Alternatively spliced VEGF receptor-2 is an essential endogenous inhibitor of lymphatic vessels

Romulo J.C. Albuquerque[1,2], **Takahiko Hayashi**[3,14], **Won Gil Cho**[1,14], **Mark E. Kleinman**[1,14], **Sami Dridi**[1], **Atsunobu Takeda**[1], **Judit Z. Baffi**[1], **Kiyoshi Yamada**[1], **Hiroki Kaneko**[1], **Martha G. Green**[1], **Joe Chappell**[4], **Jörg Wilting**[5], **Herbert A. Weich**[6], **Satoru Yamagami**[7], **Shiro Amano**[7], **Nobuhisa Mizuki**[3], **Jonathan S. Alexander**[8], **Martha L. Peterson**[9], **Rolf A. Brekken**[10], **Masanori Hirashima**[11], **Seema Capoor**[1], **Tomohiko Usui**[7], **Balamurali K. Ambati**[12,13], and **Jayakrishna Ambati**[1,2]

[1]Department of Ophthalmology & Visual Sciences, University of Kentucky, Lexington, Kentucky 40536, USA

[2]Department of Physiology, University of Kentucky, Lexington, Kentucky 40536, USA

[3]Department of Ophthalmology, Yokohama City University, Yokohama, 221-0044, Japan

[4]Department of Plant & Soil Sciences, University of Kentucky, Lexington, Kentucky 40536, USA

[5]Department of Anatomy and Cell Biology, Georg-August-University, Goettingen, Germany

[6]Department Gene Regulation and Differentiation, Helmholtz Centre for Infection Research, Braunschweig, Germany

[7]Department of Ophthalmology, University of Tokyo School of Medicine, Tokyo, 113-8655, Japan

[8]Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center, Shreveport, Louisiana 71130-3932, USA

[9]Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky, Lexington, Kentucky 40536, USA

[10]Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, Texas 75390-8593, USA

[11]Division of Vascular Biology, Department of Physiology and Cell Biology, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan

[12]Department of Ophthalmology and Visual Sciences, Moran Eye Center, University of Utah School of Medicine, Salt Lake City, Utah 84132, USA

[13]Department of Ophthalmology, Veterans Affairs Salt Lake City Healthcare System, Salt Lake City, Utah 84148, USA

Abstract

Correspondence should be addressed to J.A. (jamba2@email.uky.edu)..
[14]These authors contributed equally to this work.

Disruption of the precise balance of positive and negative molecular regulators of blood and lymphatic vessels can lead to myriad diseases that affect one in four people worldwide. Although dozens of natural inhibitors of hemangiogenesis have been identified, an endogenous selective inhibitor of lymphatic vessels has not yet been described. We report the existence of a secreted, splice variant of vascular endothelial growth factor receptor-2 (sVegfr-2) that inhibits developmental and reparative lymphangiogenesis by blocking Vegf-c. Tissue-specific loss of sVegfr-2 in mice induced, at birth, spontaneous lymphatic invasion of the normally alymphatic cornea and hyperplasia of skin lymphatics without accompanying changes in blood vasculature. sVegfr-2 inhibited lymphangiogenesis but not hemangiogenesis induced by corneal suture injury or transplantation, enhanced corneal allograft survival, and suppressed lymphangioma cellular proliferation. Naturally occurring sVegfr-2 is a molecular uncoupler of blood and lymphatic vessels whose modulation might have a therapeutic role in lymphatic vascular malformations, transplantation, and potentially in tumor lymphangiogenesis and lymphedema.

The blood and lymphatic vessel networks are jointly essential for development, wound healing, and immune surveillance and activation. Pathological responses of these parallel circulatory systems can lead to diseases as varied as age-related macular degeneration, atherosclerosis, cancer, lymphedema, rheumatoid arthritis, and tumor metastasis[1]. Collectively, exuberant or inadequate responses of hemangiogenesis and lymphangiogenesis are estimated to affect nearly two billion people[2],[3]. The prevalence of such diseases has fueled intense efforts to develop proand anti-angiogenic therapeutics. Several anti-hemangiogenic drugs are FDA-approved but not one specific lymphangiogenesis inhibitor is even in clinical trials. As such there is great interest in identifying such specific inhibitors both to alleviate disease burden and to better understand lymphatic vascular biology. However, despite concerted efforts, it has been challenging to approach lymphangiogenesis selectively due to the difficulty in mechanistically disassociating it from hemangiogenesis.

The vascular endothelial growth factors (Vegf) family of molecules is indispensable for growth of blood[4],[5] and lymphatic[6] vessels. Hemangiogenesis requires a precise balance of positive and negative regulators[7]; however, the mechanisms governing lymphangiogenesis equilibria remain nebulous. Soluble splice variants of Vegf receptor-1 (sVegfr-1) act as potent natural inhibitors of hemangiogenesis by trapping the blood endothelial mitogen Vegf-a (refs. [8]-[10]) and are the only reported secreted splice variants of any of the Vegf receptor tyrosine kinases since their discovery in the early 1990s. Here we describe the detection and function of a new secreted splice variant within the Vegf receptor family, which we demonstrate is a critical endogenous antagonist of Vegf-c and a physiological regulator of lymphatic vessels.

RESULTS

Cloning of sVegfr-2

During our studies uncovering the non-redundant function of sVegfr-1 in corneal avascularity[11], which is critical for optimal vision, we observed, on western blotting, anomalous migration of a 75 kDa protein species that was immunoreactive to an antibody (T014; ref. [12]) recognizing the amino-terminus of Vegfr-2 (Supplementary Fig. 1a). Given

the homology in the exon-intron structure between *Vegfr1* and *Vegfr2*, we hypothesized that this unidentified protein represented a novel truncated splice variant of the 230 kDa membrane-bound form of Vegfr-2 (mbVegfr-2) and that it might have anti-lymphangiogenic activity because the lymphatic mitogen Vegf-c (ref. [13]) is capable of binding mbVegfr-2 (ref. [14]).

By modeling the putative alternative splicing event of *Vegfr2* to that of *Vegfr1* (ref. [8]), we found that retention of intron 13 would yield a truncated transcript variant whose protein product would lack the transmembrane and intracellular tyrosine kinase domains of mbVegfr-2 due to the presence of an in-frame early termination TAA codon 39 nucleotides downstream from the aforementioned exon/intron junction (Supplementary Fig. 1c). To verify the existence of this novel soluble splicing variant in the mouse cornea, we devised primers targeting intron 13 and exon 12 (Supplementary Fig. 1c and Supplementary Fig. 2a). Targeting exon 12 allowed us to distinguish between amplification of mRNA-derived cDNA and genomic DNA contamination based on amplicon size. PCR yielded a 393-bp product encompassing the locus of the splicing event (Supplementary Fig. 1d). Bioinformatic analysis of intron 13 revealed three potential polyadenylation (polyA) signal sequences (Supplementary Fig. 1c). Using rapid amplification of cDNA 3′ ends PCR, we found the third potential polyA signal at position 3956-61 to be active (Supplementary Fig. 1c,e and Supplementary Fig. 2a). From mouse cornea cDNA, we cloned the 2022-bp open reading frame of *sVegfr2* encoding a polypeptide of 673 amino acids (Supplementary Fig. 2b, for detailed sequence of the *sVegfr2* transcript see Supplementary Fig. 2a). This novel protein contained a unique 13-aa carboxyl-terminus sequence (Supplementary Fig. 1b) not present in mbVegfr-2 or any other known protein and against which we raised a rabbit polyclonal antibody (AA21127; Supplementary Fig. 3).

sVegfr-2 is expressed in the cornea

This *sVegfr2* transcript was localized by *in situ* hybridization principally to the corneal epithelium (Fig. 1a). Immunolocalization using AA21127 in the newborn mouse demonstrated the presence of sVegfr-2 in the corneal epithelium and stroma (Supplementary Fig. 4a). In the adult cornea, sVegfr-2 was more abundant in the epithelium than in the stroma (Fig. 1b and Supplementary Fig. 4a). sVegfr-2 was distributed uniformly in the cornea with enhanced expression near the limbus (Supplementary Fig. 4b). In contrast, sVegfr-2 was not immunolocalized in the conjunctiva (Supplementary Fig. 4b,c). sVegfr-2 was identified in the cornea as a 75 kDa species by western blotting both by AA21127 and T014 (Supplementary Fig. 4d). However, 230 kDa mbVegfr-2 was not detected in the cornea by western blotting using T014 (Supplementary Fig. 1a) or by immunofluorescence using an antibody targeting the carboxyl-terminus of mbVegfr-2, which is not present in sVegfr-2 (Supplementary Fig. 4e). Neither was the *mbVegfr2* transcript detected in the cornea by RT-PCR (Supplementary Fig. 4f). Thus, the mouse cornea expresses sVegfr-2 but not mbVegfr-2.

sVegfr-2 is essential for alymphatic cornea

To define the function of sVegfr-2 in the cornea, we targeted it using multiple strategies. Because *Vegfr2*^{−/−} mice die *in utero*[15], we conditionally ablated corneal sVegfr-2 using a

Cre-loxP strategy. This strategy enabled the specific targeting of sVegfr-2 because mbVegfr-2 is not expressed in the cornea (Supplementary Fig. 4e,f). We created *Vegfr2^{loxP/loxP}* mice by targeting exon 1 (Supplementary Fig. 5) and interbred them with *LeCre* mice that constitutively and uniformly express Cre recombinase in the cornea[16]. Mice of all genotypes were born at the expected mendelian ratios and were macroscopically indistinguishable from one another. Strikingly however, all *LeCre/Vegfr2^{loxP/loxP}* mouse corneas (*n* = 30), which verifiably lacked sVegfr-2 expression (Supplementary Fig. 6a), were densely covered with lymphatic vessels at birth (P0) (Fig. 1c). These vessels were identified as lymphatics by virtue of their intense Lyve-1 reactivity, moderate Cd31 reactivity, nuclear Prox1 expression, and blind-ended morphology (Fig. 1c-e and Supplementary Fig. 6b). Although Lyve-1⁺ macrophages have been described in the cornea[17], co-expression of Prox1 in these vessels confirms their lymphatic endothelial fate (Fig 1d,e). Furthermore, ultrastructural examination showed that these vessels lacked erythrocytes, did not have a continuous basement membrane, and contained partly overlapping thin endothelial cells free of pericyte coverage, all features typical of lymphatics (Fig. 1f). Surprisingly these corneas were not invaded by blood vessels as demonstrated by the absence of Cd31⁺ Lyve-1⁻ vessels (Fig. 1c,g). We confirmed this independently by demonstrating that the vessels in these corneas did not express Meca-32, a blood vessel-specific marker (Supplementary Fig. 6c,d). All littermate control corneas (*n* =30) were, like wild-type mice, devoid of both lymphatic and blood vessels. *LeCre/Vegfr2^{loxP/loxP}* mice had normal limbal blood vessel morphology and an unimpaired corneal hemangiogenesis response to suture injury (Supplementary Fig 6d,e), confirming intact vascular endothelial cell Vegfr-2 function.

