Reassessment of the role of VEGF-D in health and disease

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Tissue:	Vascular system
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Reassessment of the role of VEGF-D in health and disease

Short title: Endogenous VEGF-D in health and disease

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ABSTRACT

Vascular endothelial growth factor-D (VEGF-D) is one of the two ligands of the VEGFR-3 receptor on lymphatic endothelial cells. Gene-silencing studies in mice and *Xenopus* tadpoles recently showed that the role of endogenous VEGF-D in lymphatic development is moderate. By contrast, exogenous VEGF-D is capable of stimulating lymphangiogenesis. Nonetheless, its endogenous role in pathological conditions remains largely unknown. Hence, we reassessed its role in disease, using *Vegf-d*^{null} mice. *Vegf-d*^{null} mice were generated, which under physiological conditions, displayed normal embryonic and postnatal lymphangiogenesis and lymphatic remodeling, efficient lymphatic functioning and normal health. *Vegf-d*^{null} mice also reponded normally in models of skin wound healing and healing of infarcted myocardium, despite enhanced expression of VEGF-D in these models in wild type mice. Furthermore, *Vegf-d*^{null} mice were indistinguishable from their wild type littermates in a model of lymphangioma formation. Together, our data indicate that endogenous VEGF-D in mice is dispensible for lymphangiogenesis, not only during development, but also in several pathological conditions during adult life.

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Key words: VEGF-D, lymphatic, gene targeting, pathology, mouse, wound healing, lymphangioma, myocardial infarction

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INTRODUCTION

The lymphatic vascular system is of vital medical importance, as it is critical to reabsorb extravasated fluid and dietary fat into the circulation [1-3]. Thus, when insufficient, lymphedema develops. By transporting immune cells to lymphoid organs, it also plays a critical role in inflammation, infection and immunity, transplant rejection, and in numerous other diseases [1,2]. Importantly, malignant tumors can directly promote lymphangiogenesis within the primary tumor and in draining lymph nodes, leading to enhanced cancer metastasis to lymph nodes and to distant organs [2]. Lymphedema currently is still incurable (apart from the symptomatic and archaic use of supportive stockings, etc), while cancer metastasis is one of the leading causes of mortality in our societies. Thus, efforts to identify factors involved in lymphatic growth, and of approaches to stimulate or inhibit the growth of lymphatics [4-9] remain warranted.

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The vascular endothelial growth factor (VEGF) homologue VEGF-D constitutes one of the two known ligands of VEGF receptor-3 (VEGFR-3) on lymphatic endothelial cells (LEC) [1]. Deficiency of VEGF-C, the second VEGFR-3 ligand, in mice and *Xenopus* tadpoles impairs lymphatic endothelial cell sprouting and leads to severe lymphatic defects in tadpoles and embryonic lethality in mice [10-12]. VEGF-D appears to have a more moderate role in physiologic embryonic lymphangiogenesis, as recently reported by us and others in mice or *Xenopus* tadpoles, suggesting that VEGF-D might be more important in pathology rather than in development [12-14]. Various studies have implicated both VEGF-D and VEGF-C in pathological lymphangiogenesis. Expression levels of VEGF-D and VEGF-C correlate with lymph node metastasis in several human cancers [15,16], and addition or overexpression of exogenous VEGF-C or VEGF-D induces lymphatic growth and tumor metastasis in preclinical cancer models [17-25]. Addition of exogenous VEGF-D or VEGF-C also induces the formation of functional, mature collecting lymphatic

vessels after the surgical removal of axillary lymph nodes and their associated collecting lymphatic vessels in mice [6]. Similarly, adenoviral or transgenic overexpression of VEGF-D corrects defective lymphangiogenesis in airway inflammation in wild type mice [26] or in the skin of VEGF-C$^{+/-}$ lymphedema mice [14]. Vice versa, strategies to prevent VEGF-C/D mediated signaling block tumor (lymph)angiogenesis and metastasis in cancer, or lymphangiogenesis in corneal inflammation [5,21,23,27,28].

These data however either do not discriminate between VEGF-C and VEGF-D or involve effects of exogenous VEGF-D growth factor, but leave the question about the role and relevance of endogenous VEGF-D in pathological situations in the adult unresolved. Here, we evaluated the importance of endogenous VEGF-D in lymph vessel formation in stress or pathological conditions during adult life in gene deficient mice.

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METHODS

GENERATION, GENOTYPING AND EXPRESSION ANALYSIS OF *VEGF-D*^{NULL} MICE

Mice deficient in VEGF-D were generated using a targeting vector, p*PNT.Vegf-d*^{null}, designed to replace exon 2 through exon 4 of the *Vegf-d* gene (8.5-kb deletion) with the LacZ cDNA, fused in frame to the first codon of exon 2 (Figure 1A-D), as detailed in the Supplementary Information. All experiments except the lymphangioma model (see below) were performed on wild type and knockout littermates from a colony with overall 50:50 mixed 129SvEv:Swiss background. The lymphangioma experiment was performed on wild type and *Vegf-d*$^{0/-}$ males from a pure 129SvEv colony established from offspring of the chimeric founder mated to wild type 129SvEv females. Genotyping and expression analysis was as described in Supplemental Information. Housing and procedures involving experimental animals were approved by the Institutional animal Care and Research Advisory Committee of the K.U.Leuven, Belgium.

PHENOTYPING OF THE *VEGF-D*^{NULL} MICE IN BASELINE

Phenotyping of *Vegf-d*^{null} mice in baseline (see Supplementary Information for details) included analysis of the formation or remodeling of lymph sacs and lymph vessels during embryogenesis and in neonatal or adult skin and intestine using immunofluorescence microscopy. Functionality of lymph vessels was tested using lymphangiography, via feeding with fluorescent lipid tracers, and by determination of serum total cholesterol and total neutral glycerolipids levels.

PHENOTYPING OF *VEGF-D*^{NULL} MICE DURING STRESS OR PATHOLOGICAL CONDITIONS

Lymphatic vascular remodeling was studied in a model of skin wound healing as described before [33]. For skin wounding, a standardised 15 mm full-

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thickness skin incision was made on the back of the mice, taking care not to damage the underlying muscle. Wound healing was quantified by daily measuring the width and the length of the wound. New lymphatic vessel formation was analysed on skin sections harvested 15 days after wounding.

Myocardial infarction was performed by ligation of the LAD as described [34]. After 7 days, infarcted hearts were dissected and prepared for sectioning and visualization of lymphatic vessels.

Lymphatic endothelial tumor (lymphangioma) formation was induced by intraperitoneal injection of incomplete Freund's adjuvant as described [35,36]. After 4 weeks, lymphangioma masses were measured and counted and livers containing lymphangioma masses were collected for immunohistochemical analysis.

