SUPPLEMENTAL MATERIAL

DETAILED METHODS:

Mouse Genetic Models:

Ndst1$^{f/f}$*TekCre*$^{+}$ mutants were generated as described[1]. Syndecan-4 homozygous-null mice (*Sdc4-/-)*[2] (kindly provided by P. Goetinck, Massachusetts General Hospital) were used to generate oil-granuloma/lymphangioma lesions[3]. For some studies, lesions were examined in compound (*Sdc4-/-*)*Ndst1*$^{f/f}$*TekCre*$^{+}$ mutants (after crossing *Ndst1*$^{f/f}$*TekCre*$^{+}$ onto the *Sdc4-/-* background). For carcinoma models, transgenics expressing polyoma middle T-antigen (*PyMT*) under control of the mammary tumor virus (*MMTV*) promoter (*MMTV-PyMT*; Jackson) were backcrossed onto C57Bl/6. *MMTV-PyMT* heterozygosity was sufficient for development of palpable breast tumors in 12-week females. Compound male heterozygotes (*Ndst1*$^{f/f}$*TekCre*$^{+}$*PyMT*$^{+/-}$) were crossed with wildtype *Ndst1*$^{f/f}$ females (which had been extensively backcrossed onto the C57Bl/6 background). Among *PyMT*$^{+/-}$ tumor-susceptible female offspring, this generated ~50% *Ndst1*$^{f/f}$*TekCre*$^{+}$ mutants. Mutant (*Ndst1*$^{f/f}$*TekCre*$^{+}$) versus wildtype (*Ndst1*$^{f/f}$*TekCre*$^{-}$) *MMTV-PyMT*$^{+/-}$ females were sacrificed at 12 weeks for tumor-pathologic studies. To generate *Ndst1*$^{f/f}$*Prox1*$^{+/Cre\text{ERT2}}$ mutants and *Ndst1*$^{f/f}$*Prox1*$^{-/Cre\text{ERT2}}$ controls, *Prox1*$^{+/Cre\text{ERT2}}$ mice (provided generously by G. Oliver, Memphis,TN)[4] were bred to *Ndst1*$^{f/f}$ mice after backcrossing onto C57Bl/6. Tamoxifen (Sigma; dissolved in corn oil) was injected intraperitoneally into *Prox1*$^{+/Cre\text{ERT2}}$ mutants (and *Prox1*$^{-/Cre\text{ERT2}}$ littermate-controls) daily (0.12 mg/g body weight) for 5d to induce *Cre*-recombinase. *Prox1*$^{+/Cre\text{ERT2}}$ mice were bred to *Rosa26R* reporter mice to assess *Cre* localization. LLC tumors were generated by subcutaneous delivery of 5 x10^5 LLCs in 100μL PBS into the inguinal skin-fold. Cells were either VEGF-C overexpressing (LLC-VC) or empty-vector control (LLC-ev) LLCs simultaneously injected into the opposite inguinal region of the same animals, injected 7d following the first tamoxifen dose. Tumors were isolated for pathologic analysis 10d after cell-injection. Mice were housed in AAALAC-approved vivaria following IACUC standards, maintained on a 12hr light-dark cycle, weaned at 3-4 weeks age, and fed standard chow *ad libitum*. For injections, animals were anesthetized using isoflurane (2.5%) through an oxygen-supplemented vaporizer system.

Pathologic tissue processing and analysis: Tumor/tissue specimens for some carcinoma and tissue-based were formalin-fixed, paraffin-embedded, and H&E stained. Sections were immunostained with rabbit-anti-mouse LYVE-1 (5μg/ml; Millipore) followed by biotin-conjugated anti-rabbit secondary (1μg/ml; Jackson), alkaline-phosphatase conjugated streptavidin (2μg/ml; Jackson), and Vector-Blue substrate (Vector). Endogenous peroxidases and avidin/biotin binding were blocked (Vector-kit), with proteinase-K used for antigen retrieval (Dako). Nuclear-Fast-Red counterstain was used. Mean lymphatic vessel density (LVD) was quantified as average number of lymphatic processes per high-power microscopic field. For each tumor, multiple fields were quantified from 2 macroscopically separated levels, with fields selected by a staff pathologist blinded to genotype. Images were photographed (Nikon Eclipse-80i; 40X objective at RT). Peroxidase-based TUNEL was used following manufacturer protocol (Roche). For VEGF-C staining, blocked sections were incubated in rabbit-anti-VEGF-C overnight (10μg/ml; Abcam, or rabbit-IgG control; 4^{o}C) in blocking buffer; and treated with biotin anti-rabbit (1.5μg/ml; Vector) and streptavidin-Cy3 (1μg/ml; Jackson).

