

A Novel Gene Expression Profile in Lymphatics Associated with Tumor Growth and Nodal Metastasis

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Abstract

Invasion of lymphatic vessels is a key step in the metastasis of primary tumors to draining lymph nodes. Although the process is enhanced by tumor lymphangiogenesis, it is unclear whether this is a consequence of increased lymphatic vessel number, altered lymphatic vessel properties, or both. Here we have addressed the question by comparing the RNA profiles of primary lymphatic endothelial cells (LEC) isolated from the vasculature of normal tissue and from highly metastatic T-241/vascular endothelial growth factor (VEGF)-C fibrosarcomas implanted in C57BL/6 mice. Our findings reveal significant differences in expression of some 792 genes (i.e., ≥ 2 -fold up- or down-regulated, $P \leq 0.05$) that code for a variety of proteins including components of endothelial junctions, subendothelial matrix, and vessel growth/patterning. The tumor LEC profile, validated by immunohistochemical staining, is distinct from that of normal, inflammatory cytokine, or mitogen-activated LEC, characterized by elevated expression of such functionally significant molecules as the tight junction regulatory protein endothelial specific adhesion molecule (ESAM), the transforming growth factor- β coreceptor Endoglin (CD105), the angiogenesis-associated leptin receptor, and the immunoinhibitory receptor CD200, and reduced expression of subendothelial matrix proteins including collagens, fibrillin, and biglycan. Moreover, we show similar induction of ESAM, Endoglin, and leptin receptor within tumor lymphatics in a series of human head and neck and colorectal carcinomas, and uncover a dramatic correlation between ESAM expression and nodal metastasis that identifies this marker as a possible prognostic indicator. These findings reveal a remarkable degree of phenotypic plasticity in cancer lymphatics and provide new insight into the processes of lymphatic invasion and lymph node metastasis. [Cancer Res 2008;68(18):7293–303]

Introduction

Early lymph node metastasis is a common clinical finding in many human cancers, and one that is associated both with aggressive disease and poor prognosis. Clearly, treatments to specifically block dissemination through the lymphatic network

would be desirable either as independent therapies or as adjuncts to existing chemotherapy (1). However, the ability to develop such approaches is currently handicapped by poor understanding of lymphatic vessel molecular biology and ignorance of the molecular interactions that occur between migrating cells and lymphatic vessel endothelium (see ref. 2 for recent review). This contrasts with the blood vascular system, where at least the basic mechanisms regulating vessel wall transmigration have been well studied and extensively characterized (3).

The target vessels for invasion by lymph-metastasizing tumor cells include preexisting tissue lymphatics abutting the tumor mass either through coincidence or mutual chemoattraction (4), as well as new lymphatic vessels that proliferate either within or around the tumor as a result of lymphangiogenesis (5, 6). The latter process is regulated primarily by the lymphangiogenic growth factors VEGF-C and VEGF-D generated by tumor cells or by host cells including tissue macrophages (6). Indeed, recent reports show an association between elevated lymphatic vessel density and either nodal metastasis or reduced 5-year survival in several human cancers (2). Moreover, in animal tumor models, the induction of lymphangiogenesis by engineered overexpression of VEGF-C/D can be sufficient to trigger invasion and lymph node metastasis (7–9). Nevertheless, it remains controversial as to whether this metastasis-enhancing effect results simply from an increase in the number of target vessels due to mitogenesis or if other properties of the newly dividing tumor lymphatics, such as increased permeability, increased lymph flow, or specific changes in lymphatic surface phenotype, predispose them to invasion by tumor (10–13).

In the tumor blood vasculature, recent RNA profiling of immunoselected human tumor endothelial cells using Sequential Analysis of Gene Expression analysis has revealed significant alterations in gene expression that seem to correlate with propensity for hematogenous metastasis (14). This has led to the identification of a number of candidate tumor endothelial markers that are mechanistically implicated in tumor metastasis (15, 16), some of which are currently being evaluated for prognostic and therapeutic targeting applications (14, 17). Thus far, no similar studies have been reported for tumor lymphatics and there is currently no prospect of corresponding markers for the prediction of lymphogenic metastasis.

Here we present the first comparative profiling of normal and tumor-associated lymphatic endothelial cells (LEC) isolated from lymph node metastasizing T-241/VEGF-C mouse fibrosarcomas, using combined GeneChip microarray and immunohistochemical analyses. We reveal an expression pattern in metastasizing tumor LEC distinct from that of nonmetastasizing tumor LEC, normal dermal LEC, or mitogen (VEGF-C)-activated or inflammation-activated LEC, particularly with respect to molecules associated

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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with interendothelial junction and subendothelial matrix integrity. Importantly, we validate key aspects of these findings in human cancers by showing selective expression in tumor lymphatics of the endothelial specific adhesion molecule (ESAM), the transforming growth factor- β (TGF β) coreceptor Endoglin, and the angiogenesis associated leptin receptor (leptin-R). Lastly, we present new evidence of a strong association between ESAM expression and lymph node metastasis in a series of head and neck squamous cell carcinomas (HNSCC) and colorectal carcinomas, indicating the possible value of this marker as a prognostic indicator for disseminated disease. Together these findings identify a pool of novel candidate biomarkers for tumor lymphatics whose evaluation should provide powerful insight into the mechanisms of lymphogenic metastasis.

Materials and Methods

Cells

Murine T241 fibrosarcoma cells transfected with green fluorescent protein (GFP) and either empty vector (nonmetastatic T-241 control) or a construct encoding human recombinant VEGF-C (T-241/VEGF-C/GFP) were cultured in RPMI 1640 supplemented with 10% FCS as described previously (18). Primary mouse dermal LEC and T-241 fibrosarcoma LEC (see below) were grown in gelatin-coated tissue culture flasks in EGM-2 medium (Clonetics).

Antibodies

Antibodies to human and mouse LYVE-1 (19) and mouse ESAM (20) have been described previously. Polyclonal goat anti-human ESAM, goat anti-human LYVE-1, and goat anti-mouse VEGF receptor 3 (VEGFR3) antibodies were from R&D Systems Europe; mouse anti-human Endoglin was from DAKO; polyclonal rabbit anti-human leptin-R antibodies were from Abcam; rat anti-mouse CD31, Endoglin, and MECA-32 monoclonal antibodies were from BD Pharmingen; rabbit anti-Prox1 was from Research Diagnostics Inc.; goat anti-mouse biglycan was from Santa Cruz Biotechnology; and rabbit anti-human CD200 was from Serotec UK. Rabbit anti-mouse podoplanin and rat anti-mouse CD200 were kind gifts from D. Kerjaschki and N. Barclay, respectively. Alexa Fluor 488 and 568/594 conjugates were from Invitrogen.

Mouse Fibrosarcoma Lymphatic Metastasis Model

T241/VEGF-C/GFP fibrosarcoma cells (2×10^6 /mL) in sterile PBS (pH 7.5) were injected s.c. (100 μ L) to the right mid-dorsum of 8-wk-old female C57BL/6 mice. After the 7th day, mice were checked regularly for primary tumor growth and sacrificed before the tumor exceeded ethical limits (generally day 14). Metastasis to the draining axillary lymph node was confirmed postmortem after dissection either by visualization of GFP-containing tumor cells within frozen sections of lymph nodes or by *in vitro* outgrowth of GFP-containing tumor cells from individual nodes.