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IN SITU HYBRIDIZATION, HISTOLOGY AND IMMUNOSTAINING

Mouse tissues were fixed in 1% paraformaldeyde overnight, dehydrated, embedded in paraffin and sectioned at 8 µm thickness. After deparaffinization and rehydratation, sections were incubated in antigen retrieval solution (Dako) for 20 min at 95 °C or incubated with a trypsin solution (Sigma) at 37°C. Immunostaining for VEGFR-3 (e-Bioscience; dilution 1/50), Lyve (Upstate; dilution 1/100), CD45 (Becton Dickinson; dilution 1/100) and CD31 (Pharmingen; dilution 1/100) was done as described [11,37,38]. Samples of intestine and ear skin were also fixed in ice cold Dent's fixative overnight, blocked with 2% BSA in TBS (10mM Tris.HCl pH 7.4 containing 150mM NaCl). Samples of intestine were incubated overnight with LYVE-1 antibody (Upstate, dilution 1/500), followed by FITC-labeled goat anti-rabbit IgG (Rockland Immunochemicals). Samples of ear skin were incubated with Cy3 conjugated anti-human SMC (Sigma, dilution 1/200), with Podoplanin antibody (Reliatech, dilution 1/100) followed by anti-hamster FITC conjugated secondary antibody (Jackson Immunoresearch Laboratories, dilution 1/200), and with LYVE-1

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antibody (Upstate, dilution 1/500) followed by Alexa 644 anti-rabbit antibody (dilution 1/300). Both sections and whole-mounts were visualized on a Zeiss Axioplan 2 imaging microscope.

MORPHOMETRIC ANALYSIS OF THE LYMPHATIC OR BLOOD VASCULATURE

Lymphatic or blood vessel densities of the infarcted heart, skin wound, normal skin, gut and lymphangioma sections and whole mounts of the intestine or ears of mice were determined after immunostaining for VEGFR-3, podoplanin or LYVE1 (lymph vessels) or for CD31 (blood vessels), and were analysed using the KS300 morphometric software (Zeiss). The lymphatic network in the whole mount preparation of the intestine was analysed by counting the number of branches per mm^2, the average distance between two branches and the number of vessels penetrating the villi per mm^2. Leukocyte infiltration in skin wounds and in lymphatic endothelial tumors was quantified after immunostaining for CD45.

STATISTICAL ANALYSIS

Data represent mean ± SEM unless otherwise stated. Statistical significance was calculated by Student's t test (Prism v4.0b), considering $p<0.05$ as statistically significant.

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RESULTS

NOTE: Supplementary information is coded with the prefix "S" (e.g. Figure S1).

GENERATION OF *VEGF-D*^{NULL} MICE

We recently reported that silencing of endogenous VEGF-D transiently delays lymphangiogenesis in *Xenopus* tadpoles, without, however, causing overt lymphatic defects or lymph vessel dysfunction. Here, we evaluated the role of endogenous VEGF-D in the mouse model, using various assays. Therefore, we applied standard homologous recombination technology in embryonic stem cells to generate mice with inactivation of the *Vegf-d* gene, which maps to the X-chromosome (Figure 1A-D). Homozygous *Vegf-d*$^{-/-}$ females and hemizygous *Vegf-d*$^{0/-}$ males (from hereon referred to as *Vegf-d*^{null} mice) were born at the expected Mendelian inheritance, indicating that Vegf-d deficiency did not compromise embryonic development, consistent with previous findings [13]. Moreover, *Vegf-d*^{null} mice appeared healthy at birth, gained weight normally, survived and were fertile. They had no swollen feet or neck, showed no signs of lymphedema upon macroscopic inspection of the abdomen and thorax, and their dermis and subcutaneous adipose tissue were not thickened (Figure 1E,F and not shown).

NORMAL EMBRYONIC AND POSTNATAL LYMPHANGIOGENESIS IN *VEGF-D*^{NULL} MICE

We then examined lymphatic development before and after birth in more detail. In the mouse embryo, CD31^{+} venous endothelial cells transdifferentiate into Prox1^{+} lymphatic endothelial cells, which then loose CD31 expression [39,40]. When lymphatic commitment is impaired, such as in Prox1 knockout embryos, CD31 expression persists while Prox1 expression never appears in endothelial cells at these prospective lymphatic sites [39,40]. Immunostaining of cross-sections through the jugular region revealed that transdifferentiation of

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$CD31^+$ venous endothelial cells into lymphatic vessel hyaluron receptor-1 positive ($LYVE\text{-}1^+$) LECs proceeded normally in mid-gestation *Vegf-d*^{null} embryos (Figure 1G,H) By immunostaining, these lymphatics also expressed the lymphatic marker VEGFR-3 (Figure S1A,B). VEGF-D was also not required for the phenotypic shift in lymphatic identity during remodeling of the primitive lymphatic capillary plexus into a hierarchical vessel network of lymphatic collectors and capillaries such as during postnatal development of the dermal lymphatic system in the ear skin (Note S1).

The lymphatic vasculature also appeared normal and functional in adult *Vegf-d*^{null} mice. Indeed, lymphangiography using FITC-dextran revealed a normal lymphatic network in the ear and tail in adult mice of both genotypes (Figure 1I-L). Moreover, Evan's blue dye, intradermally injected into the hind footpads, was normally transported via the deeper collecting lymph vessels alongside the ischiatic vein (not shown).

Lymphatic uptake of the ingested fluorescent lipid tracer BODIPY® FL C16 by the mesenteric lymphatics was also normal without signs of leakage in adult *Vegf-d*^{null} mice and confirmed that the intestinal lymphatic vessels developed their typical radial pattern (Figure S2I,J). By immunostaining for LYVE-1, the superficial lymphatics in the skin (not shown) and the deep lymph vessels in the intestines exhibited a normal pattern, density and size in adult *Vegf-d*^{null} mice (Figure S2K,L). Intestinal lymphatics must function properly to absorb fat. Serum levels of the total cholesterol (TC) and total neutral glycerolipids (TNL) were normal in *Vegf-d*^{null} mice, indirectly confirming that the intestinal lymphatics function normally in the absence of VEGF-D (levels of TC and TNL in nmol/5µl, respectively: 10.4 ± 3.8 and 4.1 ± 2.1 in WT mice versus 8.7 ± 3.7 and 5.8 ± 2.9 in *Vegf-d*^{null} mice; n=7-10; P=NS). Thus, VEGF-D deficiency in mice in baseline conditions did not cause overt abnormalities or lymphatic defects during embryonic and postnatal life.