Lymphangiogenesis assessments in tissue from LLC tumor bearing mice: Formalin-fixed paraffin-embedded tumor sections were heat-denatured in citrate buffer, blocked (1%BSA/TTBS), treated with goat-anti-LYVE-1 (5μg/ml; R&D) and Cy3 anti-goat (2μg/ml; Abcam) antibodies, cover-slipped with Vectashield (Vector), and photographed (Nikon Eclipse-80i; 40X objective at RT). Lymphangiogenesis only occurred along the tumor periphery in this model: LVD was calculated as average number of vessels/field for all tandem high-power fields along the tumor-periphery (acquired and quantified blinded to genotype). In some studies, LLC-VC cells were injected intravenously ($1x10^5$ cells/mouse) post-tamoxifen to establish orthotopic lung-tumor foci. At 10d post-injection, mice were sacrificed, and lungs digested (0.2% type-I collagenase; Sigma), with washed suspensions subjected to immuno-labeling described separately.

Neonate lymphatic whole-mount analyses: For whole-mount analyses, ear tissue from sacrificed newborn mice within age 1-week was examined. Following fixation (4% paraformaldehyde), dermis was mechanically exposed, permeabilized with triton, and blocked overnight in 3% goat serum in labeling buffer (0.3% triton X-100/ PBS; 4°C). Rabbit anti-mouse LYVE-1 was applied overnight in labeling buffer (1μg/ml; Abcam; 4°C), followed overnight again by goat anti-rabbit Cy3 (1μg/ml; Abcam). Tissue was mounted (Vectashield), and fields (imaged with 10X objective; RT) were analyzed for lymphatic vessel density by the method of grid intersection[5]. Adobe CS2-photoshop software was used to quantify and analyze grid images (blinded to genotype). Lymphatic vessel density (grid-intersection) values for each genogype were calculated, with normalized values (+/-SD) plotted on corresponding graph. Values were compared with appropriate t-test statistic used to calculate p-value reported in corresponding Figure legend.

Wound lymphangiogenesis: was examined in the setting of an early wound-healing model[6] initiated through a standard 3mm cylindrical full-thickness punch biopsy on the dorsal skin of anesthetized mutant versus wildtype littermates. After four days, mice were sacrificed, and the entire wound from each mouse, including a margin of normal surrounding skin, was excised using a wide (8mm) cylinder biopsy. Samples were then split coronally with respect to the wound-crater center, with a piece from each transferred to paraffin embedding so as to allow microtome coronal-sections to be taken for histology. Paraffin sections treated with Hemo-De clearing agent (Fisher) were rehydrated. Antigen retrieval was performed through a 10 min Proteinase K incubation; and endogenous avidin and biotin were blocked. Samples were then blocked in 1%BSA/TTBS blocking buffer (1 hr at RT) followed by addition of goat anti-LYVE-1 (5μg/ml; R&D) and incubation overnight at 4ºC. Samples were then incubated in Biotin anti-goat (20μg/ml; R&D) for 1 hr in blocking buffer, followed by streptavidin-AP (1μg/ml; Vector) for 1 hr at RT. Vector Blue (Vector Labs) substrate was added to slides and incubated for 15 min. Vectashield (Vector Labs) was added to the slides, and images were photographed (Nikon Eclipse 80i; 40X objective at RT). Lymphatic vessels in the column of adjacent high-power (40X objective) fields covering the wound border on each side of the wound crater (from epidermis to base of dermis) were quantified while blinded to genotype, and used to calculate the density or index of total lymphatic vessels per wound margin.

