

Identification of Vascular Lineage-Specific Genes by Transcriptional Profiling of Isolated Blood Vascular and Lymphatic Endothelial Cells

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In mammals, the lymphatic vascular system develops by budding of lymphatic progenitor endothelial cells from embryonic veins to form a distinct network of draining vessels with important functions in the immune response and in cancer metastasis. However, the lineage-specific molecular characteristics of blood vascular versus lymphatic endothelium have remained poorly defined. We isolated lymphatic endothelial cells (LECs) and blood vascular endothelial cells (BVECs) by immunomagnetic isolation directly from human skin. Cultured LECs but not BVECs expressed the lymphatic markers Prox1 and LYVE-1 and formed LYVE-1-positive vascular tubes after implantation *in vivo*. Transcriptional profiling studies revealed increased expression of several extracellular matrix and adhesion molecules in BVECs, including versican, collagens, laminin, and N-cadherin, and of the growth factor receptors endoglin and vascular endothelial growth factor receptor-1/Flt-1. Differential immunostains of human skin confirmed the blood vessel-specific expression of these genes. During embryonic development, endoglin expression was gradually down-regulated on lymphatic endothelium whereas vascular endothelial growth factor receptor-1 was absent from lymphatics. We also identified several genes with specific expression in LECs. These results demonstrate that some lineage-specific genes are only expressed during distinct developmental stages and they identify new molecular markers for blood vascular and lymphatic endothelium with important implications for future studies of vascular development and function. (*Am J Pathol* 2003, 162:575–586)

The lymphatic system consists of a vascular network of thin-walled capillaries that drain protein-rich lymph from the extracellular spaces within most organs and that play major roles in the immune response and in tumor metastasis.^{1,2} Lymphatic vessels provide the conduit for antigen-presenting cells from the organ exposed to antigens to the regional lymph nodes, involving active recruitment of antigen-presenting cells by chemokines and other mediators secreted by lymphatic endothelium.³ Moreover, the early dissemination of malignant tumors frequently occurs via lymphatic vessels to regional lymph nodes, and the recent discovery of active tumor lymphangiogenesis and its role in cancer metastasis has drawn considerable attention to the molecular mechanisms that control activation and proliferation of lymphatic endothelium.^{2,4} In particular, overexpression of the lymphangiogenesis factors vascular endothelial growth factor (VEGF)-C and VEGF-D by tumor cells has been shown to promote tumor lymphangiogenesis by activation of VEGF receptor-3 (VEGFR-3) on tumor-associated lymphatic endothelium, resulting in enhanced rates of lymph node metastasis.^{5–8}

In contrast to the rapid progress made in elucidating the formation and molecular control of the blood vascular system,^{9,10} the mechanisms controlling the normal development of lymphatic vessels and the molecular regulation of their biological function have remained poorly understood, mainly because of the lack of molecular tools to specifically distinguish lymphatic vessels from blood vessels and to functionally characterize the lymphatic endothelium.¹¹ Consequently, our understanding of the function of the lymphatic system and its role in disease is still rudimentary. Recently, several novel markers have been reported to be predominantly expressed by lymphatic endothelium. VEGFR-3, a receptor for the lymphangiogenesis factors VEGF-C and VEGF-D, is expressed by both blood vascular and lymphatic endothelium during embryonic development, whereas its

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expression becomes restricted to lymphatic vessels in adult life.¹² However, VEGFR-3 has been detected on blood vascular endothelium associated with tumors and healing wounds,^{13,14} and blockade of VEGFR-3 also resulted in inhibition of tumor angiogenesis.¹⁵ Recently, podoplanin, a transmembrane mucoprotein, has been reported as a novel lymphatic-vessel marker,¹⁶ and both podoplanin and VEGFR-3 have been used for the isolation of lymphatic endothelial cells (LECs).^{17,18} The hyaluronan receptor LYVE-1¹⁹ is specifically expressed by LECs, but not by blood vessels in most organs including the skin,^{5,20-22} and recent studies have identified the transcription factor Prox1,² a homeobox gene required for the development of the lymphatic system,²³ as a master control gene in the program specifying LEC fate.^{22,24} However, besides these few markers with specific expression in lymphatic endothelium, little is known about the distinct molecular characteristics of blood vascular endothelial cells (BVECs) versus LECs, and a comprehensive comparison of the lineage-specific differentiation of these cell types has been lacking.

To identify novel lineage-specific molecules involved in the biological function of these distinct vascular cell types, we developed a new protocol for the selective isolation of LECs and BVECs directly from human skin. Here, we report that immunomagnetic selection of CD34⁻/CD31⁺ cells yields pure cultures of LECs that maintain expression of the specific lymphatic markers Prox1 and LYVE-1, whereas BVECs remain negative for these markers. Importantly, gene array profiling, combined with quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and double-immunofluorescence stains of normal skin, identified several novel genes with specific expression in lymphatic or blood vascular endothelium. Together, these results provide important new tools for molecular and functional studies of lymphatic and blood vascular endothelium in vascular development, tumor progression, and the immune response.

Materials and Methods

Isolation of Human Dermal BVECs and LECs

Neonatal human foreskins were obtained after routine circumcisions. After enzymatic digestion, the epidermis was removed and dermal cells were mechanically released as previously described.²⁵ CD34-positive BVECs were isolated by immunomagnetic purification²⁵ with an anti-human CD34 antibody (BD Pharmingen, San Diego, CA) conjugated to immunomagnetic beads (Dynal, Lake Success, NY). Thereafter, the remaining CD34-negative cells were incubated with an immunomagnetic beads-conjugated anti-human CD31 antibody (Dynal) to isolate LECs. LECs were seeded onto fibronectin-coated (10 μ g/ml; BD Biosciences, Bedford, MA) culture dishes and were propagated in endothelial cell basal medium (Bio-Whittaker, Walkersville, MD), supplemented with 10 μ g/ml of hydrocortisone acetate, 2.5×10^{-2} mg/ml N-6,2'-O-dibutyryl-adenosine 3',5'-cyclic monophos-

phate (Sigma, St. Louis, MO), 2 mmol/L L-glutamine, 20% fetal bovine serum (Life Technologies, Inc., Grand Island, NY), antibiotics, and 20 ng/ml of recombinant human VEGF165 (R&D Systems, Minneapolis, MN). VEGF was omitted after the first passage. LECs remained negative for CD34 expression for at least eight passages, as evaluated by immunocytochemistry. BVECs were cultured in the same medium without addition of VEGF. Confluent primary BVEC cultures were further purified by immunomagnetic E-selectin selection after 6 hours of stimulation with recombinant human tumor necrosis factor- α as described.²⁵

Immunostains

Immunofluorescence stainings were performed on 6- μ m cryostat sections of neonatal human foreskin or on 10- μ m sections of mouse embryos as previously described,^{22,26} using antibodies to Prox1,²² murine or human LYVE-1 (kindly provided by Dr. D. Jackson, John Radcliffe Hospital, Oxford, UK²⁰), human CD34 and CD31 (BD Pharmingen), mouse VEGFR-1 (MF1; kindly provided by Dr. DJ Hicklin, Imclone Systems, New York, NY) and human VEGFR-1/Fit-1 (Sigma), N-cadherin (Transduction Laboratories, San Diego, CA), versican (clone 12C5; Developmental Studies Hybridoma Bank, University of Iowa, Ames, IA), mouse endoglin (BD Pharmingen) and human endoglin (Neomarkers, Fremont, CA), macrophage mannose receptor (BD Biosciences), desmoplakin (Serotec, Raleigh, NC), MIP-3- α (Santa Cruz Biotechnology, Santa Cruz, CA), and corresponding secondary antibodies labeled with AlexaFluor488 or AlexaFluor594 (Molecular Probes, Eugene, Oregon). Nuclei were counterstained with 20 μ g/ml of Hoechst bisbenzimidazole.²⁷ Sections were examined by using a Nikon E-600 microscope (Nikon, Melville, NY) and images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI). Antibody staining of cultured cells was observed using a Leica TCS NT4D confocal imaging system (Leica, Heidelberg, Germany).

