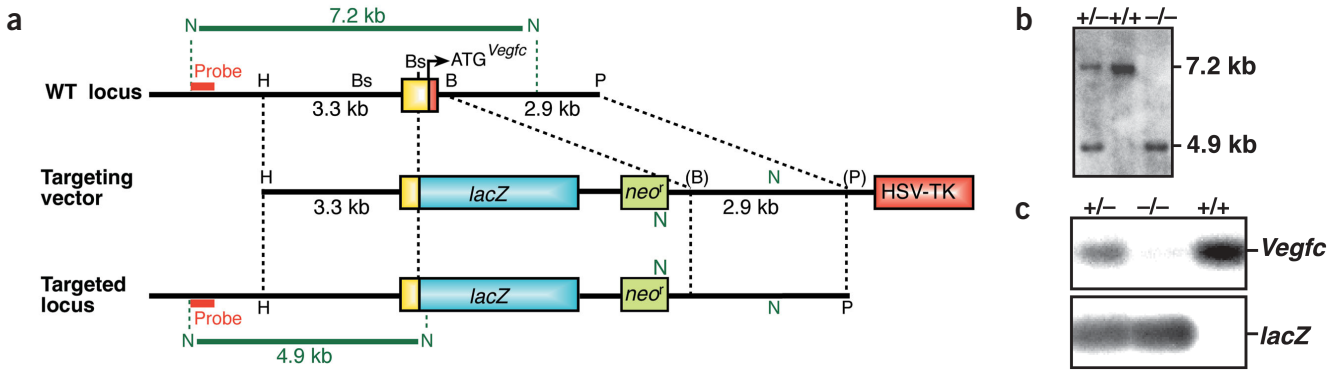

Figure 1 Genetic targeting of the *Vegfc* locus. (**a**) The homologous recombination event deletes sequences encoding the translation initiation site and the signal sequence of *Vegfc*, and places *lacZ* under the control of the *Vegfc* regulatory region. H, *Hin*dIII; N, *Nco*I; P, *Pst*I; Bs, *Bsm*BI; *neo*^{r}, neomycin resistance; HSV-TK, herpes simplex virus thymidine kinase; WT, wild-type. (**b**) Southern blot of amniotic DNA after *Nco*I digestion and hybridization with the 5′ external probe indicated by the red bar in **a**. Right margin, molecular sizes of the digested fragments. (**c**) *Vegfc* and *lacZ* expression analyzed by RT-PCR from E11.5 embryos.

Because of the early death of *Vegfr3*-deficient mice, the function of VEGFR-3 in the initial development of the lymphatic vessels has remained unknown[13]. In addition, the functions of VEGF-C and VEGF-D have not been analyzed in this process. For these reasons we targeted the *Vegfc* locus in mouse chromosome 8 and analyzed the lymphatic vascular development in embryos deficient in VEGF-C.

RESULTS

Lymphatic vascular formation fails in *Vegfc*-deficient embryos

We generated mice in which *Vegfc* was replaced by the *lacZ* marker gene (**Fig. 1**). *Vegfc*$^{-/-}$ embryos seemed edematous from E12.5 onward (**Fig. 2a**). Approximately half of the mutant embryos died between E15.5 and E17.5 in the mixed (ICR/129Sv) background, and there were no live-born *Vegfc*$^{-/-}$ pups. Using β-galactosidase as a marker for endogenous *Vegfc* expression, we found that *Vegfc* was strongly expressed from E8.5 onward in the jugular region where the first lymph sacs form[23] (**Fig. 2b**). Accordingly, staining for β-galactosidase activity and VEGFR-3 immunoreactivity in sections of E10.5 *Vegfc*$^{+/-}$ embryos indicated that *Vegfc* was abundantly expressed in the mesenchyme dorsolateral to the VEGFR-3-positive jugular veins, which give rise to the lymphatic endothelium (**Fig. 2c**). *Vegfc* was also expressed in the smooth muscle cells surrounding large arteries (**Fig. 2c**).