Immunoblotting: Serum-starved cells were stimulated with mature VEGF-C (100ng/mL,15min for most studies). Cells were lysed in RIPA (50mM Tris (pH 7.4), 0.15M NaCl, 10mM $MgCl_2$, 10mM $CaCl_2$, 1mM EDTA, 1μl/ml protease inhibitors (Sigma), 1mM PMSF, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, and 1mM sodium orthovanadate), followed by freezing. Thawed samples were separated on 4-15% gradient gels with rabbit-polyclonal antibodies against phospho-(Ser^{473})Akt (1:1000; Cell Signaling), total-Akt, phospho-Erk1/2 (1:1000; Cell

Signaling), or total-Erk to probe membranes, followed by IRdye-conjugated anti-rabbit antibody (1:10000; LI-COR). Bands were normalized to the ratio of phospho-/Total Akt for baseline-starved cells on a Odyssey/LI-COR infrared system. For cleaved-caspase-3 studies, to examine the effect of VEGF-C on apoptotic signaling in cultured serum-starved siRNA treated hLECs, hLECs were starved for 6hr in the presence/absence of 1μg/mL mature VEGF-C, lysed in RIPA; and run on 4-15% gradient gels (BioRad). In some cases during pilot studies, starvation was varied to include other non-VEGF-C growth factors +/- serum in the basal medium. Blots were labeled with rabbit-anti-caspase (1:1000; Cell Signaling) or cleaved-caspase-3 (1:500; Cell Signaling) antibodies. Bands were quantified following anti-rabbit IRdye labeling.

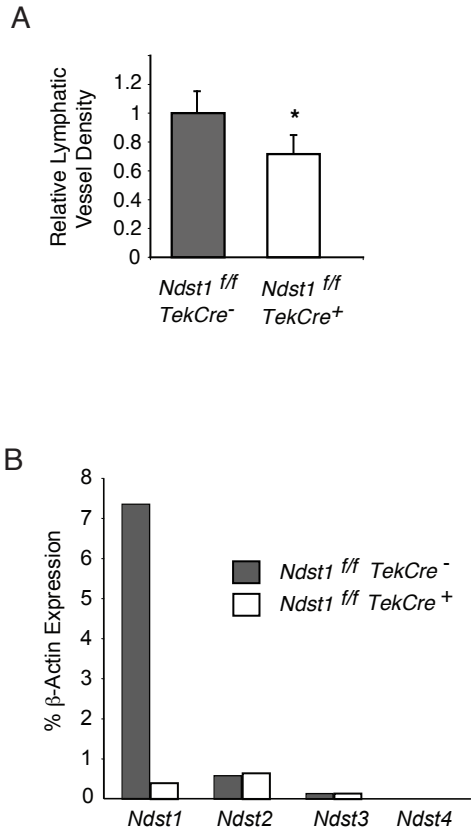
Phospho-MAPK Array: For array studies, human lung lymphatic primary endothelial cells (hLEC) were grown to 70-80% confluency in a 60mm dishes. Cells were transfected with 20nM of siRNA using Lipofectamine RNAiMAX (ThermoFisher) following manufacturer recommendations. Briefly, cells were rinsed with PBS, and transfection complex was added in Opti-Mem (ThermoFisher) and incubated for 6 hours. The cells were rinsed with PBS and normal growth media was added, followed by overnight recovery. Cells were then serum starved for a total of 5 hours in DMEM with a 30 min media change at the end of starvation. Human recombinant VEGF-C (R&D Systems) was then added at 100ng/mL and incubated for 15 min. Cells were then lysed in 0.5mL NP-40 lysis buffer. Using starved (baseline) versus post-VEGF-C stimulation lysates, the array was completed following manufacturer instructions (R&D). Reference dots (on 3 slide-corners) confirmed for each slide that streptavidin-HRP had been appropriately incubated during the procedure.

Proteoglycan core-protein blotting: Cultured near-confluent hLEC pre-treated +/- heparinase to destroy heparan sulfate chains were lysed, electrophoresed on SDS-PAGE, and examined for HSPG core proteins by probing blots with an antibody directed against HS “stubs” (anti-ΔHS). The latter remain on HSPG core proteins as neo-epitopes, consisting of the non-reducing (glucuronate) glycan termini generated by digestion of cell-surface HSPGs with heparinase, with methodology as published[7]. Lysates were run on a 4-15% SDS-PAGE gel, transferred, and blotted with mouse anti-ΔHS antibody (1:1000 overnight at 4^{o}C; clone 3G10, a kind gift from Dr G. David) which recognizes the stub neo-epitopes on HSPG core proteins. After incubation in IRdye 680-conjugated anti-mouse antibody (1:10,000; LI-COR), blots were visualized/ photographed using a Odyssey/LI-COR infrared system. In a separate blot using the same SDS PAGE-gel and reagents, instead of treating with 3G10 antibody, the lysate from heparinase-treated cells was incubated solely with rabbit anti-human syndecan-4 antibody (1:1000, Abcam), and examined in the LI-COR system after incubation with anti-rabbit IRdye 800 (1:10,000; LI-COR).

