

Distinct vascular endothelial growth factor signals for lymphatic vessel enlargement and sprouting

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Lymphatic vessel growth, or lymphangiogenesis, is regulated by vascular endothelial growth factor-C (VEGF-C) and -D via VEGF receptor 3 (VEGFR-3). Recent studies suggest that VEGF, which does not bind to VEGFR-3, can also induce lymphangiogenesis through unknown mechanisms. To dissect the receptor pathway that triggers VEGFR-3-independent lymphangiogenesis, we used both transgenic and adenoviral overexpression of placenta growth factor (PIGF) and VEGF-E, which are specific activators of VEGFR-1 and -2, respectively. Unlike PIGF, VEGF-E induced circumferential lymphatic vessel hyperplasia, but essentially no new vessel sprouting, when transduced into mouse skin via adenoviral vectors. This effect was not inhibited by blocking VEGF-C and -D. Postnatal lymphatic hyperplasia, without increased density of lymphatic vessels, was also detected in transgenic mice expressing VEGF-E in the skin, but not in mice expressing PIGF. Surprisingly, VEGF-E induced lymphatic hyperplasia postnatally, and it did not rescue the loss of lymphatic vessels in transgenic embryos where VEGF-C and VEGF-D were blocked. Our data suggests that VEGFR-2 signals promote lymphatic vessel enlargement, but unlike in the blood vessels, are not involved in vessel sprouting to generate new lymphatic vessels in vivo.

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Abbreviations used: E, embryonic day; P, postnatal day; PAE, porcine aortic endothelial; PECAM, platelet endothelial cell adhesion molecule; PIGF, placenta growth factor; SMA, smooth muscle α -actin; SMC, smooth muscle cell; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

The discovery of vascular endothelial growth factors (VEGFs) and their receptors has facilitated the understanding of the development and function of the vasculature (1–3). Each VEGF family member appears to have a specific function. Whereas VEGF induces angiogenesis, i.e., growth of new blood vessels from preexisting ones, placenta growth factor (PIGF) mediates both angiogenesis and arteriogenesis, i.e., the formation of collateral arteries from preexisting arterioles (1, 2). VEGF-C and -D are primarily lymphangiogenic factors, which can also trigger angiogenesis in some conditions (3). Overall, the members of the VEGF

family and their receptors appear to provide promising and versatile tools for therapeutic manipulation of the vascular system (1–3).

VEGF is one of the most important regulators of both physiological and pathological angiogenesis, and its activity is mediated via VEGF receptor (VEGFR) 1 and 2. VEGF, acting mainly via VEGFR-2, is an endothelial cell mitogen, motogen, chemoattractant, and survival factor that increases the permeability of blood vessels (1). The importance of VEGF in the development of the vascular and hematopoietic systems is exemplified by the fact that inactivation of even one *VEGF* allele leads to early embryonic lethality caused by defects in angiogenesis and hematopoiesis (4, 5).

Genes with sequence homology to VEGF have been discovered in Orf and pseudocowpox

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viruses. These virus -encoded VEGFs, which are commonly called VEGF-E, cause highly vascularized and pustular dermatitis in sheep, in goats, and, occasionally, in humans (6–8). The virus-encoded VEGF-Es can be separated into two groups, with VEGF-E_{D1701} and -E_{NZ2} most closely related to VEGF and PlGF, whereas VEGF-E_{NZ7} is similar to VEGF-C and -D (9). In this article, VEGF-E refers to VEGF-E_{NZ7}. The virus-encoded VEGFs bind to VEGFR-2 and induce its autophosphorylation to almost the same extent as VEGF, but do not bind to VEGFR-1 (7, 8, 10). Although VEGF-E does not play a role in vascular physiology, it can be used as a VEGFR-2-specific agonist in experimental models of angiogenesis in vitro and in vivo. Such studies have indicated that VEGF-E expression in the skin of transgenic mice results in an angiogenic phenotype (11).

Recent studies have suggested that at least the most commonly expressed isoform of VEGF can also induce lymphatic hyperplasia (12, 13). However, the signaling mechanisms mediating this response have been unclear. In this study, we wanted to determine if signals mediated via VEGFR-1 or -2 can trigger lymphangiogenesis in embryonic or adult tissues. For our analysis, we used adenoviral transduction of VEGF-E, as well as transgenic overexpression of PlGF and VEGF-E, to activate VEGFR-1 and -2, respectively.

RESULTS

Characteristics of VEGF-induced lymphatic hyperplasia

VEGF-C has been shown to induce excessive sprouting of lymphatic vessels 4 d after adenoviral delivery and new

lymphatic vessels after 14 d (14, 15). To study the lymphatic vascular effects of VEGF, we transduced the ear skin of mice with AdVEGF164 or AdVEGF165, encoding the mouse and human isoforms, respectively, and performed whole-mount immunofluorescence analysis 4 d later. As a positive control for lymphangiogenesis, we used AdVEGF-C (Fig. 1 A). Consistent with the data of Nagy et al. (12), we detected enlarged lymphatic vessels and small lymphatic vessel sprouts in the skin of AdVEGF164-transduced ears (Fig. 1 B). In contrast, lymphatic hyperplasia, but no sprouting, was observed in the AdVEGF165-transduced ears (Fig. 1 C) compared with ears transduced with AdLacZ (Fig. 1 D). These experiments indicated that human VEGF165 is capable of stimulating only circumferential lymphatic vessel growth, whereas mouse VEGF164 induced some lymphatic vessel sprouting as well. This difference between the two factors was not caused by an increased inflammatory response, as we detected similar numbers of CD11b+ inflammatory cells in the AdVEGF164- and AdVEGF165-transduced ears (unpublished data).