sVegfr-2 is a Vegf-c antagonist

These results indicate that the developing mouse cornea is exposed to pro-lymphangiogenic stimuli that are counterbalanced by sVegfr-2 to create an alymphatic cornea. Indeed wild-type P0 corneas, unlike adult corneas, expressed Vegf-c (Supplementary Fig. 7). We reasoned that sVegfr-2 ablation led to spontaneous corneal lymphangiogenesis because sVegfr-2 trapped Vegf-c. Indeed, the levels of sVegfr-2 (1,655.8±44.62 pg per mg of total protein) in the wild-type P0 cornea were sufficiently in excess of Vegf-c (45.4±3.2 pg per mg of total protein) to fulfill a trapping function. sVegfr-2, which contains the Vegf-c binding Ig-like domain 2 of mbVegfr-2 (ref. [18]), interacted with Vegf-c by immunoprecipitation and inhibited both Vegfr-3 phosphorylation and proliferation of lymphatic endothelial cells (LECs) stimulated by Vegf-c (Fig. 1h-j). Also, Vegf-c-induced corneal lymphangiogenesis was inhibited by intracorneal administration[11] of a plasmid encoding for sVegfr-2 (psVegfr-2) (Supplementary Fig. 8). Collectively, these data are consistent with a model of sVegfr-2 acting as an endogenous sink for Vegf-c during corneal development and thereby establishing an alymphatic cornea.

Consistent with the report that mouse Vegfr-2 binds human VEGF-D but not mouse Vegf-d (ref. [19]), psVegfr-2 inhibited corneal lymphangiogenesis induced by human VEGF-D but not mouse Vegf-d (Supplementary Fig. 8). Also, unlike Vegf-c, Vegf-d was not expressed in the newborn mouse cornea (Supplementary Fig. 9), thus accounting for the ability of sVegfr-2 to maintain the alymphatic nature of the cornea.

sVegfr-2 inhibits reparative lymphangiogenesis

To determine the function of sVegfr-2 in the adult, we studied a clinically relevant mouse model of suture-induced corneal neovascularization[20]. Suture injury increased corneal sVegfr-2 expression (in the epithelium and not in infiltrating macrophages) in wild-type mice (Supplementary Fig. 10). Ablating sVegfr-2 by intracorneal administration[11] of pCre in *Vegfr2*[*loxP/loxP*] mice markedly increased suture-induced lymphangiogenesis but not hemangiogenesis compared to pNull administration by 161±9% ($P < 0.05$, $n = 5$) (Fig. 2a-c). This outstripping of hemangiogenesis by lymphangiogenesis suggests that induction of endogenous sVegfr-2 by injury is a compensatory anti-lymphangiogenic response. Both pCre and pNull-injected corneas of wild-type mice showed similar degrees of lymphangiogenesis following suture injury, excluding a non-specific effect of Cre recombinase (Fig. 2c). pCre induced enhancement of suture-induced lymphangiogenesis in *Vegfr2*[*loxP/loxP*] mice was reduced by a Vegfr-3 tyrosine kinase inhibitor[21], supporting the concept that endogenous sVegfr-2 is an *in vivo* Vegf-c antagonist (Fig. 2c). Conversely, augmenting sVegfr-2 via *in vivo* transfection[11] of wild-type mouse corneas with psVegfr-2, but not pNull, reduced suture injury-induced lymphangiogenesis but not hemangiogenesis by 76±6% ($P < 0.05$, $n = 5$) (Fig. 2d,e).

sVegfr-2 enhances corneal transplant survival

Because lymphangiogenesis has been implicated in corneal allograft rejection[22], we studied the function of sVegfr-2 in a mouse model of corneal transplantation. A single intracorneal administration of dimeric Vegfr-2/Fc more than doubled transplant survival rate (105% increase compared to the IgG/Fc treatment and 101% compared to no treatment; $P < 0.05$, $n = 10–13$; Fig. 2f). The dramatic reduction in rejection rate induced by Vegfr-2/Fc administration (75% compared to the IgG/Fc treatment and 74% compared to no treatment) is consistent with the observed reduction in blood vessel and lymphatic sprouting through the donor-recipient interface (Fig. 2g,h). Surprisingly, a single intracorneal administration of monomeric sVegfr-2 induced the same degree of corneal allograft survival as dimeric Vegfr-2/Fc despite reducing sprouting only of lymphatic but not blood vessels through the donor-recipient interface (Fig. 2f-h). These data suggest that impairment of the establishment of the afferent arm of the immune-arc-reflex[23],[24] alone is sufficient to enhance corneal allograft survival. Further, the rate of allograft survival following a single local administration of sVegfr-2 was at least as great as the rate of survival induced by multiple systemic administrations of a Vegfr-3 antagonist in a prior study[25]. Apart from reducing lymphangiogenesis, it also is possible that sVegfr-2 promoted allograft survival by inhibiting Vegf-c-induced Vegfr-3 signaling in corneal dendritic cells and preventing their transmigration into the draining lymph node[25]. Collectively, these data provide a novel experimental strategy to uncouple hemangiogenesis from lymphangiogenesis and a new endogenous therapeutic target for improving survival of this most common solid transplant in humans.

Endogenous Vegf-c promotes lymphangiogenesis

Selective lymphangiogenesis inhibition by sVegfr-2 is consistent with the selective effect of endogenous Vegf-c on the lymphatic vasculature[6],[13],[26]-[28]. However, implantation of pellets

containing 160–200 ng of recombinant VEGF-C into the 2 μl mouse cornea has been reported to induce hemangiogenesis[29]-[32]. To reconcile these findings, we injected neutralizing anti-Vegf-c antibodies into the cornea of wild-type mice after suture injury and found that this resulted in inhibition of lymphangiogenesis ($P < 0.05$, $n = 8$) but not hemangiogenesis (Fig. 3a,b). These data directly demonstrate that endogenous Vegf-c selectively promotes lymphangiogenesis in the cornea, and help explain the selective effect of sVegfr-2. The lack of Vegfr-3 expression by conjunctival and corneal blood vessels[33],[34] also support a model where endogenous Vegf-c preferentially binds Vegfr-3 on lymphatic vessels over Vegfr-2 on blood vessels in these tissues, based on relative receptor affinities[35]. Also, Vegf-c is far less efficient at activating Vegfr-2 than Vegf-a[36], the principal driver of corneal hemangiogenesis[11],[32].

We quantified VEGF-C levels following 160 ng VEGF-C pellet implantation and found that this resulted in corneal VEGF-C levels that were 100–125 times greater than the levels observed after suture injury and 45–66 times greater than neonatal levels (Fig. 3c). Such exaggerated levels that are supra-physiological and far greater than those achieved in pathophysiologically relevant states could explain the reported hemangiogenic effects of exogenous VEGF-C in this artificial context.

Monomeric sVegfr-2 does not block hemangiogenesis

These selective anti-lymphangiogenic effects of sVegfr-2 also were unexpectedly specific because mbVegfr-2 is capable of binding Vegf-a and promoting hemangiogenesis. To explain this selectivity, we first sought to determine whether sVegfr-2 existed in monomeric or dimeric form because a recombinant form of the ectodomain of mbVegfr-2 has been shown to be a monomer that has little or no affinity for Vegf-a compared to a dimeric recombinant Vegfr-2/Fc fusion protein[37]-[39]. Expression studies revealed that sVegfr-2 secreted by mouse corneal epithelial, human embryonic kidney, and Chinese hamster ovary cells, as well as recombinant sVegfr-2, all migrated at equivalent apparent molecular masses under both non-reducing and reducing conditions of western blotting, data consistent with its existence as a monomer (Fig. 3d,e). In contrast, the migration of Vegfr-2/Fc was consistent with it being a dimer.

Next we tested the effects of monomeric sVegfr-2 and dimeric Vegfr-2/Fc on models of corneal neovascularization induced by injury or Vegf-a. Suture-induced corneal hemangiogenesis, which is driven principally by upregulation of endogenous Vegf-a (ref. [32]), was inhibited by Vegfr-2/Fc but not sVegfr-2 (Fig. 3f). Similarly, Vegf-a-induced phosphorylation of mbVegfr-2 in porcine aortic endothelial cells was inhibited by Vegfr-2/Fc but not sVegfr-2, and Vegf-a-induced corneal hemangiogenesis was inhibited by Vegfr-2/Fc but not by sVegfr-2 or psVegfr-2 (Fig. 3g,h). These functional data provide a mechanistic basis for the absence of an anti-hemangiogenic effect of endogenous sVegfr-2, and corroborate the previously reported *in vitro* differential Vegf-a binding avidity between monomeric and dimeric forms of Vegfr-2.

Non-ocular role of sVegfr-2

We found *sVegfr2* expression by Northern blotting of poly A-enriched RNA isolated from various mouse organs (Supplementary Fig. 11), suggesting that sVegfr-2 might have functions outside the eye. *sVegfr2* mRNA was abundant in the alymphatic epidermis of the skin as well as in the hair follicles of wild-type mice (Fig. 4a). sVegfr-2 protein was immunolocalized in the epidermis, hair follicles, and, consistent with its ability to diffuse, also in the dermis of wild-type mice (Fig. 4b). In contrast, mbVegfr-2 was expressed in the skin vasculature, but not in the epithelial cells or hair follicles (Supplementary Fig. 12a). As in the cornea, Vegf-c was expressed in the P0 wild-type mouse skin but undetectable in the adult (Supplementary Fig. 12b). Excess Vegf-c in the skin, achieved by either transgenic overexpression or implantation of overexpressing cells, leads to hyperplasia but not sprouting of lymphatic vessels[13],[40]. To determine the function of sVegfr-2 in the skin, we interbred *Vegfr2^loxP/loxP* mice with *K14Cre* mice that constitutively and uniformly express Cre recombinase in the epidermis and hair follicles[41]. This strategy specifically targets sVegfr-2 because the epidermis and hair follicles express sVegfr-2 but not mbVegfr-2. Strikingly, in all P0 *K14Cre/Vegfr2^loxP/loxP* mouse skin, which lacked sVegfr-2 expression (Fig. 4c), there was dramatic enlargement of lymphatic vessels compared to those in littermate control skin (Fig. 4d-f). These dilated skin lymphatics in *K14Cre/Vegfr2^loxP/loxP* mice also were hyperplastic (Fig. 4e,g). However, the density of lymphatic structures, as quantified by branch point analysis, was not greater in *K14Cre/Vegfr2^loxP/loxP* mice (Fig. 4h), just as in mouse skin exposed to excess Vegf-c (refs. [13],[40]). In contrast to the lymphatic architectural changes, there was no increase in skin blood vessel diameter or density in *K14Cre/Vegfr2^loxP/loxP* mice (Fig. 4d,e). The recapitulation of Vegf-c overexpression induced selective lymphatic hyperplasia in mice lacking sVegfr-2 supports the concept that sVegfr-2 is an *in vivo* antagonist of Vegf-c.