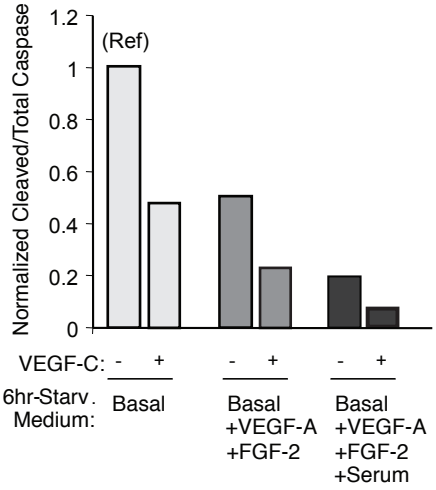
SUPPLEMENTAL REFERENCES:

1. Wang L, Fuster M, Sriramarao P and Esko JD. Endothelial heparan sulfate deficiency impairs L-selectin- and chemokine-mediated neutrophil trafficking during inflammatory responses. Nat Immunol. 2005;6:902-10.
2. Echtermeyer F, Streit M, Wilcox-Adelman S, Saoncella S, Denhez F, Detmar M and Goetinck P. Delayed wound repair and impaired angiogenesis in mice lacking syndecan-4. J Clin Invest. 2001;107:R9-R14.
3. Mancardi S, Stanta G, Dusetti N, Bestagno M, Jussila L, Zweyer M, Lunazzi G, Dumont D, Alitalo K and Burrone OR. Lymphatic endothelial tumors induced by intraperitoneal injection of incomplete Freund's adjuvant. Exp Cell Res. 1999;246:368-75.

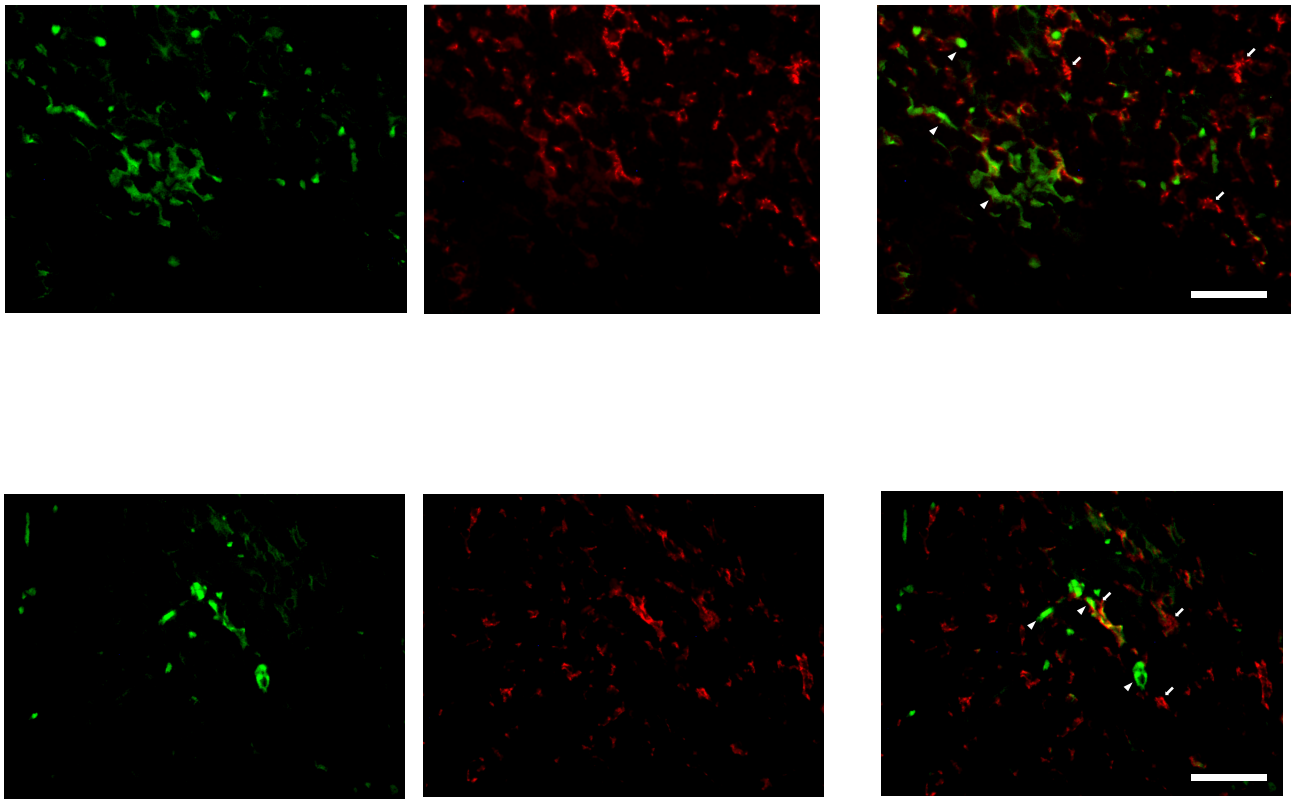
4. Srinivasan RS, Dillard ME, Lagutin OV, Lin FJ, Tsai S, Tsai MJ, Samokhvalov IM and Oliver G. Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature. Genes Dev. 2007;21:2422-32.
5. Kirchner LM, Schmidt SP and Gruber BS. Quantitation of angiogenesis in the chick chorioallantoic membrane model using fractal analysis. Microvasc Res. 1996;51:2-14.
6. Fuster MM, Wang L, Castagnola J, Sikora L, Reddi K, Lee PH, Radek KA, Schuksz M, Bishop JR, Gallo RL, Sriramarao P and Esko JD. Genetic alteration of endothelial heparan sulfate selectively inhibits tumor angiogenesis. J Cell Biol. 2007;177:539-49.
7. Govindraj P, West L, Koob TJ, Neame P, Doege K and Hassell JR. Isolation and identification of the major heparan sulfate proteoglycans in the developing bovine rib growth plate. J Biol Chem. 2002;277:19461-9.