The localization and timing of *Vegfc* expression indicated that VEGF-C is needed for the development of the lymphatic vasculature. Indeed, staining of sections from the jugular region for the lymphatic markers VEGFR-3, LYVE-1 (ref. 24) and podoplanin[25,26] showed that lymph sacs did not form in *Vegfc*$^{-/-}$ embryos, whereas they were clearly visible in the *Vegfc*$^{+/-}$ and *Vegfc*$^{+/+}$ littermates (**Fig. 3a** and data not shown). All lymphatic vessels, including the thoracic duct, were also absent at later developmental stages (data not shown). VEGFR-3 expression persisted in some erythrocyte-containing capillaries of *Vegfc*$^{-/-}$ embryos, whereas it was downregulated in the *Vegfc*$^{+/-}$ and *Vegfc*$^{+/+}$ littermates (**Fig. 3a**). However, the total amount of VEGFR-3 mRNA was reduced in the *Vegfc*$^{-/-}$ embryos, whereas the amount of VEGF-D, angiopoietin-2 or VEGFR-2 mRNA was not altered (data not shown). The veins and arteries seemed normal in sections from *Vegfc*$^{-/-}$ embryos stained for platelet endothelial cell adhesion molecule 1 (PECAM-1, also known as CD31) and smooth muscle actin (**Fig. 3b** and data not shown).

VEGF-C is required for lymphatic endothelial cell migration

Because embryos deficient in *Prox1* also fail to form the primitive lymph sacs[9,10], we studied Prox-1 expression in *Vegfc*$^{-/-}$ embryos by immunofluorescence. We isolated whole-mount explants of the axial vascular system from E10.5–E13 embryos. At E10.5, we

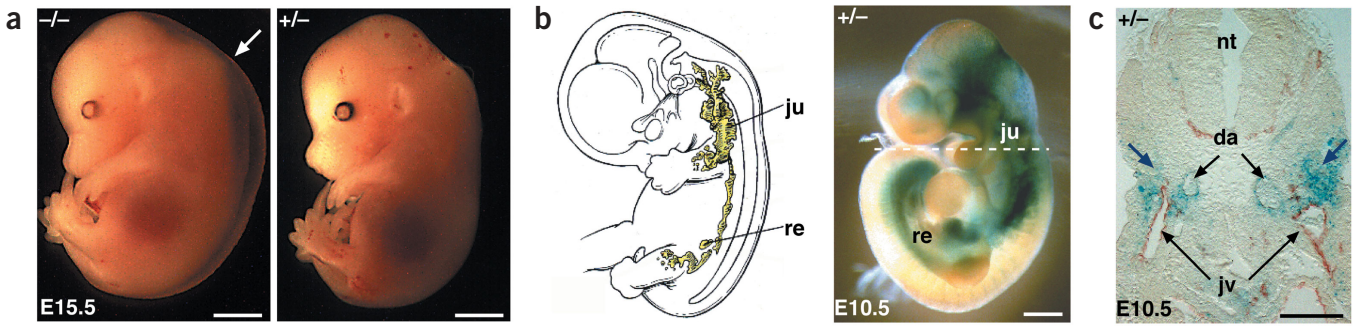

Figure 2 *Vegfc* expression is associated with lymphatic vascular development. (**a**) Severe edema (arrow) in an E15.5 *Vegfc*$^{-/-}$ embryo. (**b**) Left, location of the jugular (ju) and retroperitoneal (re) lymph sacs in a 42-day-old human fetus, corresponding to the lymph sacs of E13–E14 mouse embryos. From ref. 44; reproduced with permission. Right, β-galactosidase staining of an E10.5 *Vegfc*$^{+/-}$ embryo. There is *Vegfc* expression in regions corresponding to lymphatic vascular formation indicated in the left panel. (**c**) Transverse section of an E10.5 *Vegfc*$^{+/-}$ embryo (at dashed line in **b**). There is *Vegfc* expression in the mesenchymal cells (blue arrows), toward which the endothelial cells from the jugular vein (jv; stained red for VEGFR-3) eventually sprout to form the jugular lymph sacs. da, dorsal aorta; nt, neural tube. Scale bars: **a**, 2 mm; **b**, right, 500 μm; **c**, 250 μm.