Extension and maintenance of tip cell filopodia in the blood vascular endothelium has been shown to depend on VEGF signaling via VEGFR-2 (16). To determine if VEGFR-2 is also needed for the sprouting of lymphatic vessels in adults, we first injected mice intraperitoneally with blocking monoclonal antibodies against VEGFR-2 or -3, and then stimulated sprouting of the lymphatic vessels with AdVEGF-C, AdVEGF164, or AdVEGF165. Quantification

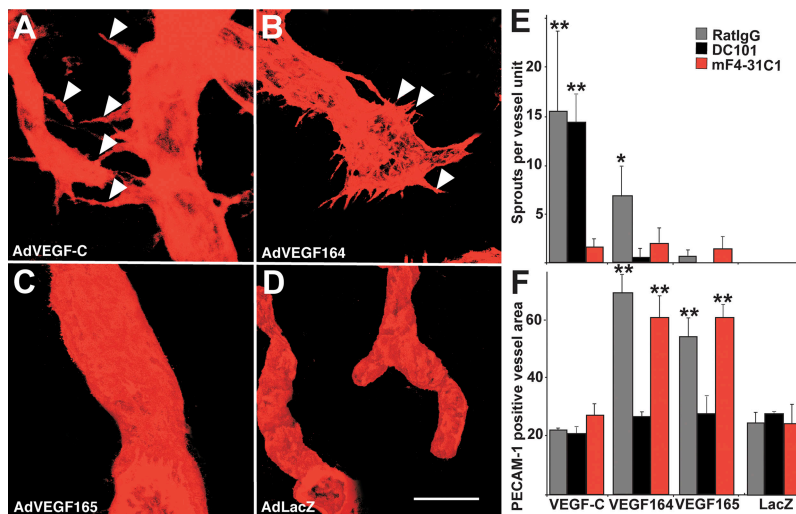


Figure 1. VEGF- and receptor-specific signals for lymphatic hyperplasia and sprouting. Whole-mount staining for LYVE-1 of indicated adenovirus vector-transduced ears. For comparison, AdVEGF-C-induced active sprouting and enlargement of the lymphatic vessels (A). Ad-VEGF164 also induces lymphatic sprouting (B), but AdVEGF165-transduced ears show only circumferential growth of the lymphatic vessels (C) in comparison with AdLacZ-transduced ears (D). Arrowheads indicate sites of sprouting. Quantitation of the number of lymphatic sprouts in the indicated adenovirus vector-transduced ears (E). Note that sprouting of lymphatic vessels after VEGF-C transduction is blocked by intraperitoneally

injected blocking antibodies against VEGFR-3 (mF4-31C1, red bars), but not by antibodies against VEGFR-2 (DC101, black bars). The VEGF164-induced sprouting is blocked by intraperitoneally injected blocking antibodies both against VEGFR-2 (DC101) and -3 (mF4-31C1). No sprouting can be detected after VEGF165 or LacZ transduction with or without antibody treatment. Quantitation of the PECAM-1-positive vessel density in the indicated adenovirus vector-transduced ears (F). The asterisks indicate significantly increased amounts of lymphatic sprouts (E) and significantly increased vessel density areas (F). *, P < 0.05; **, P < 0.01. Results represent the means ± the SD of six mice per group. Bar, 150 μm.

of lymphatic sprouting showed that the sprouts induced by AdVEGF-C were not inhibited by nonspecific rat IgG or antibodies against VEGFR-2 (DC101; Fig. 1 E), but were strongly inhibited (16-fold) by antibodies against VEGFR-3 (mF4-31C1). Both antibodies blocked the considerably weaker sprouting induced by AdVEGF164, whereas essentially no sprouting was obtained after AdVEGF165 or AdLacZ transduction. Quantification of the platelet endothelial cell adhesion molecule (PECAM) 1-positive vessel density in the AdVEGF164- and AdVEGF165-transduced ears indicated that both VEGF vectors promoted angiogenesis (Fig. 1 F). In contrast, AdVEGF-C or AdLacZ had very little or no effect on the blood vessels. Collectively, these results suggested that VEGFR-2 signaling may not be necessary for the sprouting of lymphatic vessels in adults. To study this further, we chose to overexpress VEGF-E in the skin.

VEGF-E_{NZ7} does not act via the VEGFR-3 pathway

VEGF-E has been shown to bind to VEGFR-2, but not to VEGFR-3, in stimulated cells (7, 16). To ensure that VEGF-E is not able to induce phosphorylation of VEGFR-3 in endothelial cells, we stimulated porcine aortic endothelial (PAE) cells stably expressing either VEGFR-2 or -3 with increasing amounts of recombinant VEGF-E and analyzed receptor phosphorylation. Although VEGF-E induced VEGFR-2 phosphorylation at low concentrations, it was not able to induce phosphorylation of VEGFR-3, even at high concentrations (Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20062642/DC1>). Furthermore, soluble VEGFR-2-Ig fusion proteins or rat monoclonal antibodies against human VEGFR-2 inhibited VEGFR-2 stimulation by VEGF-E, whereas VEGFR-3-Ig did not. In contrast, all three reagents blocked VEGFR-2 stimulation by VEGF-C

(Fig. S1 B). These results indicate that VEGF-E does not interact with the VEGFR-3 signal transduction pathway *in vitro*.

Adenovirally transduced VEGF-E induces lymphatic hyperplasia *in vivo*

To determine if the VEGFR-2 transduction pathway can trigger lymphatic sprouting, we expressed an adenovirus encoding VEGF-E in mouse skin and studied the lymphatic vessels by whole-mount immunofluorescence analysis 4 d later. We detected enlarged lymphatic vessels, but no lymphatic vessel sprouts in the skin of AdVEGF-E-transduced ears (Fig. 2 A, red signal, and 2 K) compared with ears transduced with AdLacZ (Fig. 2 B, red signal). Simultaneous PECAM-1 staining of the blood vessels showed excessive angiogenesis in AdVEGF-E-transduced ears (Fig. 2 A, green signal) whereas AdLacZ had essentially no effect on the blood vasculature (Fig. 2 B, green signal).

To determine if the effects of VEGF-E on the lymphatic endothelium indirectly involved the VEGFR-3 ligand receptor system, we injected mice with AdVEGF-3-Ig, expressing a VEGF-C and -D “trap” (17). As shown in Fig. 2 C, the hyperplasia of lymphatic vessels induced by AdVEGF-E was not inhibited by systemic VEGFR-3-Ig delivery. Furthermore, this treatment did not inhibit the lymphatic vessel hyperplasia induced by AdVEGF165, whereas it substantially decreased lymphangiogenesis induced by AdVEGF-C treatment. However, the lymphatic area density in the VEGF-E-transduced ears was considerably decreased by anti-VEGFR-2 treatment (Fig. 2, D, E, and G), but not by anti-VEGFR-3 treatment (Fig. 2, F and G). Also, the AdVEGF-C-induced lymphatic vessel sprouting was inhibited in this experiment only by anti-VEGFR-3 blocking antibodies (Fig. 2, H–K).