A protein immunoreactive to an antibody recognizing the amino-terminus of Vegfr-2 has been detected in plasma and shown to be a surrogate biomarker of tumor growth[42]-[44]. However, its molecular identity (whether it is encoded by a splice variant of *Vegfr2* or derived from ectodomain shedding or proteolytic cleavage of mbVegfr-2) and cellular source have been elusive. Circulating Vegfr-2 in plasma was immunoreactive to both AA21127 and T014 but not an antibody recognizing the carboxyl-terminus of mbVegfr-2 (Fig. 5a), suggesting that it was encoded by the *sVegfr2* splice variant. Mouse blood endothelial cell (BEC) lines derived from the microvasculature of the brain, pancreas, or skin all synthesized and secreted sVegfr-2, far in excess of LEC production (Fig. 5b,c). Also sVegfr-2 was detected by immunofluorescence using AA21127 in mouse lung microvasculature, identifying BECs as sources of plasma sVegfr-2 (Fig. 5d). The production of anti-lymphangiogenic sVegfr-2 by BECs could be one of the mechanisms underlying the observation that lymphangiogenesis typically lags behind hemangiogenesis in many neovascular models. Surprisingly, plasma levels of sVegfr-2 were significantly lower in *K14Cre/Vegfr2^loxP/loxP* mice compared to littermate controls (Fig. 5e), suggesting that the skin epithelium also is a source of circulating sVegfr-2. An earlier report showing that *K14*-driven recombinant soluble Vegfr-3 led to measurable circulating levels of the engineered protein[27] supports the concept that endogenous skin-derived sVegfr-2 also can enter the circulation.

sVEGFR-2 exists in humans and inhibits lymphangioma cellular proliferation

We confirmed the existence of *sVEGFR2* in humans using RT-PCR to clone the 2159-bp open reading frame of a transcript in human umbilical vein endothelial cells (Fig. 6a), that, as with mouse *sVegfr2*, resulted from retention of intron 13 also containing an in-frame early termination stop codon in human *VEGFR2*. Translation of *sVEGFR2* results in a protein containing 679 amino acids with a unique 16-aa carboxyl-terminus sequence not present in mbVEGFR-2 and against which we developed a polyclonal antibody (AA21129, Supplementary Fig. 13). sVEGFR-2 was expressed in human cornea with a distribution similar to that in mouse (Fig. 6b), suggesting that it plays a similar role in maintaining an alymphatic state. The potential anti-lymphangiogenic property of sVEGFR-2 in humans was tested *in vitro*. Lymphangioma is a common disfiguring childhood neoplasia whose etiology is unknown. Human lymphangioma endothelial cells (LaECs) produce VEGF-C and express VEGFR-3 (refs. [45]-[47]), suggesting that their growth could be inhibited by disrupting this axis. Previously we described the isolation of LaECs from axillary lymphangiomas in two human infants[47]. We found that sVEGFR-2 abolished VEGF-C-induced proliferation of both these LaECs (Fig. 6c,d), raising the exciting possibility that these pediatric tumors could be molecularly targeted.

DISCUSSION

As an endogenous uncoupler of the intertwined blood and lymphatic circulatory systems, sVegfr-2 represents a new molecular tool to selectively dissect the individual contributions of these parallel vasculatures in development and disease. More importantly the identification of sVegfr-2 can enable new therapeutic strategies by selectively modulating aberrant lymphatic proliferation without encountering the potential adverse effects of non-specific anti-angiogenic therapy.

The importance of pure lymphangiogenesis suppression is nowhere more apparent than in the setting of lymphangioma, which occurs in roughly 1 in 50 children[48]. These tumors, although classified as benign, can be locally invasive and terribly disfiguring. Their pediatric preponderance highlights the need to develop selective anti-lymphangiogenic therapy. Our finding that sVEGFR-2 can prevent VEGF-C induced proliferation of human lymphangioma endothelial cells raises the exciting possibility that these pediatric tumors could be targeted on a molecular level.

Our discovery of sVegfr-2 in the cornea endorses this tissue as a rich source of angiogenesis modulators, and introduces the yin-yang concept of opposing natural molecular forces that regulate blood vessel growth[7] to lymphangiogenesis. Just as sVegfr-1 is required for corneal avascularity[11],[49], we now show that sVegfr-2 is essential for corneal alymphaticity. The dual presence of sVegfr-1 and sVegfr-2 attests to the premium placed by the cornea on impeding both blood and lymphatic vessel growth to preserve optical clarity and relative immunological privilege, and illustrates the conservation of alternative splicing in generating these tandem traps.

The surprising finding that sVegfr-2 enhanced corneal allograft survival despite not inhibiting hemangiogenesis suggests that impairment of the establishment of the afferent

arm of the immune-arc-reflex[23],[24] alone is sufficient to enhance corneal allograft survival. Interestingly, the rate of allograft survival following a single local administration of sVegfr-2 was at least as great as the rate of survival induced by multiple systemic administrations of a Vegfr-3 antagonist in a prior study[25]. Apart from reducing lymphangiogenesis, it also is possible that sVegfr-2 promoted allograft survival by inhibiting Vegf-c-induced Vegfr-3 signaling in corneal dendritic cells and preventing their transmigration into the draining lymph node[25]. In addition to identifying a new endogenous therapeutic target for improving survival of this most common solid transplant in humans, our data also provide a mechanistic basis for evaluating selective lymphangiogenesis suppression in other transplants, such as kidney, whose rejection is associated with lymphangiogenesis[50].

Unlike collagen XVIII fragments, semaphorin 3F, and transforming growth factor-β, the only other reported endogenous inhibitors of lymphangiogenesis[51]-[54], native sVegfr-2 is a specific and direct lymphangiogenesis inhibitor that did not inhibit hemangiogenesis in multiple models, which often is intimately intertwined with lymphangiogenesis. One reason for this specificity is that native sVegfr-2 as a monomer, unlike dimeric mbVegfr-2, has poor avidity for Vegf-a. Nevertheless, we cannot exclude a minor anti-hemangiogenic role for sVegfr-2 that might be masked by the dominant role of coexisting molecules such as sVegfr-1 that have much greater affinity for Vegf-a, or in experimental contexts where high levels of exogenous Vegf-c promote hemangiogenesis. A complete molecular resolution of why endogenous sVegfr-2 binds Vegf-c and not Vegf-a must await structure solutions of the Vegfr-2 ectodomain alone and in complex with Vegf-a as well as of Vegf-c. Collectively our findings demonstrate that sVegfr-2 is a broad and non-redundant physiological regulator of lymphatic vessels. Further studies will reveal whether sVegfr-2 can arrest solid tumor lymphangiogenesis and metastasis, which can be promoted by Vegf-c overexpression[55]-[57] and inhibited by blocking Vegf-c activity[58],[59].

METHODS

Animals

Balb/C, C57Bl/6J, and *K14Cre* mice were purchased from The Jackson Laboratory. *Vegfr2^loxP/loxP^* (flox) mice were generated as described below. *LeCre* mice[16], which constitutively express Cre recombinase in the cornea were a gift of R. Ashery-Padan and P. Gruss via D.C. Beebe. For all procedures, anesthesia was achieved by intraperitoneal injection of 50 mg/kg ketamine hydrochloride (Fort Dodge Animal Health, Wyeth) and 10 mg/kg xylazine (Phoenix Scientific, San Marcos, CA). All animal procedures were approved by the Animal Care and Use Committees at the University of Kentucky or Yokohama City University, and conformed to the Association for Research in Vision and Ophthalmology Statement on Animal Research.

Conditional *Vegfr2* gene ablation

We achieved embryonic conditional genetic ablation of *Vegfr2* in the cornea by cross-breeding *Vegfr2^loxP/loxP^* mice with *LeCre* mice[16]. Alternatively, to target *Vegfr2* in the adult mouse cornea, we performed intrastromal injections of naked plasmids[11] containing the

sequence for Cre recombinase (pCre; 20 μg; gift of R.K. Nordeen, University of Colorado) or an empty plasmid (pNull; 20 μg) in fellow eyes of *Vegfr2^loxP/loxP* or BALB/c mice 3 days prior to suture placement. To achieve embryonic conditional genetic ablation of *Vegfr2* in the epidermis, we cross-bred *Vegfr2^loxP/loxP* mice with *K14Cre* mice that constitutively express Cre recombinase in the skin epidermis and hair follicle.

Transmission electron microscopy

Eyes were enucleated from wild-type and *LeCre/Vegfr2^loxP/loxP* mice and fixed in 3.5% glutaraldehyde/4% paraformaldehyde for 2 h followed by preparation of uranyl acetate- and lead citrate-stained ultrathin sections for transmission electron microscopy studies (Phillips Biotwin).

Corneal injury

Two intrastromal 11-0 sutures (Mani, Japan) were placed in the mouse cornea 180° from each other. All sutures were placed in the midpoint between the limbus and the corneal apex. They were left in place for up to 14 days. Naked plasmids coding for Cre-recombinase (gift of R.K.Nordeen, University of Colorado), mouse sVegfr-2 (psVegfr-2), were utilized for *in vivo* enforced expression studies as shown earlier[11]. To block Vegfr-3 activity, daily intraperitoneal injections of Vegfr-3 tyrosine kinase inhibitor (MAZ51, EMD Chemicals, 8 mg/kg) were performed in pCre treated *Vegfr2^loxP/loxP* mice after corneal suture placement for 14 consecutive days. Vehicle only (DMSO) was administered as control treatment.

Vegf-c neutralization

Neutralizing rabbit anti-Vegf-c (Angio-Proteomie, pV1006R-r, 16 μg) antibody and control rabbit IgG (16 μg) were injected intrastromally into fellow eyes on the day of suture placement and every 3 days thereafter for 14 days.