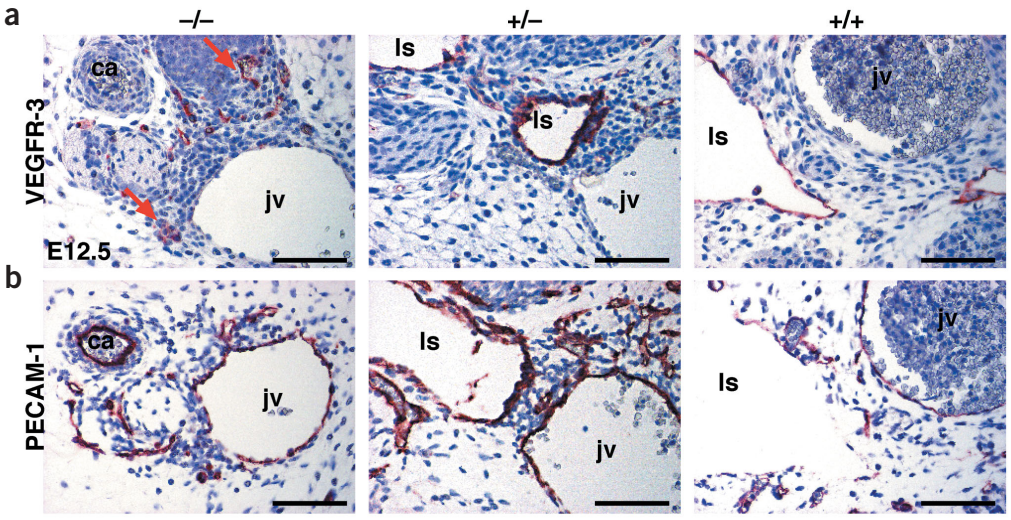

Figure 3 *Vegfc* deficiency results in failure of lymphatic vascular development. (**a**) VEGFR-3 staining of the jugular region at E12.5. The *Vegfc*$^{-/-}$ embryo lacks the jugular lymph sac (ls). In the *Vegfc*$^{-/-}$ embryo, the small VEGFR-3-expressing vessels contain erythrocytes (red arrows). (**b**) PECAM-1 staining of blood vessels from sections adjacent to those in **a**. The lymphatic endothelium of the jugular lymph sac also stains for PECAM-1 in the *Vegfc*$^{+/-}$ and *Vegfc*$^{+/+}$ embryos. jv, jugular vein; ca, carotid artery. Scale bars, 100 μm.

detected strong endothelial Prox-1 staining bilaterally in the jugular veins from all embryos (**Fig. 4a**). These Prox-1-expressing lymphatic endothelial cells had started sprouting in *Vegfc*$^{+/-}$ and *Vegfc*$^{+/+}$ embryos, whereas in the *Vegfc*$^{-/-}$ embryos they remained confined to the wall of the cardinal vein (**Fig. 4a** and data not shown). Subsequently, in the *Vegfc*$^{+/+}$ and *Vegfc*$^{+/-}$ embryos, the Prox-1-expressing endothelial cells formed the jugular lymph sacs, which were clearly present at E13 (**Fig. 4a** and data not shown). However, at that time point there were only a few Prox-1-expressing endothelial cells left in the cardinal veins of the *Vegfc*$^{-/-}$ embryos, and we found no lymph sac–like structures (**Fig. 4a** and data not shown). Instead, we found macrophage-like cells infiltrating the jugular region, consistent with the clearance of apoptotic cells (data not shown). Prox-1 expression in cardiomyocytes and hepatocytes seemed normal in *Vegfc*$^{-/-}$ embryos at all stages analyzed (**Fig. 4a** and data not shown).

To more directly determine if the Prox-1-expressing endothelial cells could be rescued by VEGF-C, we added agarose beads soaked in growth factor or human serum albumin to the whole-mount explants of E11.5 *Vegfc*$^{-/-}$, *Vegfc*$^{+/-}$ and *Vegfc*$^{+/+}$ embryos. After 48 h of culture, Prox-1-expressing endothelial cells had migrated into intimate contact with the beads containing VEGF-C but not those containing human serum albumin (**Fig. 4b** and data not shown). Mouse VEGF-D, which is a selective ligand of VEGFR-3 (ref. 27), also induced a weak migratory response (data not shown), whereas VEGF did not attract the Prox-1-expressing endothelial cells in this assay (**Fig. 4b**).

Vegfc$^{+/-}$ mice have lymphedema

Whereas lymph sac formation in *Vegfc*$^{+/-}$ embryos was normal or only slightly reduced, newborn *Vegfc*$^{+/-}$ pups had accumulation of chylous, milky fluid in the abdomen, indicating defects in intestinal lymphatic vessel function (**Fig. 5a**). Biochemical analysis of this fluid showed that it

