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***Vegfr3-CreER^{T2}* mouse, a new genetic tool for targeting the lymphatic system.**

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Abstract

The lymphatic system is essential in many physiological and pathological processes. Still, much remains to be known about the molecular mechanisms that control its development and function and how to modulate them therapeutically. The study of these mechanisms will benefit from better-controlled genetic mouse models targeting specifically lymphatic endothelial cells. Among the genes expressed predominantly in lymphatic endothelium, *Vegfr3* was the first one identified and is still considered to be one of the best lymphatic markers and a key regulator of the lymphatic system. Here we report the generation of a *Vegfr3-CreER^{T2}* knockin mouse by gene targeting in embryonic stem cells. This mouse expresses the tamoxifen-inducible CreER^{T2} recombinase under the endogenous transcriptional control of the *Vegfr3* gene without altering its physiological expression or regulation. The *Vegfr3-CreER^{T2}* allele drives efficient recombination of floxed sequences upon tamoxifen administration specifically in *Vegfr3*-expressing cells, both *in vitro*, in primary lymphatic endothelial cells, and *in vivo* at different stages of mouse embryonic development and postnatal life. Thus our *Vegfr3-CreER^{T2}* mouse constitutes a new powerful genetic tool for lineage tracing analysis and for conditional gene manipulation in the lymphatic endothelium that will contribute to improve our current understanding of this system.

Introduction

Lymphatic vessels control tissue fluid homeostasis, immune cell trafficking and absorption of dietary fats. Malformation and/or dysfunction of the lymphatic network is a feature of numerous pathological conditions such as lymphedema, inflammation, obesity, hypertension, defective cholesterol metabolism, failure of lung inflation at birth and tumour dissemination and metastasis [1-3]. Its widespread involvement in disease has raised an increasing interest in lymphatics. However, until recently, lack of genetic tools to allow specific targeting of lymphatic vessels has hampered the study of this complex system.

To date, several Cre-expressing mouse models, based on gene-targeted BAC/PAC, knockin, or conventional transgenic constructs, have been used to target the lymphatic vasculature. Some of these lines such as the *Cdh5-CreER^{T2}* [4,5] and the *Pdgfrb-CreER^{T2}* [6,7] lines target both the blood endothelial cell (BEC) and the lymphatic endothelial cell (LEC) compartments to similar extent. Identification of genes differentially expressed in the lymphatic and blood vasculatures [8,9] has prompted the emergence of genetic models for studying specifically the lymphatic system. Still, some of these lines target also BECs to significant extent, such as the *Lyve1-Cre* line [10] or exhibit extra-vascular expression of the recombinase: stromal cells of lymph nodes and secondary lymphoid organs (*Podoplanin-Cre* [11]) or neurons, cardiomyocytes and hepatocytes (*Prox1-Cre* [12]). This is observed even with a time-controlled induction of the recombinase in the *Prox1-CreER^{T2}* line [13,14], the only lymphatic inducible Cre line available up to now, limiting its optimal application for LEC-specific conditional targeting. All together, these models have contributed to a better knowledge of the molecular mechanisms regulating lymphatic development and function, however, the generation of a more LEC-specific and time-controlled mouse Cre line was still under demand.

First discovered in 1990, *Vegfr3* is one of the best-characterized LEC markers, and a master key regulator of lymphatic development and function. *Vegfr3* expression becomes mostly restricted to LECs during early development [15], although is also induced in the tip endothelial cells of the blood capillaries during angiogenesis [16]. *Vegfr3* has also been detected in fenestrated endothelium [17] and in monocytes and macrophages [18] however restricted to minor subpopulations of these cells. It is precisely this more lymphatic-specific expression pattern what makes *Vegfr3* a good candidate for the generation of an inducible mouse model to target lymphatic vessels. So far, only a constitutive *Vegfr3-Cre* mouse is available [19]. Here we report the generation of the first Cre-inducible knockin model driven by *Vegfr3* and characterize it at different times of lymphatic development.

Results and discussion

To create the *Vegfr3-CreER^{T2}* knockin mouse we used the targeting strategy already described for the generation of the *Vegfr3-GFP^{Luc}* reporter mouse [20]. Briefly, an

IRES-CreER^{T2}-encoding sequence was introduced into the the 3'-UTR of the mouse *Vegfr3* gene by homologous recombination in embryonic stem cells (Figure 1a-c). The internal ribosomal entry site (IRES) allows bicistronic translation of the knockin allele driving CreER^{T2} expression from the endogenous *Vegfr3* promoter and regulatory sequences [21]. Insertion of the *IRES-CreER^{T2}* sequence into the 3'-UTR does not detectably change the expression of *Vegfr3* at mRNA or protein level (Figure 1d-e). As previously described for the *Vegfr3-EGFP^{Luc}* line [20], no phenotype is observed in mice carrying the *Vegfr3-CreER^{T2}* allele in homozygosis.