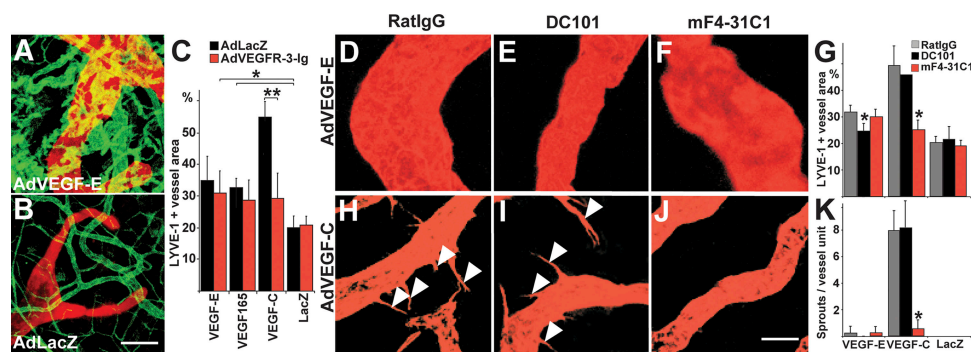


Figure 2. VEGFR-2 is not involved in lymphatic vessel sprouting.

Whole-mount staining for LYVE-1 (red) and PECAM-1 (green) of indicated adenovirus vector-transduced ears. Note that AdVEGF-E-transduced ears show circumferential growth of the lymphatic vessels and a strong angiogenic effect in the blood vessels (A) compared with AdLacZ-transduced ears (B). (C) Quantitation of the LYVE-1-positive vessel density in the indicated adenovirus vector-transduced ears. No sprouting can be detected after VEGF-E transduction with or without antibody treatment, but blocking antibodies against VEGFR-2 (DC101) reduces the hyperplasia of the lymphatic vessels (D–F). Sprouting of lymphatic vessels can be detected

after VEGF-C transduction (H). The sprouting is not blocked by intraperitoneally injected blocking antibodies against VEGFR-2 (DC101) (I), but is blocked by intraperitoneally injected blocking antibodies against VEGFR-3 (mF4-31C1) (J). Arrowheads indicate sprouts. Quantitation of the LYVE-1-positive vessel density (G) and the number of lymphatic sprouts (K) in the indicated adenovirus vector-transduced ears. The asterisks indicate significant differences in vessel density area (C and G) or the amount of lymphatic sprouts (K). *, $P < 0.05$; **, $P < 0.01$. Results represent the means \pm the SD of six mice per group. Bars: (A and B) 300 μ m; (D–F and H–J) 150 μ m.

Expression of VEGFR-2 and -3 in lymphatic capillaries and collecting vessels

Under normal conditions, VEGFR-3 is expressed strongly in lymphatic capillaries, whereas VEGFR-2 is expressed predominantly in blood vessels, but also, to a smaller extent, in lymphatic vessels. VEGFR-2 was prominently expressed, particularly in lymphatic valves, as seen in Fig. 3. AdVEGF-C transduction induced enlargement of the collecting lymphatic vessels and disrupted the typical morphology of the valve leaflets (Fig. 3, A–F). In contrast, AdVEGF-E transduction did not have major effects on the collecting lymphatic vessels or their valves (Fig. 3, G–L). In high magnification, VEGFR-2 internalization could be seen after AdVEGF-C and -E transduction, but not after AdLacZ transduction (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20062642/DC1>, and not depicted). VEGFR-3 was prominently expressed, particularly in the tips of lymphatic sprouts, and was internalized in the lymphatic capillaries after AdVEGF-C transduction (Fig. S2, A–C, and not depicted), but not after AdVEGF-E or AdLacZ transduction (Fig. S3, G–I and M–O). This result confirmed that VEGF-E does not interact with the VEGFR-3 signal transduction pathway *in vivo*, and that the effect of VEGF-E on the lymphatic capillaries is, at least in part, directly mediated via VEGFR-2.

K14-VEGF-E transgenic mice have hyperplastic lymphatic vessels in the skin

To study the role of VEGFR-2 signaling in lymphangiogenesis under conditions not involving an inflammatory response to the adenovirus and minor tissue injury caused by vector injection, we next analyzed transgenic mice that express VEGF-E driven by the keratin 14 (K14) promoter in basal epidermal cells (11). These mice were shown to exhibit a substantial increase in the blood vessel density of their skin.

We found by whole-mount staining of lymphatic vessels that the transgenic mice also have enlarged cutaneous lymphatic capillaries when compared with wild-type mice (Fig. 4, A and F). The K14-VEGF-E lymphatic vessels absorbed fluorescent dextran from sites of intradermal injection in fluorescent lymphangiography, indicating that they were functional (Fig. S3, A and B). In contrast to the capillaries, the collecting lymphatic vessels or vessels in the lymph nodes were not hyperplastic in the K14-VEGF-E mice (Fig. S3, A–F). Shown for comparison are lymphatic vessels induced by the VEGFR-3-specific ligand VEGF-C156S (18), which is expressed under the same K14-promoter (Fig. 4 B) (19). It is notable that the lymphatic vessels in K14-VEGF-C156S mice were mostly located around the hair follicles that produce high levels of the transgene-encoded protein, whereas no such relationship was apparent in the hyperplastic lymphatic vessels of the K14-VEGF-E mice. In contrast, the lymphatic vessels in the skin of the K14-PIGF mice were similar to those of littermate wild-type mice (Fig. 4, C and F).