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VEGF-D IS DISPENSIBLE FOR LYMPHANGIOGENESIS DURING WOUND HEALING

Lymph vessels are quiescent in normal health, but start to grow during tissue regeneration and wound healing [41]. Viral transfer or transgenic overexpression of the *VEGF-D* gene induces lymphangiogenesis in uninjured muscle and lymphatic hyperplasia in neonatal skin and rescues lymphatic hypoplasia in VEGF-$C^{+/-}$ lymphedema mice [14,17,42], while delivery of exogeneous VEGF-C has been shown to increase lymphangiogenesis in wounds [8,43,44]. A role for the endogenous VEGF-D in wound healing had however not been documented yet. We therefore examined whether endogenous VEGF-D might also regulate lymphangiogenesis in models of wound healing.

As a first approach, we evaluated the consequences of VEGF-D deficiency in skin wound healing, as VEGF-D expression was reported to be enhanced in healing skin wounds in mice [45]. Therefore, we inflicted a full skin incision on the back of the mice and monitored the wound width as a measure of wound closure and healing. Healing progressed similarly in wild type and *Vegf-d*^{null} mice as indicated by comparable wound closure time curves (Figure 2A). Quantification of lymph vessel perfusion area and lymph vessel density of wound sections after immunostaining for LYVE-1 (Table S1; Figure 2B,C) or VEGFR-3 (not shown) at 15 days after skin wounding revealed no differences between the *Vegf-d*^{null} and wild type wounds. In accordance with sprouting from the uninjured adjacent dermis [41], lymphangiogenesis was mainly concentrated at the periphery of the wound (Figure 2B,C). Blood vessel densities in wound area and border zone, quantified on CD31-stained sections, were also comparable between genotypes (Table S1). As VEGF-C and VEGF-D have been implicated in the recruitment of VEGFR-3^{+} leukocytes [26,46], we also counted the number of $CD45^{+}$ leukocytes in the skin wounds. No genotypic differences were, however, observed ($CD45^{+}$ area in % of total wound area: 4.3

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± 1.55 in *Vegf-d*^{null} mice *versus* 5.3 ± 1.4 in WT mice; mean ± SEM; *n*=5; *P*=NS).

Lymph vessels are present in the heart and have been implicated in pericardial fluid homeostasis and drug delivery. Pilot experiments revealed an increase in lymph vessel regeneration at one week after ligation of the left coronary artery. VEGF-D expression in the heart of wild type mice was increased 2.5-fold in the zone bordering the infarct after ligation (mRNA copies of VEGF-D per 10^3 mRNA copies of β-actin: 0.92 ± 0.12 in the infarct border zone after ligation *versus* 0.39 ± 0.032 in the left ventricle in baseline; mean ± SEM, *n*=4, *P*<0.05 versus baseline). In contrast, VEGF-C expression was not enhanced but tended to be reduced after ligation (mRNA copies of VEGF-C per 10^3 mRNA copies of β-actin: 1.1 ± 0.1 in the infarct border zone after ligation *versus* 1.52 ± 0.3 in the left ventricle in baseline; mean ± SEM, *n*=4, *P*<0.05 versus baseline for the infarct zone). We therefore assessed lymph vessel regeneration after infarction in wild type and *Vegf-d*^{null} mice. Immunostaining of myocardial cross-sections for VEGFR-3 or LYVE-1 at seven days after coronary artery ligation revealed a comparable number of lymph vessels in the infarcted heart in WT and *Vegf-d*^{null} mice (total VEGFR-3$^+$ vessels/mm^2: 55 ± 8.5 in WT mice *versus* 56 ± 5.1 in *Vegf-d*^{null} mice; *n*=8-7; *P*= NS). Morphometric analysis also did not reveal genotypic differences in the densities of small, medium and large lymphatic vessels (lumens respectively smaller or equal to 250, between 250 and 500, or equal or larger than 500 μm^2) (not shown). Overall, these results suggest that VEGF-D deficiency did not affect lymphangiogenesis and healing of skin wounds or of regenerating infarcted myocardium.

ROLE OF VEGF-D IN LYMPHANGIOGENESIS IN PATHOLOGICAL CONDITIONS

Recent studies have shown that certain angiogenic molecules have a restricted activity in pathological but not in physiologic conditions [33]. As VEGF-D is expressed in aggressive tumors such as melanoma and

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inflammatory breast cancer [47,48] and overexpression of VEGF-D has been shown to increase lymphangiogenesis in cancer [18,19], we examined whether endogenous VEGF-D regulates lymph vessel growth in a model of lymphangioma growth. This model is based on intraperitoneal injection of incomplete Freund's adjuvant, inducing white, non-invasive, VEGFR-3- and podoplanin positive tumor lesions growing along the thoracic duct and on the surface of the diaphragm and liver, with leukocyte recruitment toward the lesion area [35,36]. Lymphangioma induction in the liver of *Vegf-d*^{null} mice yielded lesions of similar appearance, number and area as compared to wild type mice (area of lymphangioma tissue in cm^2: 0.46 ± 0.15 in wild type *versus* 0.48 ± 0.18 in *Vegf-d*^{null} mice; mean ± SEM; n=5; P=NS). Quantification of VEGFR-3 (not shown) or LYVE1$^+$ vessels in the lesions after immunostaining of lymphangioma sections, showed comparable vessel densities in wild type and *Vegf-d*^{null} mice (liver: LYVE1$^+$ total vessel densities in number per μm^2: 40 ± 19 in wild type *versus* 36 ± 8 in *Vegf-d*^{null} mice, mean ± SEM; n=5; P=NS; Figure 3A,B*)*. No difference in leukocyte recruitment to the lesion areas was observed between wild type and *Vegf-d*^{null} mice liver lesions (Figure 3C,D). These results indicate that endogenous VEGF-D is not critically involved in lymphatic endothelial tumor formation in this mouse model of lymphangioma.

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DISCUSSION

In this study, we analyzed the role of endogenous VEGF-D, one of the ligands of the lymphangiogenic receptor VEGFR-3 [1], in pathological conditions using gene deficient mice. While indeed, endogenous VEGF-D appears to play only a moderate role in development and in postnatal lymphangiogenesis (this study and [12-14]), its role in lymphangiogenesis in pathologicalal conditions was not extensively addressed to date. Nevertheless, VEGF-D stimulates lymphangiogenesis in adulthood [6,7,14,17,19] and is involved in metastasis in experimental tumors and various human cancers (reviewed in [15]). We therefore assessed whether endogenous VEGF-D might have a role that is more restricted to pathological challenges than to normal health, as shown previously for other angiogenic molecules such as placental growth factor (PlGF) and VEGF-B (unpublished results and refs [33]).