Immunomagnetic Isolation of Fibrosarcoma and Normal Murine LEC

Normal dermal LEC were isolated from the tail skin of 8-wk-old female C57BL/6 mice (8–12 mice per preparation). Briefly, skin was incubated at 4°C overnight in PBS containing 2 mg/mL dispase to remove epidermis, and endothelial cells were released from dermis by scraping into a Petri dish containing EBM-2 medium. The mixed dermal cell populations were collected by centrifugation and cultured in gelatin-coated flasks (24 h) before detachment with Accutase; purification of LEC was done by immunomagnetic selection with rabbit anti-mouse LYVE-1 antibodies and goat anti-rabbit MACS beads according to the manufacturer's instructions. LEC were then expanded in culture (3–4 d) in six-well multiwell dishes (total of 1×10^6 – 1.5×10^6 cells per preparation) before isolation of RNA and phenotypic analysis.

For isolation of LEC from T241/VEGF-C/GFP fibrosarcomas, primary tumors from mice exhibiting nodal metastases were first minced and

incubated (1 h, 37°C) with shaking in PBS (pH 7.5) containing collagenase (1 mg/mL), elastase (0.1 mg/mL), hyaluronidase (2 mg/mL), and DNase (0.1 mg/mL). The dispersed tissue was then filtered and washed by centrifugation/resuspension in EGM-2 medium before culture in gelatin-coated flasks. The majority of contaminating tumor cells were then removed by selective detachment with PBS, 5 mmol/L EDTA before lifting of the more strongly adherent LEC with Accutase, purification by LYVE-1 immunomagnetic bead selection, and expansion in culture (3–4 d; total yield, 0.5×10^6 – 1×10^6 cells) for RNA isolation and phenotyping as described for dermal LEC.

Phenotyping of LEC by Immunofluorescence Microscopy and Flow Cytometry

For single/double immunofluorescent staining of cultured LEC, primary antibodies were applied in PBS supplemented with FCS (5%, v/v), typically at 10 μ g/mL, and incubated at 25°C for 30 min. Cells were washed briefly with PBS (pH 7.5) before incubation with Alexa Fluor secondary antibodies at 10 μ g/mL in PBS-5% FCS. Samples were fixed in 2% formaldehyde-PBS (v/v) for 10 min, mounted in VectaShield-4',6-diamidino-2-phenylindole (VectaShield-DAPI; Vector Laboratories Inc.), and then viewed under a Zeiss Axiovert fluorescence microscope and captured with a Hamamatsu Orca digital video camera and ImproVision Openlab software.

For flow cytometry, cultured LEC were lifted with Accutase (PAA Laboratories), suspended in incubation buffer (PBS-5% FCS, 0.1% azide), and incubated (30 min, 5°C) with primary antibody followed by washing and reincubation (30 min, 5°C) with the appropriate Alexa Fluor 488 goat conjugate before analysis on a FACScalibur flow cytometer using CellQuest software.

Affymetrix GeneChip Microarray Analysis

Total cellular RNA was isolated (RNeasy, Qiagen) from freshly derived primary LEC ($3 \times$ independent normal LEC cultures and $3 \times$ T-241/VEGF-C tumor LEC cultures; 0.5×10^6 – 1×10^6 cells per culture) before analysis and quantitation with NanoDrop ND-1000 and an Agilent Bioanalyzer 2100 (Agilent). cRNA probe preparation (50 ng input RNA) and hybridization to high-density oligonucleotide mouse MOE430_2.0 arrays (Affymetrix) were carried out using procedures described in the GeneChip expression technical manual (Affymetrix) as previously reported (19). Following hybridization, arrays were processed using a GeneChip Fluidics Station 450 according to the recommended protocols (EukGE-WS2v5, Affymetrix) of double staining and posthybridizations washes. Fluorescent images were captured using gene Array Scanner 3000 (Affymetrix). All experiments were designed and all information was compiled in compliance with MIAME (21) guidelines to facilitate correct interpretation and independent verification of microarray data.

Data Analysis and Bioinformatics

Gene transcript levels were determined from data image files using algorithms in Gene Chip Operating Software (GCOS1.2, Affymetrix) with global scaling. Data analysis was done using Data Mining Tool (DMT 3.1, Affymetrix) and GeneSpring 7.2 (Silicon Genetics). The Robust Multiarray Average expression measurement was also used with help of probe sequence (GC content) information (GC Robust Multiarray Average; refs. 22, 23) as implemented in BioConductor R statistics.⁵ Cell Intensity files (.CEL) were processed into expression values for all the 45,101 probe sets (transcripts) on each array and following the respective normalization step. Differentially expressed genes were selected if they passed Welch's *t* test and parametric test (variance not assumed equal, $P < 0.05$) and showed at least 2-fold changes between normal and tumor-associated LEC. Differentially expressed genes are classified according to the Gene Ontology functional category and GenMAPP and KEGG pathways. The array data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO)⁶ and are accessible through GEO Series accession no. GSE6255.

⁵ <http://www.bioconductor.org>

⁶ <http://www.ncbi.nlm.nih.gov/geo/>

Semiquantitative PCR

cDNA was prepared from total RNA by reverse transcription using oligo dT primers. PCR primer sequences were obtained from PrimerBank (24). Reaction cycle numbers for amplification were optimized to ensure that the reaction mix was sampled during the log phase.

Validation of Affymetrix Array Analyses by Immunofluorescence Microscopy and Immunohistochemistry

Mouse tissues. Thin frozen sections of normal mouse skin, T-241 control, or T-241/VEGF-C tumor tissue were prepared by freezing in optimum cutting temperature embedding medium (purchased from R.A. Lamb Laboratory Supplies) before cutting 8- μ m or thinner sections by cryostat. Nonspecific binding was blocked by incubation with 10% goat serum in PBS (pH 7.5) before application of primary antibodies (10 μ g/mL) in 5% FCS in PBS followed by appropriate Alexa Fluor 488/586 conjugates (10 μ g/mL). Sections were mounted in VectaShield-DAPI and images captured as above.

Human tissues. A panel of 28 cases of HNSCC, 28 cases of primary colorectal carcinoma, and 2 cases each of cutaneous melanoma and primary breast ductal adenocarcinoma (grade 2) was assembled by random selection from the histopathology database at the John Radcliffe Hospital Pathology Department, with full professional approval and in accordance with local ethic committee guidelines.

Colorectal carcinoma cases varying in stage from T₂ to T₄ (equal numbers) were divided into two groups of 14 cases based on the presence or absence of lymph node metastasis at time of resection. Lymph node metastasis was defined as histologic identification of tumor cells in regional or distant lymph nodes that were free from local extension by primary tumor; only cases where evidence of lymph node involvement was confirmed by subsequent histologic assessment were included. Due to size limitations with HNSCC sections, lymph node metastasizing and nonmetastasizing tumors were approximately matched by maximum microscopic dimension and grade rather than tumor stage. Serial sections (4 μ m) were cut onto silanized glass slides before dewaxing, rehydration, and microwave retrieval as described previously (5). Sections were then stained with antibodies to ESAM (1 μ g/mL), Endoglin (0.6 μ g/mL), or leptin-R (0.5 μ g/mL) and developed using the peroxidase-catalyzed biotin-tyramide signal amplification procedure (CSA system, DakoCytomation) for light microscopy or detection with streptavidin Alexa Fluor 488, followed by staining with goat anti-human LYVE-1/donkey anti-goat Alexa Fluor 586 or mouse anti-human LYVE-1/goat anti-mouse Alexa Fluor 586 and mounting in VectaShield-DAPI for fluorescence microscopy.