PIGF coexpression inhibits VEGF-E-induced lymphatic hyperplasia

Surprisingly, when we crossed the K14-VEGF-E mice with K14-PIGF mice to simultaneously activate VEGFR-1 and -2 receptor homodimers, we found that the lymphatic hyperplasia induced by VEGF-E was inhibited (Fig. 4 D). Quantification of LYVE-1-positive vessel area confirmed that the lymphatic vessels of the compound K14-VEGF-E + K14-PIGF transgenic mice were considerably less hyperplastic than those of K14-VEGF-E mice (Fig. 4 G). Furthermore, when adenoviruses expressing AdPIGF and AdVEGF-E were injected separately into the ear skin of nude mice, the VEGF-E-induced lymphatic hyperplasia was partially halted (unpublished data). As the adenoviral vectors in these conditions

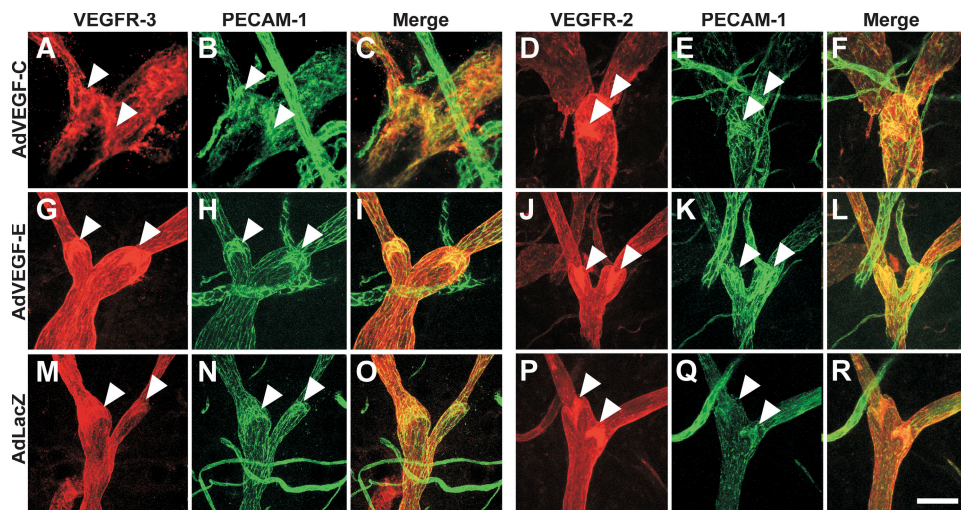


Figure 3. VEGFR-2 and -3 are expressed in the collecting lymphatic vessels and in the valves. Whole-mount stainings for VEGFR-2 or -3 (red) and PECAM-1 (green) of the indicated adenovirus vector-transduced ears. Note that AdVEGF-C transduction enlarges the collecting

lymphatic vessels (and stimulates internalization of VEGFR-3; A–F and not depicted), whereas AdVEGF-E transduction does not have any effect on the collecting vessels or the valves (G–L) compared with the AdLacZ-transduced ears (M–R). The arrowheads indicate lymphatic valves. Bar, 150 μ m.

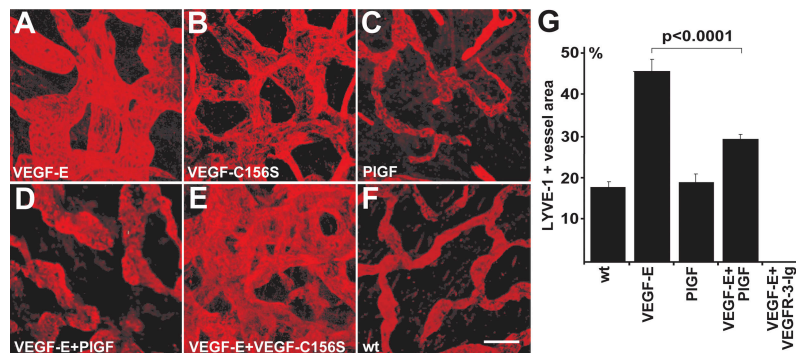


Figure 4. VEGF-E induces lymphatic vessel hyperplasia in the skin of transgenic mice. Whole-mount staining for LYVE-1 in the ears of K14-transgenic mice expressing the indicated growth factors and wild-type

mice (wt; A–F). Quantitation of the LYVE-1–positive vessel density in the ears of the transgenic mice (G). Results represent the means \pm the SD of six mice per group. Bar, 150 μ m.

transduced predominantly nonoverlapping cell populations in the skin (unpublished data), the inhibition of lymphangiogenesis by PIGF was not dependent on heterodimer formation between PIGF and VEGF-E. In contrast, additional hyperplasia was seen in the lymphatic vessels of the compound K14-VEGF-E + K14-VEGF-C156S transgenic mice that should activate both VEGFR-2 and -3 receptor homodimers (Fig. 4 E). No considerable differences were detected in the accumulation of CD11b–positive inflammatory cells in the skin of K14-VEGF-E mice in comparison to K14-PIGF mice, which is consistent with the idea that the lymphatic phenotype in the K14-VEGF-E mice is not caused by infiltration of inflammatory cells producing VEGF-C or -D (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20062642/DC1>).

VEGF-E cannot rescue lymphatic regression induced by blocking VEGF-C and -D

VEGFR-3-Ig was previously shown to inhibit embryonic and tumor lymphangiogenesis (17, 20). The lymphatic vessels of K14-VEGFR-3-Ig mice regress after the onset of K14 promoter expression, and the mice lack cutaneous lymphatic vessels (17). To study if the lack of lymphatic vessels in these mice could be rescued by a VEGFR-2 ligand, we crossed the K14-VEGF-E mice with the K14-VEGFR-3-Ig mice. However, no lymphatic vessels were detected in the skin of the compound K14-VEGF-E + K14-VEGFR-3-Ig mice (Fig. 4 G and not depicted), which indicated that VEGFR-2 signals cannot rescue the lymphatic phenotype caused by lack of VEGFR-3 signals.

Angiogenesis and lymphangiogenesis in K14-VEGF-E mice occur during the first postnatal weeks

To investigate the lymphatic and blood vascular effects of VEGF-E during embryonic development, the back skin of staged compound heterozygotic K14-VEGF-E + VEGFR-3^{+/*LacZ*} and K14-VEGF-E + VEGFR-2^{+/*LacZ*} embryos was stained for β -galactosidase activity to visualize lymphatic and blood vessels. To our surprise, no differences could be found in

the lymphatic and blood vessel staining patterns or in lymphatic sprouting in comparison with the VEGFR-3^{+/*LacZ*} and -2^{+/*LacZ*} embryos at embryonic day (E) 17.5 (Fig. 5, A–D, arrowheads). RT-PCR of RNA extracted from the skin of staged K14-VEGF-E embryos indicated that VEGF-E is already expressed at E14.5 (unpublished data). This indicated that K14-VEGF-E had essentially no effect on either the lymphatic or the blood vasculature during embryonic development.