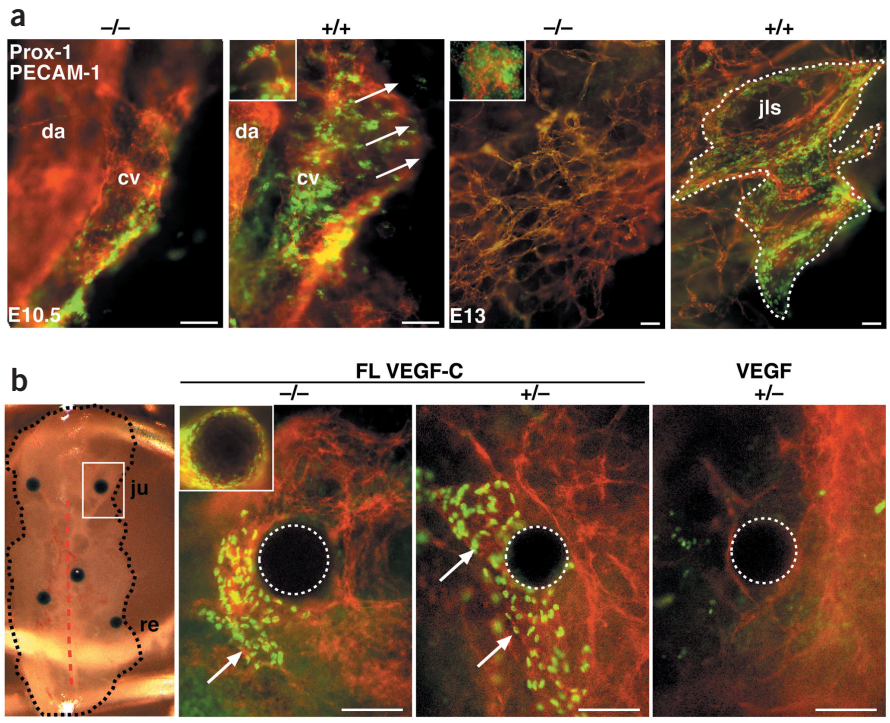

Figure 4 VEGF-C is required for migration of Prox-1-expressing endothelial cells. (**a**) Prox-1-expressing endothelial cells (green) sprout out (arrows) from the cardinal vein (cv) in E10.5 *Vegfc*$^{+/+}$ embryos; the corresponding cells are confined to the wall of the cardinal vein in *Vegfc*$^{-/-}$ embryos. Inset, left, PECAM-1 expression (red) in sprouting Prox-1-expressing cells. At E13, the jugular lymph sac (jls; dashed line) has formed in *Vegfc*$^{+/+}$ embryos but not in *Vegfc*$^{-/-}$ embryos. Inset, right, Prox-1 staining in the cardiomyocytes of a *Vegfc*$^{-/-}$ embryo. da, dorsal aorta. (**b**) Far left, effect of VEGF-C protein in *Vegfc*$^{-/-}$ embryos, analyzed by placement of growth factor–containing agarose beads (blue) into the jugular (ju) and retroperitoneal (re) regions of whole-mount explants (dashed black line). Dashed red line, dorsal aorta. Middle and right, immunofluorescence staining from the region of the boxed area shown at far left. The lymphatic endothelial cells (Prox-1, green; PECAM-1, red; indicated by arrows) have migrated toward the VEGF-C beads in *Vegfc*$^{-/-}$ and *Vegfc*$^{+/-}$ embryos, whereas VEGF does not induce such response. Inset shows, at a different focus level, how the Prox-1-expressing cells have covered the VEGF-C-containing bead in a *Vegfc*$^{-/-}$ embryo. Scale bars: **a**, 100 μm; **b**, far left, 700 μm; **b**, middle, right, 100 μm. FL, full-length.