The efficiency and specificity of Cre-mediated recombination in *Vegfr3-CreER^{T2}* mice, at different stages of mouse development, has been tested using two different Cre-reporter lines (Figure 2a). The *R26-LSL-lacZ* reporter exhibits Rosa26-driven expression of the E-coli β -galactosidase (lacZ) gene upon Cre-mediated excision of a floxed STOP sequence [22]. In parallel, the *R26-mTmG* double fluorescent reporter mediates Rosa26/CAG-driven expression of a membrane-bound green fluorescent protein (GFP) upon Cre-mediated excision of a floxed mTomato coding sequence [23].

In embryos, *Vegfr3* is first detected in BECs at E8.5, thus preceding the differentiation of the lymphatic vasculature occurring at E9.75-E10.0 [15,24]. Around E10.5, initial LECs bud and sprout from the cardinal vein and inter-somitic vessels forming the first lymphatic structures, the peripheral longitudinal lymphatic vessel (PLLV) and the ventral primordial thoracic duct (pDT), both structures mostly known as the jugular lymph sacs (LS) [24]. Upon formation of the first lymphatic network, from E10.0-E11.0 onwards, *Vegfr3* expression is high in the developing lymphatic endothelium and down-regulated in blood vessels [15,24]. To validate the *Vegfr3-CreER^{T2}* model during these initial stages of lymphatic development we first treated pregnant wild type females crossed with homozygous males for both alleles *Vegfr3-CreER^{T2}* and *R26-LSL-lacZ* or *Vegfr3-CreER^{T2}* and *R26-mTmG* with tamoxifen (TAM) or 4-hydroxy-tamoxifen (4OHT) at E8.5-E10.5 and analyzed the embryos at different times of gestation. Induction of CreER^{T2} is detectable in *+Vegfr3-CreER^{T2}* embryos as early as E8.5-9.5 before lymphatic specification of the embryonic endothelium and upon a single intraperitoneal (IP) injection of TAM (3mg) to the pregnant mother (Figure 2b). At E10.5, when the first LECs are specified from the cardinal vein (CV), the same dose of 4OHT, 3 mg via IP to the gestating mother, induced a highly efficient recombination at the jugular LS (Figure 2c). As expected at this stage of embryonic development, Cre-mediated recombination was also detected in a low proportion of BECs (Lyve1⁻, Endomucin⁺) (arrows in Figure 2c).

We then studied the efficiency of recombination in two embryonic lymphatic beds with different origin, the mesenteric and the dermal lymphatic vessels [25,26]. When inducing for 4 consecutive days, starting at E10.5, the mesenteric lymphatic vessels (Nrp2⁺, Endomucin⁻) were efficiently targeted (analysis at E14.5 and E17.5, Figure 2d). Likewise, analysis of the dermal lymphatic vessels showed efficient recombination and co-expression of GFP with the lymphatic markers Nrp2 and Prox1 (Figure 2e). We have already reported elsewhere the recombination efficiency in dermal and mesenteric lymphatics as 86% and 79% respectively at E15.0 under this induction protocol [26]. A small proportion of BECs are also recombined (Figure 2e) reflecting remaining expression of *Vegfr3* in embryonic vessels at this stage of development.

In summary, the *Vegfr3-CreER^{T2}* allele allows efficient and highly specific targeting of *Vegfr3* expressing cells in heterozygous embryos, by 4OHT or TAM administration to the pregnant mother, from the earlier stages of embryonic development at which *Vegfr3* is expressed, and even before the onset of lymphatic specification, providing a powerful and unique tool for genetic analysis of the developing lymphatic system at this early stage.

After birth, *Vegfr3* expression is downregulated as lymphatic vessels mature [20,27]. Therefore we wanted to analyse the efficiency of the *Vegfr3-CreER^{T2}* allele for targeting the lymphatic system after birth, both at early postnatal development and in adult animals.

Initially, Cre was induced in *Vegfr3-CreER^{T2}*; *R26-LSL-lacZ* double heterozygous pups by feeding the lactating mother with TAM-diet from the day of delivery (postnatal day 0 or P0) up to weaning time (P21) when pups were analyzed. X-Gal staining of whole-mount preparations of diaphragm, peritoneum and mesentery, revealed an efficient recombination in lymphatic vessels but not in blood vessels in these conditions (Figure 3a). Accordingly, X-Gal staining analysis of different frozen sections from various tissues and organs showed a high level and widespread induction of CreER^{T2} in lymphatic vessels (data not shown).