Vegfr-2 phosphorylation assay

Porcine aortic endothelial cells stably transfected with Vegfr-2 (PAE-KDR) were cultured in DMEM (Invitrogen) containing 10% FBS, penicillin G (100 units/ml), streptomycin sulfate (0.1 mg/ml) (all from Sigma Aldrich) at 37 °C, 10% CO_2 and 90% room air. Upon attaining 80% confluence these cells were serum starved for 24 h, after which time they were exposed to Vegf-a (R&D Systems, 2.5 n*M*) or media alone (negative control) and monomeric sVegfr-1 (Reliatech, 15 n*M*) , monomeric sVegfr-2 (Reliatech, 75 n*M*), dimericVegfr-2/Fc (R&D Systems, 75 n*M*) or IgG/Fc control (Jackson ImmunoResearch, 75 n*M*) for 15 min. Cell lysates were processed in RIPA buffer and resolved by SDS 8% polyacrylamide gradient gel electrophoresis (PAGE) and transferred to nitrocellulose (NC) membranes. Immunoblotting was performed using a rabbit antibody against phosphorylated Vegfr-2 (1:500, Upstate, 36-019), and loading was assessed using an antibody against Vegfr-2 (1:1000, R&D Systems, AF357) and Vinculin (1:2000, Sigma, V4139).

Silver staining

Vegfr-2/Fc or sVegfr-2 were resolved by SDS 10% PAGE and stained using SilverSNAP (R) Stain (Pierce) according to manufacturer instructions.

Immuno-morphometric analyses

Immunostaining and flat mounting were performed as previously shown[11] using rabbit anti-mouse Lyve-1 antibody (Abcam; 1:333), rat anti-mouse Cd31 antibody (BD Biosciences; 1:50), rat-anti-mouse Meca-32 antibody (BD Biosciences; 1:10), goat anti-mouse Lyve-1 antibody (R&D, 1:100), and rabbit anti-mouse Prox-1 (Angiobio;1:500) for 48 h at 4 °C. Alexa Fluor 488 (goat anti-rabbit; 1:200) and Alexa Fluor594 (goat anti-rat; 1:200), Alexa Fluor488 (donkey anti-goat; 1:200, Invitrogen), or Cy3 conjugated donkey anti-rabbit (Jackson ImmunoResearch; 1:400) for 24 h were used as secondary antibodies. Tissue mounts were visualized under fluorescent microscopy (Leica SP-5) and analyzed with ImageJ (NIH). Mean percentage Lyve-1$^+$ (lymphatic vessels) or Cd31$^+$/Lyve-1$^-$ (blood vessels) areas were calculated for corneal flat mounts and skin whole mounts using ImageJ software. The number of Prox1$^+$ nuclei within Lyve-1$^+$ skin lymphatic vessels were counted in 12 random fields and expressed as LEC density per 100 μm. The density of lymphatic structures was determined by counting lymphatic vessel branch points per unit area (750 μm × 750 μm) on standard low-magnification Lyve-1-stained images of the mouse skin.

Mouse endothelial cell culture

Mouse blood endothelial cells from brain (Bend3, gift of C.D. Kontos, Duke University), pancreas (MS1) and skin (Py4, both gifts from J.L. Arbiser, Emory University) and mouse lymphatic endothelial cells (mLEC; ref. [60]) were cultured in DMEM (Invitrogen) containing 10% FBS, penicillin G (100 units/ml), streptomycin sulfate (0.1 mg/ml) (all from Sigma Aldrich) at 37 °C, 10% CO_2 and 90% room air. Upon attaining 80% confluence these cells were serum starved for 24 h when supernatant fractions were collected for sVegfr-2 protein quantification by ELISA. Total RNA was also extracted for RT-PCR.

Human lymphatic microvascular endothelial cell proliferation assay

Cultured human lymphatic microvascular endothelial cells (Cambrex) were maintained in EGM-2 MV (Clonetics) supplemented with 10%FBS and antibiotics at 37 °C under 5% CO2. Once confluent cells were plated at 96-well plate at a density of 30,000 cells/well. Cells were serum starved for 2 h and then exposed to media alone (MCDB 131 + 5% FBS), VEGF-C (200 ng/ml; R&D Systems) enriched media or VEGF-C enriched media with sVEGFR-2 (13.6 μg/ml). This concentration of sVEGFR-2 corresponds to a molar ratio of approximately 11-13:1 compared to VEGF-C, and is therefore physiological in view of our finding that sVegfr-2 levels in the cornea are ~20-fold higher than Vegf-c levels on a molar basis. Proliferation was quantified using BrdU uptake (Chemicon International) at 36 h after incubation with VEGF-C.

Lymphangioma proliferation assay

Lymphatic endothelial cells were isolated from lymphangiomas from two individuals, 4 and 10 months of age[47], with the approval of the ethics committee of the Georg-August-University and with the informed consent of the patients' parents. These cells were grown in EGM2-MV growth media containing 5% FBS. Cells were passaged onto a 96-well plate (5000 cells/well) in basal media (MCDB131) containing 2% FBS, and allowed to adhere overnight. Cultures were then treated with 200 ng/ml recombinant human wild-type (WT)

VEGF-C (Reliatech) alone or together with 25 μg/ml of sVegfr-2 (Reliatech) in basal media with 0.1% FBS. Cell proliferation was measured after 24 h by using BrdU cell proliferation kit (Chemicon) according to the manufacturer's instructions.

Western blotting

Mouse cornea lysates as well as cell culture supernatant were resolved by SDS 8% or 4-20% polyacrylamide gradient gel electrophoresis (PAGE) and transferred to nitrocellulose (NC) membranes. Immunoblotting was performed using a rabbit antibody against the amino terminus of mouse Vegfr-2 (1:1000; clone T014; ref. [12]), custom made sVegfr-2 specific antibody (1:1000; AA21127) and loading was assessed using rabbit antibody against human GAPDH (1:2000; Abcam).

Immunoprecipitation studies

Recombinant mouse sVegfr-2 (2 μg) was incubated with mouse recombinant Vegf-c (100 ng, Biovision) in PBS at 4 °C for 1 h. Immunoprecipitation was carried out with 2 μg of an anti-Vegfr-2 antibody or an isotype control IgG. Immobilized protein A/G beads (20 μl, Pierce) were used for precipitation. Samples were boiled, resolved by SDS-PAGE with respective positive controls (rsVegfr-2 and rVegf-c) and transferred to a NC membrane. Rabbit antibodies against Vegf-c (1:1000, Santa Cruz) and Vegfr-2 (1:1000, T014) were used to probe for Vegf-c and Vegfr-2 respectively. Immunoprecipitation of Vegfr-2 from mouse plasma was performed as described previously by others [42]. Rabbit antibody against Vegfr-2 (T014, 2 μg) was employed for immunoprecipitation. T014 (1:1000), goat anti-Vegfr-2 (C) antibody (1:1000, Abcam-ab2349) or rabbit anti-sVegfr-2 antibody (1:1000, AA21127) was used for immunoblotting. Mouse LECs were incubated with media only or Vegf-c (200 ng/ml, Biovision) with or without sVegfr-2 (13.8 μg/ml) or Vegfr-1/Fc (20 μg/ml, R&D Systems) for 15 min. The sVegfr-2 and Vegfr-1/Fc concentrations are equimolar. The lysates were immunoprecipitated with anti-Vegfr-3 antibody (Santa Cruz, C-20), immunoblotted with anti-phosphotyrosine (Millipore, 4G10, 1:1000), and reblotted with anti-Vegfr-3 antibody (eBioscience, AFL4, 1:500).

Statistical analyses

Mann Whitney U test with Bonferroni correction was used for statistical comparison of multiple variables. Comparison of corneal transplant survival was performed by Kaplan-Meier Survival. The null hypothesis was rejected at $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

References

1. Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. Nature. 2005; 438:967–974. [PubMed: 16355214]

2. Carmeliet P. Angiogenesis in life, disease and medicine. Nature. 2005; 438:932–936. [PubMed: 16355210]

3. Fenwick A. Waterborne infectious diseases--could they be consigned to history? Science. 2006; 313:1077–1081. [PubMed: 16931751]

4. Carmeliet P, et al. Abnormal blood vessel development and lethality in embryos lacking a single Vegf allele. Nature. 1996; 380:435–439. [PubMed: 8602241]

5. Ferrara N, et al. Heterozygous embryonic lethality induced by targeted inactivation of the Vegf gene. Nature. 1996; 380:439–442. [PubMed: 8602242]

6. Karkkainen MJ, et al. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nat Immunol. 2004; 5:74–80. [PubMed: 14634646]

7. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med. 1995; 1:27–31. [PubMed: 7584949]

8. Kendall RL, Thomas KA. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci U S A. 1993; 90:10705–10709. [PubMed: 8248162]

9. Sela S, et al. A novel human-specific soluble vascular endothelial growth factor receptor 1: cell-type-specific splicing and implications to vascular endothelial growth factor homeostasis and preeclampsia. Circ Res. 2008; 102:1566–1574. [PubMed: 18515749]

10. Rajakumar A, et al. Novel soluble Flt-1 isoforms in plasma and cultured placental explants from normotensive pregnant and preeclamptic women. Placenta. 2009; 30:25–34. [PubMed: 19010535]

11. Ambati BK, et al. Corneal avascularity is due to soluble Vegf receptor-1. Nature. 2006; 443:993–997. [PubMed: 17051153]

12. Huang X, Gottstein C, Brekken RA, Thorpe PE. Expression of soluble Vegf receptor 2 and characterization of its binding by surface plasmon resonance. Biochem Biophys Res Commun. 1998; 252:643–648. [PubMed: 9837760]

13. Jeltsch M, et al. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science. 1997; 276:1423–1425. [PubMed: 9162011]

14. Joukov V, et al. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. Embo J. 1996; 15:290–298. [PubMed: 8617204]

15. Shalaby F, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 1995; 376:62–66. [PubMed: 7596435]

16. Ashery-Padan R, Marquardt T, Zhou X, Gruss P. Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye. Genes Dev. 2000; 14:2701–2711. [PubMed: 11069887]

17. Maruyama K, et al. Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. J Clin Invest. 2005; 115:2363–2372. [PubMed: 16138190]

18. Jeltsch M, et al. Vascular endothelial growth factor (Vegf)/VEGF-C mosaic molecules reveal specificity determinants and feature novel receptor binding patterns. J Biol Chem. 2006; 281:12187–12195. [PubMed: 16505489]