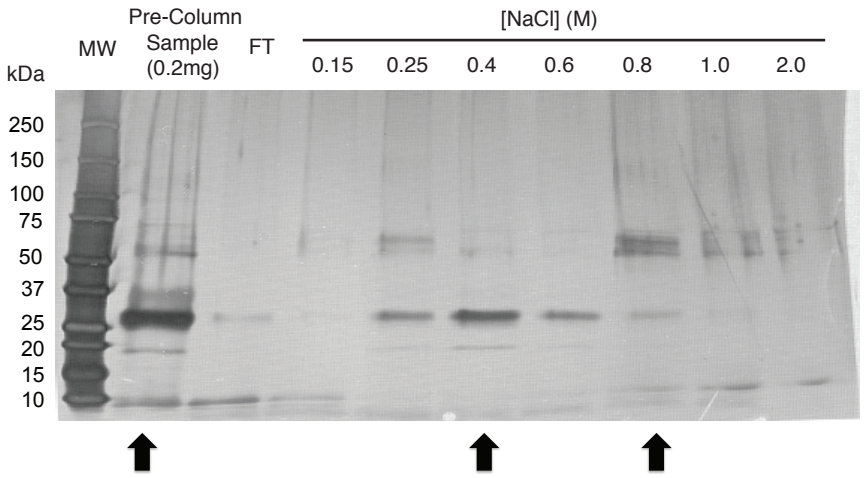
Online Figure I. Neonatal ear-bud lymphangiogenesis in Ndst1f/fTekCre+ mutant mice and their Cre- littermates.
(A) To examine early developing lymphatic trunks/ vasculature in dermis of the neonatal ear-bud, whole-mount sections were stained for LYVE-1 (from n=10 Ndst1f/fTekCre+ mutants versus n=7 Cre- wildtype littermates; *P<0.001 for difference in lymphatic vessel density). (B) To assess deletion efficiency of the mutation in lymphatic vasculature, primary lymphatic endothelial cells purified from the lungs of either Ndst1f/fTekCre+ mutant mice or their Cre- littermates were examined for the expression of major heparan sulfate proteoglycan core proteins using quantitative PCR. RNA was isolated, reverse transcribed, amplified using gene specific primers to each proteoglycan core protein, and quantified relative to expression of β-actin. Ct values from duplicate assays were used to calculate % expression.