We then tested if shorter induction protocols were also efficient for activating the *Vegfr3-CreER^{T2}* allele in early postnatal development. In this case, *Vegfr3-CreER^{T2}*; *R26-mTmG* double heterozygous pups were treated immediately after birth with 50 µg of 4OHT injected IP at P1 and P2 (Figure 3b-d). Whole mount fluorescence images at P6 showed that most lymphatic vessels were targeted in all organs analyzed (Figure 3b). Following the same induction scheme, in both P6 mesentery and P21 skin, co-localization of GFP⁺ cells with Nrp2 expression (Figure 3c) or Lyve1 expression (Figure 3d) confirms that the recombination occurred specifically in LECs and not in BECs. Highly efficient recombination in valves of lymphatic collectors is also observed at these stages (asterisks in Figures 3b-d).

We then examined the Cre-induction efficiency in post-weaning animals, heterozygous for the reporter *R26-LSL-LacZ* allele and either heterozygous or homozygous for the *Vegfr3-CreERT2* allele. Initially, mice were fed with TAM-diet for 3 weeks, starting at 4 weeks of age, immediately after weaning. An efficient recombination in lymphatic vessels in all organs and tissues analyzed was observed by X-Gal staining (Figure 4a) in both groups of mice. A Cre gene-dose effect was observed in homozygous animals where the efficiency of recombination was significantly increased under the same induction scheme in all tissues examined (Figure 4a). Nevertheless, the level of recombination in adult heterozygous mice can be increased by extending the length of the induction period. After 5 weeks of TAM-diet, recombination in heterozygous mice was similar to the one observed in homozygous mice after a 3 weeks induction, and did not significantly increase after 2 more weeks of treatment (Supplementary Figure 1). Interestingly, although not at the same level as in LECs, to some extent recombination of the *R26-LSL-lacZ* reporter was also observed in BECs under this prolonged induction protocol, mainly in blood capillaries. Recombination in BECs is also more prominent in *Vegfr3-CreER^{T2}* homozygous mice, something observed also during embryonic development (data not

shown). However, shorter induction schemes in our model allowed specific targeting of LECs. A single topical application of a low dose of 4OHT (25 μ g) in the mouse ear at 5 weeks of age lead to an almost complete and specific recombination in LECs, (Figure 4b) both in heterozygous and homozygous mice. Fluorescence signal in valves indicates that those are also efficiently targeted.

In order to better characterize the relative recombination efficiency in BEC and LEC compartments in our model, we performed FACs analysis of different organs from *Vegfr3-CreER^{T2}; R26-mTmG* double heterozygous mice treated with 1mg of Tamoxifen, via IP, at 4 weeks of age for 5 consecutive days and analysed one week later (Figure 4c-d and Supplementary Figure 2). We observed a high proportion of GFP⁺ LECs (Pecam1⁺, Cd45⁻, Cd11b⁻, Podoplanin⁺) in all organs examined: skin (96.4 $\pm$ 1.4%) liver (88.9 $\pm$ 14.4%), pancreas (83.4 $\pm$ 11.6%) and heart (92.6 $\pm$ 1.5%), while the proportion of GFP⁺ BECs (Pecam1⁺, Cd45⁻, Cd11b⁻, Podoplanin⁻) showed variability among different organs. While in the skin the proportion of recombined BECs was low (5.7 $\pm$ 0.1%), in pancreas and heart increased up to 16.6 $\pm$ 1.5% and 13.6 $\pm$ 2.2% respectively. In the spleen, a LEC population was not detected using our FACs protocol. This may reflect that mouse spleen mostly contains efferent collecting lymphatic vessels around the central artery, but is low in capillary lymphatic vessels [28]. However, recombination in the BEC compartment of the spleen was 41.5 $\pm$ 2.4%. In the liver, two different BEC populations were distinguished based on Pecam1 expression. Recombination in Pecam1-high BECs was 32.7 $\pm$ 2.5% while recombination in Pecam1-intermediate BECs was 90 $\pm$ 5.4%. This last population probably corresponds to the described liver sinusoidal ECs that express high levels of *Vegfr3* [29]. In addition, FACs analysis for immune cells (Pecam1⁻, CD45⁺, Cd11b⁺) showed a minor recombination rate (<1.55%) of this cell population in all the organs analysed (Figure 4c-d and Supplementary Figure 2).