To address this question, we generated *Vegf-d*^{null} mice and verified embryonic and postnatal physiological lymphangiogenesis, and finally, assessed lymphangiogenesis during models of pathology. VEGF-D deficiency did not prevent normal lymphangiogenesis during embryonic and postnatal development. The *Vegf-d*^{null} mice did not develop lymphedema nor other histologic or functional abnormalities or health problems. This was in accordance with earlier findings in mice [13] and with our recent results in *Xenopus* tadpoles which indicated a role of VEGF-D as a modifier of other lymphangiogenesis factors such as VEGF-C or Prox-1 during development [12]. Unlike human VEGF-D, mouse VEGF-D does not bind the blood endothelial cell receptor VEGFR-2 [49]. In accordance with this, no angiogenic defects were observed in the *Vegf-d*^{null} mice (Figure 1G,H; Figure S2E,H and not shown). This normal phenotype, in contrast with the prenatal lethal phenotype of VEGF-C deficiency [10], was not due to a compensatory upregulation of VEGF-C in *Vegf-d*^{null} mice (Note S2), in accordance with similar observations reported for the independently generated VEGF-D deficient mice [13]. These findings

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confirm a lesser importance for VEGF-D, and might in part be explained by a different expression pattern or timing of VEGF-C and VEGF-D. For instance, whereas VEGF-C is expressed in mouse embryos at the site where the jugular lymph sac will develop [10], no evidence is available for a substantial parallel expression of VEGF-D at this site [50]. Furthermore, recent results from transgenic studies in mice indicate that VEGF-D overexpression promotes lymphangiogenesis predominantly after birth [42].

VEGF-D neutralization experiments indicate that VEGF-D is involved in pathological conditions in the adult [16,23,51], supporting the concept that its role might be more disease related. As VEGF-D was found to induce lymphatic hyperplasia in neonatal skin [42] and VEGF-D expression was reported to be elevated during healing of skin wounds [45], the lymphangiogenic response after skin wounding was analyzed. No genotypic differences in the rate of healing and development of lymph vessels were however observed. This suggests that endogenous VEGF-C – or other lymphatic growth factors – might suffice for the healing response in this model. Recent findings that VEGFR-2 is more important than VEGFR-3 in lymphatic capillary formation in a skin regeneration model in mice [52] support this notion, given the inability of murine VEGF-D to bind VEGFR-2 [49].

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As we also observed enhanced expression of VEGF-D in the infarcted heart, we evaluated whether endogenous VEGF-D is involved in the lymphatic vessel regeneration, which occurs in the healing myocardium in mice at one week after infarct induction. However, no differences in lymphatic vessel regeneration between wild type and *Vegf-d^{null}* mice were found. In human patients with myocardial infarction, VEGF-C is consistenlty expressed by viable cardiomyocytes around the lesion at all stages of healing [53]. We speculate that threshold levels of VEGF-C, perhaps in combination with other lymphatic factors, might have sufficed or compensated for the absence of VEGF-D.

Furthermore, in a mouse lymphatic endothelial tumor model, VEGFR-3 positive lymphangiomas grew in *Vegf-d*^{null} mice to similar sizes and with comparable gross and histological appearance as compared to wild type mice. Thus, somewhat unexpectedly, no critical role of endogenous VEGF-D was observed in the stress or pathological models presently studied, including in models associated with enhanced VEGF-D expression such as myocardial infarction and skin wound healing. The extent of upregulation observed (ca 3-fold; this study and [45]), although statistically significant, was moderate and its biological significance therefore perhaps remains limited, explaining the similar responses in wild type and *Vegf-d*^{null} mice in these models. In constrast, for other growth factors that were identified as dispensible for normal development and health, but found to be critically involved in pathology, such as PlGF [33,37], the upregulation in pathological conditions was much more pronounced and of apparent biological relevance. For instance, over 50-fold enhanced PlGF antigen levels were observed in infarcted as compared to healthy myocardium, and up to 20-fold enhanced levels of PlGF were measured in tumor tissue as compared to corresponding healthy tissue in wild type mice ([33] and unpublished results). These pronounced elevations were associated with distinct impairment of respectively infarct revascularization or tumor growth In PlGF deficient mice [33]. Forced overexpression of VEGF-D, via transgenesis, gene transfer, or using overexpressing tumor cells, has profound biological effects on angiogenesis and/or lymphangiogenesis [14,17,19,20,42,54], raising the question whether endogenous levels of VEGF-D are insufficient to be of functional relevance to stimulate lymphangiogenesis. Defining the precise biological role of endogenous VEGF-D on the other hand, remains an outstanding, intriguing question.

This genetic study in mice indicated that endogenous VEGF-D is only minimally involved in embryonic development, and in normal health after birth in mice, in accordance with previous findings in the mouse [13,14] as well as in

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Xenopus tadpoles [12]. We now also showed that VEGF-D deficiency does not cause overt lymphatic defects or dysfunction in specific physiologic stress models or pathological conditions. Whether endogenous VEGF-D is redundant, is involved in specific disease conditions, or might have other functions beyond (lymph)angiogenesis – albeit dispensable for normal development and health – needs to be further explored.

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FIGURE LEGENDS

FIGURE 1: Generation and lymphatic phenotyping of *Vegf-d*^{null} mice

(A-D) *Vegf-d* gene inactivation strategy. **A,** Scheme, showing the genomic structure of the wild type (WT) and inactivated (*Vegf-d^{null}*) murine *Vegf-d* gene, with relevant diagnostic restriction sites and the fragments (with their respective length). The gene-inactivation targeting vector *pPNT.Vegf-d^{null}* (top) contains a neomycine selection cassette (neo) and a LacZ gene fused in frame to the first codon of exon 2. The introns (shaded bar), exons (dark boxes), probes (black bars under the genes) and PCR primers (black arrowheads under the genes) are indicated. **B,** Southern blot, revealing restriction fragments of the expected size, after StuI-DraIII digestion of genomic DNA from wild type (*Vegf*-d$^{0/+}$) and mutant (*Vegf-d*$^{0/-}$) ES cells, hybridized with the 5' probe. As *Vegf-d* maps to the X-chromosome and male XY ES cells were used, only a single mutant but no wild type band is visible in correctly targeted ES cells. **C,** Southern blot of EcoRI-digested genomic DNA from female germline offspring of a male chimera, hybridized with the 3' probe, identifying restriction fragments of the expected size for a wild type (*Vegf-d*$^{+/+}$) and a heterozygous null (*Vegf-d*$^{+/-}$) female. **D,** RT.PCR on RNA isolated from lungs of wild type and *Vegf-d^{null}* mice using primers x and y annealing in exon 4 and 5, respectively (see panel A), amplifying a 504-bp band on wild type VEGF-D cDNA only (top). RT.PCR for HPRT (bottom) confirmed equal loading. **(E-L)** Lymphatic phenotyping of wild type (WT) and VEGF-D^{null} embryos and adult mice. Panels E,F: The feet of *Vegf-d*^{null} mice have a normal size and show no signs of swelling. Panels G,H: Immunostaining for LYVE-1, revealing a normal size, number and pattern of the lymph vessels in the jugular regions in E12.5 WT and *Vegf-d*^{null} embryos. Panels I,J: Lymphangiography with FITC-dextran, revealing normal pattern and density of lymph vessels in the ear of *Vegf-d*^{null} mice. Panels K,L: Lymphangiography using FITC-dextran revealing a normal lymphatic vascular

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network in the tail of adult WT and *Vegf-d*^{null} mice. LV: lymph vessel; ACV: anterior cardinal vein. Bars: 100 µm in panels G,H; 700 µm in panels I,J; 500 µm in panels K,L.