19. Baldwin ME, et al. The specificity of receptor binding by vascular endothelial growth factor-d is different in mouse and man. J Biol Chem. 2001; 276:19166–19171. [PubMed: 11279005]

20. Streilein JW, Bradley D, Sano Y, Sonoda Y. Immunosuppressive properties of tissues obtained from eyes with experimentally manipulated corneas. Invest Ophthalmol Vis Sci. 1996; 37:413–424. [PubMed: 8603847]

21. Kirkin V, et al. Characterization of indolinones which preferentially inhibit VEGF-C- and VEGF-D-induced activation of VEGFR-3 rather than VEGFR-2. Eur J Biochem. 2001; 268:5530–5540. [PubMed: 11683876]

22. Cursiefen C, Chen L, Dana MR, Streilein JW. Corneal lymphangiogenesis: evidence, mechanisms, and implications for corneal transplant immunology. Cornea. 2003; 22:273–281. [PubMed: 12658100]

23. Liu Y, Hamrah P, Zhang Q, Taylor AW, Dana MR. Draining lymph nodes of corneal transplant hosts exhibit evidence for donor major histocompatibility complex (MHC) class II-positive dendritic cells derived from MHC class II-negative grafts. J Exp Med. 2002; 195:259–268. [PubMed: 11805152]

24. Yamagami S, Dana MR. The critical role of lymph nodes in corneal alloimmunization and graft rejection. Invest Ophthalmol Vis Sci. 2001; 42:1293–1298. [PubMed: 11328742]

25. Chen L, et al. Vascular endothelial growth factor receptor-3 mediates induction of corneal alloimmunity. Nat Med. 2004; 10:813–815. [PubMed: 15235599]

26. Yoon YS, et al. VEGF-C gene therapy augments postnatal lymphangiogenesis and ameliorates secondary lymphedema. J Clin Invest. 2003; 111:717–725. [PubMed: 12618526]

27. Makinen T, et al. Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble Vegf receptor-3. Nat Med. 2001; 7:199–205. [PubMed: 11175851]

28. Karpanen T, et al. Lymphangiogenic growth factor responsiveness is modulated by postnatal lymphatic vessel maturation. Am J Pathol. 2006; 169:708–718. [PubMed: 16877368]

29. Cao Y, et al. Vascular endothelial growth factor C induces angiogenesis in vivo. Proc Natl Acad Sci U S A. 1998; 95:14389–14394. [PubMed: 9826710]

30. Kubo H, et al. Blockade of vascular endothelial growth factor receptor-3 signaling inhibits fibroblast growth factor-2-induced lymphangiogenesis in mouse cornea. Proc Natl Acad Sci U S A. 2002; 99:8868–8873. [PubMed: 12070340]

31. Cao R, et al. Comparative evaluation of FGF-2-, VEGF-A-, and VEGF-C-induced angiogenesis, lymphangiogenesis, vascular fenestrations, and permeability. Circ Res. 2004; 94:664–670. [PubMed: 14739162]

32. Cursiefen C, et al. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. J Clin Invest. 2004; 113:1040–1050. [PubMed: 15057311]

33. Cursiefen C, et al. Lymphatic vessels in vascularized human corneas: immunohistochemical investigation using LYVE-1 and podoplanin. Invest Ophthalmol Vis Sci. 2002; 43:2127–2135. [PubMed: 12091407]

34. Hamrah P, et al. Expression of vascular endothelial growth factor receptor-3 (VEGFR-3) on monocytic bone marrow-derived cells in the conjunctiva. Exp Eye Res. 2004; 79:553–561. [PubMed: 15381039]

35. Joukov V, et al. Proteolytic processing regulates receptor specificity and activity of VEGF-C. Embo J. 1997; 16:3898–3911. [PubMed: 9233800]

36. Bernatchez PN, Rollin S, Soker S, Sirois MG. Relative effects of VEGF-A and VEGF-C on endothelial cell proliferation, migration and PAF synthesis: Role of neuropilin-1. J Cell Biochem. 2002; 85:629–639. [PubMed: 11968003]

37. Roeckl W, et al. Differential binding characteristics and cellular inhibition by soluble Vegf receptors 1 and 2. Exp Cell Res. 1998; 241:161–170. [PubMed: 9633524]

38. Fuh G, Li B, Crowley C, Cunningham B, Wells JA. Requirements for binding and signaling of the kinase domain receptor for vascular endothelial growth factor. J Biol Chem. 1998; 273:11197–11204. [PubMed: 9556609]

39. Wiesmann C, et al. Crystal structure at 1.7 A resolution of Vegf in complex with domain 2 of the Flt-1 receptor. Cell. 1997; 91:695–704. [PubMed: 9393862]

40. Goldman J, Le TX, Skobe M, Swartz MA. Overexpression of VEGF-C causes transient lymphatic hyperplasia but not increased lymphangiogenesis in regenerating skin. Circ Res. 2005; 96:1193–1199. [PubMed: 15890974]

41. Vasioukhin V, Degenstein L, Wise B, Fuchs E. The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. Proc Natl Acad Sci U S A. 1999; 96:8551–8556. [PubMed: 10411913]

42. Ebos JM, et al. A naturally occurring soluble form of vascular endothelial growth factor receptor 2 detected in mouse and human plasma. Mol Cancer Res. 2004; 2:315–326. [PubMed: 15235107]

43. Ebos JM, et al. Vascular endothelial growth factor-mediated decrease in plasma soluble vascular endothelial growth factor receptor-2 levels as a surrogate biomarker for tumor growth. Cancer Res. 2008; 68:521–529. [PubMed: 18199548]

44. Ebos JM, Lee CR, Christensen JG, Mutsaers AJ, Kerbel RS. Multiple circulating proangiogenic factors induced by sunitinib malate are tumor-independent and correlate with antitumor efficacy. Proc Natl Acad Sci U S A. 2007; 104:17069–17074. [PubMed: 17942672]

45. Kaipainen A, et al. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci U S A. 1995; 92:3566–3570. [PubMed: 7724599]

46. Huang HY, Ho CC, Huang PH, Hsu SM. Co-expression of VEGF-C and its receptors, VEGFR-2 and VEGFR-3, in endothelial cells of lymphangioma. Implication in autocrine or paracrine regulation of lymphangioma. Lab Invest. 2001; 81:1729–1734. [PubMed: 11742043]

47. Norgall S, et al. Elevated expression of VEGFR-3 in lymphatic endothelial cells from lymphangiomas. BMC Cancer. 2007; 7:105. [PubMed: 17584927]

48. Wilting J, et al. Embryonic development and malformation of lymphatic vessels. Novartis Found Symp. 2007; 283:220–227. discussion 227-229, 238-241. [PubMed: 18300425]

49. Azar DT. Corneal angiogenic privilege: angiogenic and antiangiogenic factors in corneal avascularity, vasculogenesis, and wound healing (an American Ophthalmological Society thesis). Trans Am Ophthalmol Soc. 2006; 104:38.

50. Kerjaschki D, et al. Lymphatic endothelial progenitor cells contribute to de novo lymphangiogenesis in human renal transplants. Nat Med. 2006; 12:230–234. [PubMed: 16415878]

51. Brideau G, et al. Endostatin overexpression inhibits lymphangiogenesis and lymph node metastasis in mice. Cancer Res. 2007; 67:11528–11535. [PubMed: 18089781]

52. Kojima T, Azar DT, Chang JH. Neostatin-7 regulates bFGF-induced corneal lymphangiogenesis. FEBS Lett. 2008; 582:2515–2520. [PubMed: 18570894]

53. Bielenberg DR, et al. Semaphorin 3F, a chemorepulsant for endothelial cells, induces a poorly vascularized, encapsulated, nonmetastatic tumor phenotype. J Clin Invest. 2004; 114:1260–1271. [PubMed: 15520858]

54. Oka M, et al. Inhibition of endogenous TGF-beta signaling enhances lymphangiogenesis. Blood. 2008; 111:4571–4579. [PubMed: 18310502]

55. Skobe M, et al. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat Med. 2001; 7:192–198. [PubMed: 11175850]

56. Mandriota SJ, et al. Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. Embo J. 2001; 20:672–682. [PubMed: 11179212]

57. Brakenhielm E, et al. Modulating metastasis by a lymphangiogenic switch in prostate cancer. Int J Cancer. 2007; 121:2153–2161. [PubMed: 17583576]

58. He Y, et al. Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. J Natl Cancer Inst. 2002; 94:819–825. [PubMed: 12048269]

59. Hirakawa S, et al. VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. Blood. 2007; 109:1010–1017. [PubMed: 17032920]