Altogether, these results show that BEC recombination in *Vegfr3-CreER^{T2}* mice is dependent on- and can be modulated by both TAM dose and time of exposure, as well as the gene dose of the *CreER^{T2}* allele. Moreover, consistent with previous studies [30], our model shows that *Vegfr3* expression is not fully restricted to lymphatic vessels beyond embryonic development but it is also expressed, at lower level, in capillary BECs throughout adulthood. BEC recombination efficiency is organ-specific in post-weaning mice. This specificity may reflect different levels of *Vegfr3* expression in BECs from different tissues and organs and needs to be further explored. Furthermore, no detectable recombination is observed in immune cells in our model.

Finally, in order to assay the recombination mediated by the *Vegfr3-CreER^{T2}* allele also *in vitro*, we established primary LECs from double homozygous *Vegfr3-CreER^{T2};R26mTmG* mice. LEC purity and identity was confirmed by FACs analysis and showed to be 97.2% in the primary culture based on Prox1 and *Vegfr2* expression (Figure 5a). LEC identity was further confirmed by Prox1 immunostaining in culture (Figure 5b). Upon 4OHT treatment of our primary LECs, efficient recombination of the reporter allele (approximately 75% of the cells were GFP⁺) was achieved as early as 12 hours after 4OHT addition to the culture medium (Figure 5c). FACs analysis confirms a 100% recombination of LECs after 72 hours of 4OHT treatment (Figure 5a).

In summary, the *Vegfr3-CreER^{T2}* mouse provides a unique, powerful and highly versatile tool for conditional gene manipulation and lineage tracing analysis of the lymphatic endothelium, *in vitro* and *in vivo*, at all stages of embryonic development and through adulthood. The model responds to a whole variety of induction protocols and the response can be modulated by gene dose and by the intensity of induction. Importantly, the *Vegfr3-CreER^{T2}* mouse adds to the only previously available LEC-targeting and Cre-inducible model *Prox1-CreERT2* [13,14] a more specific targeting of LECs and the possibility of studying signalling pathways controlling cell fate and differentiation before *Prox1* specification in the cardinal vein. Thus, our *Vegfr3-CreER^{T2}* mouse will contribute to shed new light into the molecular mechanisms regulating lymphangiogenesis, both *in vivo* and *in vitro*.

Methods

Mice

The *R26-mTmG* line [23] was obtained from the Jackson Laboratory. The *R26-LSL-LacZ* line [22] was a gift from Philippe Soriano (Mount Sinai School of Medicine, New York, NY) and the Tg.CAG-Flpe [31] line was kindly provided by Francis Stewart (TU Dresden, Germany). Mice were maintained in a mixed 129/SvJae, C57Bl/6J and CD1 background for these studies. All the experimental procedures involving the use of animals were approved by the CNIO Institutional Animal Care and Use Ethics Committee and the Uppsala Laboratory Animal Ethical Committee.

Gene targeting and genotyping

For building the targeting vector, the 3' and 5' homology arms (3.3Kb and 3.7Kb respectively) were amplified by PCR from the BAC RP23-445K3 (Chori BAC/PAC Resources Centre) using primer pairs 5'-TTTGCGGCCGCGCACCCCTCACCCGCACATTC-3' / 5'-TTTGCGGCCGCGGGAAGCAAGACACCACCT-3' and 5'-TTTGTCGACAGGCTTGGCTGCTTCTCTC-3' / 5'-TTTGTCGACGCCCTCGTTGTCTGAGTTTG-3' respectively. The targeting was designed to introduce an *IRES-CreER^{T2}* cassette 168 nucleotides downstream of the STOP codon of the endogenous *Vegfr3* coding sequence. The targeting vector (20 µg) was linearised and electroporated into V6.4 embryonic stem cells, of F1(C57BL/6J x 129/SvJae) background [32], obtained from J. Schimenti (Cornell University, Ithaca, NY). After selection in Neomycin (G418 200 µg/ml) and Gancyclovir (2µM) for one week, 298 colonies were picked, 57 were analysed by Southern Blot and 25 (44%) were true homologous recombinants. The probes, P1 (262 bp) and P2 (342 bp) used for the screening were amplified by PCR from the BAC DNA using primers 5'-TTTGTCGACGAATACAGATACCTAAGC-3' / 5'-TTTGTCGACCCACATATGAGGCTTTCC-3' and 5'-TTTGTCGACCAAGCCTCTCATGTGCAG-3' / 5'-TTTGTCGACGTCTCATGGCCAGTATC-3' respectively. Chimeras were generated from two independent positive clones by aggregation with CD1 morulae following standard procedures [33]. For removing the neo cassette, germ line transmitting chimeras were crossed with Tg.CAG-Flpe females. The offspring was analysed by tail DNA genotyping by PCR using primers: forward 5'-CACTATGCTCCGTGTCTTG-3' and reverse 5'-GAGGAACTGCTTCCTTCAC-3' that amplify a 562bp band only when the PGK-neo cassette has been excised. Protocols for genotyping the *R26-LSL-LacZ* and *R26-mTmG* alleles have already been described [22,23]. The *Vegfr3-*