FIGURE 2: Lymphangiogenesis during skin wound healing

(A), Healing proceeded at a comparable rate in wild type (WT) and *Vegf-d*^{null} mice. Standardised 15-mm full-thickness skin incisions were made on the back of the mice, taking care not to damage the underlying muscle. Wound healing was quantified by monitoring the width of the wound daily. Data represent mean ± SEM, n= 15-16; P=NS by multivariate analysis. **(B, C)** Immunostaining for LYVE-1 of skin wounds of wild type (B) and *Vegf-d*^{null} mice **(**C**)** 15 days after incision, revealing formation of lymph vessels (arrows) in the wound area at numbers comparable to those in wild type controls. The wound area (W) is demarkated by a dashed line; note the thicker epidermis in the wound (arrowheads) as compared to adjacent area. Bars: 100µm.

FIGURE 3: Pathological lymphangiogenesis during lymphangioma formation

(A-D) Immunostaining for LYVE-1 (A,B)) or CD45 (C,D) on sections of lymphangioma (Ly) growing on the surface of the liver (Li) at 30 days after induction of tumor formation in wild type (A,C) or *Vegf-d*^{null} mice (B,D) by i.p. injection of incomplete Freund's adjuvant. The results show a similar appearance and staining of the lesions in both genotypes. Bars: 5 µm in all panels.

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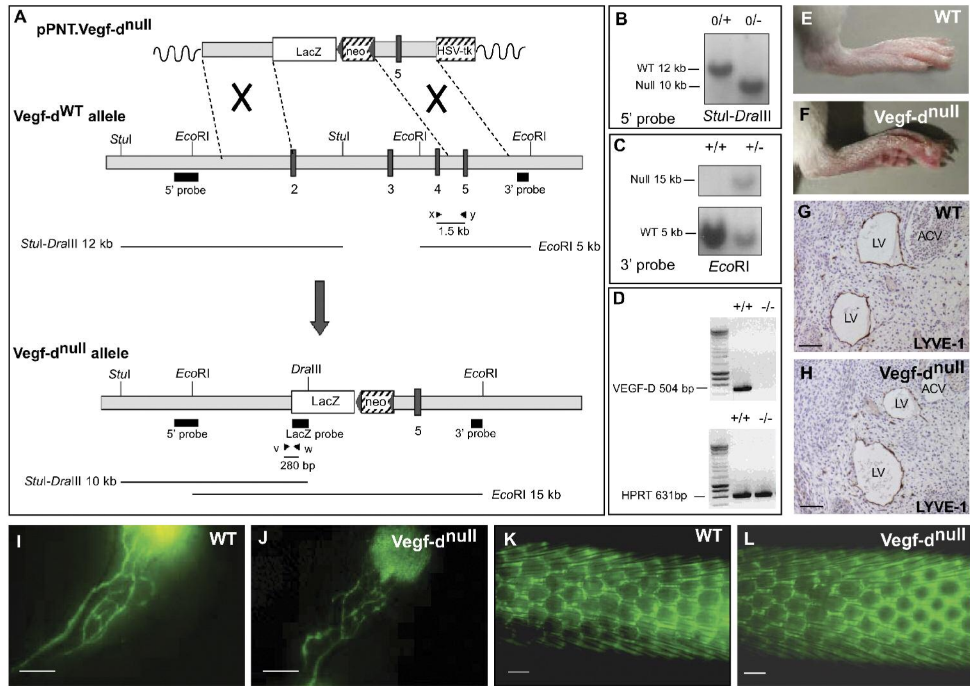

Figure 1

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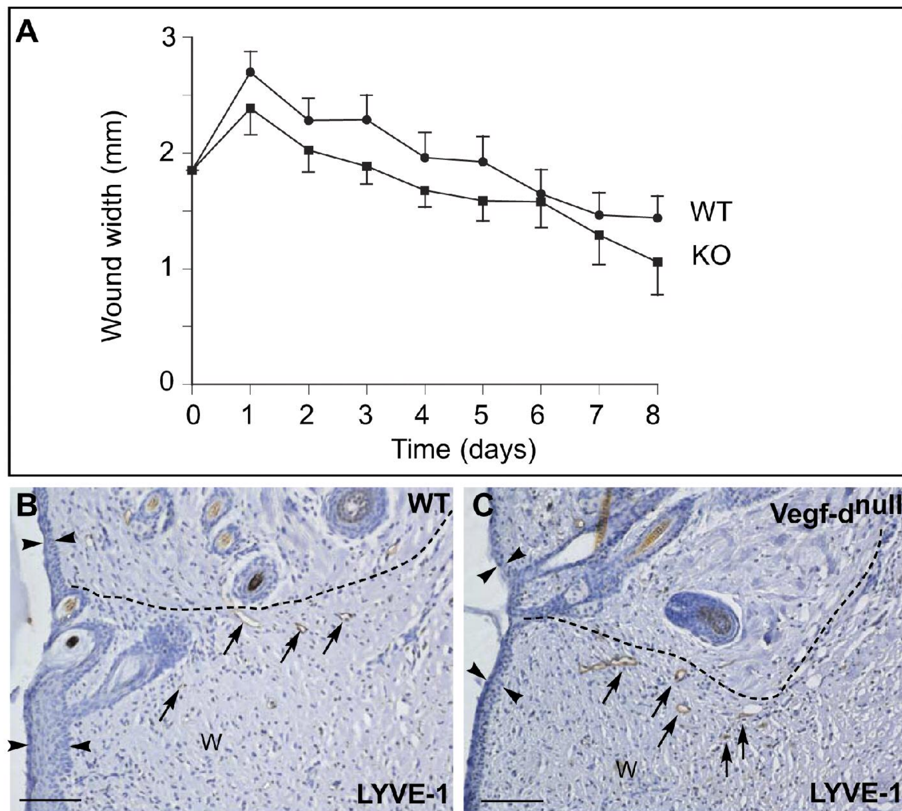