Northern and Western Blot Analyses

Total cellular RNA was isolated from confluent LEC and BVEC cultures at passages 4 and 5, using the TRIzol reagent (Invitrogen, Carlsbad, CA). Samples of RNA (10 μ g each) were subjected to Northern blot analyses as described,²⁶ using a 2.0-kb human Prox1 cDNA probe and a 956-bp human type XVIII collagen probe (kindly provided by Dr. Y. Ninomiya). A 36B4 ribosomal-associated protein cDNA probe was used as a control for equal RNA loading.²⁸ For Western blot analyses, confluent LEC and BVEC cultures were homogenized in lysis buffer as described.⁵ Ten μ g of protein per sample were analyzed by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were immunoblotted with polyclonal antibodies against human VEGFR-2/KDR or VEGFR-3/Fit4 (Santa Cruz Biotechnology) as described.⁵

Real-Time Quantitative RT-PCR

The ABI Prism 7000 Sequence Detection System was used to perform either SYBR-Green based or dual-labeled probe based real-time RT-PCR reactions as described.²⁹ Sequences of the primers used for this study are provided in Table 3. For SYBR-Green based reactions, at least three sets of primers were used for each gene of interest. SYBR-Green PCR Master Mix was used for all SYBR-Green reactions with the addition of MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA). For dual-labeled probe-based real-time RT-PCR reactions, probes labeled with 6-FAM and TAMRA at their 5' and 3' end, respectively, were multiplexed with a GAPDH probe labeled with Joe and TAMRA at the 5' and 3' end as an internal control. TaqMan EZ RT-PCR Core Reagent was used for dual-labeled probe based reactions. Total RNAs were isolated as described above and were treated with RNase-free RQ-DNase (Promega, Madison, WI) before analyses. Twenty ng of total RNA were used for each reaction. The primers and probes were designed using Primer Express software (Perkin Elmer Life Sciences, Boston, MA) and were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Expression data were normalized based on the expression levels of GAPDH mRNA.

In Vitro and in Vivo Tube Formation Assays

Confluent LEC and BVEC cultures at passage 5 were labeled with 5-chloromethylfluorescein diacetate (CMFDA, Cell Tracker Green; Molecular Probes) or with 5-(and-6)-((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR; Cell

Tracker Orange) according to the manufacturer's instructions. LECs or BVECs (2.5×10^5) were seeded onto each well of Matrigel-coated (Becton Dickinson, Franklin Lakes, NJ) 24-well plates and were incubated for 24 hours at 37°C. Cells were analyzed by using a Nikon TE-300 microscope and images were captured with a SPOT digital camera. *In vivo* tube formation was investigated by mixing 1×10^6 LECs or BVECs with 500 μ l of Matrigel, followed by subcutaneous injection into SCID CV17 mice (Charles River Laboratories, Wilmington, MA). All experiments were performed three times with comparable results. After 7 days, mice were sacrificed and tissue samples were fixed with 4% paraformaldehyde overnight and embedded in paraffin. Six- μ m paraffin sections were either stained with hematoxylin and eosin or were double-stained with antibodies against human CD31 and LYVE-1 as described above.

Oligonucleotide Array Analyses

Total cellular RNA was extracted from confluent fifth passage LEC and BVEC cultures, maintained in complete endothelial growth medium without addition of VEGF. Oligonucleotide array analyses were performed using the human 95Av2 (12,625 genes) gene arrays (GeneChip; Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Arrays were scanned using an Affymetrix confocal scanner and analyzed by the Microarray Suite 5.0 software (Affymetrix). Intensity values were scaled so that the overall fluorescence intensity of each chip of the same type was equivalent. Genes with a *P* value of ≤ 0.002 were considered as significantly increased or decreased.

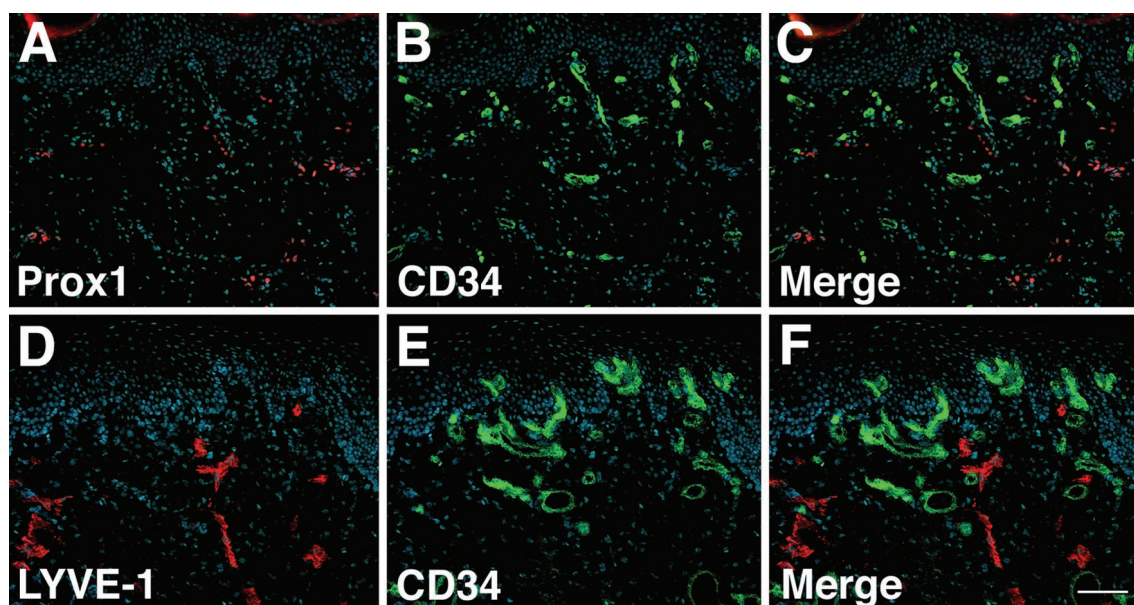


Figure 1. Specific detection of lymphatic and blood vessels in normal human skin. **A–C:** The homeobox gene Prox1 is specifically expressed by LECs (**A**, red), whereas blood vessels express CD34 (**B**, green). Double immunostains for Prox1 (red) and CD34 (green) reveal mutually exclusive vascular expression in lymphatic and blood vessels (**C**). Nuclei are labeled blue (Hoechst stain). **D–F:** Specific staining of lymphatic vessels with an antibody against the hyaluronan receptor LYVE-1 (**D**, red) and of blood vessels with an anti-CD34 antibody (**E**, green). **F:** Double immunostains for LYVE-1 (red) and CD34 (green) demonstrate mutually exclusive expression in lymphatics and blood vessels. Scale bar, 100 μ m.

Proliferation Assays

BVECs or LECs (3.5×10^4) were seeded onto fibronectin-coated six-well plates. Triplicate dishes were treated without or with 100 ng/ml of recombinant human placental growth factor-1 (PIGF-1, R&D Systems), 100 ng/ml human VEGF-C (kindly provided by Dr. K. Alitalo), 100 ng/ml human VEGF-D (R&D Systems), or 10 ng/ml recombinant human VEGF-A (VEGF165, R&D Systems) in endothelial growth medium. After 48 hours, cells were trypsinized and cell numbers were determined using a hemacytometer. For thymidine incorporation assays, 2×10^4 cells were seeded into quadruplicate wells of 24-well plates in the presence or absence of the above growth factors. Thymidine incorporation was assessed after 36 hours as previously described.³⁰ For apoptosis assays, 1×10^4 cells were seeded into triplicate wells of 96-well plates in endothelial cell basal medium containing 2% fetal calf serum, and were treated with the above growth factors for 72 hours. Apoptosis rates were determined by the Cellular DNA Fragmentation ELISA kit (Roche, Germany) according to the manufacturer's instructions. Statistical analyses were performed using the paired Student's *t*-test.