60. Ando T, et al. Isolation and characterization of a novel mouse lymphatic endothelial cell line: SV-LEC. Lymphat Res Biol. 2005; 3:105–115. [PubMed: 16190815]
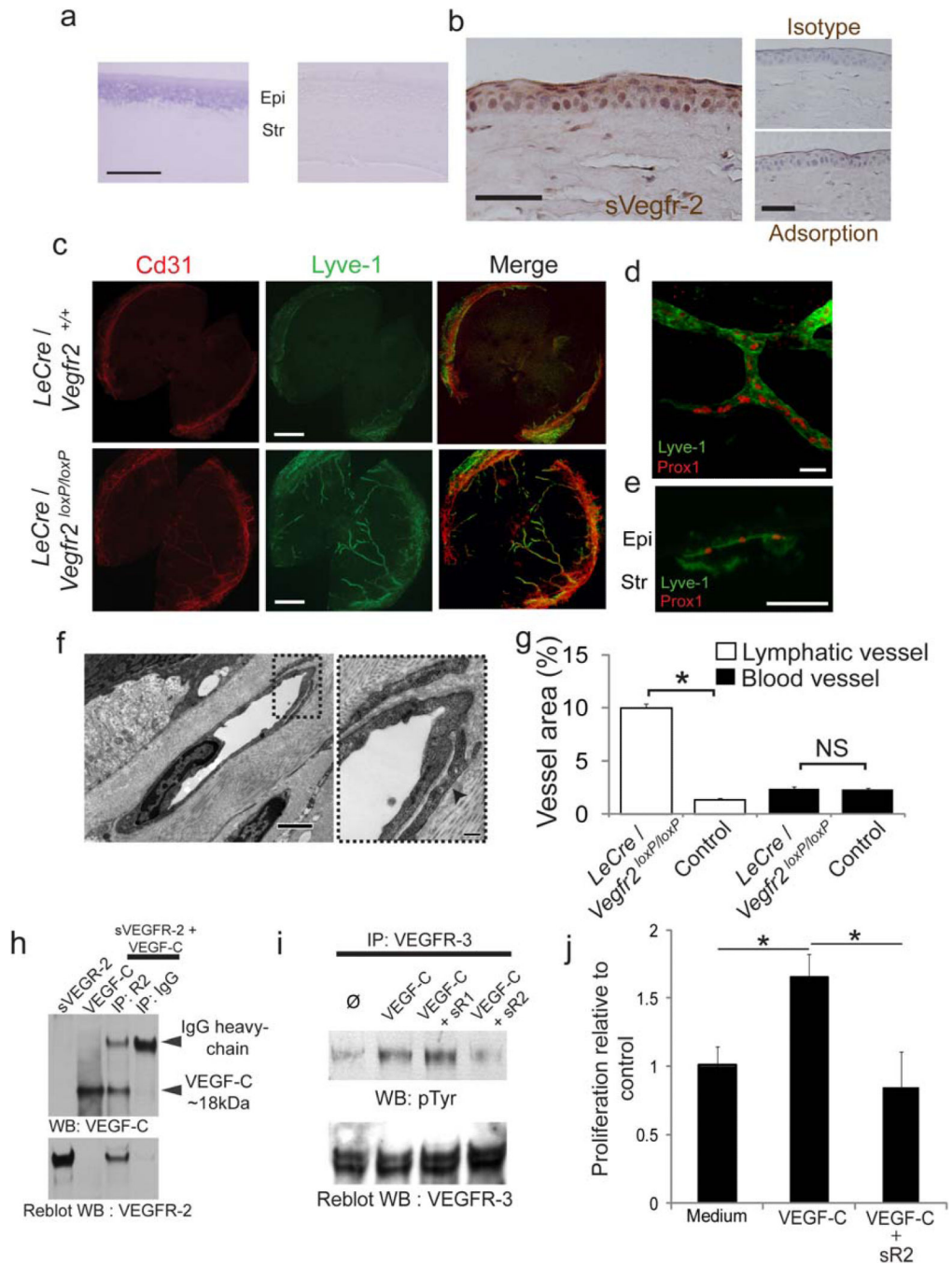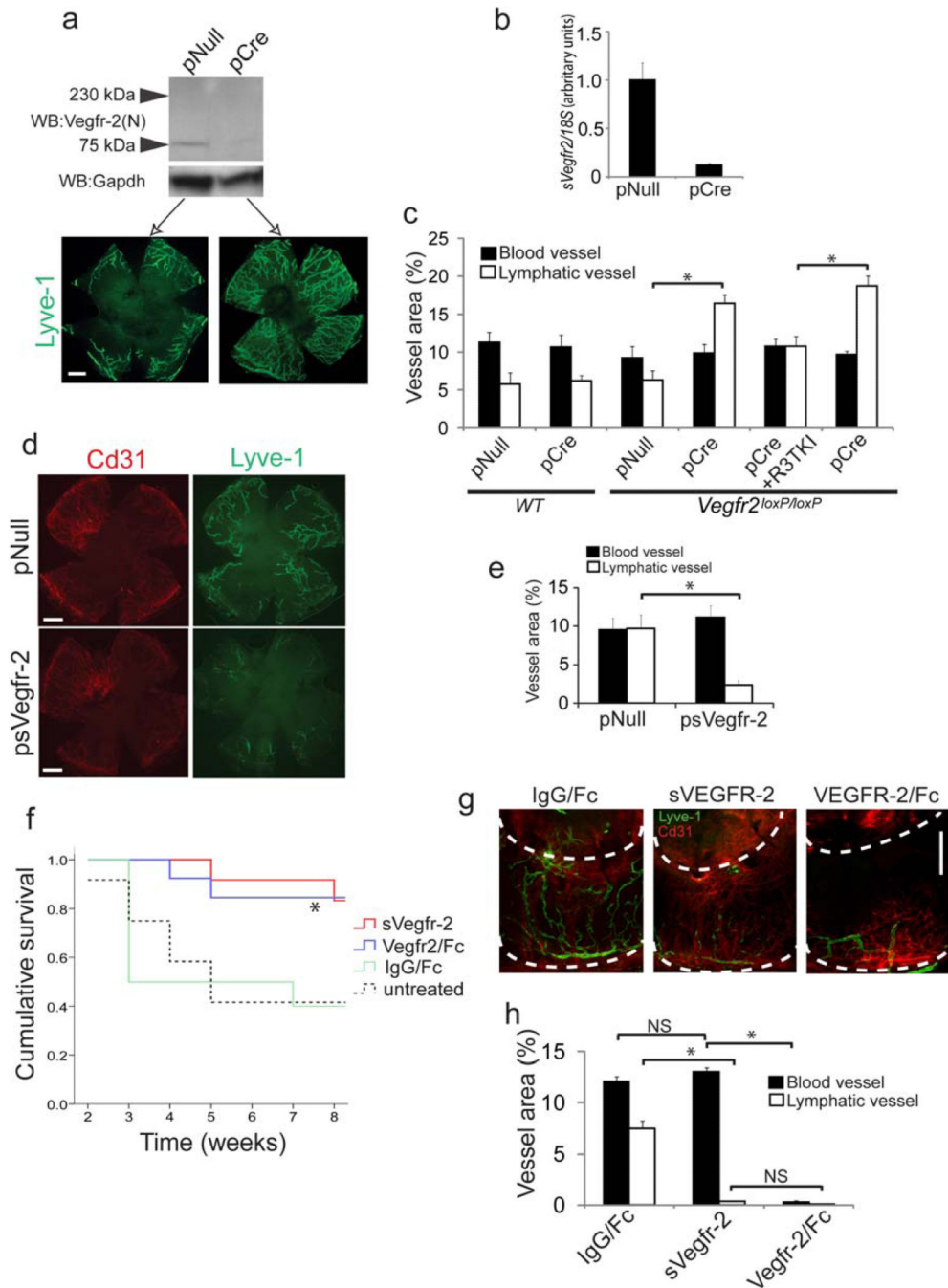
Figure 1.

Loss of endogenous sVegfr-2, which antagonizes Vegf-c, leads to spontaneous corneal lymphangiogenesis. (**a**) *sVegfr2* mRNA detection (purple-blue) by *in situ* hybridization in the mouse cornea. Epi, epithelium; Str, stroma. (**b**) sVegfr-2 immunolocalization (brown) in the mouse cornea using AA21127. Cell nuclei stained blue by hematoxylin. (**c**) Representative corneal flat mounts of a *LeCre/Vegfr2$^{loxP/loxP}$* mouse ($n = 30$). Lyve-1$^+$ (green) lymphatic and Cd31$^+$ (red) Lyve-1$^-$ blood vessels are shown. (**d,e**) Immunofluorescent detection of Lyve-1$^+$ (green) Prox1$^+$ (red) lymphatic vessels in corneal

whole mounts (**d**) and cross sections (**e**) of *LeCre/Vegfr2loxP/loxP* mice. **(f)** Transmission electron microscopy of lymphatic vessel in cornea of *LeCre/Vegfr2loxP/loxP* mice. Inset (right). (**g**) Quantification of vessel area in corneal flat mounts ($n = 20$). Error bars depict s.e.m. **(h)** Representative western blot of samples of recombinant sVegfr-2 (rsVegfr-2) incubated with recombinant Vegf-c (rVegf-c) and immunoprecipitated with either anti-Vegfr-2 (3rd lane from the left) antibody or isotype control IgG (4th lane from the left) and immunoblotted with anti-Vegf-c antibody. sVegfr-2 and Vegf-c resolution shown on first two lanes from the left. **(i)** Representative immunoblot of Vegf-c-induced Vegfr-3 phosphorylation of mouse lymphatic endothelial cells. Vegfr-3 re-blot is shown. φ, media only. **(j)** Proliferation of human lymphatic microvascular endothelial cells, quantified by BrdU uptake. (**f,j**), NS, not significant ;* $P < 0.05$, Significance by Mann Whitney U test. Error bars depict s.e.m. Scale bars: (**a,b,d**), 50 μm; (**c**) 500 μm; (**e**) 25 μm; left panel of (**g**), 1 μm; right panel of (**g**), 200 nm.

Figure 2.
sVegfr-2 inhibits reparative corneal lymphangiogenesis and rejection of corneal allografts.
(a) Representative western blot, 5 days after suture placement in *Vegfr2^loxP/loxP* mouse
corneas treated with plasmid coding for Cre recombinase (pCre) or empty vector (pNull) (*n*
= 8). Loading control, Gapdh. Bottom panel (arrows) shows Lyve-1+ vessels-green 14 days
after suture placement. **(b)** Real-time PCR of *sVegfr2* in the *Vegfr2^loxP/loxP* mouse corneas
(*n* = 4). **(c)** Quantification of vessel area in corneal flat mounts of *Vegfr2^loxP/loxP* and Balb/C
wild-type (WT) corneas (*n* = 5-6) and *Vegfr2^loxP/loxP* mice corneas injected with pCre