Figure 2

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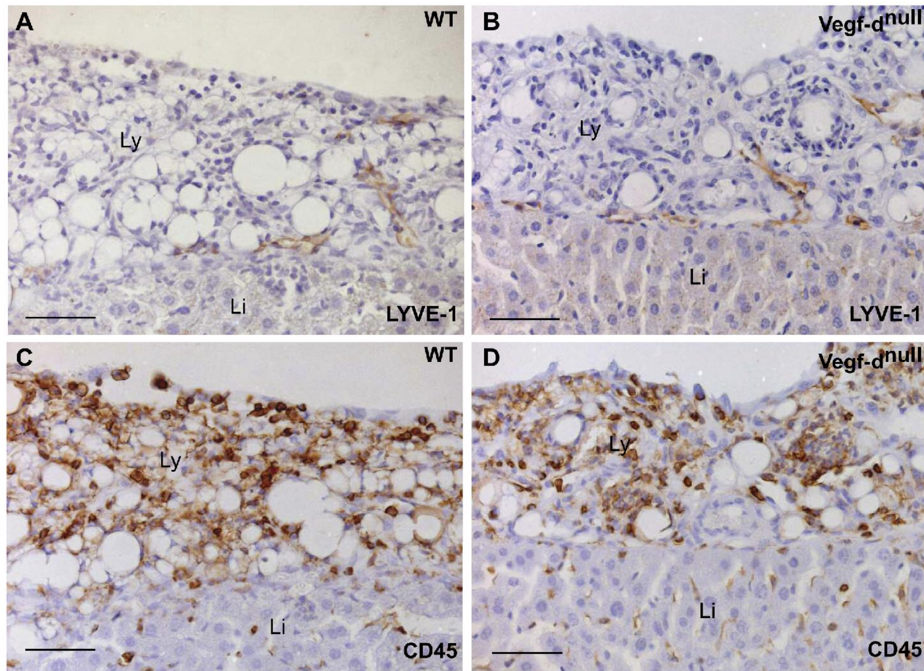

Figure 3

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Review

SUPPLEMENTARY INFORMATION

Reassessment of the role of VEGF-D in health and disease

Koch et al.

SUPPLEMENTARY METHODS

GENERATION OF VEGF-D NULL MICE

A targeting vector, p*PNT.Vegf-d*^{null}, was constructed to replace exon 2 through exon 4 of the *Vegf-d* gene (8.5-kb deletion) with the LacZ cDNA, fused in frame to the first codon of exon 2 and followed by the SV40 polyA, and a *neo*^{r} selection marker cassette (Figure 1A of the main text). Genomic fragments used as homologous flanks were isolated from subclones of the murine *Vegf-d* gene. The 5' homology is a 4-kb fragment comprising part of intron 1 up to the first codon of exon 2. The 3'homology is a 3.3-kb fragment comprising the downstream 2 kb of intron 4, exon 5 and 1.3 kb of intron 5. With the designed deletion of exon 2-4, potential aberrant splicing of the resulting RNA from exon 1 (harboring the start codon) to exon 5 would result in an out-of-frame sequence with numerous in-frame stop codons, the first one occurring at codon 11 of the out-of-frame exon 5 sequence. The linearized targeting vector was electroporated in 129SvEv embryonic stem (ES) cells and correctly targeted *Vegf-d*^{null} ES cell clones were identified by appropriate Southern blot analysis and were used for embryo aggregation with Swiss morula stage embryos as described previously 83 to generate chimeric and transgenic animals. Given the X-chromosomal location of the *Vegf-d* gene [1], the VEGF-D deficient colony was established using wild type (*Vegf-d*$^{0/+}$) and hemizygous null (*Vegf-d*$^{0/-}$) males mated to heterozygous *Vegf-d*$^{+/-}$ females to obtain the different genotypes.

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Genotyping of ES cell clones and of the VEGF-D null mice

DNA was isolated from ES cell clones or from mouse tail tips for genotype analysis by Southern blotting or by PCR. Correct homologous recombination of the (male) VEGF-$D^{0/-}$ ES cell clones at the 5' and 3' side was confirmed by Southern blot analysis using restriction digests and external probes giving hybridization patterns discriminating the wild type and targeted allele (Figure 1 of the main text). The 5' probe used for hybridisation was a 0.9-kb *Bst*EII-*Not*I fragment located 2 kb upstream of the 5' homology; the 3' probe was a 0.6-kb PCR amplified fragment located immediately downstream of the 3' homology. To exclude additional random integration of the targeting vector, a 0.9-kb *Bsu*36I-*Dra*III fragment corresponding to the 5' part of the *LacZ* gene (Figure 1A of the main text) was used to verify the presence of a single 15-kb *Eco*RI or a single 10-kb *Stu*I-*Dra*III fragment of the mutated allele.

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PCR genotyping of the final *Vegf-d*^{null} colony was performed using Ready-to-go PCR Beads (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) with a double primer set (forward primer X 5'-GAG ATA TCA GTG CCT CTG ACA TCA GTG-3' corresponding to nt 796-822 of the murine *Vegf-d* cDNA, Genbank accession number NM010216; reverse primer Y 5'-AAT AGG ACA GAG TTT CTT GGA ATG AGG-3' corresponding to nt 969-943 of the murine *Vegf-d* cDNA) amplifying a 1.5-kb band on the wild type allele only (Figure 1A of the main text), and primerset V/W (forward primer V 5'-GTT AGC AAC CAT GGT TAT CAA G-3' located 27 nucleotides upstream of exon 2; reverse primer W 5'-GAC AGT ATC GGC CTC AGG AAG ATC GCA CTC CAG C-3' annealing in the *LacZ* gene at nt 1196-1163 in pcmvβGal, Genbank accession number U02451) amplifying a 280-bp band on the *Vegf-d*^{null} allele only (Figure 1A of the main text).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

RNA ANALYSIS OF VEGF-DNULL MICE

Poly-adenylated RNA was extracted from adult lung or heart using the Quick Prep mRNA purification kit (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), and was submitted to first-strand cDNA synthesis by oligo(dT) priming using the Ready-to-Go T-primed First strand kit (Amersham Pharmacia Biotech). For semi-quantitative expression analysis, the first-strand cDNA was used for PCR amplification with an exon 2-exon4 primer set amplifying a 504-bp band (primers 5'-TTT GAG CGA TCA TCC CGG TCC ATG TTG-3' and 5'–GGG GCC CGT GGG CAA GCA CTT ACA ACC-3' corresponding to nt 382-408 and 885-849 of the Vegf-D mRNA, respectively). RT.PCR for the HPRT gene was used as a reference control. The RT-PCR products were analyzed by electrophoretic separation on a 1% agarose gel (Figure 1D of the main text). Real-time quantitative RT.PCR for *muVegf-c* and *muVegf-d* was performed with the primers and probes listed in Supplement table 2.