Results

Selective Isolation of Lymphatic and Blood Vascular Endothelial Cells from Human Skin

Using the lymphatic-specific transcription factor Prox1²² as a specific marker for lymphatic endothelium, we found that CD34 was selectively expressed by Prox1-negative blood vessels in human skin, whereas Prox1-positive lymphatic vessels did not express CD34 (Figure 1; A to C). These results were confirmed by immunofluorescence double stains with antibodies against the lymphatic-specific hyaluronan receptor LYVE-1 and against CD34, demonstrating mutually exclusive staining of lymphatic vessels with LYVE-1 and of blood vessels with CD34 (Figure 1; D to F). All Prox1-, LYVE-1-, or CD34-positive vessels also expressed the pan-vascular marker CD31 (data not shown). These results revealed that CD34 was specifically expressed by blood vascular, but not by lymphatic, endothelium. We next isolated CD34-positive BVECs and CD34-negative/CD31-positive LECs by immunomagnetic purification directly from enzymatically digested neonatal human foreskins. After seeding on fibronectin-coated culture dishes, both LECs and BVECs maintained a typical cobblestone-like endothelial morphology for at least eight passages (Figure 2, A and B).

Human Dermal LECs and BVECs Maintain Their Lineage-Specific Differentiation *in Vitro*

All cultured LECs maintained expression of the hyaluronan receptor LYVE-1 during the first eight passages (Figure 2D), whereas LYVE-1 expression was absent from BVEC cultures (Figure 2C). Double-immunofluorescence

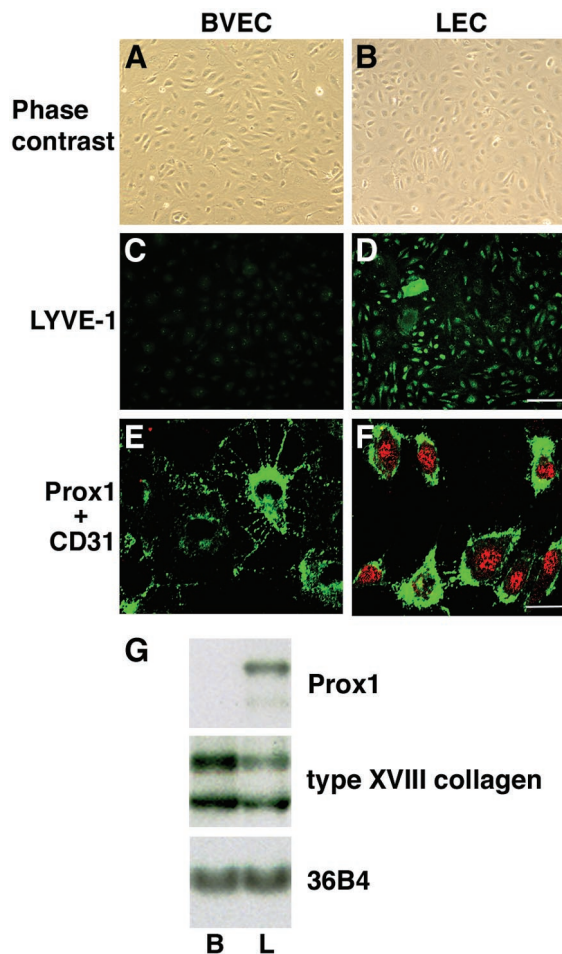


Figure 2. Cultured human dermal LECs and BVECs maintain their lineage-specific differentiation *in vitro*. **A** and **B**: Cultured BVECs and LECs (passage 4) show comparable endothelial morphology with characteristic cobblestone appearance. Phase-contrast micrograph. **C** and **D**: All LECs express the lymphatic marker LYVE-1 *in vitro* (**D**), whereas BVECs are not labeled (**C**). **E** and **F**: Double immunostains for CD31 (green) and Prox1 (red) demonstrate CD31 membrane labeling in both cell types whereas Prox1 (nuclear stain, red) is selectively expressed in LECs. **G**: Northern blot analysis confirms selective Prox1 mRNA expression in cultured LECs (**L**), whereas no expression is found in BVECs (**B**). In contrast, the basement membrane component type XVIII collagen is expressed at higher levels in BVECs. Hybridization with a probe to ribosomal protein-associated RNA 36B4 demonstrates equal loading. Scale bars: 100 μ m (**D**); 25 μ m (**F**).

stains with antibodies to the endothelial junction molecule CD31 (PECAM-1³¹) and the lymphatic-specific transcription factor Prox1 revealed membrane labeling for CD31 in all cultured cells, whereas only LECs showed nuclear staining for Prox1 (Figure 2, E and F). Northern blot analyses confirmed that Prox1 mRNA was exclusively expressed in LECs but not in BVECs (Figure 2G). In contrast, the vascular basement membrane molecule type XVIII collagen was more strongly expressed in BVECs than in LECs (Figure 2G), in accordance with the only rudimentary basement formation in lymphatic vessels *in vivo*.¹ To further investigate the distinct mRNA expression of marker genes that have been previously reported to be specific for lymphatic vessels in normal human skin, we performed TaqMan quantitative real-time RT-PCR analysis of cultured LECs *versus* BVECs. We found a 74.1-fold higher expression of Prox1 mRNA ex-

Table 1. TaqMan Real-Time RT-PCR Analysis of Lineage-Specific Vascular Markers in Cultured LEC and BVEC

	Fold increase in LEC over BVEC	Fold increase in BVEC over LEC
Prox1	74.1	
Podoplanin	54.6	
LYVE-1	6.7	
CCL21	4.6	
VEGFR-3/ Flt4	4.4	
VEGF-C		3.7

pression in LECs, as compared with BVECs (Table 1). Moreover, LECs showed highly increased expression of the lymphatic markers podoplanin, LYVE-1, VEGFR-3,

and CCL21/secondary lymphoid chemokine, whereas the levels of VEGF-C were higher in BVECs than in LECs (Table 1). Western blot analyses confirmed increased VEGFR-3 protein expression in LECs, whereas comparable levels of VEGFR-2 were found in both cell types (data not shown).

Isolated LECs and BVECs Maintain Their Lineage-Specific Differentiation during Tube Formation in Vivo

Twenty-four hours after seeding onto Matrigel-coated culture tissues, both BVECs and LECs efficiently formed a network of tube-like structures *in vitro* (Figure 3, A and B).