Vessels doubly positive for Endoglin and LYVE-1, ESAM and LYVE-1, or leptin-R and LYVE-1 were scored as tumoral if present within the main tumor mass or a perimeter zone of one $\times 10$ field of magnification (3.14 mm²) from the main tumor edge. Marginal tissue was defined as normal tissue (with no significant inflammation or fibrosis) >1 mm from the outer margin of this perimeter zone. For Endoglin/LYVE-1 double-positive vessels, five fields of view containing lymphatic vessel hotspots (each at $\times 20$ magnification = 0.79 mm²) were assessed for vessel counts in tumoral and marginal tissue in each case. For ESAM/LYVE-1 and leptin-R/LYVE-1 double-positive vessels, the total number of vessels within 10 random fields ($\times 10$ magnification) was counted. In some experiments, vessels were also scored according to staining intensity and pattern as either weak (granular patchy staining) or strong (bright continuous or discontinuous wall staining).

Statistics

Statistical analyses were carried out for the association between Endoglin, ESAM, and leptin-R positive lymphatic vessel number and tumoral/normal marginal location and for Endoglin expression level with tumoral/normal marginal location within the group of 28 HNSCC and colorectal cancer cases using the *t* test. Similar analyses were also carried out for the association between Endoglin-, ESAM-, and leptin-positive vessel number and presence or absence of lymph node metastasis.

Results

Isolation and characterization of LEC derived from murine T-241/VEGF-C tumors. As a model system to study the effects of tumor environment on LEC phenotype, we used the GFP-transfected T241/VEGF-C tumor line (18) that consistently forms lymph node metastasizing fibrosarcomas when implanted in the dermis of C57BL/6 mice, characterized by formation of extensive intratumoral and peritumoral LYVE-1⁺/⁺Podoplanin⁺/⁺PROX-1⁺ lymphatic vessels (see Supplementary Fig. S1). For the analyses, we isolated LEC both from T-241/VEGF-C fibrosarcomas harvested 12 to 14 days after initial implantation (tumor LEC) and from normal dermis (normal LEC) using immunomagnetic bead selection with affinity-purified LYVE-1 antisera. The resulting populations in each case coexpressed the lymphatic markers LYVE-1, podoplanin, VEGFR3, and Prox-1, together with the pan-endothelial marker CD31, indicating their authentic LEC phenotype (Fig. 1A and B). As expected, the cultures displayed some heterogeneity in LYVE-1 expression—a characteristic shared by the receptor in intact tissue lymphatics (see, e.g., Supplementary Figs. S1, S3, and S4) that is partly due to regulated LYVE-1 uptake and lysosomal degradation (25). Overall purity was estimated to be in excess of 98% as assessed by fluorescence-activated cell sorting (FACS) analysis for either podoplanin or LYVE-1, indicating the absence of any significant contamination with stromal tissue or tumor cells. Furthermore, normal dermal and T-241/VEGF-C tumor LEC were uniformly negative for MECA-32, a murine blood vascular endothelial marker (see Supplementary Fig. S1 and data not shown), indicating the absence of contaminating blood vascular endothelial cells. Lastly, the three-dimensional culture properties of the two LEC populations were similar insofar as both showed identical capacities for tube formation in Matrigel (data not shown).

Metastatic T-241/VEGF-C tumor LEC have a distinct transcriptional profile. Having confirmed the authenticity of the LEC populations by immunofluorescence microscopy and flow cytometry, we next compared their transcriptional profiles by generating cRNA probes for hybridization to Affymetrix mouse 430 GeneChip arrays. The results (Fig. 1C; NCBI GEO database accession GSE6255) revealed differential expression of some 792 genes including 271 that were up-regulated and 521 that were down-regulated in T-241/VEGF-C tumor compared with normal dermal LEC by ≥ 2 -fold ($P \leq 0.05$). Based on potential relevance to metastasis, we focused initially on transcripts associated with cell-cell adhesion and endothelial junction formation, as well as others encoding extracellular matrix components and vessel growth/patterning factors, yielding the representative panel shown in Table 1. The changes in levels of 15 transcripts chosen from this panel were independently confirmed by a limited cycle semiquantitative reverse transcriptase PCR (Supplementary Fig. S2) and in selected examples by comparative immunohistochemical staining of frozen tissue (Fig. 2).

Changes in endothelial junction and adhesion molecules. Most notably among endothelial cell adhesion molecules, the tight junction-associated molecule JAM-A (*F11r*) and the closely related molecules Coxsackie adenovirus receptor (*Cxadr*), ESAM (*Esam1*), the nectin family adherens junction component CD112, and the adhesion-associated α actinin-binding protein Ajuba showed up-regulation in tumor LEC, whereas JAM-B (VE-JAM, *Jam-2*) was down-regulated. Given that these molecules mediate steps in the transmigration of discrete leukocyte populations across blood and