To analyze the lymphatic vessel density in the K14-VEGF-E mice, we counted the number of LYVE-1–positive lymphatic vessels in histological sections from 5–7-wk-old adult mice as well as from 1-, 7-, and 14-d-old pups. The number of lymphatic vessels was not increased in the K14-VEGF-E mice at any of these time points (unpublished data). Also, no considerable differences in the LYVE-1–positive lymphatic vessel area were detected between the K14-VEGF-E and wild-type littermate mice on postnatal day (P) 1 or P7 (Fig. 5 E). On day 14, however, the lymphatic vessel area was 2.2- \pm 0.2-fold greater in K14-VEGF-E mice than in the wild-type littermates, and this difference was further increased to 3.6- \pm 0.2-fold in the adult mice. Quantitative RT-PCR of RNA extracted from the back skin at day 7 showed no differences in the amounts of either VEGF-C or -D expression between K14-VEGF-E pups and wild-type littermates (unpublished data), suggesting that these ligands do not mediate the lymphatic phenotype postnatally.

VEGF-E stimulation of lymphatic endothelial cells in vitro has previously been shown to increase their proliferation (21). To investigate if overexpression of VEGF-E also increased proliferation of lymphatic endothelial cells in vivo, we injected the proliferation marker BrdU intraperitoneally into pregnant K14-VEGF-E or wild-type mice at E17.5, P7 pups, or adult mice. We then counted the number of BrdU + LYVE-1 double-positive cells from frozen skin sections. Although we could not detect any differences in the amount of double-positive BrdU/LYVE-1 cells between K14-VEGF-E embryos and wild-type embryos (Fig. 5 F), we observed a significant increase in BrdU/LYVE-1 double-positive cells in K14-VEGF-E pups at P7 compared with

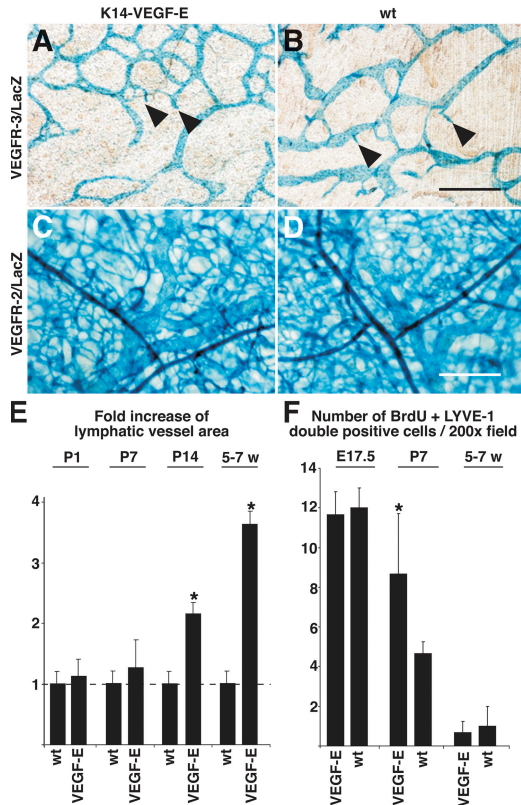


Figure 5. The lymphatic and blood vascular phenotypes in K14-VEGF-E mice occur postnatally. (A and B) Skin lymphatic vessels of K14-VEGF-E + VEGFR-3^{+LacZ} compound heterozygote and VEGFR-3^{+LacZ} (wt) embryos were analyzed by β -galactoside staining at E17.5. Note the similar degree of lymphatic vessel sprouting (arrowheads). Embryonic skin blood vessels of a K14-VEGF-E + VEGFR-2^{+LacZ} compound heterozygote and VEGFR-2^{+LacZ} mice were analyzed by β -galactoside staining at E17.5 (C and D). (E) Comparison of the lymphatic vessel areas of 1-, 7-, and 14-d-old pups and adult K14-VEGF-E mice. (F) Comparison of BrdU + LYVE-1 double-positive cells in E17.5 embryos, P7 pups, and adult K14-VEGF-E mice. The asterisks indicate significantly increased vessel density areas. *, $P < 0.05$. Results represent the means \pm the SD of six embryos/mice per group. Bars: (A and B) 500 μ m; (C and D) 200 μ m.

their wild-type littermates. Only a few double-positive cells were stained per field in adult mice, and no differences between K14-VEGF-E and wild-type mice could be detected. These results indicate that the proliferation of lymphatic endothelial cells is increased in the K14-VEGF-E mice compared with the wild-type littermates, but that this difference is restricted to the postnatal period, when the lymphatic vessels are still actively growing.

Angiogenesis induced by VEGFR-2 stimulation lags behind that induced by VEGFR-1 stimulation

We also analyzed the blood vasculature from histological sections of 1-, 7-, and 14-d-old pups stained for PECAM-1 (Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20062642/DC1>). At day 1, the blood vessel area in K14-VEGF-E mice was not substantially increased as compared

with wild-type littermates, whereas the blood vessel area was increased by 3.2 ± 0.3 -fold in K14-PIGF mice and by 3.5 ± 0.2 -fold in K14-VEGF-E + K14-PIGF mice. On P7 and P14, the blood vessel area in K14-VEGF-E mice was increased by 3.5 ± 0.2 -fold and 3.6 ± 0.1 -fold, respectively, compared with wild-type littermates. This indicates that whereas newborn K14-PIGF mice already have blood vessel hyperplasia, the angiogenic effect of K14-VEGF-E starts during the first postnatal week, and it may in fact precede the onset of lymphatic vessel hyperplasia.

Vessel leakage is inhibited in the double-transgenic K14-VEGF-E + K14-PIGF mice

Overexpression of VEGF-E via an adeno-associated virus vector promotes blood vessel leakage (22). K14-PIGF mice also show increased vessel permeability (23). As vessel leakage results in interstitial edema that may provoke lymphatic vessel enlargement, we wanted to study in more detail the relationship between edema and lymphatic hyperplasia in the transgenic mice. To compare vessel permeability in the transgenic mice, we injected high molecular weight FITC-dextran into the tail vein and monitored the efflux of the dye at different time points. FITC-dextran leaked out much more rapidly from the vessels of the K14-VEGF-E and K14-PIGF mice compared with the K14-VEGF-E + K14-PIGF mice (Fig. 6, A–D). We also injected Evans blue dye into the tail vein of the transgenic mice and their wild-type littermates and monitored its extravasation. Spectrophotometric quantification of the dye indicated that although both K14-VEGF-E and K14-PIGF exhibited increased vessel permeability, the K14-VEGF-E + K14-PIGF mice had comparatively reduced vessel permeability (Fig. 6 E). The results agreed with those from the FITC-dextran experiments, and they indicate that PIGF, possibly by stimulating VEGFR-1 homodimer formation, can at least partially block the increased capillary permeability induced by VEGF-E through VEGFR-2 homodimer formation.