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PHENOTYPING OF THE *VEGF-D*^{NULL} MICE IN BASELINE

The baseline phenotype of *Vegf-d*^{null} mice during embryonic, postnatal and adutl life was as follows. Embryos were harvested at E12.5 and analysed for the formation of lymph sacs and lymph vessels. Ears from P13 mice were dissected in order to evaluate the remodeling of the lymphatic plexus and the recruitment of smooth muscle cells (SMCs) into the lymphatics collectors of the skin. Skin and gut tissues were also collected from adult mice in physiologic conditions. The lymphatic network in the ear of the adult mice was analyzed by fluorescence microscopic imaging of lymphatic vessels after intradermal injection of FITC-dextran (Mr ~2x106 Da) [2]. Filling of the deeper lymphatic vessels in the region of the ischiatic vein was analyzed after intradermal injection of Evans blue (Sigma, 3 mg/ml in PBS) into the hind footpads and removal of the skin, as described [3]. Adult mice were fed with 0.1 mg of fluorescent lipid tracer BODIPY® FL C16 (Molecular Probes Europe BV, Leiden, the Netherlands) and sacrificed 2 hours after. Intestines were exposed under the fluorescence microscopy and analysed for the lipid uptake [4]. Serum

samples were prepared from whole blood collected from adult mice by tail bleeding, and were analyzed for total cholesterol [5] and total neutral glycerolipids [6] using fluorimetric coupled enzyme indicator assays.

SUPPLEMENTARY NOTES

NOTE S1: We studied whether loss of VEGF-D affected the remodeling of the primitive lymphatic capillary plexus into a hierarchical vessel network of lymphatic collectors and capillaries, using postnatal development of the dermal lymphatic system in the ear skin as a model [7]. At P5, the initial lymphatic capillary plexus extends sprouts into the upper layer of the dermis, resulting in a two-layered lymphatic vasculature by P10: a capillary plexus below the epidermis and a collecting vessel network in the medial inner part of the ear. At this stage, the collecting vessels form luminal valves and acquire smooth muscle cell (SMC) coverage. Interestingly, while LYVE-1 is initially expressed in all lymph vessels in the primitive plexus, its expression becomes gradually restricted to the lymphatic capillaries in the mature vasculature at the time of initiation of SMC recruitment, whereas podoplanin remains expressed constitutively in all types of lymph vessels [7]. Whole mount immunofluorescent staining of P13 ears for podoplanin and SMC revealed a normal capillary plexus and collecting lymphatic network (Figure S2A,C-E,G,H). Moreover, LYVE-1 was expressed mainly in the capillaries but down-regulated in the collecting lymphatics in *Vegf-d*^{null} mice (Figure S2B,F), indicating that VEGF-D was not required for the phenotypic shift in lymphatic identity.

NOTE S2: Quantitative real-time RT.PCR on RNA isolated from adult lung did not reveal compensatory upregulation of VEGF-C expression in *Vegf-d*^{null} mice (mRNA copies of VEGF-C per 10^3 mRNA copies of β-actin: 0.5 ± 0.1 in *Vegf-d*^{null} mice *versus* 0.8 ± 0.2 in wild type mice; mean ± SEM, *n*=2-3, *P*=NS). Similarly, no compensatory upregulation of

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VEGF-C expression was noticed by Baldwin et al. in their independently generated *Vegf-d*^{null} mice [8.].

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SUPPLEMENTARY FIGURES

FIGURE S1: Lymphangiogenesis in wild type and *Vegf-d*^{null} embryos

Immunostaining of paraffin sections for VEGFR-3 revealing a normal size, number and pattern of the lymph vessels in the jugular regions in E12.5 WT **(A)** and *Vegf-d*^{null} embryos **(B)**. LV: lymph vessel; ACV: anterior cardinal vein. Bars: 100 µm in both panels.

FIGURE S2: Normal development of lymphatic capillaries and collecting lymph vessels in *Vegf-d*^{null} neonates.

(A-H) Whole-mount triple immunofluorescent staining for smooth muscle α-actin (SMA) (A,E; red), podoplanin (B,F; green), and LYVE-1 (C,G; blue) of dissected ears from wild type (A-D) and *Vegf-d*^{null} neonates (E-H*)* at postnatal day 13. Merged images are shown in panels D (wild type) and H (Vegf-d^{null}). The stainings reveal comparable formation of the lymphatic capillary (podo^{+}/LYVE-1^{+}; blue arrows in panels B,C,F,G) and collecting lymphatic network (podo^{+}/SMA^{+}; green arrows in panels A,B,E,F). SMA also stains blood vessels (red arrows in A,E). Note that in contrast to podoplanin, LYVE-1 at postnatal day 13 is only expressed in the lymphatic capillaries but not or minimally in the collecting lymph vessels. **(I, J)** Normal lymphatic uptake of the ingested fluorescent lipid tracer BODIPY® FL C16 by the mesenteric lymphatics in both adult wild type (I) or *Vegf-d*^{null} mice (J) showing their typical radial patterning (arrows). **(K, L)** Whole-mount immunostaining for LYVE-1, revealing normal size, number and pattern of deep lymph vessels in the intestines of adult WT and *Vegf-d*^{null} mice. Analysis of LYVE-1-positive lymph vessels in whole mount intestines revealed no genotypic differences in the number of branches per mm^2 (58 ± 11 in WT *versus* 59 ± 3 in *Vegf-d*^{null} mice), the average distance between two branches (103 ± 7 µm in WT *versus* 104 ± 2 µm in *Vegf-d*^{null} mice), and the number of vessels penetrating the villi per mm^2 (32 ± 1 in WT *versus* 30 ± 1 in *Vegf-d*^{null} mice). Bars: 25 µm in panels A-H; 100 µm in panels K,L.

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SUPPLEMENTARY TABLES

TABLE S1: Lymph vessel counts and density in the wound area and in the area adjacent to the wound (border area) of wild type (WT) and *Vegf-d*^{null} mice 2 weeks after skin wounding.