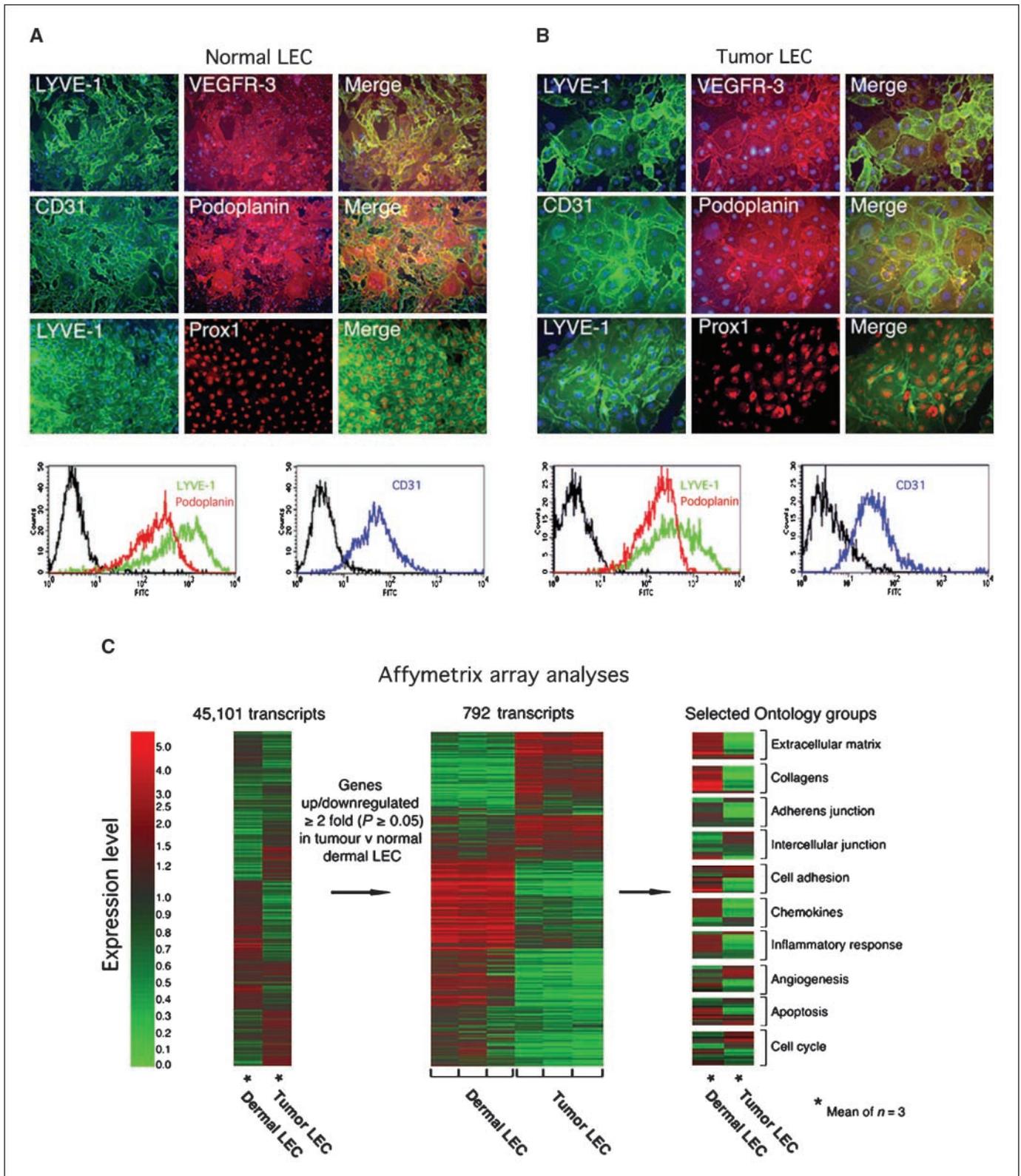


Figure 1. Characterization and transcriptional profiling of mouse T-241/VEGF-C fibrosarcoma LEC and normal dermal LEC. Primary cultures of LYVE-1 immunomagnetic bead–selected normal dermal LEC (A) and T241/VEGF-C fibrosarcoma LEC (B) are shown dual stained for LYVE-1 and either VEGFR3 or Prox-1, or for the pan-endothelial marker CD31 and podoplanin, followed by Alexa 488 (green) or Alexa 568 (red) conjugates as indicated. Original magnification, $\times 20$. Representative FACS histograms show expression levels of LYVE-1 (green), podoplanin (red), CD31 (blue), and irrelevant immunoglobulin control (black). Variation in LYVE-1 expression reflects heterogeneity seen in intact tissues (see text and Supplementary Fig. S1). C, Affymetrix mouse 430 GeneChip array analyses showing the 792 transcripts up- or down-regulated >2 -fold ($P \leq 0.05$) in T241/VEGF-C (Tumor) LEC compared with normal (Dermal) LEC. Data are represented as heatmaps using GeneTree software. Expression levels relative to the mean chip value are graded from red (above average) through green (below average) according to the calibration scale shown on the left.

Table 1. Genes up-regulated or down-regulated in T241/VEGF-C-derived LEC compared with normal skin-derived LEC ($P \leq 0.05$)

| Affymetrix probe ID | Transcript | Gene | Fold change |
|--|---|----------------|-------------|
| Cell adhesion and junctional proteins | | | |
| 1453282_at | Coxsackie adenovirus receptor | <i>Cxadr</i> | +5.3 |
| 1460356_at | Endothelial specific adhesion molecule | <i>Esam1</i> | +3.9 |
| 1421344_a_at | Ajuba | <i>Jub</i> | +2.9 |
| 1424595_at | Junctional adhesion molecule A | <i>F11r</i> | +2.6 |
| 1417703_at | CD112 | <i>Pvrl2</i> | +2.2 |
| 1450757_at | Cadherin 11 | <i>Cdh11</i> | -108 |
| 1449244_at | N-Cadherin | <i>Cdh2</i> | -78 |
| 1428547_at | CD73 | <i>N5te</i> | -13 |
| 1436568_at | Junctional adhesion molecule B | <i>Jam2</i> | -9.5 |
| Extracellular matrix | | | |
| 1416342_at | Tenascin-C | <i>Tnc</i> | +5.3 |
| 1450857_a_at | Procollagen I α 2 | <i>Col1a2</i> | -954 |
| 1448323_a_at | Biglycan | <i>Bgn</i> | -625 |
| 1427883_a_at | Procollagen III α 1 | <i>Col3a1</i> | -315 |
| 1451978_at | Lysyl oxidase like 1 | <i>Loxl1</i> | -121 |
| 1416121_at | Lysyl oxidase | <i>Lox</i> | -73 |
| 1426285_at | Laminin α 2 | <i>Lama2</i> | -65 |
| 1426947_x_at | Procollagen VI α 2 | <i>Col6a2</i> | -61 |
| 1451527_at | Procollagen C-endopeptidase enhancer 2 | <i>Pcolce2</i> | -44 |
| 1448590_at | Procollagen VI α 1 | <i>Col6a1</i> | -41 |
| 1437165_at | Procollagen C-endopeptidase enhancer | <i>Pcolce</i> | -37 |
| 1416740_at | Procollagen V α 1 | <i>Col5a1</i> | -18 |
| 1418061_at | Latent transforming growth factor β binding protein 2 | <i>Ltbp2</i> | -18 |
| 1427257_at | Versican | <i>Cspg2</i> | -16 |
| 1435264_at | Elastin microfibril interfacier 2 | <i>Emilin2</i> | -14 |
| 1424131_at | Procollagen VI α 3 | <i>Col6a3</i> | -10 |
| 1448870_at | Latent transforming growth factor β binding protein 1 | <i>Ltbp1</i> | -3.6 |
| 1425896_at | Fibrillin-1 | <i>Fbn1</i> | -3.3 |
| 1450625_at | Procollagen V α 2 | <i>Col5a2</i> | -3.0 |
| Vascular growth and patterning | | | |
| 1415874_at | Sprouty homologue 1 | <i>Spry1</i> | +9.0 |
| 1450414_at | Platelet-derived growth factor B | <i>Pdgfb</i> | +8.8 |
| 1425875_a_at | Leptin receptor | <i>Lepr</i> | +8.3 |
| 1460302_at | Thrombospondin-1 | <i>Thbs1</i> | +5.8 |
| 1451924_at | Endothelin-1 | <i>Edn1</i> | +5.3 |
| 1450922_a_at | Transforming growth factor β 2 | <i>Tgfb2</i> | +4.7 |
| 1416077_at | Adrenomedullin | <i>Adm</i> | +4.1 |
| 1418415_at | Homeobox B5 | <i>Hoxb5</i> | +3.3 |
| 1432176_a_at | TGF β 1R3 Endoglin (CD105) | <i>Eng</i> | +3.1 |
| 1435110_at | Unc 5 homologue B | <i>Unc5b</i> | +2.8 |
| 1448831_at | Angiopoietin 2 | <i>Angpt2</i> | +2.8 |
| 1435458_at | Proviral integration site 1 | <i>Pim1</i> | +2.6 |
| 1417455_a_at | Transforming growth factor β 3 | <i>Tgfb3</i> | +2.5 |
| 1438658_a_at | Endothelial differentiation sphingolipid G-protein coupled receptor 3 | <i>Edg3</i> | -402 |
| 1429348_at | Semaphorin 3C | <i>Sema3c</i> | -78 |
| 1448606_a_at | Endothelial differentiation sphingolipid G-protein coupled receptor 2 | <i>Edg2</i> | -72 |
| 1448925_at | Twist 2 | <i>Twist2</i> | -18 |
| 1415943_at | Syndecan 1 | <i>Sdc1</i> | -9.5 |
| 1427231_at | Roundabout homologue 1 | <i>Robo1</i> | -5.3 |
| 1418261_at | Spleen tyrosine kinase | <i>Syk</i> | -4.9 |
| 1417011_at | Syndecan 2 | <i>Sdc2</i> | -4.2 |
| 1416693_at | Forkhead box C2 | <i>Foxc2</i> | -3.5 |
| 1417389_at | Glypican 1 | <i>Gpc1</i> | -3.4 |
| Miscellaneous | | | |
| 1448788_at | CD200 | <i>Cd200</i> | +6.0 |
| 1419728_at | ENA-78 | <i>Cxcl5</i> | -62 |
| 1460227_at | Tissue inhibitor of metalloproteinase 1 | <i>Timp1</i> | -7.0 |
| 1441855_x_at | GRO- α | <i>Cxcl1</i> | -6.0 |
| 1416180_a_at | Radixin | <i>Rdx</i> | -2.5 |