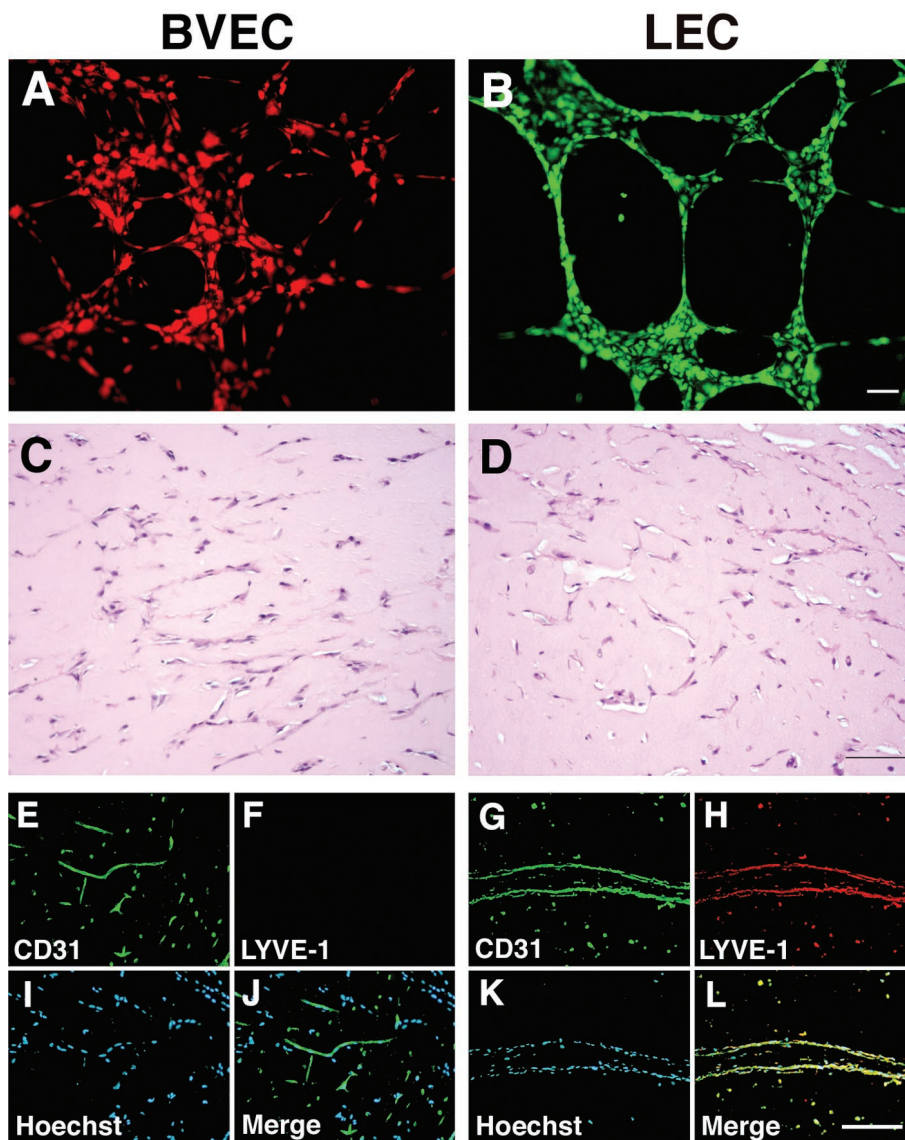


Figure 3. BVECs and LECs maintain lineage-specific differentiation after formation of tube-like structures *in vivo*. **A** and **B**: Cultured BVECs and LECs were fluorescently labeled with CMTMR (orange) or CMFDA (green), respectively, and were seeded onto Matrigel. Both BVECs (**A**) and LECs (**B**) demonstrate formation of tube-like structures *in vitro*, a characteristic feature of endothelial cells. **C–L**: Isolated BVECs or LECs were mixed with Matrigel and were implanted subcutaneously into SCID mice. Efficient formation of tube-like structures by BVECs (**C**) and LECs (**D**) after 7 days *in vivo*. H&E stains. Both BVECs (**E**) and LECs (**G**) express CD31 (green) *in vivo*, whereas only tubes formed by LECs (**H**), but not by BVECs (**F**) are also positive for LYVE-1 (red). **I** and **K**: Nuclear stain with Hoechst (blue). **J** and **L**: Double immunostains for CD31 (green) and LYVE-1 (red), combined with nuclear stains (blue), confirm lineage-specific tube formation in Matrigel. Scale bars: 50 μ m (**B**); 100 μ m (**D**, **L**).

Table 2. Genes with Significantly ($P \leq 0.002$) Increased Expression in LEC or BVEC

	Accession no.	Fold increase in LEC over BVEC	Fold increase in BVEC over LEC
Growth factors and chemokines			
Vascular endothelial growth factor-C*	X94216		42.2
Placenta growth factor	X54936		3.0
Vascular endothelial growth factor-B	U43368		1.7
Transforming growth factor-beta	M60315		1.4
Platelet-derived growth factor-B	M12783		1.4
Platelet-derived growth factor-A	X06374		1.2
Melanoma growth stimulatory activity (Gro1alpha)	X54489		1.2
MCP-1	M26683		1.2
Exodus-1 (CCL20, MIP-3alpha)	U64197	39.4	
Fibroblast growth factor-12	AL119322	14.9	
Transforming growth factor-alpha	X70340	4.0	
Thrombospondin-1	U12471	3.2	
Follistatin	M194812.6		
Stromal-cell derived factor-1 (SDF-1)	L36033	2.0	
RANTES	M21121	1.6	
Angiopoietin-2	AF004327	1.5	
Receptors			
G protein-coupled receptor 39*	A1936826		11.3
Chemokine receptor X (CKRX)	AF014958, AF410948		9.2, 9.2
VEGF receptor-1/Flt1*	U01134		6.5
Interleukin-13 receptor	U70981		6.1
CD44*	AF098641, M59040		3.5, 2.8
Latent TGF-beta binding protein	Z37976		3.2
CXCR4	L06797		3.0
Colony stimulating factor 2 receptor, beta	H04668		2.5
Thrombin receptor	M62424		2.3
Complement Iq receptor	U94333		2.1
Endoglin	X72012		1.7
Insuline-like growth factor II receptor	Y00285		1.6
Interleukin 4 receptor	X52425		1.6
TRAIL receptor 2	AF016266		1.4
Jagged2	AF029778		1.2
Macrophage mannose receptor	M93221	22.6	
Proteinase-activated receptor-2	U34038	4.6	
Membrane glycoprotein gp 130*	M57230	3.7	
46 kDa Coxsackievirus and adenovirus receptor	Y07538	3.5	
Orphan G protein-coupled receptor	A1674208	3.5	
Glycine receptor beta subunit	U33267	2.5	
Intracellular hyaluronic acid binding protein	AF032862	1.7	
Jagged1	U77914, AF003837	1.6, 1.3	
CGRP type 1 receptor	L76380	1.5	
Extracellular matrix molecules			
Chondroitin sulfate proteoglycan 2 (versican)*	D32039, X15998		59.7, 13.9
Collagen type III, alpha 1 chain	AB007902		45.2
Collagen type VI, alpha3 chain	X52022		8.0
Collagen type I, alpha 2 chain*	J03464, V00503		4.9, 4.3
Collagen type VI, alpha 1 chain	X15580		3.0
Bone morphogenetic protein 1	M22488		2.6
Fibulin 5	AF093118		2.6
Fibulin-like extracellular matrix protein 2	AF093119		2.1
SPARC/osteonectin	J03040		2.0
Laminin, beta 2 chain	M55210, X79683		1.9, 1.7
Laminin, beta 1 chain	M61916		1.7
Fibronectin	M10905, X02761		1.7, 1.5
Procollagen type V, alpha 2 chain	Y14690		1.7
Perlecan	M85289		1.3
Proteoglycan 1	X17042		1.3
Collagen type IV, alpha 2 chain	X05610		1.2
Reelin*	U79716	26.0	
Extracellular matrix protein (MFAP3)	L35251	3.0	
Adhesion Molecules			
Integrin alpha4*	X16983		13.0
Desmoglein 1	AF097935		13.0
N-cadherin*	M34064		4.0
Integrin beta3 (GPIIIa, CD61)*	M35999		2.5
Integrin alpha 5	X06256		2.1
Platelet/endothelial cell adhesion molecule (CD31)	AA100961, L34657		1.7, 1.5
Neural cell adhesion molecule (NrCAM)	AB002341		1.7
H-cadherin	U59289		1.7
Protocadherin 42	L11370		1.7
Integrin beta5	X53002, M35011		1.6, 1.5
CD151	D29963		1.6
Intercellular adhesion molecule-2 (ICAM-2)	X15606		1.6
VE-cadherin	X79981		1.3
CEA-CAM*	S71326, X16354	59.7, 3.5	
Desmoplakin-1	AL031058	4.6	
Galectin 8	L78132	2.6	
Integrin alpha 6B	S66213	1.9	
Plakoglobin	M23410	1.3	
Miscellaneous			
Von Willebrand factor	M10321		2.3
Endothelial cell-specific molecule 1	X89426		2.3
Podoplanin	A1660929, AF030428	16.0, 113	
Proxl	U44060	4.6	
Intestinal trefoil factor*	A1985964	3.2	
Down syndrome critical region gene 2	AJ006291	1.7	

*Confirmed by quantitative real-time RT-PCR. Several genes were represented twice on the arrays.