followed by systemic administration of a Vegfr-3 tyrosine kinase inhibitor (R3TKI) (*n* = 8*)*. Lyve-1$^+$, lymphatic vessel - white bars; Cd31$^+$/Lyve-1$^-$, blood vessel – black bars. **(d)** Representative corneal flat mounts of C57Bl/6J wild-type mouse corneas. Lyve-1$^+$, lymphatic vessel - green;Cd31$^+$/Lyve-1$^-$, blood vessel – red. **(e)** Quantification of vessel area in flat mounts of C57Bl/6J wild-type mouse corneas (*n* = 5). Lyve-1$^+$, lymphatic vessel - white bars; Cd31$^+$/Lyve-1$^-$, blood vessel – black bars. **(f)** Kaplan-Meier survival curves showing survival of corneal grafts in BALB/c hosts (*n* = 10–13). **(g)** Representative corneal flat mounts of transplanted mouse corneas. Lyve-1$^+$, lymphatic vessel - green;Cd31$^+$/ Lyve-1$^-$, blood vessel – red. Limbus, bottom dotted-line; Recipient-donor interface, top dotted-line. **(h)** Quantification of corneal graft vessel area in flat mounts of transplants (*n* = 5). Lyve-1$^+$, lymphatic vessel - white bars;Cd31$^+$/Lyve-1$^-$, blood vessel – black bars. (NS, non-significant; *, *P* < 0.05; Significance by Mann Whitney U test). Error bars depict s.e.m. Scale bars: (**a,d,g**), 500 μm.
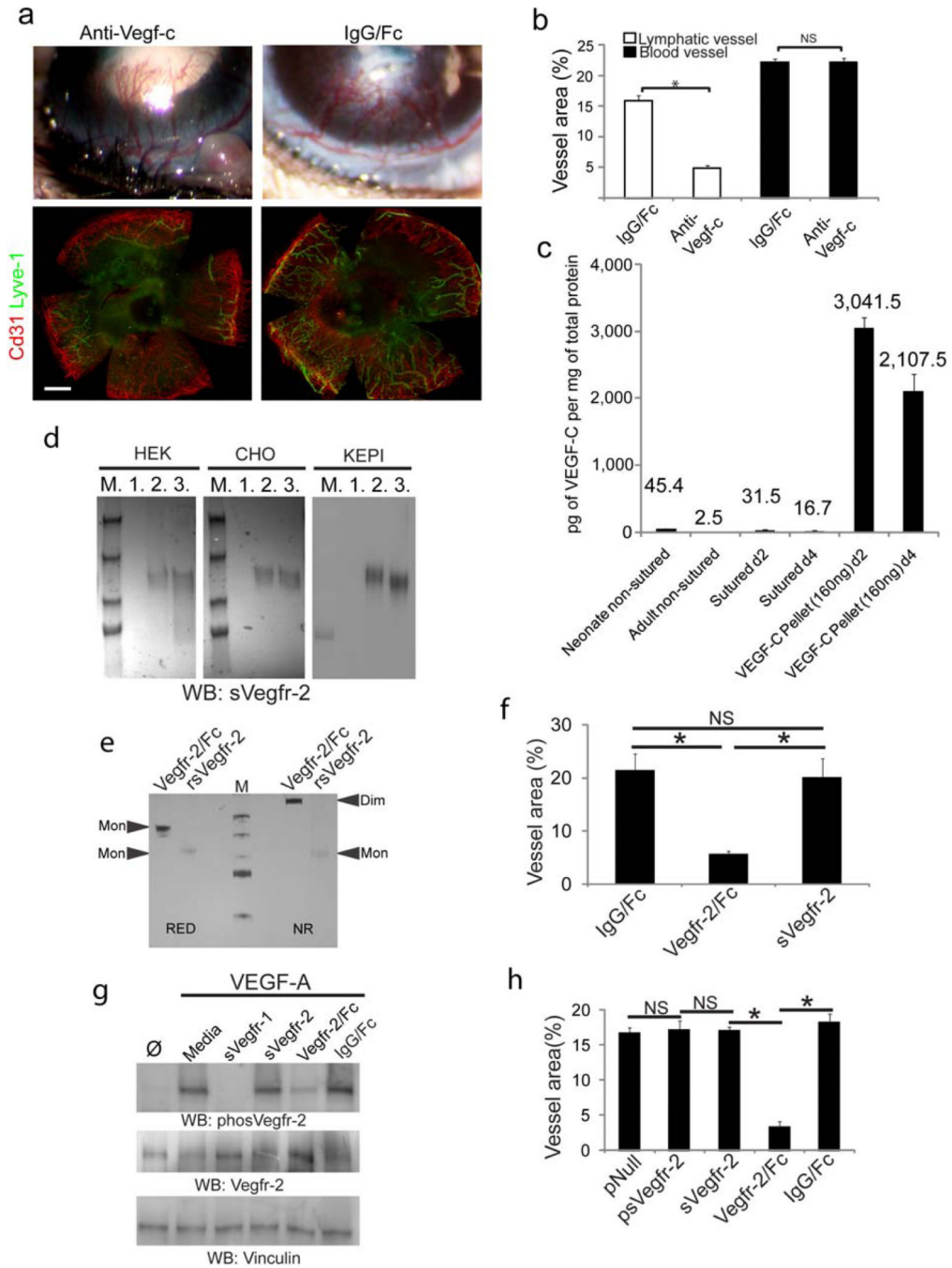
Figure 3.
Endogenous Vegf-c and sVegfr-2 selectively modulate corneal lymphangiogenesis. **(a)** Representative color photographs of sutured wild-type mouse corneas (top). Representative flat mounts of sutured corneas. Lyve-1^{+}, lymphatic vessel - green;Cd31^{+}/Lyve-1^{−}, blood vessel – red. **(b)** Quantification of corneal vessel area of sutured corneas of wild-type mice ($n = 8$). Lyve-1^{+}, lymphatic vessel - white bars; Cd31^{+}/Lyve-1^{−}, blood vessel – black bars. **(c)** ELISA quantification of Vegf-c/VEGF-C in wild-type mouse corneas 2 and 4 days after suture placement or implantation of pellets containing 160 ng recombinant human VEGF-C.

(d) Immunobloting of sVegfr-2 secreted by transfected human embryonic kidney cells (HEK), Chinese hamster ovary cells (CHO), and mouse corneal epithelial cells (KEPI) under reducing (RED) and non-reducing (NR) conditions. M, marker; lane 1. pNull; lane 2. psVegfr-2 (RED); lane 3. psVegfr-2 (NR). **(e)** Silver staining of polyacrylamide gel loaded with Vegfr-2/Fc and recombinant sVegfr-2 (rsVegfr-2) and resolved under reducing versus non-reducing conditions. Dim, dimer; Mon, monomer; M, marker. **(f)** Corneal area occupied by blood vessels (Cd31$^+$/Lyve-1$^-$) following suture injury in wild-type mice. **(g)** Representative immunoblot of phosphorylated Vegfr-2 (phosVegfr-2) of porcine aortic endothelial cells stably transfected with Vegfr-2 (PAE-KDR). Reblotting for total Vegfr-2 and Vinculin are shown. φ, no Vegf-a. **(h)** Corneal area occupied by blood vessels (Cd31$^+$/Lyve-1$^-$) following pVegf-a injection. NS, not significant; *, $P < 0.05$; Significance by Mann Whitney U test. n = 4–8. Error bars depict s.e.m.(**b,c,f,h**). Scale bar: (**a**), 500μm.
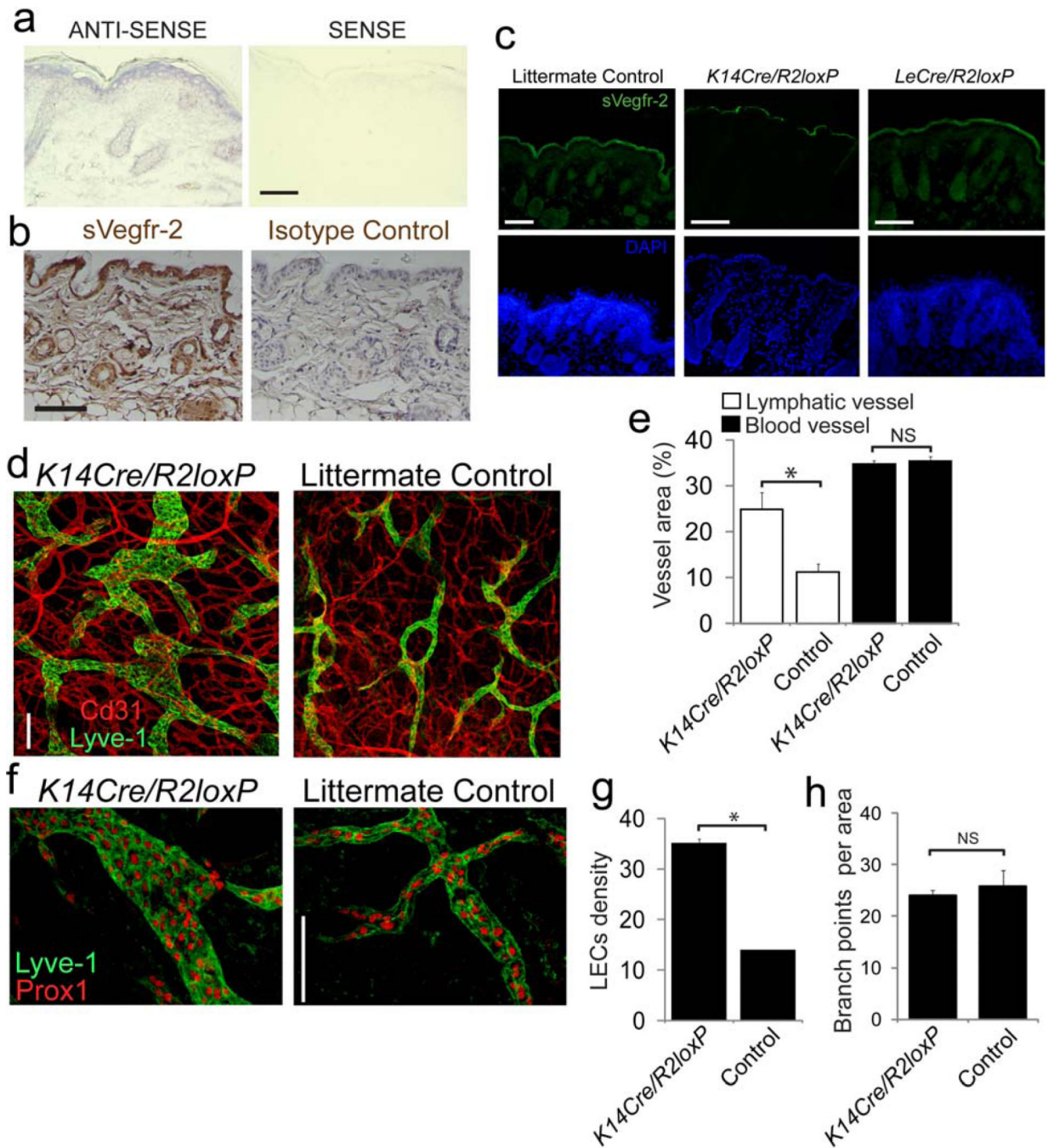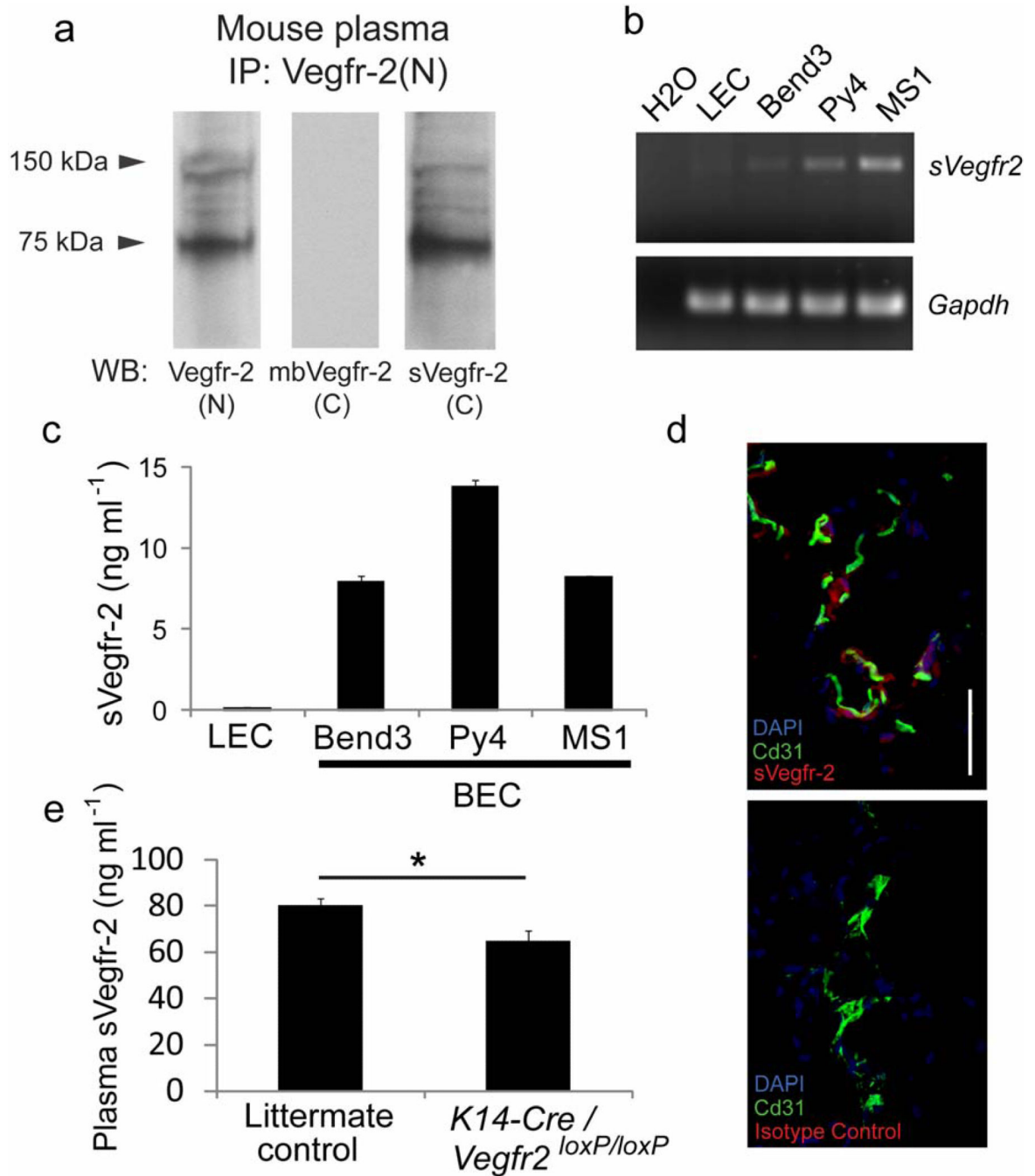
Figure 4.
Loss of *sVegfr2* in the skin induces lymphatic hyperplasia. **(a)** *sVegfr2* mRNA detection
(purple-blue) by *in situ* hybridization in the mouse skin. (**b**) sVegfr-2 immunolocalization
(brown) in the mouse skin using AA21127. Cell nuclei stained blue by hematoxylin. (**c**)
Immunofluorescence of sVegfr-2 in the skin of newborn mice. Superficial keratin
autofluorescence is seen in all instances. Cell nuclei stained with DAPI. Scale bar, 50 μm.
(d) Representative whole mount of the ventral skin of P0 mice. Lyve-1^{+} (green) lymphatic
and Cd31^{+} (red) Lyve-1^{−} blood vessels are shown. **(e)** Skin area occupied by blood vessels