Correlation of blood flow with vascular leakage in the transgenic mice

Using a laser Doppler flowmeter, we measured ear tissue blood flow in the transgenic mice at 5 wk of age. Both the K14-VEGF-E and K14-PIGF mice had considerable increased blood flow (fold increase of 3.5 ± 0.2 and 3.9 ± 0.1 , respectively) compared with the wild-type littermates (Fig. 6 F). To our surprise, the K14-VEGF-E + K14-PIGF mice showed only 2.0 ± 0.1 -fold increased blood flow compared with the wild-type littermates, even though these mice have an increased density of blood vessels compared with the single transgenic mice (Fig. S5 and not depicted).

To investigate if the decreased blood flow in K14-VEGF-E + K14-PIGF mice was correlated with an increase in the smooth muscle cell (SMC) coating around the blood vessels, we measured the ratio of smooth muscle α -actin (SMA)-positive cells to PECAM-1 positive cells from frozen sections of the skin. As shown by representative images and ratio quantification, the SMC layer around the blood vessels in

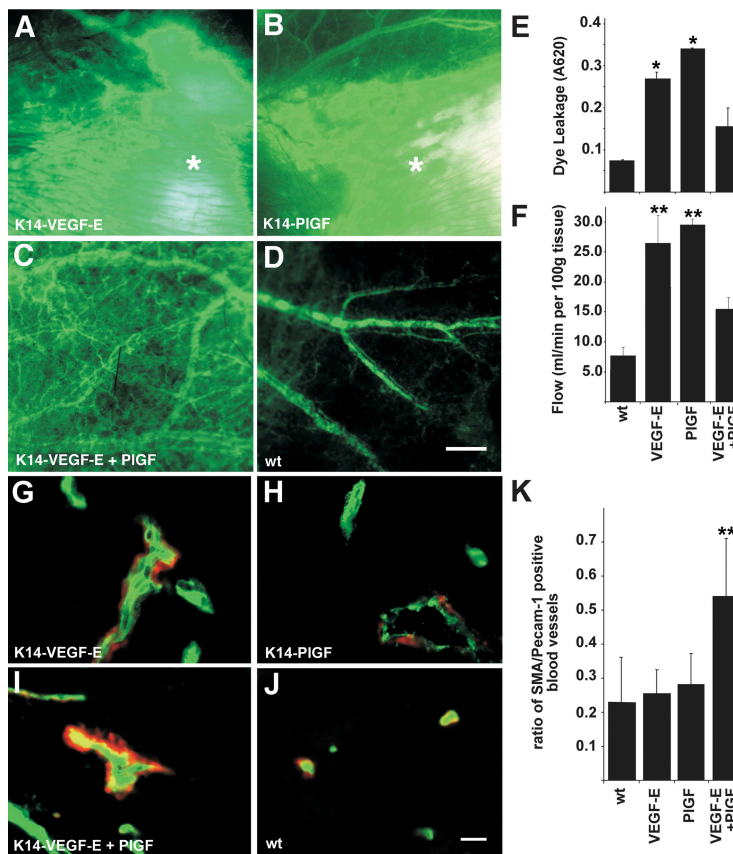


Figure 6. Vessel leakage is inhibited in double-transgenic K14-VEGF-E + K14-PIGF mice. Immunofluorescent images of mouse ears 10 min after FITC-dextran injection (A–D). Both K14-VEGF-E (A) and K14-PIGF mice (B) display increased permeability (asterisk) and leakiness of the vessels in comparison to wild-type littermates (D). In the double K14-VEGF-E + K14-PIGF mice, much of the leakage is halted (C). Quantitation of blood vessel leakage 10 min after dye injection in the modified Evans blue permeability assay (E). Laser Doppler flowmetric analyses for blood

flow in the ears of the transgenic mice (F). Immunofluorescence staining for PECAM-1 (green) and SMA (red) in the skin of transgenic mice (G–J). Note the thickened SMC layer around the blood vessel in the K14-VEGF-E + K14-PIGF mice (I). Quantitation of the SMA/PECAM-1 ratio in the skin of transgenic mice (K). The asterisks indicate significantly increased vessel leakage (E), flow (F), or SMA/PECAM-1 ratio (K). *, $P < 0.05$; **, $P < 0.01$. Results represent the means \pm the SD of six mice per group. Bars: (A–D) 250 μ m; (G–J) 10 μ m.

K14-VEGF-E + K14-PIGF mice appeared to be denser than in single transgenic and wild-type mice (Fig. 6, G–K). This suggested that the K14-VEGF-E + K14-PIGF mice have a thicker SMC layer around the blood vessels, and hence decreased blood flow and capillary exchange rate compared with the single-transgenic K14-VEGF-E and K14-PIGF mice. In contrast, K14-VEGF165 mice did not show enhanced SMC coverage over that seen in wild-type littermates or K14-VEGF-E or K14-PIGF single transgenic mice (unpublished data). Because the effects of VEGF are mostly mediated by VEGFR-2, and because K14-VEGF165 mice have an enhanced vessel leakage, probably caused by a loosely attached SMC layer around the arteries (24), our results suggest that PIGF-induced VEGFR-1 homodimers play a role in the reduction of VEGF-E/VEGFR-2-mediated leakiness.

DISCUSSION

We have analyzed the roles of VEGF receptors in lymphangiogenesis by using adenoviral and transgenic overexpression

of VEGF family members in mouse skin, combined with the use of antibodies blocking specific VEGF receptors. In particular, overexpression of PIGF and VEGF-E allowed specific stimulation of VEGFR-1 and -2. Our data shows that stimulation of VEGFR-2 induces hyperplasia of the lymphatic vessels, but very few vessel sprouts, in postnatal mice. On the other hand, we could not detect any lymphatic phenotype when only the VEGFR-1 was activated by overexpression of PIGF in the skin. This indicates that the lymphatic vascular effects induced by overexpression of VEGF as seen by us and others (12, 13), are indeed mediated mainly via VEGFR-2.