CreER^{T2} knockin mice were genotyped using primers: forward 5'-CACTATGCTCCGTGTCTTG-3' and reverse 5'-GTGACTCTCAGACATATG-3' (591 bp, WT allele) and primers: forward 5'-CATGGAGCATCTGTACAGCATGAAG-3' and reverse 5'-GTGACTCTCAGACATATG-3' (716 bp, knockin allele).

Tamoxifen administration

For induction of CreERT2 in embryos, pregnant females were injected, via intraperitoneal (IP), with tamoxifen (TAM) or 4-hydroxy-tamoxifen (4OHT) (Sigma) in peanut oil (10mg/ml) at the times and doses indicated, considering day E0.5 of development the day of vaginal plug detection. For systemic induction in adult animals, mice were fed with a TAM-containing diet (0.4 mg/g of TAM, Teklad CRD TAM⁴⁰⁰/CreER, Harlan) for the indicated periods of time preceded by 10 days of an adaptation diet (2019S Teklad Global, Harlan) or injected via IP with 1mg of TAM in peanut oil (20mg/ml) during 5 consecutive days via IP. Lactating females were given adaptation diet starting 10 days before delivery time at which TAM-diet was administered. Pups were injected 50 µg of 4OHT in ethanol (25mg/ml) via IP at P1 and P2. For local induction in the ears, 25µg of 4OHT in acetone (5mg/ml) was applied topically to the ear skin.

X-Gal staining

For whole mount staining, embryos were fixed for 30 min at room temperature (RT) in 0.1M phosphate buffer, pH 7.2, containing glutaraldehyde 0.2%, paraformaldehyde (PFA) 1.5%, MgCl₂ 2 mM and EGTA 5 mM. For OCT-cryosection staining, sections (10 µm thick) were attached to a glass coverslip (Superfrost, Thermo Scientific), dried for 1 hour at RT and fixed in the same conditions. After permeabilisation at RT (2 x 30 min) in 0.1M phosphate buffer, pH 7.2, containing Nonidet P-40 0.02%, sodium deoxycholate 0.01%, samples were stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) for 12-24 hours at 37°C following standard protocols. After taking images of the whole mount stained samples, they were embedded in OCT for sectioning. Frozen sections were counterstained for nuclear fast red using standard histological techniques.

Immunofluorescence

For whole-mount immunostaining, tissue was fixed in 4% PFA for 2h at RT, permeabilised in 0.3% Triton-X100 in PBS (PBSTx) and blocked in PBSTx plus 3% milk. Primary antibodies were incubated at 4°C overnight in blocking buffer. After washing in PBSTx, the samples were incubated with fluorochrome-conjugated secondary antibodies in blocking PBSTx plus 1% milk, washed and mounted in Mowiol. The following antibodies were used: rat anti-mouse LYVE-1 (R&D systems), rat anti-mouse PECAM1 (BD), rabbit anti-human Prox1 (generated against human Prox1 C-terminus (567-737aa), Prox1-GST construct provided by Dr. T. Petrova, University of Lausanne), rabbit anti-GFP (Invitrogen), chicken anti-GFP (Abcam), rat anti-mouse Endomucin (Santa Cruz Biotechnology), hamster anti-mouse Podoplanin (Developmental Studies Hybridoma Bank), goat anti-mouse Neuropilin-2 (all from R&D Systems). Secondary antibodies conjugated to DyLight 405, AF488, Cy3 or AF647 were obtained from Jackson ImmunoResearch.

For primary LECs immunostaining, cells were fixed with 4 % PFA/PBS for 20 min at RT and permeabilized using 0.5% Triton-X-100/PBS for 5 min at RT followed by blocking with 3% BSA/0.3% Triton for 1h and incubation with primary antibodies

(GFP-antibody; VE-cadherin C19, SantaCruz). Primary antibodies were detected with AlexaFluor 488 and AlexaFluor 647 - coupled secondary antibodies (Jackson ImmunoResearch).

Imaging

Confocal images were acquired using Zeiss 710 confocal microscope and Zen 2009 software or Leica SP8 inverted microscope and software. All confocal images represent maximum intensity projections of z-stacks. Stereomicroscope images were acquired using a Leica MZ16F fluorescence microscope with a Leica DFC420C camera and Leica Microsystems software.