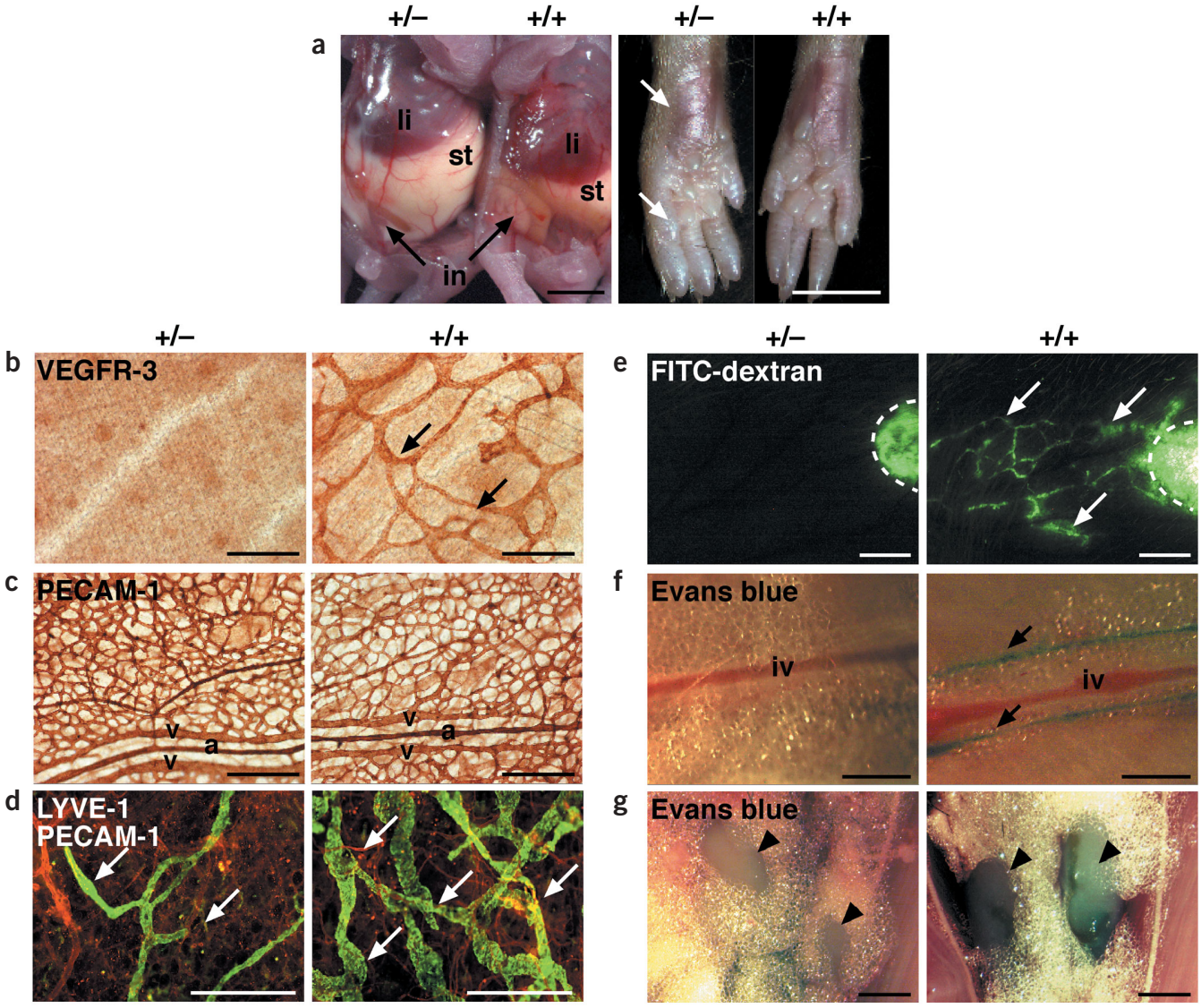

Figure 5 Developmental and functional defects of the lymphatic vessels in *Vegfc*$^{+/-}$ mice. (**a**) Chylous ascites in the abdominal cavity of newborn *Vegfc*$^{+/-}$ pup and swollen paws of an adult *Vegfc*$^{+/-}$ mouse (white arrows). li, liver; in, intestine; st, stomach. (**b**) VEGFR-3 immunostaining of whole-mount skin preparations showing hypoplasia of the cutaneous lymphatic vessels in an E16.5 *Vegfc*$^{+/-}$ embryo compared with a *Vegfc*$^{+/+}$ littermate. (**c**) PECAM-1-stained blood vessels. v, vein; a, artery. (**d**) Immunofluorescence staining for LYVE-1 (green; arrows) and PECAM-1 (red) in ear skin of adult *Vegfc*$^{+/-}$ and *Vegfc*$^{+/+}$ mice. (**e**) Fluorescence micro-lymphography of the ear. Cutaneous lymphatic vessels (arrows) are not functional in the adult *Vegfc*$^{+/-}$ mice. Dashed lines, site of the fluorescein isothiocyanate–dextran injection. (**f**,**g**) Transport of Evans blue dye into collecting lymphatic vessels (arrows) alongside the ischiatic vein (iv, **f**) and farther into the para-aortic lymph nodes (arrowheads, **g**) is detected in *Vegfc*$^{+/+}$ but not in *Vegfc*$^{+/-}$ mice. Scale bars: **a**, 5 mm; **b**–**d**, 500 μm; **e**,**f**, 2 mm; **g**, 1 mm.