		WT	*Vegf-d*^{null}
Total lymphatic perfusion area	Wound	4,480 ± 685	4,740 ± 1,040
(um^2)	Border	10,100 ± 1,950	7,430 ± 1,420
Total lymph vessel density	Wound	40 ± 6.2	54 ± 5.5
($LYVE1^+$ vessels/mm^2)	Border	29 ± 1.8	26 ± 2.3
Total blood vessel density	Wound	280 ± 19	300 ± 26
($CD31^+$ vessels/mm^2)	Border	160 ± 14	180 ± 15

Lymphatic and blood vessels were immunostained for LYVE-1 or CD31, respectively. The border area is constituted of the two optical fields flanking the wound area. Data represent mean ± SEM (*n*=7-16). *P*=NS versus wild type for all data.

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TABLE S2: Primer and probe sequences used for quantitative real-time RT.PCR expression analysis of mouse tissue.

Mouse primers	
muVEGF-C.forward	5'-AAG ACC GTG TGC GAA TCG A-3'
muVEGF-C.reverse	5'-ACA CAG CGG CAT ACT TCT TCA-C-3'
muVEGF-C.probe	5'-FAM-AAA GGA CAG TCC TGG ATC ACA ATG CTT CA-TAMRA-3'
muVEGF-D.forward	5'-CTC TGT GGA CCG CAC ATG AC-3'
muVEGF-D.reverse	5'-TCT CCC GGA CAT GGT GCT-3'
muVEGF-D.probe	5'-FAM-TAC AGA CGC ACT CAC AGC GAT CTT CAT CA-TAMRA-3'
HPRT.forward	5'-TTA TCA GAC TGA AGA GCT ACT GTA ATG ATC-3'
HPRT.reverse	5'-TTA CCA GTG TCA ATT ATA TCT TCA ACA ATC-3'
HPRT.probe	5'-FAM-TGA GAG ATC ATC TCC ACC AAT AAC TTT TAT GTC CC-TAMRA-3'

The house keeping gene HPRT was used as internal control.

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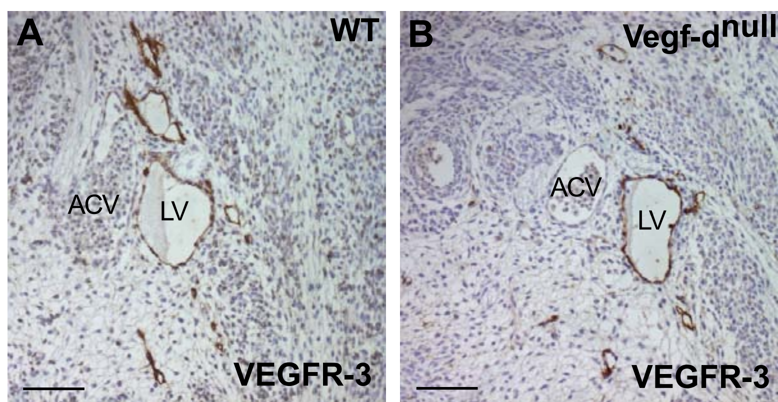

Figure S1

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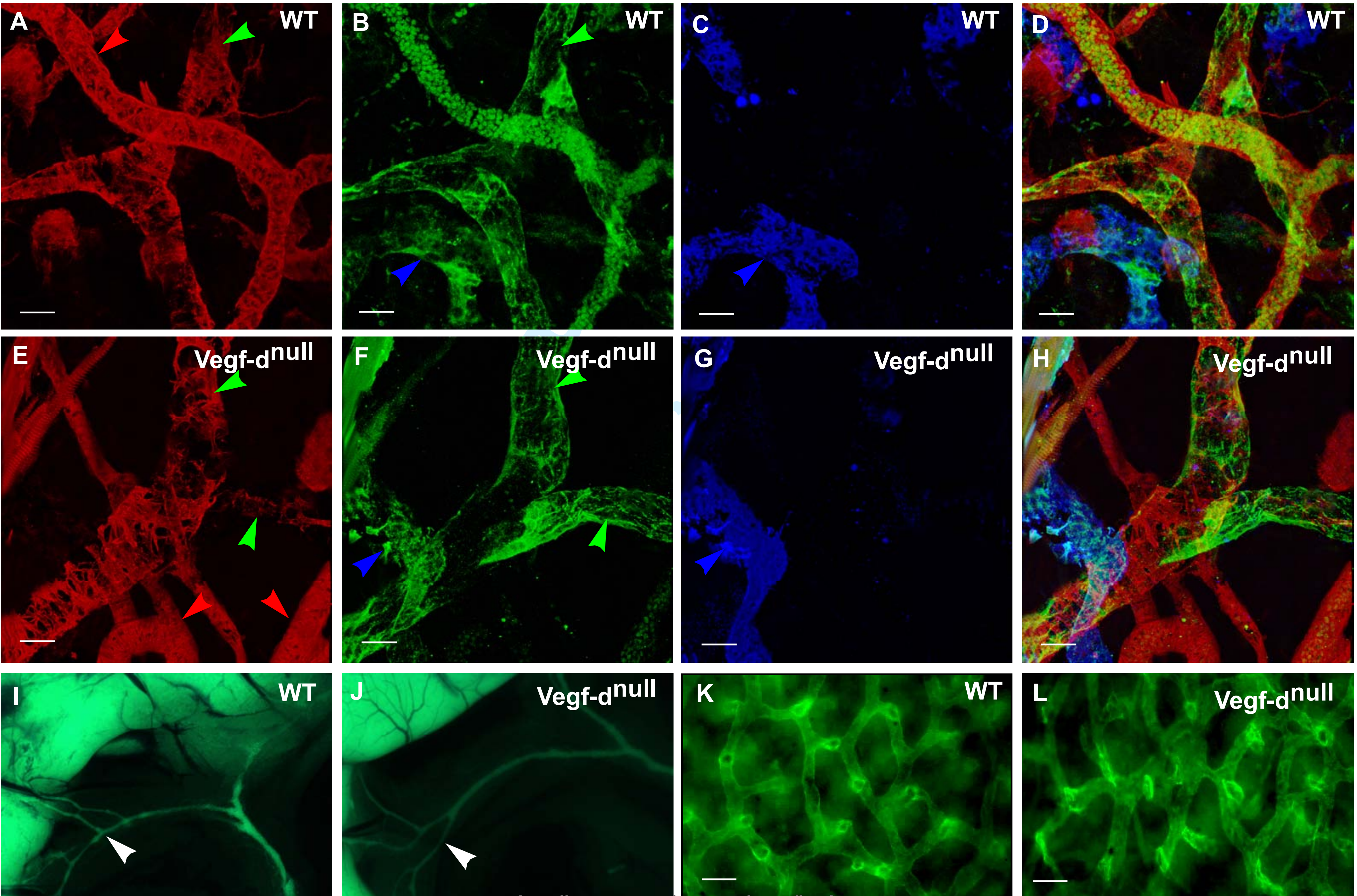

Figure S2