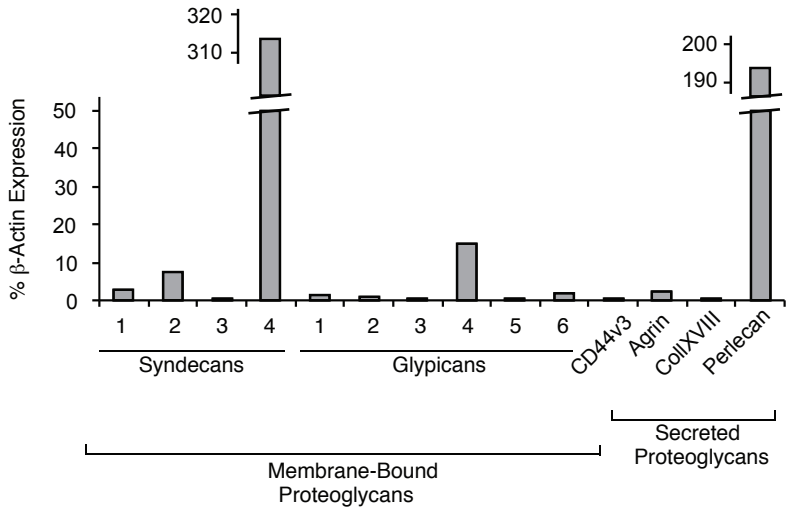
Online Figure II. VEGF-C mediated protection from apoptotic stress in primary human lung lymphatic endothelial cells. Cultured primary human lymphatic endothelial cells (hLEC) grown in fully supplemented medium (including VEGF-A, FGF-2, and 5% serum) were tested for the ability of human recombinant VEGF-C to protect from apoptosis following exposure to a variety of media conditions over a 6 hour period, including full starvation ("Basal" medium) and additional supplementation, as indicated on the left graph. Measurements are based on the ratio of cleaved- to total caspase-3, as measured by Western blotting (with densitometry normalized to the value for fully-starved untreated cells).



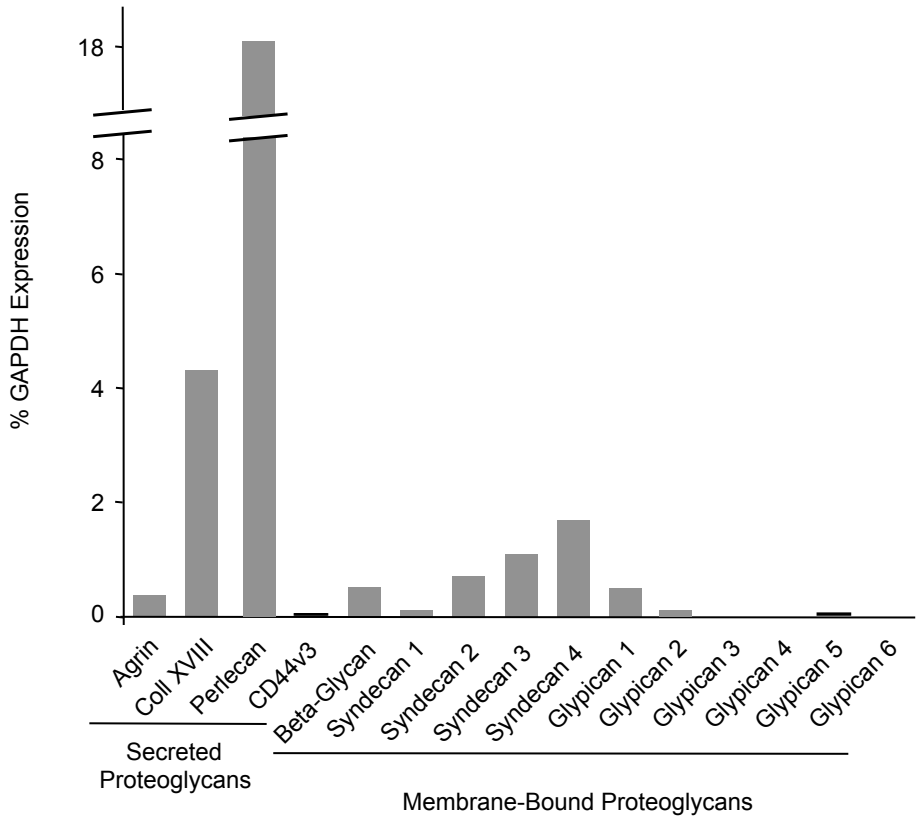
Online Figure III. LYVE-1 and F4/80 labeling in periphery of LLC-VC tumors. Sections from VEGF-C over-expressing tumors (as used for tumor analysis in Figure 2) were labeled with anti-mouse LYVE-1 (green) and F4/80 (red) antibodies, and photomicrographs were taken using a 40X objective, with merge images to right. Representative images are shown for periphery of such tumors (wherein lymphangiogenesis was quantified using LYVE-1 analysis in Fig.2); demonstrating essentially independent LYVE-1 structures bordered (occasionally in close-proximity) with F4/80+ macrophages. (Bar = 50 μm).