contained high concentrations of triglycerides (20–149 mmol/l) compared with normal plasma triglyceride concentrations (2.3 mmol/l). The subserosal intestinal lymphatic vessels were hypoplastic and occasionally enlarged in the *Vegfc*$^{+/-}$ pups, and their lymphatic capillaries were also hypoplastic in all the other organs studied (**Supplementary Fig. 1** online). In less than 10% of the *Vegfc*$^{+/-}$ pups, the chylous fluid persisted. The stagnant chylous ascites produced an inflammatory response in the abdominal cavity of these mice and led to severe adhesions and fibrosis of the internal organs, which necessitated that these mice be killed during the first postnatal weeks. Despite the severe hypoplasia of the lymphatic vasculature in newborn *Vegfc*$^{+/-}$ pups, the lymphatic capillaries eventually grew into most organs within the first postnatal weeks (data not shown). However, adult *Vegfc*$^{+/-}$ mice showed swelling of the paws, indicating that lymphatic defects in the skin persisted (**Fig. 5a**). Examination of *Vegfc*$^{+/-}$ embryos and adult mice indicated that these mice had severe hypoplasia of the cutaneous lymphatic vessels, whereas the blood vessels were normal (**Fig. 5b–d**). We evaluated the function of the cutaneous lymphatic vessels in the adult mice by microlymphography. After intradermal injection of large-molecular-weight fluorescent dextran into the ears of the mice, the dextran was taken up by the cutaneous lymphatic vessels of *Vegfc*$^{+/+}$ mice, whereas there was no uptake of dextran in the *Vegfc*$^{+/-}$ mice (**Fig. 5e**). We also studied lymphatic fluid transport by intradermal injection of Evans blue dye to the hind limbs; this resulted in the appearance of the dye in the collecting lymphatic vessels alongside the ischiatic veins and subsequently in the retroperitoneal para-aortic

lymph nodes in the *Vegfc*$^{+/+}$ mice. Consistent with the microlymphography analysis, there was no transport of the dye in the *Vegfc*$^{+/-}$ mice (**Fig. 5f,g**).

DISCUSSION

We have shown here that VEGF-C signaling is essential for the formation of the lymphatic vessel sprouts from the embryonic veins, which is one of the first crucial steps in the development of the lymphatic vascular system. Our results indicate that VEGF-C is not needed for cell commitment to the lymphatic endothelial lineage, but that paracrine VEGF-C signaling is required for the migration and eventual survival of Prox-1-expressing endothelial cells from the cardinal vein and for the subsequent formation of lymph sacs.

VEGF-C was expressed in the regions toward which the endothelial cells expressing Prox-1 and VEGFR-3 sprout from the cardinal veins to form the primary lymph sacs[9,14,23]. This suggests that a paracrine VEGF-C signal forms a concentration gradient toward which the first lymphatic endothelial cells migrate to form the lymph sacs. In our rescue experiment, VEGF-C and mouse VEGF-D but not VEGF were able to rescue the migration of the committed lymphatic endothelial cells. Thus, signaling through VEGFR-3 is required in this process, whereas signaling through the two related receptors, VEGFR-1 and VEGFR-2, is without effect. Although VEGFR-3 is initially expressed in all venous endothelial cells, only Prox-1-expressing cells start the sprouting process and simultaneously upregulate other lymphatic markers such as podoplanin and LYVE-1 (refs. 10,11,26). However, the signals that result in polarized Prox-1 expression in the cardinal vein and in the downregulation of lymphatic markers such as VEGFR-3 on the blood vascular endothelium remain unknown.

Our observations indicate that VEGF-C is not essential for blood vessel development, although both of its receptors, VEGFR-2 and VEGFR-3, have been shown to be essential for this process[13,28]. As VEGF-C and VEGF-D are the only known ligands for VEGFR-3, the seemingly normal blood vascular development in *Vegfc*-deficient mice indicates that VEGF-D or an as-yet-unknown ligand is sufficient for the remodeling of the blood vessels. However, endogenous VEGF-D does not compensate for VEGF-C in embryonic lymphatic development, although recombinant mouse VEGF-D was active in our rescue experiment. This may reflect the fact that in embryos, VEGF-D is not expressed at the critical sites of lymph sac formation[29] and thus it is not able to accomplish paracrine VEGFR-3 signaling in this region. In addition, VEGF-C also binds to neuropilin-2 (NRP-2)[30], and *Nrp2*-deficient mice show an absence or severe reduction of lymphatic capillaries[31]. Thus, it is possible that VEGF-C signaling through VEGFR-3 is enhanced by NRP-2, similar to what has been shown for VEGF signaling through VEGFR-2 and NRP-1 (ref. 32). The phenotypes in our VEGF-C mutant mice may therefore result from lack of both VEGFR-3 and NRP-2 signals in the lymphatic endothelium.