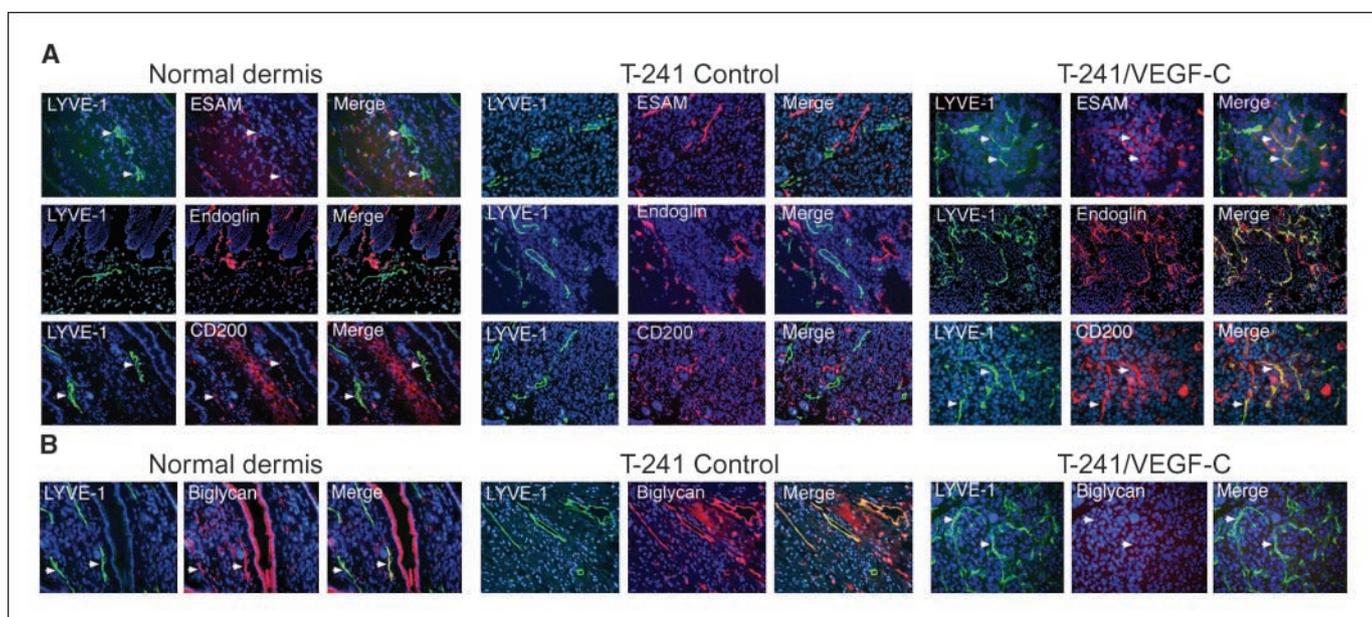


Figure 2. Confirmation of altered gene expression in lymphatics of T241/VEGF-C fibrosarcomas by comparative immunostaining. *A*, representative frozen sections of normal dermis and primary tumors from mice transplanted with either T-241 control or T-241/VEGF-C fibrosarcomas were dual immunostained for LYVE-1 (Alexa 488; green) and ESAM, Endoglin, or CD200 (Alexa 568; red) to confirm the up-regulated gene expression seen in Affymetrix microarray analyses of isolated LEC (Fig. 1C). ESAM, Endoglin, and CD200 expression is restricted to LYVE-1-negative blood capillaries in normal skin and nonmetastatic control T-241 tumors but selectively up-regulated in intratumoral/peritumoral LYVE-1-positive lymphatic capillaries in metastatic T-241/VEGF-C fibrosarcomas (original magnification, $\times 20$). *B*, dual immunostaining for LYVE-1 and biglycan shows that the proteoglycan is abundant in the subendothelial matrix of LYVE-1-negative blood vessels and small LYVE-1-positive lymphatic capillaries in normal dermis and control nonmetastatic T-241 tumors but is absent from LYVE-1-positive lymphatics in metastatic T241/VEGF-C fibrosarcomas (original magnification, $\times 20$). Arrowheads in selected images are included as points of reference for appropriate single or dual stained lymphatic capillaries.

lymphatic vessels during normal immune trafficking (20, 26, 27), their induction in tumor lymphatics may well have functional significance for invasion by metastasizing T-241/VEGF-C tumor cells. Other notable changes included down-regulation of the adherens junction proteins N-cadherin (*Cdh2*) and cadherin-11 (*Cdh11*; see Table 1). Curiously, tumor LEC also showed up-regulation of RNA for CD200, an immunoglobulin superfamily receptor reported to inhibit immune activation through binding of its receptor CD200R on myeloid cells (28). Immunostaining for ESAM and CD200 confirmed that both proteins were indeed present in LYVE-1-positive lymphatics within T-241/VEGF-C fibrosarcomas whereas neither was detected in lymphatics within normal mouse dermis (Fig. 2A). Moreover, neither protein was detected within the lymphatics of control T-241 fibrosarcomas lacking VEGF-C, which grow as localized nondisseminating tumors (18), consistent with an association between marker expression and nodal metastasis.

Down-regulation of subendothelial matrix proteins. A number of genes encoding key components of the extracellular matrix were differentially expressed in T-241/VEGF-C tumor-associated LEC. For example, transcripts for individual α chains of the fibrillar collagens, procollagens I α 2, III α 1, and V α 2, and all three α -chain subunits of the network-forming fibril-associated collagen (procollagen VI) were down-regulated. We also noted down-regulation of transcripts encoding the procollagen-C endopeptidase enhancer proteins, lysyl oxidase and the small proteoglycan biglycan, which control the mechanical properties of collagen fibrils (29), and reduced expression of RNA encoding the lymphatic vessel anchoring filament protein fibrillin-1, together with the microfibril-associated proteins EMILIN-2 and Latent TGF Binding Proteins (30). In contrast, there was up-regulation of

tenascin-C, a modulator of matrix adhesion commonly found in the tumor stroma and associated with promotion of tumor growth (31). We confirmed down-regulation of biglycan by immunofluorescence microscopy, which showed that the protein was present within LYVE-1-positive lymphatic vessels of normal skin and control nonmetastasizing T-241 tumors, but absent from lymphatics of nodal metastasizing T-241/VEGF-C fibrosarcomas (Fig. 2B). These changes in extracellular matrix and associated components suggest a possible alteration in the interaction between tumor lymphatics and the underlying fibrosarcoma matrix that may have consequences for metastasis.