Primary LEC isolation and 4OHT treatment

Tails were cut at the base and transferred into sterile HBSS/1% Pen/Strep before the skin was removed from the tails and cut into 30 mm long fragments. The fragments were incubated in 5% Dispase (Roche)/PBS for 45 min at 37 °C with gentle agitation to separate epidermis from dermis. Dermal fragments were washed in HBSS/1% Pen/Strep, transferred into Collagenase A (1 mg/ml) (Roche)/PBS solution, filtered and then seeded on 0.5% gelatin-coated dishes. When reached confluence, cells were detached with Accutase and sorted with dynabeads (sheep-anti-rat) and Pecam1 (Mec13.3) antibody (Pharmingen) for 45min at 4°C. One passage later, cells were again sorted as above using Lyve1 (Aly7) antibody (Abnova). Primary LECs were then seeded on gelatin-coated chamber slides (Lab-Tek, 8 well, glass), grown to low confluence and incubated for the indicated time points with 1µM 4OHT (Sigma).

Vegfr3 Northern Blott

Total RNA was prepared from E13.5 embryos in TriZol reagent (Life Technologies) following manufacturer's instructions. 15µg of total RNA was blotted. The 500bp fragment used as probe was contained in the 3'-UTR region of *Vegfr3*, and amplified by PCR with primers: forward, 5'-GCGGCCGCACCCTCACCCGCA-3' and reverse, 5'-GCGGCCGCAGCAGGAGCTG-3'.

Vegfr3 immunoprecipitation and Western Blott

Whole tissue extracts were prepared in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS), containing protease inhibitors. In all cases, the same amount of total protein (2 mg) were incubated with anti-Vegfr3 antibodies (Becton Dickinson) for 2 hours at 4°C with orbital rotation, followed by incubation with 50µl of protein G-Agarose beads (Santa Cruz Biotechnology) overnight. After washing in 0,1M NaCl; 0,05% NP-40; 50 mM Tris-HCl, pH 8,0, the immune complexes were loaded on a 7.5% SDS-PAGE gel and blotted using the Bio-Rad system. The membrane was incubated overnight at 4°C with Vegfr3 antibody (rat-anti mouse, BD), 1/50 in blocking buffer. AF680 (Invitrogen) was used as secondary antibody. Gel was scanned on an Odyssey Infrared Imaging System (LI-COR Biosciences) using the 700-nm channel.

Flow cytometry

Organs were dissected in cold PBS. Heart, liver (mid lobe) and spleen were digested in a mixture of 0,2 mg/ml Collagenase P (Roche), 0,8 mg/ml Dispase II (Roche) and 0,2 mg/ml DNase (Roche) in RPMI medium (Gibco). Ear skins were digested in Collagenase IV (Life technologies) 10 mg/ml and DNase (Roche) 0,2 mg/ml in PBS. Pancreas was digested in Collagenase A (Roche) in PBS. All digests were incubated for 20-40 min at 37°C, depending on the tissue, quenched by adding 2 mM EDTA

and filtered through a 70 μ m nylon filter (BD Biosciences). Cells were washed with FACS buffer (PBS, 0.5% FBS, 2 mM EDTA) and immediately processed for staining in 96 well plates. Fc receptor binding was blocked with rat-anti mouse CD16/CD32 (93), (eBiosciences). Samples were thereafter stained with anti-Podoplanin e660 and anti-CD31/Pecam-1 (390) PE-Cy7, CD45 (30-F11) PERCP-Cy5.5 and anti-CD11b (M1/70) PERCP-Cy5.5 (eBiosciences). Erythrocytes were excluded using anti-TER-119 (TER-119) e450, together with the cell death dye Sytox blue (Life technologies), both detected by the violet laser as one dump channel. For compensation, the AbC anti-rat/hamster compensation bead kit (Life Technologies) was used. Cells were analyzed on a FACSaria cell sorter (BD biosciences). Data were processed using FlowJo software (TreeStar). Single cells were gated using FSC-H/FSC-W and SSC-H/SSC-W followed by exclusion of dead cells and erythrocytes in the violet dump channel (data not shown). For FACS staining of cultured primary LECs, cells were isolated and cultured as described above. Cre recombination was induced for 72h with 1 μ M 4OHT (Sigma) supplemented to the culture medium. Recombined primary lymphatic endothelial cells were detached with Accutase (Sigma) for 10 min at 37°C. After centrifugation cells were stained with the Foxp3 Staining Buffer Set according to the manufacturer's protocol (eBiosciences). Cells were stained with goat anti-Prox1 (R&D), rabbit anti-GFP (Invitrogen, A11122), biotinylated mouse anti-VEGFR2 (eBiosciences) and secondary antibodies: donkey anti-goat Alexa647, donkey anti-rabbit Alexa488 (both Jackson ImmunoResearch) and Streptavidin PerCP-Cy5.5 (ebiosciences). Cells were analyzed on a FACSaria cell sorter (BD Biosciences). Data were processed using FlowJo software (TreeStar).