Lymphatic vascular development was also defective in *Vegfc*$^{+/-}$ mice, indicating that a normal concentration of VEGF-C is essential for the proper formation of the lymphatic vessels. The defects in the *Vegfc*$^{+/-}$ mice resemble those in Chy mice, which have lymphedema due to a heterozygous inactivation of the tyrosine kinase VEGFR-3 (refs. 18,30). In both mouse mutants, the lymphatic hypoplasia persists in the skin. Our results indicate that mutations that inactivate *VEGFC* could predispose a person to primary lymphedema, which is a disabling swelling of the extremities due to poorly functional and/or hypoplastic cutaneous lymphatic vasculature. The *Vegfc*$^{+/-}$ mice described here provide a preclinical model for the development of therapies aimed at regenerating damaged or hypoplastic lymphatic vessels.

The lymphatic phenotype of the *Vegfc*$^{+/-}$ mice resembles that of angiopoietin-2-deficient mice[33]. Like *Vegfc*$^{+/-}$ mice, mice lacking angiopoietin-2, a ligand for the Tie-2 receptor (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains), show chylous ascites formation and defects in the intestinal and cutaneous lymphatic vessels, However, the relationship between the angiopoietin and VEGF-C and VEGF-D signaling pathways in lymphatic vascular formation remains to be determined.

Loss of a single *Vegf* allele results in embryonic lethality due to impaired angiogenesis and blood island formation[34,35]. In contrast, mice deficient in other members of the VEGF family (placenta growth factor, VEGF-B or VEGF-D) do not show obvious vascular abnormalities during embryonic development (refs. 36–38 and M. Baldwin and M. Achen, personal communication). Thus, VEGF and VEGF-C are the only essential members of the VEGF family and, as with what has been described for VEGF in embryonic angiogenesis, VEGF-C is nonredundant and indispensable for embryonic lymphangiogenesis.

METHODS

Generation of mice lacking VEGF-C. All animal experiments described here were approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. In the targeting vector, the *lacZ* reporter gene replaces the first exon of *Vegfc*, placing *lacZ* under the control of the *Vegfc* regulatory regions. The *Vegfc* region was isolated from a 129Sv mouse genomic library. To construct the 3′ arm, a 2.9-kilobase *Bam*HI–*Pst*I fragment was cloned into the *Bam*HI site of the pNTPloxP targeting vector. The 3.3-kilobase 5′ arm was excised by *Hin*dIII and partial *Bsm*BI digestion and was inserted into the pSDKlacZ plasmid 5′ of a cassette of *lacZ* and the neomycin-resistance gene (*neo*^{r}). Subsequently, a *Sal*I cassette of this construct was cloned into the *Xho*I-opened pNTPloxP plasmid containing the 3′ arm to generate the final targeting vector. The 5′ arm was designed to delete the first exon, including a 125-base-pair fragment 5′ of the translation initiation site, the first 147 base pairs (49 codons) of the coding region and 143 base pairs of the first intron. The construct was electroporated into R1 (129/Sv × 129/SvJ) embryonic stem cells. Positive clones were aggregated with ICR morulas to obtain chimeric mice, which were bred with ICR mice. Pups were genotyped by Southern blotting or by PCR with primers 5′-TCCGGTTTCCTGTGAGGC-3′ (forward), 5′-AAGTTGGGTAACGCCAGG-3′ (reverse for targeted allele) and 5′-TGACCTCGCCCCCGTC-3′ (reverse for the first exon of *Vegfc*).

RT-PCR. Total RNA from embryos was extracted with the RNeasy kit (Qiagen). The RT reaction used the SuperScript cDNA Synthesis kit (Invitrogen) and random hexanucleotide primers. Base pairs 202–371 of the *Vegfc* cDNA were amplified with primers 5′-GCGCTGATCCCCAGTCCG-3′ and 5′-AGGACAGACATCAGCTCATC-3′; *lacZ* cDNA was amplified with primers 5′-CGT CAGTATCCCCGTTTACAG-3′ and 5′-AGACCAGACCGTTCATACAG-3′. Amplified products were detected by Southern blotting.