(Cd31$^+$/Lyve-1$^-$, black bars) and lymphatic vessels (Lyve-1$^+$, white bars) in P0 mice. **(f)** Representative whole mount immunofluorescent image showing Lyve-1$^+$ (green) and Prox1$^+$ (red) lymphatic vessels of P0 mice (n = 14). **(g)** Lymphatic endothelial cell (LEC) density, quantified by number of Prox1$^+$ (red) nuclei per 100 μm of lymphatic vessel (Lyve-1$^+$, green) length, in P0 mice (n = 12). **(h)** Quantitative branch point analysis of Lyve-1$^+$ lymphatic vessels per unit area (750 μm × 750 μm) is shown (n = 8). Scale bar**: (a-d,f)**, 50 μm; (**e,g,h**) NS, not significant; *, P < 0.05; Significance by Mann Whitney U test. Error bars depict s.e.m.

Figure 5.

sVegfr-2 is produced by blood endothelial cells (BECs) and skin epithelium and circulates in plasma. (**a**) Representative western blots of mouse plasma immunoprecipitated with an antibody against the amino terminus of Vegfr-2 (N) and immunoblotted with either anti-Vegfr-2 (N; left), anti-sVegfr-2 (C; right) and an antibody against the carboxyl terminus of mbVegfr-2 (C; center). (b) PCR amplification of *sVegfr2* mRNA using cDNA derived from mouse lymphatic endothelial cells (LECs) and mouse BECs isolated from the brain (Bend3), Skin (Py4) and pancreas (MS1). Adjacent lane (H2O) shows template negative control.

Gapdh was loading control (lower band), (*n* = 5). (**c**) ELISA quantification of sVegfr-2 protein in supernatant of blood and lymphatic endothelial cells (*n* = 3). (**d**) Immunofluorescence of sVegfr-2 (red) in the pulmonary microvasculature (Cd31^{+} vessel – green). Cell nuclei stained with DAPI. Scale bar, 50 μm. (**e**) ELISA quantification of sVegfr-2 levels in plasma (*n* = 16). *, *P* < 0.05; Significance by Mann Whitney U test. (**c,e**) Error bars depict s.e.m.
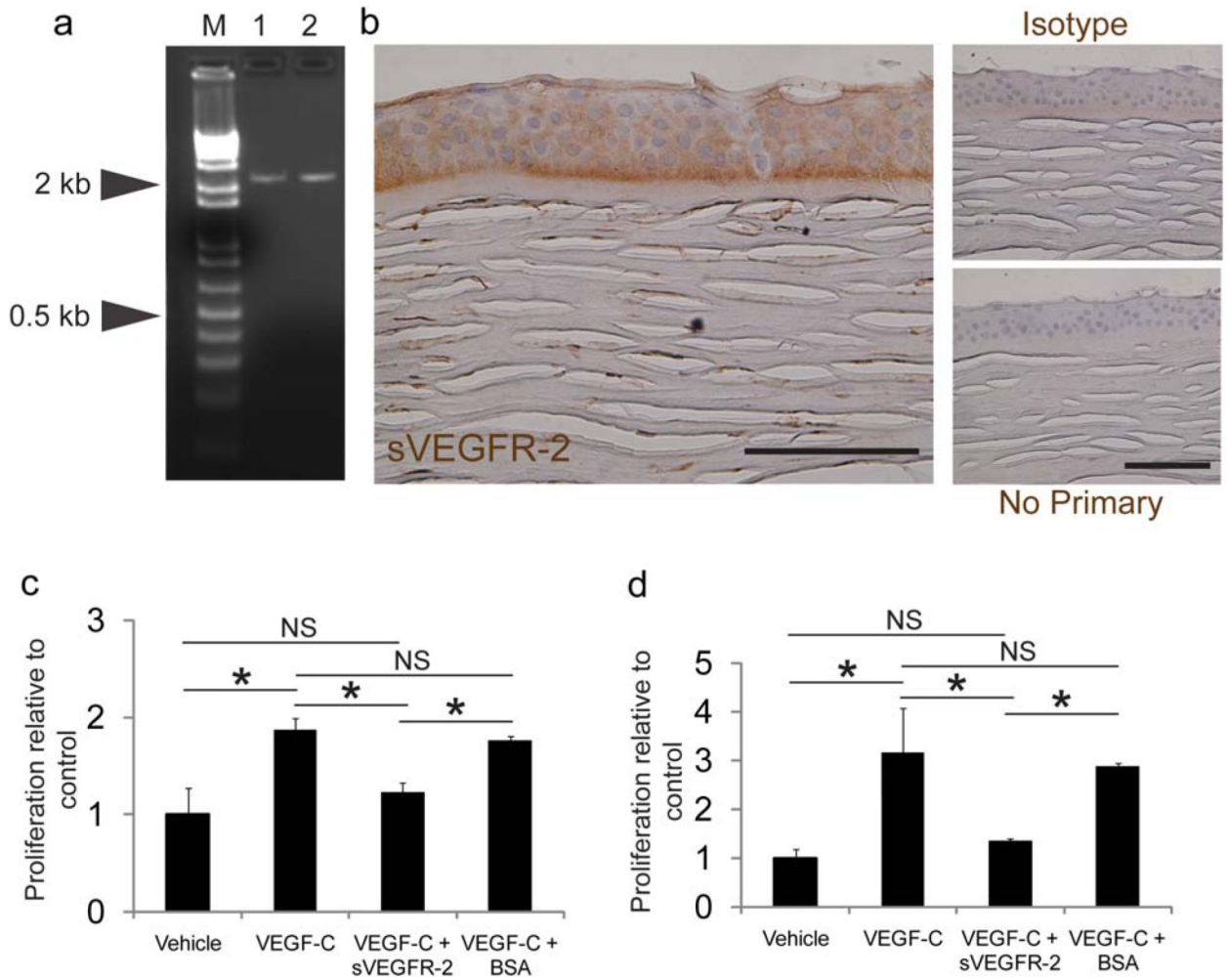
Figure 6.
sVEGFR-2 exists in humans and inhibits human lymphangioma cell proliferation. **(a)** PCR cloning of open-reading-frame of *VEGFR2* from human umbilical vein endothelial cells. **(b)** sVEGFR-2 immunolocalization (brown) in the human cornea using AA21129. Cell nuclei stained blue by hematoxylin. **(c,d)** Proliferation of lymphatic endothelial cells isolated from two children with lymphangioma, stimulated by VEGF-C and treated with sVEGFR-2 or bovine serum albumin (BSA), quantified by BrdU uptake. (n = 6–9); **c**, Patient #1 is a 4-month old child; **d**, Patient #2 is a 10-month-old child. NS, not significant; *, $P < 0.05$; Significance by Mann Whitney U test. Error bars depict s.e.m.