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Figure legends

Figure 1: Generation of the *Vegfr3-CreER^{T2}* allele.

a) Schematic of the gene targeting strategy used for the generation of *Vegfr3-CreER^{T2}* mice. The *Vegfr3* gene, *Vegfr3*-targeting vector and the recombinant *Vegfr3-neo-CreER^{T2}* allele, before and after Flpe-mediated excision of the PGK-neo cassette, are shown. Black boxes: coding exons; grey box: 3'untranslated region (3'-UTR); black triangles: frt sites; black arrows and arrow heads: position of primers used for PCR analysis of neo-cassette excision after Flpe mediated recombination; P1 and P2: position of the probes used for screening of recombinant clones by Southern Blot. **b)** Southern Blot analysis of recombinant clones. The ScaI restriction enzyme was used for digestion of clone genomic DNA. The expected size of the bands is indicated for each allele after probing with external 5'(P1) and 3'(P2) probes. Wild type (+/+), recombinant (+/rec) **c)** PCR analysis of PGK-neo cassette excision after Flpe-mediated recombination with primers shown in a). **d)** Northern Blot and **e)** Western Blott analysis of *Vegfr3* expression in *Vegfr3-CreER^{T2}* heterozygous (+/KI) or homozygous (KI/KI) E13.5 embryos. For Northern Blot (d) a probe homologous to the 3'-UTR region of the *Vegfr3* gene was used. Size of the wild type and KI mRNAs is indicated. *Gapdh* mRNA levels are shown as loading control. The Western Blot (e) shows Vegfr3 protein (125 Kd) after immunoprecipitation with Vegfr3-specific antibodies of embryo total protein extracts.

Figure 2: *Vegfr3-CreER^{T2}* induction in embryonic development.

a) Schematic of the *Vegfr3-CreER^{T2}* and the *R26-LSL-lacZ* and *R26-mTmG* reporter alleles used in this work. **b)** Whole mount Lacz staining of an E9.5 embryo treated with TAM at E8.5 (3mg single IP injection to the pregnant mother). Blue staining indicates Cre activity. **c)** Immunofluorescence of a transverse vibratome section of an E13.5 embryo treated with 4OHT at E10.5. Note the GFP⁺ lymph sac (LS), identified by Lyve1⁺ and Endomucin⁻ staining. The position of the Endomucin⁺ and Lyve1⁻ cardinal vein (CV) is shown. White arrows indicate recombination in BECs (GFP⁺, Endomucin⁺, Lyve1⁻). **d)** Whole mount GFP/immunofluorescence analysis of mesenteric vessels after induction with 4OHT at E10.5-13.5. Note that the lymphatic vessels (Nrp2⁺ single channel, Endomucin⁻) are highly recombined (GFP⁺). **e)** Whole mount GFP/immunofluorescence of dermal lymphatic vessels after the same induction scheme as in d). High level of recombination (GFP⁺) is observed in Prox1⁺ or Nrp2⁺, lymphatic vessels (single channels). Valves in lymphatic vessels are also

highly recombined (arrowheads). Blood vessels in d) and e) show some Nrp2⁺ staining but are mostly not recombined at this stage. However, some recombination is also observed in BECs (GFP⁺, Prox1⁻) (white arrows at E14.5). All embryos are heterozygous for both the *Vegfr3-CreER^{T2}* and the reporter alleles. Scale bars: 100 μ m (c and d); 200 μ m (e).

Figure 3: Postnatal *Vegfr3-CreER^{T2}* induction.