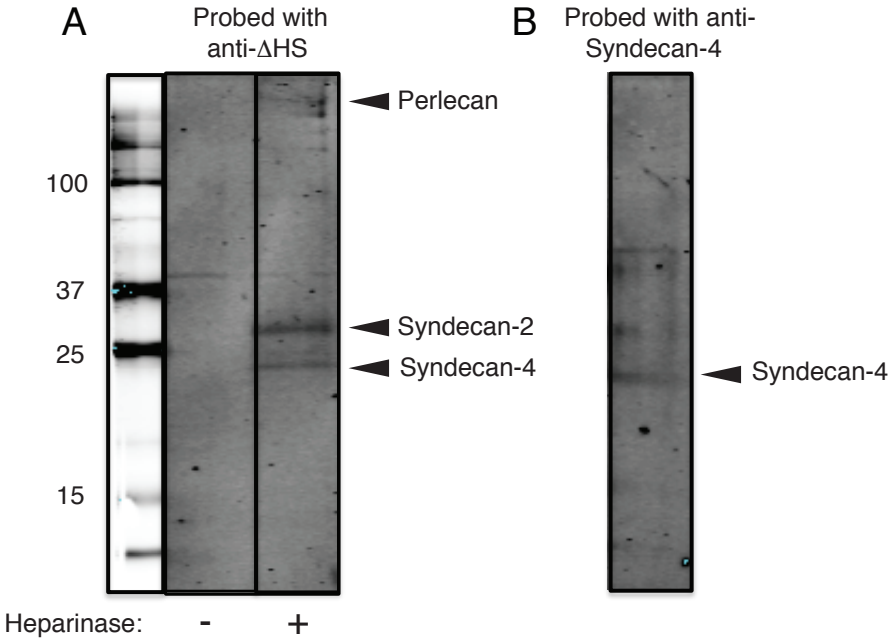
Online Figure IV. Heparin Sepharose Affinity Chromatography: Pro-VEGF-C Binding Profile. To assess binding of Pro-VEGF-C to commercial heparin, and to assess the molecular weight of NaCl-eluted species, affinity chromatography using heparin sepharose columns was carried out, and elutions were silver stained through standard methodology. Fractions collected over the indicated NaCl step-concentration range were run on SDS-PAGE with silver staining to reveal the protein elution profile. The level to which native protein migrates on the gel is shown at left ("Pre-column" sample in lane 1 with kD ladder indicated to the left); column flow-through "FT" is shown in lane 2; and elution profile (subsequent lanes) is shown to the right. This preparation of "Pro-VEGF-C" produced in CHO cells (see "Methods" section) is actually a mixture composed of multiple species/bands noted in the pre-column sample (left arrow at bottom of blot), with a predominant (partially-processed) pro-peptide VEGF-C species that elutes in the 0.4M – 0.5M NaCl range (29/31kD major band; middle arrow), a much lesser amount of the most proteolytically processed mature form of VEGF-C (~18kD band), and an additional band of "full-length" unprocessed VEGF-C that mostly elutes in the 0.8M range (58kD band; right arrow).