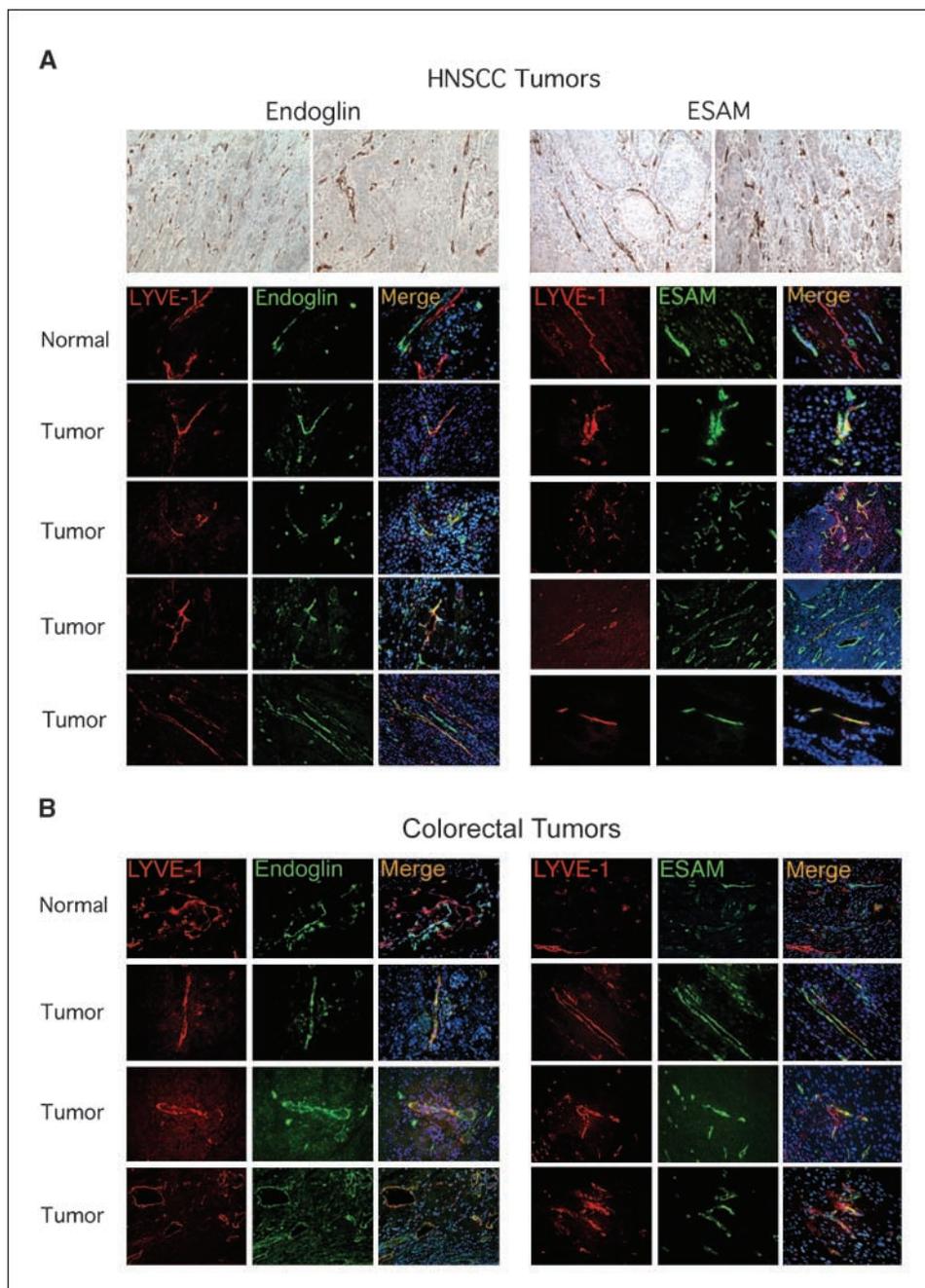
Changes in molecules related to vessel growth and patterning. Several genes associated with endothelial cell proliferation and vessel sprouting were significantly up-regulated in T-241/VEGF-C tumor LEC. These included the obesity-related leptin receptor (Ob-R), a regulator of hemangiogenesis and vascular permeability (32); platelet-derived growth factor B, a form of the smooth muscle growth factor with mitogenic activity for lymphatic vessels; TGF β 2; TGF β 3; and the endothelial specific TGF β 1/3, coreceptor Endoglin/CD105 (33). We confirmed up-regulation of Endoglin by immunostaining of frozen tissue, which showed that the protein was present in LYVE-1-positive lymphatics of nodal metastasizing T-241/VEGF-C tumors but absent from those of control T-241 tumors and normal dermis (Fig. 2A). Differentially expressed genes implicated in vessel patterning included angiotensin-2, a growth factor required for lymphatic vessel patterning (34); FoxC2, the transcription factor whose mutation leads to lymphatic vessel arterialization in lymphoedema distichiasis (35); Syk, a tyrosine kinase associated with blood and lymphatic vascular specification (36); and the G-protein-coupled endothelial lysophospholipid/sphingosine phosphate receptors *edg2* (LPA1) and

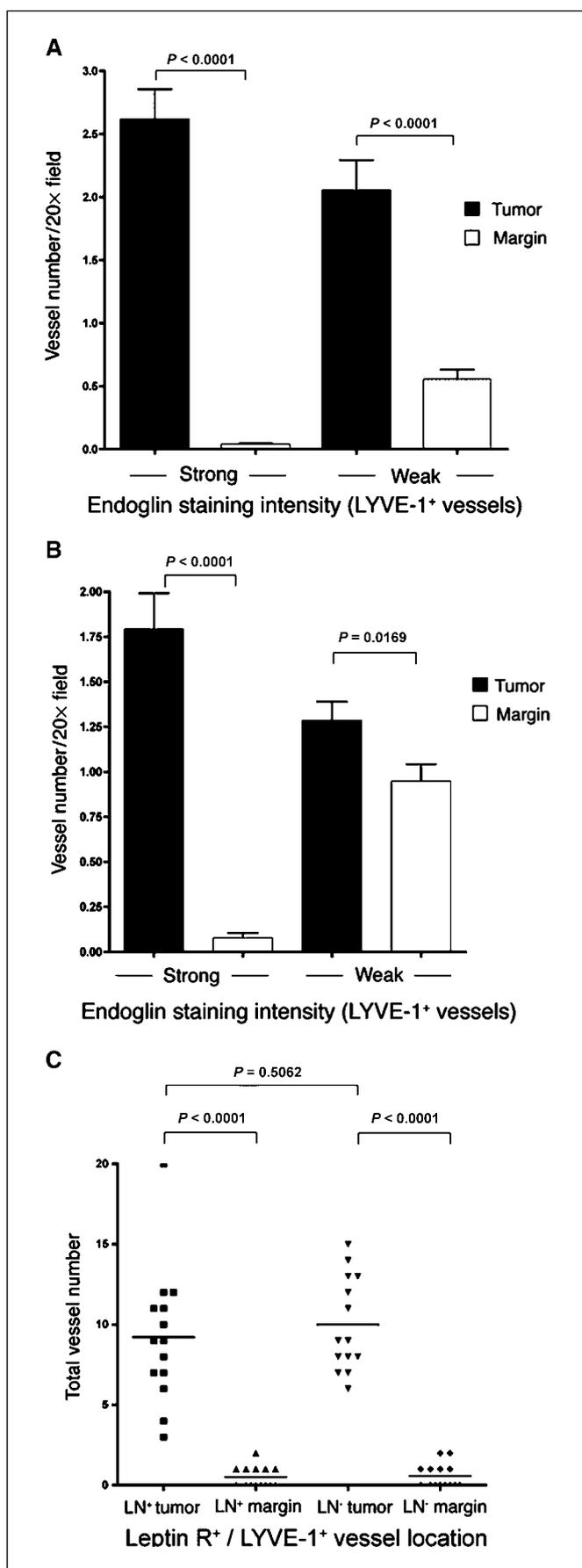
edg3 (SIP3). Changes in expression of the *edg* receptor family are particularly interesting because these molecules, which function on both leukocytes and endothelial cells, are known to regulate transmigration across both afferent and efferent lymphatics (37, 38).