a) Whole mount X-Gal staining of P21 *Vegfr3-CreER^{T2};R26-LSL-lacZ* diaphragm, peritoneum and mesentery after induction with TAM-diet given to the lactating mother from P0 to P21. Efficient recombination is observed in lymphatic vessels but not in blood vessels, identified by their erythrocyte content (white arrows) **b)** Fluorescence images of diaphragm, heart and mesentery of P6 *Vegfr3-CreER^{T2};R26-mTmG* mice induced by 2 IP injections (at P1 and P2) of 50 μ g of 4OHT. After induction, GFP⁺ lymphatic vessels are easily identified in all the organs examined without staining for specific lymphatic markers. Asterisks indicate valves. **c)** GFP/immunofluorescence imaging of P6 mesenteric vessels co-stained with Nrp2 and Pecam1. GFP is restricted to Nrp2⁺ lymphatic vessels (single channel). No recombination was observed in blood endothelial cells (Pecam⁺). White arrows point to vein and artery **d)** GFP/immunofluorescence of dermal lymphatic vessels analyzed at P21 after the same induction scheme as in b. A very efficient recombination of the Lyve1⁺ capillary lymphatic network is observed. GFP and Lyve1 are shown also as single channels. All panels in this figure correspond to double heterozygous animals. Scale bars: 500 μ m (a), 250 μ m (b), 200 μ m (c-d).

Figure 4: *Vegfr3-CreER^{T2}* induction in adult mice: *CreER^{T2}* dose effect.

a) Whole mount (diaphragm and peritoneum) or frozen sections from different organs from *Vegfr3-CreER^{T2}; R26-LSL-lacZ* mice, stained for *LacZ* expression. A direct comparison of heterozygous and homozygous mice for the Cre allele is shown for all the organs and tissues analysed. All mice were treated during 3 weeks with TAM-diet starting at 4 weeks of age (postnatal week 4 or PW4) and analysed at PW8. Red arrows in whole mount images point to some blood capillaries and blood endothelial cells that are also targeted in homozygous animals **b)** Whole mount immunofluorescence of dermal ear lymphatic vessels from *Vegfr3-CreER^{T2};R26-mTmG* mice, treated at 4 weeks of age with a single topical application of 25 μ g of TAM in the ear. One week after the treatment, all the Podoplanin⁺ lymphatic vessels in the ear were recombined (GFP single channel). **c-d)** Fluorescence-activated cell sorting (FACS) analysis of Cre-mediated recombination in endothelial cells (ECs) and immune cells from *Vegfr3-CreER^{T2};R26-mTmG* heterozygous mice treated at PW4 with 1mg of Tamoxifen/day during 5 days and analysed at PW6. Representative FACS plots and gating scheme are shown for skin analysis (c). Quantification of Cre-mediated recombination in different organs (d). Dots show % of GFP⁺ cells in individual mice. Horizontal lines represent mean (n=3). PH (Pecam1 High), PI (Pecam1 Intermediate). Scale bars: 100 μ m (a, frozen sections), 500 μ m (a, whole mount), 200 μ m (b).

Figure 5: Characterization of *Vegfr3-CreER^{T2};R26-mTmG* primary LECs

a) Fluorescence-activated cell sorting (FACS) analysis of homozygous *Vegfr3-CreER^{T2};R26-mTmG* primary LECs showing a 97.2% of purity based on Vegfr2⁺ and Prox1⁺ labelling. The right-most plot shows the recombination efficiency (GFP⁺ cells) after 72 hours of 4OHT treatment of the primary LECs **b)** VE-cadherin and Prox1

immunostaining of *Vegfr3-CreER^{T2};R26-mTmG* homozygous primary lymphatic endothelial cells to confirm lymphatic identity. c) GFP/immunofluorescence analysis of Cre-activity in homozygous *Vegfr3-CreER^{T2};R26-mTmG* primary LECs. Efficient recombination (GFP⁺) is observed after 12 hours of 4-OHT treatment. No GFP signal was detected when cells were treated with vehicle for 48 hours. Scale bars: 100 μ m (b), 50 μ m (c).

Supplementary Figure Legends

Supplementary Figure 1: Effect of prolonged TAM-diet in *Vegfr3-CreER^{T2};R26-LSL-LacZ* heterozygous mice.

a) Whole mount (diaphragm and peritoneum) or frozen sections from different organs from *Vegfr3-CreER^{T2}; R26-LSL-lacZ* mice, stained with X-Gal for *LacZ* expression. Mice were treated during 3, 5 or 7 consecutive weeks with TAM-diet, starting at 4 weeks of age. Scale bars: 100 μ m (Frozen sections), 1mm (whole mount).

Supplementary Figure 2: Quantification of postnatal *Vegfr3-CreER^{T2}, R26-mTmG* induction by FACS

Fluorescence-activated cell sorting (FACS) analysis of endothelial cells (ECs) from *Vegfr3-CreER^{T2};R26-mTmG* double heterozygous mice showing Cre-mediated recombination in lymphatic endothelial cells (LECs), blood endothelial cells (BECs) and immune cells in the different analysed organs. Tamoxifen treatment is indicated. FACS plots and gating scheme are shown for all the organs analysed. A summary of all these data is shown in Figure 4c.

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