Online Figure V. Expression of major heparan sulfate proteoglycan core proteins in SV-LEC line of lymphatic endothelial cells. Mouse SV-LEC cells, a SV40 Large T-antigen immortalized lymphatic endothelial cell line derived from mouse mesenteric lymphatic endothelia, were examined for the expression of major heparan sulfate proteoglycan core proteins using quantitative PCR. RNA was isolated, reverse transcribed, amplified using gene specific primers to each proteoglycan core protein, and quantified relative to expression of β-actin. Ct values from triplicate assays were used to calculate % expression. Bars and scale are interrupted to indicate that values for syndecan-4 and perlecan expression in this cell-line were approximately three-fold (314%) and two-fold (197%) that of beta-actin expression, respectively.



Online Figure VI. Expression of major heparan sulfate proteoglycan core proteins in primary human dermal microvascular endothelial cells (HDMEC). Cultured commercial HDMECs were examined for the expression of major heparan sulfate proteoglycan core proteins using quantitative PCR. RNA was isolated, reverse transcribed, amplified using gene specific primers to each proteoglycan core protein, and quantified relative to expression of GAPDH. Ct values from triplicate assays were used to calculate % expression. Bar and scale is interrupted to plot dominant expression of the secreted HSPG perlecan in these cells, while illustrating expression pattern for membrane-bound syndecans.



Online Figure VII. Assessment of HSPG core proteins produced by primary human lymphatic endothelial cells (hLEC). Cultured near-confluent hLEC pre-treated +/- heparinase were lysed, electrophoresed on SDS-PAGE, and examined for HSPG core proteins by probing blots with an antibody directed against HS "stubs" (anti-ΔHS). The latter remain on any HSPG core protein as neo-epitopes after heparinase digestion, with methodology as published5. (A) Lysate from heparinase-treated cells (right lane, labeled "+" at bottom) reveals multiple bands, including two dominant bands in the 20-40kD range at molecular weights characteristic for syndecan-4 (lower band, labeled) and syndecan-2 immediately above it. The heaviest band at top of blot is consistent with the secreted HSPG perlecan (also labeled). Lysate from non-heparinase treated cells (labeled "-" at bottom) served as a negative control. (B) Blot from a separate SDS-PAGE gel that was run in parallel on lysate from heparinase-treated cells, and probed for syndecan-4 (labeled) using an anti-syndecan-4 antibody. Upper thin band on the blot (unlabeled) was also noted to be present in both heparinase and non-heparinase lysates on panel (A), and thus appeared to be nonspecific.

Online Table I

Primer Sequences (Forward/ Reverse) used for Quantitative PCR of major mouse HS core proteins.

HS Core Protein:	Forward Primer:	Reverse Primer:
Syndecan1	GGAGCAGGACTTCACCTTTG	TACAGCATGAAACCCACCAG
Syndecan2	GCTGCTCCAAAAGTGGAAAC	CAGCAATGACAGCTGCTAGG
Syndecan3	GAGCCTGACATCCCTGAGAG	CCCACAGCTACCACCTCATT
Syndecan4	GAGCCCTACCAGACGATGAG	CAGTGCTGGACATTGACACC
Glypican1	AGCGAGATGGAGGAGAACCT	CTGAGTACAGGTCCCGGAAG
Glypican2	TGACTACCTGCTCTGCCTCTC	GCTTCGCTGACCACATTTCT
Glypican3	GGCAAGTTATGTGCCCATTC	ATGTAGCCAGGCAAAGCACT
Glypican4	ATGGTGGCAGAGAGGCTAGA	GGAACGAGAAATTCGTCCAG
Glypican5	AAGCCCAGTCTGGAAATCCT	TCACAGTCCCCACTGACTTG
Glypican6	CACGTTTCAGGCCCTACAAT	GTTCCAGCATTCCTCCTCGT
Agrin	AACCTGGAGGAGGTGGAGTT	CTTCTTGCAGACGCAGGAC
Perlecan	CACTCGCTCCATCGAGTACA	GATGACCCTGAGCAGCATCT
Collagen-XVIII	CTGGGAGGCTCTGTTCTCAG	CACAGTAGCTCTCGGTCAGC
Beta-glycan	TGAAGTGACTGGACGAGACG	AGTGCGGAGATTCAGGACAT
CD44v3	CTGGGAGCCAAATGAAGAAA	AGCACTTCCGGATTTGAATG
Serglycin	GAGCACCCTGCTACATTTCC	CCGCGTAGGATAACCTTGAA