VEGFR-2 expression has been shown to be high in the endothelial tip cells of blood vessels, which, through their filopodia, sense the VEGF gradient to guide blood vessel sprouting (16). We show that the sprouting mechanism of the lymphatic vessels was not dependent on VEGFR-2 in adults. Although the mouse VEGF isoform promoted some lymphatic vessel sprouting, the human isoform that binds to the

same receptors did not. However, both isoforms induced enlargement of lymphatic vessels and proliferation of blood vessels. Furthermore, overexpression of the VEGFR-2-specific ligand VEGF-E did not lead to sprouting of the lymphatic vessels, but instead to circumferential growth and the sprouting of the lymphatic vessels induced by adenoviral VEGF-C, which could not be blocked with neutralizing antibodies against VEGFR-2. These results suggest that the sprouting mechanisms of lymphatic vessels in adults do not rely on VEGFR-2, and thus differ from those of the blood vessels.

Surprisingly, VEGF-E did not induce changes in the lymphatic vasculature during embryonic development. Although VEGF-E stimulated lymphatic vessel enlargement in postnatal mice, we did not detect increased numbers of lymphatic vessels in the K14-VEGF-E mice at any age analyzed. This indicates that signaling through VEGFR-2 does not induce lymphangiogenesis during embryonic development, but its effects become apparent during the postnatal period upon maturation of the blood and lymphatic vessels. As with the adenovirus vectors, we also detected circumferential lymphatic vessel hyperplasia without additional sprouting in the transgenic mice after 1 wk of postnatal growth. One possibility was that at least some of the hyperplasia was a secondary effect resulting from the vascular leakage promoted by VEGF-E overexpression. The lymphatic hyperplasia started after the effects of VEGF-E in the blood vessels became apparent, and both the vascular leakage and the lymphatic hyperplasia could be reduced by PlGF overexpression that apparently reduced blood flow via the increased coating of small vessels by SMCs. However, from additional experiments, we learned that vascular leakage alone was not sufficient for the hyperplasia, as the lymphatic vessels of the K14-PlGF mice appeared to be normal, although their blood vessels were even leakier than in the K14-VEGF-E mice. We cannot currently explain the late onset of the effects of the K14-VEGF-E transgene, despite its high-level expression starting during the embryonic period. It may be speculated that *in vivo* particular pericellular matrix proteins and integrins are required for specific VEGFR-2 activation by VEGF-E, and that they are not present until later in development. Importantly, we also found that adenoviral coexpression of PlGF inhibits VEGF-E-induced, but not VEGF-C156S-induced, lymphatic hyperplasia (this study and unpublished data).

We also found that VEGF-E overexpression was not able to rescue the lymphatic regression induced by blocking VEGF-C and -D (the VEGF-C/D trap). This result indicates that VEGFR-2 cannot substitute for VEGFR-3 signals during the onset of lymphangiogenesis. In accordance with these data, treatment with AdVEGFR-2-Ig does not affect the postnatal development of lymphatic vessels (25). VEGFR-3 was expressed strongly in lymphatic capillaries, and it was internalized into the lymphatic endothelial cells after adenoviral VEGF-C stimulation, but not after VEGF-E stimulation, indicating that VEGF-E does not lead to signaling via this receptor. VEGFR-2 was expressed weakly in

lymphatic capillaries and more strongly in collecting lymphatic vessels in wild-type mice. The fact that essentially no VEGF-E-induced changes were observed in the collecting lymphatic vessels or lymph nodes was surprising considering the strong constitutive VEGFR-2 expression in these vessels. However, VEGFR-2 was internalized in both types of vessels upon AdVEGF-E or -C transduction, indicating that the receptor was functional.

In conclusion, we show here that overexpression of a VEGFR-2-specific ligand induces circumferential hyperplasia of the lymphatic vessels in adult, but not in embryonic, skin. However, VEGFR-2 activation is not sufficient for the generation of new lymphatic vessels, and it was not able to rescue the lymphatic regression induced by blocking the VEGFR-3 ligands VEGF-C and -D. These results specify the contribution of the different VEGFR pathways to lymphangiogenesis and reveal previously unknown postnatal changes that occur in the sensitivity of both blood and lymphatic vessels toward VEGF family ligands.

MATERIALS AND METHODS

Cell stimulation and Western blot analysis. PAE cells stably overexpressing VEGFR-2 (26) or -3 (17) were starved overnight in serum-free medium and incubated for 20 min with soluble VEGFR-2-Ig or VEGFR-3-Ig (357-kD and 349-F4; R&D Systems) fusion proteins or rat monoclonal antibodies against human VEGFR-2 (1121B; a gift from B. Pytowski, ImClone Systems, New York, NY) at a concentration of 1 μ g/ml. The cells were stimulated for 10 min with recombinant VEGF-E (27) or -C (28) at the indicated concentrations, washed with PBS, and lysed in ice-cold PLCLB lysis buffer (150 mM NaCl, 5% glycerol, 1% Triton X-100, 1.5 M MgCl₂, and 50 mM Hepes, pH 7.5) containing 2 mM sodium vanadate, 2 mM PMSF, 10 μ g/ml leupeptin, and 0.07 U/ml aprotinin. Clarified lysates were immunoprecipitated with antibodies against 2 μ g/ml VEGFR-3 (9D9f9 [29]) or 2 μ g/ml VEGFR-2, separated by SDS-PAGE, and transferred to a nitrocellulose filter. Detection was performed with phosphotyrosine-specific antibodies (clone 4G10; Millipore) and an enhanced chemiluminescence detection system (Pierce Chemical Co.). Antibodies against VEGFR-2 (C-1158; Santa Cruz Biotechnology) or -3 were used for detection of VEGFR-2 and -3 in the Western blot, respectively.

Generation and *in vitro* analysis of recombinant adenoviruses. Full-length VEGF-E cDNA (GenBank accession no. AF106020) was cloned into the pAdapt vector, and the adenovirus was produced as previously described (30). The adenoviruses encoding VEGF164, VEGF165, VEGF-C, VEGFR-3-Ig, and nuclear-targeted β -galactosidase (*LacZ*) were constructed and produced as previously described (13, 30–32). Analysis of protein expression was carried out as previously reported (32).