Table 3. Names of Genes and Primer Sequences for the SYBR-Green-Based Real-Time RT-PCR

Gene names	Forward primers	Reverse primers
CD44	AAAGGAGCAGCACTTCAGGA	CTGTCTGTGCTGTCCGGTGAT
CEA-CAM	GTAGCAAAGCCCCAAATCAA	AACGGATGGAGATTCCAGTG
Chondroitin sulfate proteoglycan 2 (versican)	TTTGCCACCCAGTTACAACA	GGGCCACAAGGACAGTAGTC
Collagen type I, alpha 2 chain	AGGAGTTGTTGGACCACAGG	TCCCTTCAATCCATCCAGAC
G protein-coupled receptor 39	AGCACAGAACAGAGGGGCTA	GAGCAGGAGGGAGAGACAGA
Integrin alpha4	GAGGAATCCCACCACTTCA	ATTTTCATGGGCACAAAACC
Integrin beta3 (GPIIIa, CD61)	TGGTCCTGCTCTCAGTGATG	GAATTTCTTTVCGGTCGTGGA
Intestinal trefoil factor	CCAGGCACTGTTTCATCTCAG	GAGCATGGGACCTTTATTCCG
Macrophage mannose receptor (MRC1)	GTGGCCGGAGTAGTCATCAT	TCTTGAGGTAGGTGCACACG
Membrane glycoprotein gp130	TGAACGAGGGGAAGAAAATG	TGTGTGTTGCCCATTCAGAT
N-cadherin	TGGAGAACCCCAATTGACATT	TGATCCCTCAGGAAGTGTCC
Reelin	AGGACCGTTATGCTGGACAC	ACATGTCAAAGGCCGATCCTC
VEGF receptor-1/Flt1	GGCCTCTGATGGTGATTGTT	GTGCTGCATCCTTGTGAGA

Names of Genes and Primer/Probe Sequences for the Taqman-Based Real-Time RT-PCR

Gene names	Forward primers	Reverse primers	Taqman probes
CCL21/SLC	GGTTCTGGCCTTTGGCATC	AGGCAACAGTCTGAGCCC	FAM-CCAGGACCCAAGGCCAGTGATGGA-TAMRA
GAPDH	GATTCACCCATGGCAAT	GAAGATGGTGATGGGATTTC	JOE-CAAGCTTCCCGTTCTCAGCC-TAMRA
LYVE-1	AGCTATGGCTGGGTTGGAGA	CCCCATTTTCCCACACTTG	FAM-TTCGTGGTCTCTCTAGGATTAGCCCAAACC-TAMRA
Podoplanin	AGGCGGCGTTGCCAT	GTCTTCGCTGGTTCCTGGAG	FAM-CCAGGTGCCGAAGATGATGTGGTC-TAMRA
Prox1	ACAAAATGGTGGCACGGA	CCTGATGTACTTCGGAGCCTG	FAM-CCCAGTTTCCAAGCCAGCGGTCTCT-TAMRA
VEGF-C	CACCACAAACATGCAGCTG	TGAAAATCCTGGCTCACAAGC	FAM-CGGCCATGTACGAACCCGCCAG-TAMRA
VEGFR3 (Flt4)	TCTGTACTAGCTCCAGGTGG	GCAGCCAGGTCTCTGTGGAT	FAM-ATGGAGTTCCCTGGCTTCCCGAAAGTG-TAMRA

We next mixed BVECs or LECs with Matrigel that was implanted subcutaneously into immunodeficient mice. Seven days after injection, both LECs and BVECs had formed tube-like structures *in vivo*, occasionally with a distinguishable lumen (Figure 3, C and D). Whereas both types of endothelial cells expressed CD31 *in vivo* (Figure 3, E and G), only LECs (Figure 3H), but not BVECs (Figure 3F) expressed the hyaluronan receptor LYVE-1. Double-immunofluorescence stains confirmed that LYVE-1 expression was restricted to human CD31-positive LECs *in vivo* (Figure 3, K and L), whereas human CD31-positive BVECs did not express LYVE-1 (Figure 3, I and J).

Identification of Genes with Specific Expression in Blood Vascular Endothelium by Gene Array Analysis of Cultured BVECs versus LECs

Comparative gene array analyses of cultured BVECs and LECs revealed that only a small percentage of genes (1.2%) showed at least twofold increased expression in BVECs (1.6% in LECs) and that only 0.37% of all genes were increased by fourfold or more in BVECs (0.39% in LECs), in agreement with the close lineage connection between both types of vascular cells. We focused our analysis on growth factors, chemokines, their receptors, extracellular matrix and adhesion molecules that likely play essential roles in the development and function of the blood vascular system. BVECs showed highly increased gene expression of several extracellular matrix molecules, including the chondroitin sulfate proteoglycan versican; type I, III, and VI collagen; SPARC; and fibronectin (Table 2). In accordance with the *in vivo* formation of a regular vascular basement membrane by BVECs, but not LECs, the expression of the basement

membrane molecules laminin, type IV collagen, and perlecan was increased in cultured BVECs. BVECs also expressed significantly higher levels of several adhesion molecules and adhesion receptors, including N-cadherin; integrins $\alpha 4$, $\alpha 5$, $\beta 3$, and $\beta 5$; and neural cell adhesion molecule (Table 2). In addition to VEGF-C, several other vascular growth factors showed increased expression by BVECs, most prominently PIGF and VEGF-B and their receptor VEGFR-1/Flt1. Moreover, endoglin, a low-affinity receptor for transforming growth factor- β , and the hyaluronan receptor CD44 were more strongly expressed by BVECs than by LECs (Table 2).

We next used double-immunofluorescence stains for the pan-vascular marker CD31 or the lymphatic marker LYVE-1 to investigate the expression of several of the BVEC-expressed genes in normal skin *in situ*. These studies revealed that VEGFR-1/Flt1, N-cadherin, and versican were exclusively expressed by LYVE-1-negative, CD31-positive blood vessels, but not by lymphatic endothelium (Figure 4; A to L). Strong endoglin expression was detected on all blood vessels, whereas its expression was low or absent in LYVE-1-positive lymphatic vessels (Figure 4; M to P).