Minimal effects of VEGF-C on lymphatic phenotype. Given that the lymph node metastasizing variant of the T-241 tumor cell line used in our study overexpresses VEGF-C, we considered the obvious possibility that the distinct expression pattern observed for tumor LEC might have resulted from direct mitogenic effects of this growth factor rather than the influence of tumor/host environment. Culture of normal murine primary dermal LEC with VEGF-C failed to induce a profile similar to T-241/VEGF-C fibrosarcoma-derived LEC when assessed by Affymetrix array

analysis (not shown); however, this may be an artifact due to the low proliferation of murine LEC in response to VEGF-C *in vitro* observed by ourselves and reported by others (see ref. 39 and data not shown). Nevertheless, comparison of the T241/VEGF-C LEC profile with that of VEGF-C-treated human dermal LEC, which do proliferate in response to the growth factor (40), showed little or no overlap (not shown). Lastly, *in vivo* lymphangiogenesis studies in mice using VEGF-C-impregnated Matrigel implants revealed no induction of ESAM or CD200 within nascent lymphatics (data not shown). Hence, we conclude that the expression profile of T-241/VEGF-C tumor LEC is not simply the result of VEGF-C signaling or mitogenesis but rather a consequence of more complex effects of tumor-endothelial or stromal cell-endothelial interactions.

Figure 3. Immunostaining for Endoglin and ESAM in human cancer lymphatics. **A**, representative sections of HNSCC stained for Endoglin or ESAM by an immunoperoxidase method to reveal extensive expression in tumor microvasculature (top; original magnification, $\times 10$ and $\times 20$, respectively) or dual immunostained for LYVE-1/Alexa 568 (red) and either Endoglin or ESAM/Streptavidin Alexa 488 (green) to show expression in intratumoral and peritumoral tissue compared with normal marginal tissue (bottom; original magnification, $\times 20$). Merged images include DAPI staining. **B**, representative sections of colorectal carcinomas and normal marginal tissue dual immunostained for LYVE-1/Alexa 568 (red) and either Endoglin or ESAM/Streptavidin Alexa 488 (green). Merged images include DAPI staining. Original magnification, $\times 20$.





Confirmation of an altered phenotype for tumor lymphatics in human cancers.

We next determined how far the lymphatic phenotype observed in the mouse fibrosarcoma model might extend to naturally occurring human cancers, and how any observed changes in the expression of candidate genes might relate to nodal metastasis. In initial studies we detected both ESAM and Endoglin immunostaining specifically in tumor lymphatics, but not normal marginal lymphatics, within a range of different cancers including melanoma and lung, ovarian, breast, endometrial, and hepatocellular carcinomas (Supplementary Fig. S3 and data not shown). Importantly, we confirmed the authenticity of both ESAM- and Endoglin-positive vessels as lymphatics in these human cancer tissues by two- and three-color immunostaining with LYVE-1 and either podoplanin or PROX-1 (Supplementary Figs. S4, S6, and S7), discounting the possibility that they represented infiltrating LYVE-1-positive macrophages or blood vessels.

For more extensive analyses, we assembled panels of paraffin-embedded tumor tissues from 28 patients with primary HNSCC (5, 41) and 28 with primary colorectal carcinoma. Both series included equal numbers of cases with or without demonstrable lymph node metastasis. Focusing on ESAM, Endoglin/CD105, and the hemangiogenesis-associated leptin-R (Ob-R; ref. 32), due to poor reactivity of antibodies to such additional markers as Cocksackie adenovirus receptor and CD200 in paraffin sections, we compared expression on LYVE-1-positive lymphatics in tumors and LYVE-1-positive lymphatics within normal marginal tissues by dual immunofluorescent antibody staining. As shown by the fluorescence micrographs in Fig. 3 and Supplementary Fig. S5, Endoglin, ESAM, and leptin-R were detected in numerous LYVE-1-positive tumor lymphatics in both HNSCC and colorectal carcinomas. In addition, some positive staining could be seen in LYVE-1-negative vessels (data not shown), consistent with known expression of these receptors in normal and neoplastic hemovascular endothelium (20, 42, 43). However, in lymphatics, all three markers were strongly expressed in peritumoral and, in some cases, intratumoral vessels, whereas only weak expression was seen in normal stromal vessels.

Quantitative analysis of immunostained vessels confirmed these initial observations and revealed a striking association between Endoglin-positive vessel incidence and tumor location among lymphatics in both HNSCC (Fig. 4A) and colorectal carcinomas (Fig. 4B), particularly among strongly Endoglin-positive vessels (2.61 ± 0.24 vessels per field in HNSCC tumor versus 0.02 ± 0.01 in marginal tissue, $P < 0.0001$; 1.79 ± 0.2 vessels per field in colorectal tumor versus 0.08 ± 0.03 in marginal tissue, $P < 0.0001$), although this was less obvious among more weakly stained vessels. Interestingly, there was also a significant association between tumor Endoglin-positive vessel number and the presence of lymph node metastases in HNSCC (Supplementary Fig. S8), although once again this was less marked among weakly stained vessels. A clear association between marker induction and neoplasia was also

Figure 4. Quantitative analysis of Endoglin- and leptin-R-positive lymphatics in HNSCC and colorectal carcinoma. Bar charts show counts for Endoglin-positive lymphatic vessel numbers present within the tumor and normal margin in 28 cases each of HNSCC (A) and colorectal carcinoma (B) assessed by dual LYVE-1 immunofluorescence microscopy. Endoglin expression is scored as either strong or weak according to staining intensity (see Materials and Methods). A scatter plot (C) shows individual counts for total leptin-R-positive lymphatic vessel numbers in tumor and normal margin of the same HNSCC series (see Materials and Methods), including comparison between cases with (LN^+) or without (LN^-) lymph node metastases. Columns, mean; bars, SE.

apparent from similar analyses of leptin-R within the lymphatics in HNSCC (9.61 ± 0.68 vessels per field in tumor versus 0.54 ± 0.13 in marginal tissue, $P < 0.0001$), although in this case there was no obvious association with nodal metastasis (Fig. 4C).

Finally, a quantitative analysis of lymphatic vessel ESAM staining confirmed similarly substantial up-regulation of this protein on tumor vessels in both HNSCC and colorectal carcinoma (Fig. 5A and B). Most notably, however, there was a dramatic association between the incidence of ESAM-positive tumor vessels and the presence of lymph node metastases (17.9 ± 2.66 vessels per field in node-positive HNSCC versus 0.57 ± 0.27 in node-negative HNSCC, $P < 0.0001$; 7.07 ± 1.34 vessels per field in node-positive colorectal tumors versus 1.36 ± 0.37 in node-negative colorectal tumors, $P = 0.0002$).