***In vivo* use of the viral vectors.** 2.5×10^8 PFU of recombinant adenoviruses encoding VEGF-C, VEGF-E, VEGF164, VEGF165, or *LacZ* were injected intradermally into 4–6-wk-old female NMRI nu/nu mice (Harlan). The skin was analyzed 4 d later. For inhibition experiments, 10^9 PFU of VEGFR-3-Ig or *LacZ* adenoviruses were injected intravenously 3 d before the ligand-encoding adenoviruses.

For sprouting inhibition experiments, the mice were injected intraperitoneally with 600 μ g of mF4-31C1, which is a rat monoclonal antibody against mouse VEGFR-3 (33), DC101, which is a rat monoclonal antibody against mouse VEGFR-2 (34), or rat IgG every second day in a volume of 200 μ l. 1 d later, 2.5×10^8 PFU of recombinant adenoviruses encoding VEGF-C, VEGF-C156S, VEGF-E, or *LacZ* were injected intradermally into the ears of the same mice. The skin was analyzed 3–4 d later.

Transgenic mice. The K14-VEGF-E, K14-PlGF, K14-VEGF-C156S, K14-VEGF-C, K14-VEGFR-3-Ig, K14-VEGF165, VEGFR-3^{+/LacZ}, and VEGFR-2^{+/LacZ} mice were previously described (11, 17, 19, 23, 24, 35–37). The Provincial State Office of Southern Finland approved all experiments involving mice, and they were performed in accordance with institutional guidelines.

Analysis of lymphatic and blood vessels. Whole-mount staining was performed as previously published (15). Blood and lymphatic vessels were stained with rabbit antiserum against LYVE-1 (38), α -hamster monoclonal anti-mouse PECAM-1 antibody (clone 2H8, MAB-13982Z; CHEMICON International, Inc.), α -goat polyclonal anti-VEGFR-2 antibody (AF644; R&D Systems), or α -goat polyclonal anti-mouse VEGFR-3 antibody (AF743; R&D Systems), followed by appropriate fluorochrome-conjugated secondary antibody (Alexa Fluor 488 or 594 [Invitrogen] or FITC [Jackson ImmunoResearch Laboratories]). Frozen sections were fixed with -20°C acetone, incubated with anti-LYVE-1, the hamster monoclonal antibody against PECAM-1 (CHEMICON International, Inc.), a rat monoclonal antibody against CD11b (BD Biosciences), or mouse anti-SMA-Cy3 (Sigma-Aldrich), followed by the appropriate fluorochrome-conjugated secondary antibodies and analysis with a compound fluorescent microscope (Zeiss 2; Carl Zeiss MicroImaging, Inc.; 10 \times objective/NA 0.30), or a confocal microscope (Zeiss LSM 510; 20 \times objective/NA 1.3 and 40 \times objective/NA 1.4). Three-dimensional projections were digitally constructed from confocal z stacks.

Paraffin sections were stained with a rabbit antibody against LYVE-1 and a rat anti-mono-clonal antibody against PECAM-1 (BD Biosciences) using the tyramide signal amplification kit (PerkinElmer). Lymphangiography and visualization of blood vessels with FITC-dextran was performed as previously described (22, 32). Quantitation of the lymphatic sprouts and the area covered by lymphatic or blood vessels in the skin was performed as previously described (25). The area covered by lymphatic or blood vessels in the skin was quantified from photomicrographs of LYVE-1- or PECAM-1-stained sections (6 photomicrographs/mouse, and 6 mice of each genotype at each time point) using the Image-Pro Plus program (Media Cybernetics). All statistical analyses were performed using the unpaired Student's *t* test. A *P* value of <0.05 was considered statistically significant.

β -Galactosidase staining of vessels. Staged embryos of the compound K14-VEGF-E + VEGFR-2^{+/LacZ} and K14-VEGF-E + VEGFR-3^{+/LacZ} mice were dissected, fixed in 0.2% glutaraldehyde, and stained with X-gal (Sigma-Aldrich) for β -galactosidase activity at 37 $^{\circ}\text{C}$.

BrdU incorporation and immunostaining. 20 mM BrdU in PBS was injected intraperitoneally at a volume of 20 $\mu\text{l/g}$ into adult mice, P7 pups, or pregnant mice at E17.5. Mice were sacrificed 4 h later, and frozen sections from the skin were stained with a monoclonal rat anti-BrdU antibody (Abcam Ltd.).

RT-PCR. RNA extracted from the skin of staged K14-VEGF-E embryos and their wild-type littermates was reverse transcribed using oligo-dT (Boehringer) and Superscript II reverse transcriptase (Invitrogen). PCR analysis using a pair of primers specific for *VEGF-E* or β -*actin* primers was performed as previously published (22).

Quantitative RT-PCR. RNA was extracted from K14-VEGF-E and wild-type littermate pups at P7, and quantitative PCR reactions were performed as previously described (25) with the DyNAmo HS SYBR Green qPCR kit (Finnzymes) using the ABI 7500 SDS real-time PCR instrument (Applied Biosystems). The oligonucleotide primers used are as follows: 5'-CACAGTGCAGGCAGCTAAC-3' and 5'-TCCACAGACATCATGGAATC-3' for VEGF-C; and 5'-CTTGCTGGAACAGAAAGACCA-3' and 5'-CTCTGAGGACTGGAAGCTGT-3' for VEGF-D, as well as 5'-ACAACCTTGGCA-TTGTGGAA-3' and 5'-GATGCAGGGATGATGTTCTG-3' for GAPDH. The expression of the genes was normalized to GAPDH expression.

Measurement of vascular permeability and tissue blood flow. A modified Evans blue permeability assay was performed as previously described (32). Microcirculation was measured from the base of the ear by a HL-N1451 Flowprobe (Transonic Systems, Inc.) connected to a laser Doppler flowmeter (model BLF21; Transonic Systems, Inc.).

Online supplemental material. Fig. S1 shows ligand-dependent VEGFR-2 and -3 phosphorylation and its inhibition by antibodies. Fig. S2 shows internalization of VEGFR-2 and -3 upon ligand stimulation in the lymphatic capillaries. Fig. S3 shows collecting lymphatic vessels and lymph node vascularity in K14-VEGF-E mice. Fig. S4 shows a CD11b staining of the skin of the different transgenic mice. Fig. S5 shows PECAM-1 staining of the skin of transgenic mice at different ages. The online version of this article is available at <http://www.jem.org/cgi/content/full/jem.20062642/DC1>.

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