β-galactosidase staining. *Vegfc* expression was monitored in embryos or adult tissues by whole-mount staining for β-galactosidase activity[39]. Paraffin-embedded samples were sectioned and stained with nuclear red or used for immunohistochemistry.

Immunohistochemistry. Paraformaldehyde-fixed paraffin sections were stained with antibodies to VEGFR-3 (rat monoclonal antibody to mouse[40]), PECAM-1 (553370; PharMingen), podoplanin (affinity-purified rabbit polyclonal antibody[25]) or LYVE-1 (rabbit antiserum[24,41]) and the Tyramide Signal Amplification system (NEL700; NEN Life Science Products). Peroxidase activity was developed with 3-amino-9-ethyl carbazole (Sigma) and sections were counterstained with hematoxylin. For whole-mount immunohistochemistry, tissues were fixed with paraformaldehyde and stained with antibodies to VEGFR-3 (BAF 743; R&D Systems) or PECAM-1 followed by the Vectastain Elite ABC reagent (avidin–horseradish peroxidase; PK-6104; Vector Laboratories). Peroxidase activity was visualized with 3,3′-diaminobenzidine (Sigma) as a substrate.

Organ culture. All dorsal structures with endodermal and intermediate mesodermal derivatives were microdissected from E10.5–E13 embryos[42]. The somites, neural tube, limbs, heart, liver and gut were then dissected from the preparations. Explants were cultured on Track-tech Nuclepore filters (pore size, 0.1 μm; Whatmann) in a Trowell-type organ culture system[42] in DMEM supplemented with 10% FCS (PromoCell). Preparations were cultured for 1–2 h at 37 °C to allow attachment to the filters and were used for immunofluorescence staining.

Whole-mount immunofluorescence staining. Tissues were fixed in methanol, blocked with 1% BSA in PBS and incubated overnight with antibodies to PECAM-1, LYVE-1 (ref. 24) and/or Prox-1. The testing and production of the Prox-1 antibodies are described in **Supplementary Figure 2** online. Carbocyanine (Cy2)-, fluorescein isothiocyanate– or tetramethylrhodamine isothiocyanate–labeled secondary antibodies (Jackson ImmunoResearch Laboratories) were used for staining. Tissues were mounted with Vectashield (Vector Laboratories) and analyzed with a Zeiss Axioplan 2 fluorescent microscope.

Agarose bead experiments. The proteins used were the full-length and 'short' (deletion of N-terminal and C-terminal regions; ΔNΔC) forms of VEGF-C, mouse VEGF-D (R&D Systems), human VEGF165 (R&D Systems) and human serum albumin. Affi-Gel Blue beads (mesh size, 100–200 nm; Bio-Rad) were incubated with the proteins at 37 °C for 45 min. The beads were washed in Dulbecco's PBS and used for the experiment. The beads were placed close to the jugular veins. Explants were cultured for 48 h, and the tissues were then used for whole-mount stainings[43].

Production of recombinant proteins. The cDNA encoding the VEGF homology domain of human VEGF-C (ΔNΔC; nucleotides 658–996) and full-length human VEGF-C (nucleotides 445–1608) were cloned into a baculoviral transfer vector between the sequences encoding the honeybee melittin signal peptide and the hexahistidine tag. The recombinant protein was isolated from serum-free conditioned supernatant of HighFive cells infected with recombinant baculovirus with nickel nitriloacetic acid–affinity chromatography. Human serum albumin (nucleotides 112–1,866) was prepared in a similar way. Because of its dimeric nature, VEGF-C has two histidine tags per molecule and thus allows for very stringent washing conditions in the affinity chromatography. To be able to use the same stringency in the chromatography of (monomeric) human serum albumin, a second histidine tag was inserted after the sequences coding for the signal peptide.

Analysis of lymphatic vessel function. To visualize the lymphatic network in the ears of adult mice, we did fluorescence microlymphography by injecting fluorescein isothiocyanate–dextran (molecular weight, 2,000 kDa; Sigma) intradermally into the ear and monitoring the progressive staining of the lymphatic network by fluorescence microscopy. Alternatively, we injected Evans blue dye (3 mg/ml in PBS; Sigma) intradermally into the hind footpads, followed by removal of the skin of the limbs to expose the region of the ischiatic vein.

GenBank accession numbers. Mouse VEGF-C, U73620; human VEGF-C, X94216; human serum albumin, V00494.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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