Expression of VEGFR-1/Flt1 and Endoglin during Embryonic Development of the Lymphatic Vascular System

Throughout the murine embryonic development of the lymphatic vascular system, VEGFR-1/Flt-1 was expressed selectively on BVECs with a particularly high expression on endothelial cells of the dorsal aorta (Figure 5A). In contrast, VEGFR-1 was not detected on Prox1-expressing lymphatic progenitor endothelial cells located in the cardinal vein or on Prox1-positive cells that had

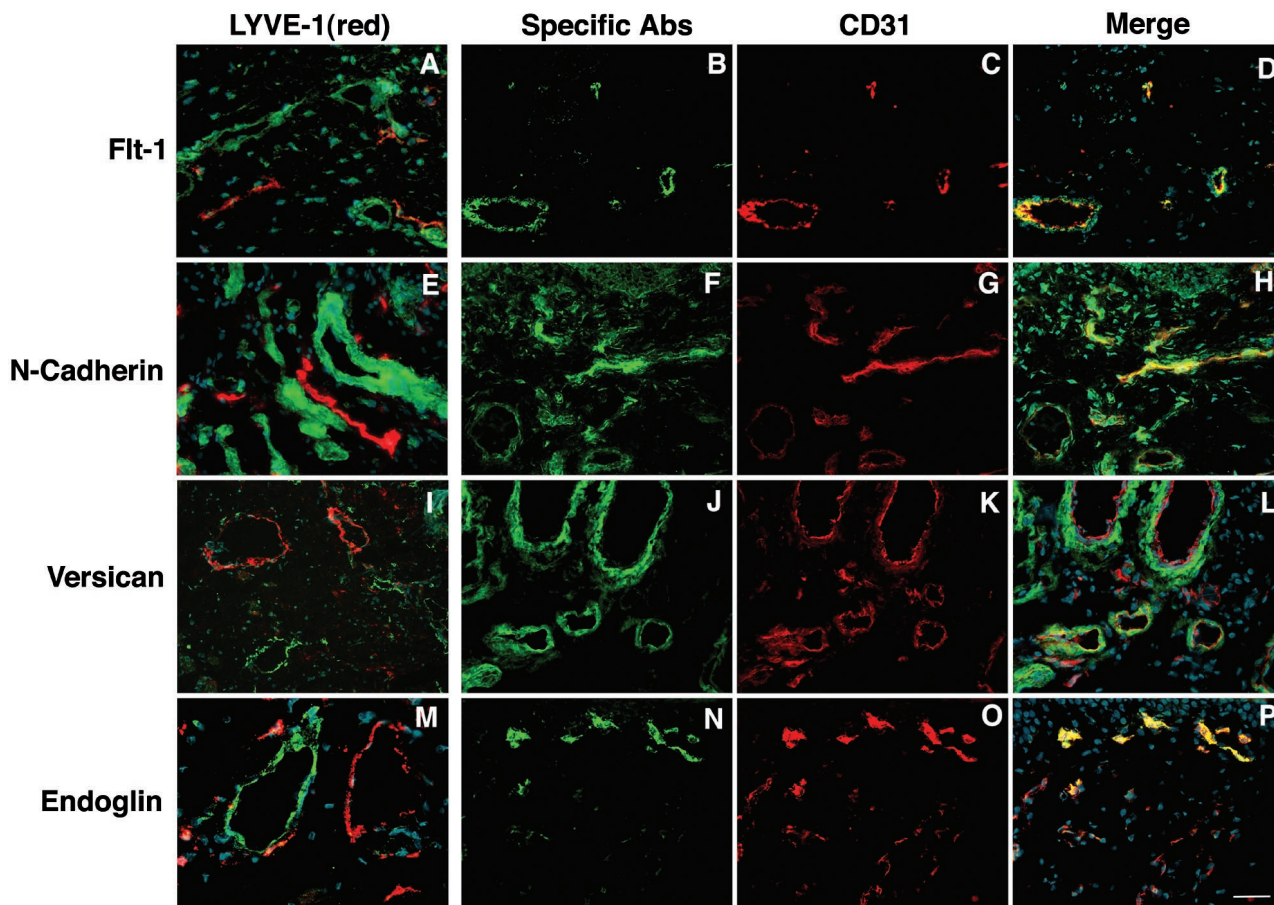


Figure 4. Blood vessel-specific expression of VEGFR-1/Flt1, N-cadherin, versican, and endoglin in normal human skin. Differential immunostains reveal mutually exclusive expression of LYVE-1 (red) on lymphatic vessels and of VEGFR-1/Flt-1 (**A**), N-cadherin (**E**), versican (**I**), and endoglin (**M**) on cutaneous blood vessels (green). Nuclear Hoechst counterstain (blue) was used to depict skin morphology. Double immunostains for the pan-endothelial marker CD31 (red) and selected blood vascular markers (green) demonstrate that only a fraction of all vessels also express VEGFR-1/Flt1 (**B–D**), N-cadherin (**F–H**), versican (**J–L**), or endoglin (**N–P**). Scale bar, 50 μ m.

already budded from the vein. In contrast, endoglin was expressed on both blood vascular and LECs at E11.5 (Figure 5B), with low or absent expression in single Prox1-positive LECs after budding. At E14.5, endoglin was strongly expressed on blood vessels, whereas lymphatic vessels show reduced expression (Figure 5C).

VEGF-A Stimulates Proliferation of Both BVECs and LECs

Both LECs and BVECs responded equally well to the mitogenic stimulation by VEGF-A (Figure 6), a ligand for both VEGFR-1 and for VEGFR-2 that was equally expressed on both cell types. Moreover, VEGF-A treatment potently stimulated DNA synthesis, as measured by 3 H-thymidine incorporation, of both BVECs (+147.4%, $P < 0.05$) and LECs (+281.9%, $P < 0.01$), and also reduced cellular apoptosis rates of both BVECs (–15.0%, $P < 0.05$) and LECs (–23.2%, $P < 0.01$). In contrast, the lymphangiogenesis factors VEGF-C and VEGF-D, ligands for VEGFR-2 and VEGFR-3, promoted LEC growth more potently than BVEC proliferation (Figure 6), in accordance with the higher levels of VEGFR-3 expression in LECs.

Identification of Genes with Predominant Expression in Lymphatic Endothelium by Gene Array Analysis

LECs expressed increased levels of proxl, podoplanin and several chemokines including stromal cell-derived factor-1 (SDF-1), RANTES, and exodus-1/CCL20/MIP3- α , a molecule involved in dendritic cell attraction to lymphatic endothelium (Table 2). Moreover, we detected increased expression of the extracellular matrix protein reelin and of several molecules involved in adhesion, including CEA-CAM, galectin 8, integrin α 6B, desmoplakin, and plakoglobin. LECs also expressed higher levels of the macrophage mannose receptor, the membrane glycoprotein gp130 and intestinal trefoil factor (Table 2). Immunofluorescence double stains of normal human skin confirmed that desmoplakin, CCL20/MIP-3 α , and macrophage mannose receptor were selectively expressed by LYVE-1-positive lymphatic vessels but not by CD31-positive/LYVE-1-negative blood vessels (Figure 7).

Discussion

We developed a novel immunomagnetic isolation technique for the direct purification of LECs and BVECs from human