In summary, our data provide clear confirmation that several of the tumor lymphatic markers we identified in experimental murine T-241/VEGF-C fibrosarcoma are also up-regulated in human cancers. Moreover, they suggest that ESAM may have significant prognostic value as an indicator of tumor metastasis.

Discussion

The identification of genes selectively expressed in tumor lymphatics represents a major step toward identifying biomarkers for metastasis and for elucidating the mechanisms by which tumors invade the microvasculature and spread to lymph nodes. In the foregoing analyses, we identified some 792 transcripts with significantly altered expression, a difference of the same order as that found in recent comparisons of lymphatic and blood endothelial cells from human dermis (44, 45). It is thus evident that an interplay between lymphatic vessels and the tumor environment can have a more significant effect on endothelial phenotype than might have been anticipated. Surprisingly, such effects could not be attributed to the direct action of VEGF-C as adduced by comparisons with the RNA profile of dermal LEC stimulated with the growth factor (40). Perhaps the low responsiveness of adult lymphatics to both VEGF-C and VEGF-D compared with embryonic and neonatal lymphatics may help explain this finding (46). We also note that conditions of acute inflammation that stimulate the exit of leukocytes from tissue to lymph induce none of the genes up-regulated in tumor LEC (19). Hence, tumors may not necessarily exploit the tactics of the immune system for spread to the lymph nodes.

Although the present array analyses were confined largely to murine fibrosarcoma lymphatics, the fact that we could validate these findings for three key markers, Endoglin, ESAM, and leptin-R, in human cancers is an indication that the alterations in LEC transcriptional profile underlie fundamental processes that cross species barriers—a property shared by tumor blood endothelial markers originally identified in Sequential Analysis of Gene Expression analyses of human cancers (47). It is interesting to note that ESAM, the closely related JAM-A and JAM-C (VE-JAM), and Coxsackie adenovirus receptor have all been shown to function as homophilic or heterophilic tight junction adhesion molecules in different contexts (see ref. 48). In particular, ESAM has been shown to mediate transendothelial migration of neutrophils through signaling via the small GTP-binding protein Rho that is implicated in the destabilization of tight junctions (20), whereas ESAM knockout mice show defective tumor angiogenesis. It is tempting to speculate that ESAM could mediate the lymphatic entry of tumor cells and subsequent nodal metastasis. The recent

availability of ESAM^{-/-} mice (20) will allow this hypothesis to be tested in the future.

It is likewise interesting that Endoglin, a component of the transforming growth factor (TGF β) receptor complex associated with hemangiogenesis (49), is a well-known marker of proliferating tumor blood vessels. Indeed, it is an independent predictor of metastasis with potential value in diagnostic imaging and therapeutic targeting (50). The up-regulated expression in tumor lymphatics observed here clearly indicates that Endoglin is not confined to the blood vasculature, although the relative

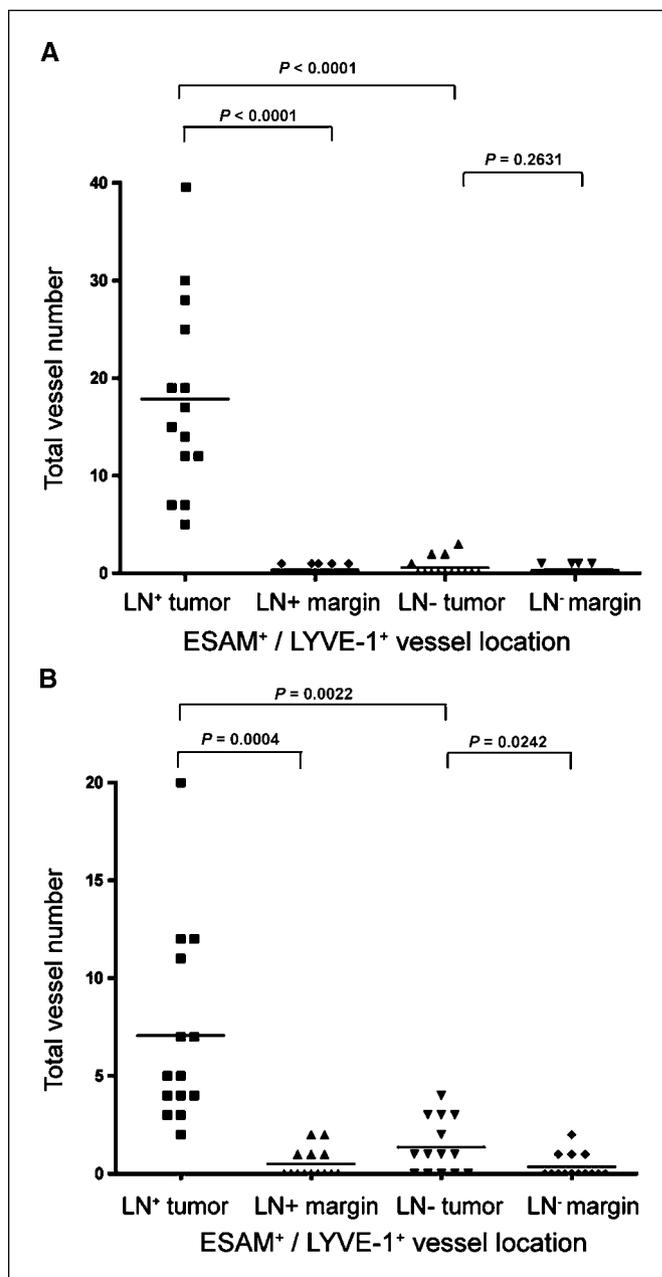


Figure 5. Association between ESAM-positive lymphatic vessel number and lymph node metastasis in HNSCC and colorectal carcinoma. Scatter plots show individual counts for total ESAM-positive lymphatic vessel numbers (see Materials and Methods) present within the tumor and normal margin in 28 cases each of HNSCC (A) and colorectal carcinomas (B) categorized according to the presence or absence of demonstrable lymph node metastases, assessed by dual LYVE-1 immunofluorescence microscopy. Points, mean; bars, SE.

heterogeneity in expression of the receptor in tumor lymphatics may complicate its use as a prognostic marker by comparison with ESAM. Such findings signal the need for further investigation into the role of Endoglin in lymphangiogenesis as well as caution in its use as a marker for immunoisolation of blood vessel endothelium (17).

Finally, the further validation of our array data through comprehensive screening of normal and pathologic tissues may well identify robust new tumor-specific lymphatic markers for both prognosis and therapy. Indeed, in this article, we have already identified one such candidate, ESAM, whose expression is strongly associated with lymph node metastasis in HNSCC and colorectal carcinoma. There is considerable need for such markers in assessing treatment strategies for cancer patients at the early stages of disease, where alteration in vessel properties may signal metastasis. Most previous studies seeking to correlate levels of lymphangiogenic growth factors and lymphatic vessel density with nodal metastasis and patient survival have made the assumption that tumor spread is limited primarily by the number, rather than the nature, of lymphatic

capillaries present within the primary tumor mass (51–54). More often than not, such studies have yielded conflicting results. The findings reported here highlight the importance of qualitative changes in the tumor lymphatics and lay the foundations for developing novel lymphatic markers that accurately assess metastatic potential and enlighten future therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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