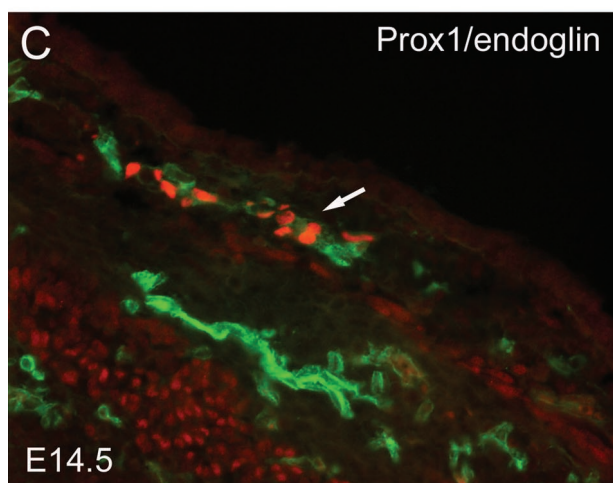
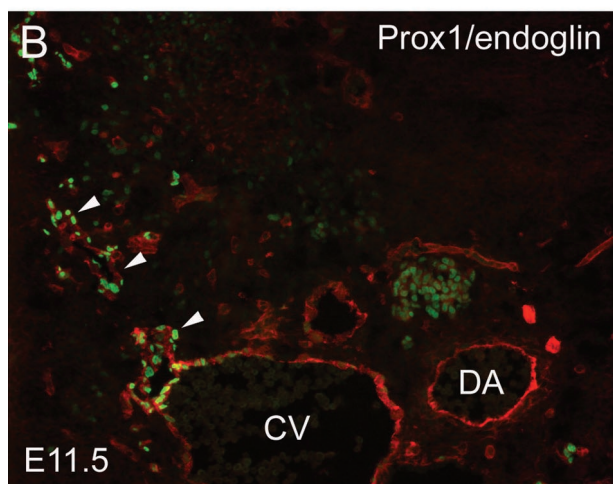
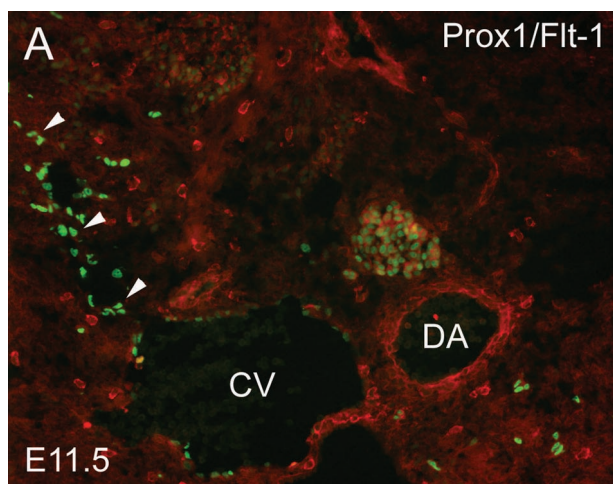


Figure 5. Expression of endoglin and VEGFR-1/Flt-1 during embryonic development of the lymphatic vascular system. Throughout the embryonic development of the lymphatic vascular system, Flt-1 is expressed selectively on BVECs, whereas endoglin is expressed on both blood vascular and LECs. **A:** Flt-1 expression (red) is limited to the vascular endothelium at E11.5 and is particularly high on endothelial cells of the dorsal aorta (DA). In contrast, Flt-1 is not detected on LECs located in or budding from (arrowheads) the cardinal vein (CV). Lymphatic endothelial cells are identified by the expression of Prox1 (green). **B:** In an adjacent section, endoglin expression (red) is detected on both blood vascular and LECs. **C:** At E14.5, endoglin is strongly expressed on blood vessels (green), whereas lymphatic vessels show reduced expression (arrow, detected by expression of Prox1, red).

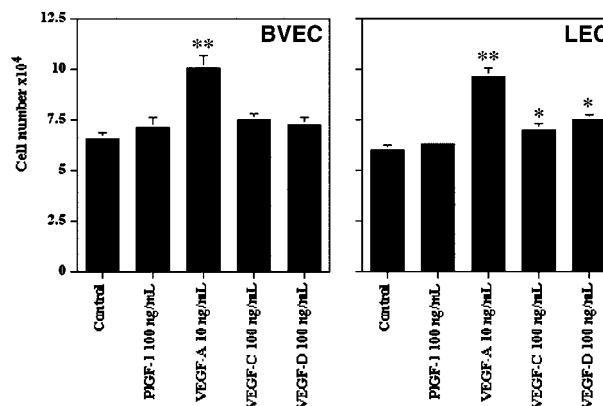


Figure 6. LEC and BVEC proliferation was equally stimulated by VEGF-A. Both VEGF-C and VEGF-D induced LEC proliferation with only minor effects on BVECs. Mean values \pm SD. *, $P < 0.05$; **, $P < 0.01$.

skin, based on the recent findings that the expression of the lymphatic hyaluronan receptor LYVE-1 and of CD34 on blood vessels was mutually exclusive in postnatal murine skin.^{5,20,22} Whereas these findings suggested that CD34 is specifically expressed by blood vascular but not by lymphatic endothelium, co-expression of the proposed lymphatic marker podoplanin and of CD34 was recently reported in a subpopulation of endothelial cells in human skin.¹⁷ However, we found that in normal human skin, CD34 expression was absent from lymphatic vessels that all expressed the specific markers Prox1^{22,32} and LYVE-1¹⁹ and, conversely, that all CD34-positive vessels were negative for these lymphatic markers. Although the function of CD34 expressed by mature blood vascular endothelium has remained poorly defined, recent studies suggest that CD34 may recognize L-selectin if decorated by 6-sulfosialyl Lewis X saccharides and the MECA-79 epitope.³³

Based on these findings, we isolated BVECs directly from human foreskins by positive selection for CD34 and LECs by consecutive positive selection of the remaining CD34-negative cells for expression of the endothelial junction molecule CD31³¹ that is expressed by both blood vascular and lymphatic endothelium.² Immunostains revealed that expression of Prox1 and of LYVE-1 was completely absent from cultured BVECs, whereas all LECs expressed both lymphatic-specific markers, confirming that the isolation procedure yielded pure cell populations and that cultured LECs and BVECs maintain cell lineage-specific differentiation even after multiple passages *in vitro*. These findings were confirmed by the highly increased expression levels of the lymphatic markers Prox1, LYVE-1, VEGFR-3/Flt4,³⁴ podoplanin,¹⁶ and CCL21 (secondary lymphoid chemokine)³⁵ in LECs. Our finding that expression of the lymphangiogenesis factor VEGF-C was markedly higher in BVECs than in LECs is in accordance with previously reported results.^{17,18}

Isolated LECs efficiently formed tube-like structures after seeding onto Matrigel *in vitro* and after subcutaneous transplantation into immunosuppressed mice *in vivo*, which maintained the expression of the lymphatic-specific hyaluronan receptor LYVE-1 for at least 1 week after transplantation. Whereas future studies are needed to investigate whether LEC tubes will also function in lymphatic drainage of tissue

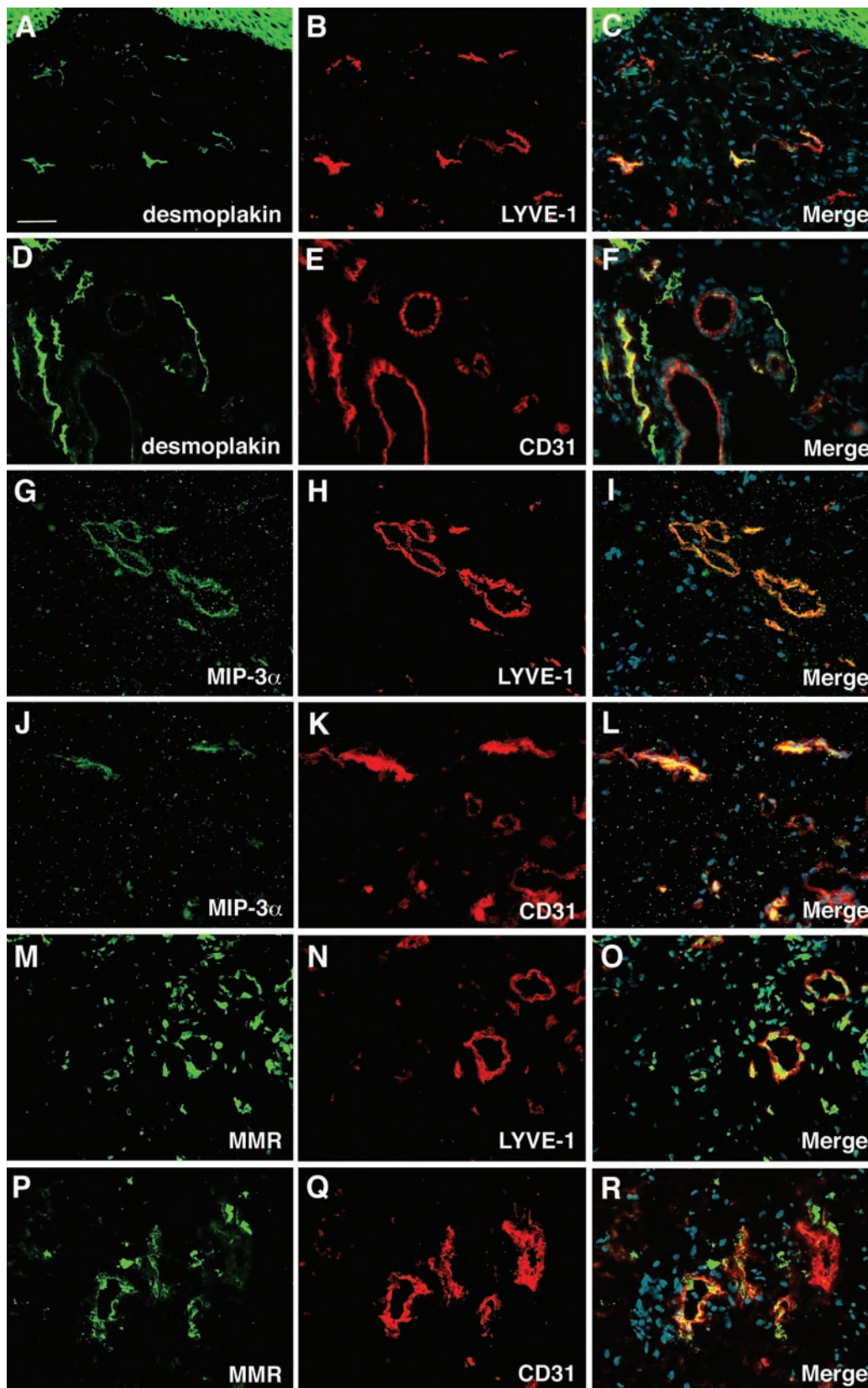


Figure 7. Lymphatic endothelium-specific expression of desmoplakin, CCL20/MIP-3 α and macrophage mannose receptor in normal human skin. **A–C:** All desmoplakin-positive lymphatic vessels also express LYVE-1, whereas only a fraction of CD31-positive vessels also expresses desmoplakin (**D–F**). **G–I:** Co-localization of MIP-3 α and LYVE-1 in cutaneous lymphatic vessels. **J–L:** Blood vessels (red in **L**) do not express MIP-3 α . **M–O:** Expression of macrophage mannose receptor (MMR) is detected on all LYVE-1-positive small lymphatic vessels (orange in **O**), whereas MMR expression is absent from blood vessels (**P–R**); blood vessels are depicted in red in **R**. Scale bar, 50 μ m.

fluid, these results demonstrate, for the first time, that transplanted LECs can reassemble to form lymphatic vascular structures *in vivo*, with possible implications for future strategies to treat lymphedemas in humans.

Our findings that only a small percentage of all genes investigated by gene profiling studies showed differential expression in LECs versus BVECs further corroborates the close relationship of the blood vascular and the lymphatic vascular system. These results are in accordance with the proposed development of lymphatic progenitor endothelial cells from embryonic veins^{2,22} and with our recent findings that ectopic expression of the transcription factor *Prox1*, a master control gene in the program specifying LEC fate, in blood vascular endothelium was sufficient to program these cells to adopt a LEC phenotype.²⁴ Importantly, we found that several genes involved in the formation of the vascular basement membrane showed increased expression in cultured BVECs, as compared with LECs. Because both types of cells were cultured under identical *in vitro* conditions, these results suggest that the absent or only rudimentary basement membrane formation of lymphatic vessels *in vivo* is not merely because of the lack of the appropriate environmental stimuli, but represents an intrinsic, lineage-specific feature of lymphatic endothelium. Indeed, recent studies in mouse embryos have revealed that budding lymphatic endothelial progenitor cells gradually down-regulate the expression of laminin, whereas blood vascular endothelium maintained strong expression of this basement membrane component.²² In contrast, *Prox1*-deficient endothelial cells failed to down-regulate laminin expression after initial budding from embryonic veins.²²

Mutations in the gene for the low-affinity transforming growth factor- β receptor endoglin have been found to be associated with the autosomal-dominant vascular malformations of hereditary hemorrhagic telangiectasia type I.³⁶ We found that endoglin expression was increased in cultured BVECs and that endoglin was strongly expressed on blood vessels, but was absent from or only sparsely expressed in lymphatic endothelium *in vivo*, in accordance with the observed vascular, but not lymphatic, abnormalities in hereditary hemorrhagic telangiectasia. VEGFR-1, a receptor for VEGF-A, VEGF-B, and PlGF, was more strongly expressed by BVECs than LECs *in vitro*, and was specifically expressed by blood vascular endothelium, but not by lymphatic endothelium, during embryonic development and postnatally *in vivo*. These results provide a potential explanation for the recent findings that intradermal injection of a VEGF-A-encoding adenovirus into mouse ears potently induced the formation of both lymphatic and blood vessels, whereas injection of an adenovirus encoding PlGF, a specific ligand for VEGFR-1,³⁷ selectively induced blood vascular angiogenesis but not lymphangiogenesis.³⁸

Lymphatic endothelium secretes the chemokine CCL21 (secondary lymphoid chemokine) that binds to CC chemokine receptor 7 (CCR7),^{3,17,35} leading to chemoattraction and migration of mature dendritic cells from the skin to regional lymph nodes³ and to enhanced lymph node metastasis of CCR7-expressing malignant melanoma xenotransplants.³⁹ Using gene array profiling studies, we found additional chemokines with enhanced expression in LECs, including stromal cell-derived factor-1, RANTES, and

CCL20 (MIP-3 α , exodus-1). Recently, low-level expression of CCL20 was detected in podoplanin-positive cultured LECs but not in podoplanin-negative cells, whereas both types of cells potently up-regulated CCL20 expression on stimulation with inflammatory cytokines.¹⁷ We confirmed our *in vitro* findings by differential immunostains of normal human skin, demonstrating that CCL20 was specifically expressed on lymphatic vessels in noninflamed skin. Our results suggest that additional chemokines, secreted by lymphatic endothelium, might participate in the recruitment of antigen-presenting cells and, possibly, tumor cells to regional lymph nodes.

It is of interest that angiopoietin-2 showed increased expression in cultured LECs since recent studies in angiopoietin-2-deficient mice suggest an important role of angiopoietin-2 for the final developmental steps of lymphatic network patterning and lymphatic vessel maturation.⁴⁰ Reelin was one of the genes with the highest increase of expression in LECs, as compared with BVECs. During the review process of this manuscript, another article also described results of gene array studies of LECs and BVECs, including several genes of the present study such as angiopoietin-2 and reelin.⁴¹ Recently, it has been shown that autosomal recessive lissencephaly with cerebellar hypoplasia is associated with human reelin mutations.⁴² Although we were unable to detect specific reelin protein expression in cutaneous lymphatic vessels with the available antibodies, some humans with reelin mutations also show congenital lymphedema,⁴² suggesting a potential function of this gene in lymphatic development.

In summary, our study reveals that LECs and BVECs maintain the distinct expression of a large number of lineage-specific genes *in vitro* and after transplantation *in vivo*, and it corroborates the close relationship between the blood vascular and lymphatic system. Moreover, these results demonstrate that some lineage-specific genes are only expressed during distinct developmental stages of vascular development, and they identify new molecular markers for blood vascular and lymphatic endothelium with important implications for vascular development, the regulation of the immune response, and the molecular control of tumor progression.

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