Neoplasia • Vol. 9, No. 12, December 2007, pp. 1038–1045 1038

www.neoplasia.com

Differential Gene Expression of Primary Cultured Lymphatic and Blood Vascular Endothelial Cells[1,2]

Gregory M. Nelson, Timothy P. Padera*, Igor Garkavtsev*, Toshi Shioda† and Rakesh K. Jain**

*Edwin L. Steele Laboratory, Department of Radiation Oncology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA; †Molecular Profiling Laboratory, Massachusetts General Hospital Center for Cancer Research, Charlestown, MA 02129, USA

Abstract

Blood vascular endothelial cells (BECs) and the developmentally related lymphatic endothelial cells (LECs) create complementary, yet distinct vascular networks. Each endothelial cell type interacts with flowing fluid and circulating cells, yet each vascular system has evolved specialized gene expression programs and thus both cell types display different phenotypes. BECs and LECs express distinct genes that are unique to their specific vascular microenvironment. Tumors also take advantage of the molecules that are expressed in these vascular systems to enhance their metastatic potential. We completed transcriptome analyses on primary cultured LECs and BECs, where each comparative set was isolated from the same individual. Differences were resolved in the expression of several major categories, such as cell adhesion molecules (CAMs), cytokines, and cytokine receptors. We have identified new molecules that are associated with BECs (e.g., claudin-9, CXCL11, neurexin-1, neurexin-2, and the neuronal growth factor regulator-1) and LECs (e.g., claudin-7, CD58, hyaluronan and proteoglycan link protein 1 (HAPLN1), and the poliovirus receptor–related 3 molecule) that may lead to novel therapeutic treatments for diseases of lymphatic or blood vessels, including metastasis of cancer to lymph nodes or distant organs.

Neoplasia (2007) **9,** 1038–1045

Keywords: Lymphangiogenesis, microarray, LEC, BEC, endothelial.

Introduction

The lymphatic system maintains tissue–fluid equilibrium while organizing the surveillance for foreign antigens by immune cells. Its smallest vessels are initial lymphatic vessels that collect interstitial fluid and immune cells from the surrounding tissue. These initial lymphatics consist of lymphatic endothelial cells (LECs) that sit on a discontinuous basement membrane and are tethered to the extracellular matrix (ECM) through anchoring filaments. The protein-rich lymph and extravasated immune cells enter the initial lymphatic vessels through intercellular junctions, are transported to a series of lymph nodes, and are then returned to the blood primarily through the thoracic duct [1]. Initial lymphatics are different from blood vessel capillaries, which are composed of blood vascular endothelial cells (BECs) encircled by pericytes and basement membrane. It is of great interest to distinguish the molecular differences between the similar but different LECs and BECs.

The lymphatic system also plays a role in pathologic states [2]. Lymphatic vessels act as a conduit for metastatic tumor cells to escape from the primary tumor. Cells can be imaged in lymphatic vessels in transit between the primary tumor and sentinel lymph node in animals [3] and sentinel lymph node biopsies positive for cancer cells serve as important determinants of cancer prognosis and for the subsequent course of therapy [4,5]. The molecular pathways involved with the development and growth of the lymphatic system are beginning to be understood. Molecules such as vascular endothelial growth factor (VEGF)-C [6,7], VEGF-D [8], VEGF-A [9,10], hepatocyte growth factor [11,12], and platelet-derived growth factor-BB [13] have been shown to be lymphangiogenic. VEGF-A and VEGF-C can also create hyperplastic tumor margin lymphatic vessels when overexpressed [9,14]. The enlarged vessels allow a greater number of metastatic cells to leave the primary tumor, thus promoting metastasis [3,15].

In addition to aiding metastasis, the lymphatic system plays a role in diseases involving lymphedema. Hereditary or primary lymphedema occurs as a result of problems with the formation of a functional lymphatic system. Some forms of primary lymphedema have been linked to gene mutations in *VEGFR-3*, i.e., the receptor for VEGF-C found on LECs [16]. Lymphedema can also occur as a result of an infection or injury to the lymphatic systems, preventing lymphatic vessels from

Abbreviations: BEC, blood vascular endothelial cell; CAM, cell adhesion molecule; DC, dendritic cell; ECM, extracellular matrix; HDMEC, human dermal microvascular endothelial cell; KEGG, Kyoto Encyclopedia of Genes and Genomes; LEC, lymphatic endothelial cell; qRT-PCR, quantitative reverse transcription–polymerase chain reaction; VEGF, vascular endothelial growth factor

Address all correspondence to: Rakesh K. Jain, Edwin L. Steele Laboratory for Tumor Biology, Department of Radiation Oncology, Massachusetts General Hospital and Harvard Medical School, 100 Blossom Street – Cox7, Boston, MA 02114, USA. E-mail: jain@steele.mgh.harvard.edu

[1]This work was supported by National Institutes of Health grants T32 CA73479 (G.N.) and R01 CA85140 (R.J.).

[2]This article refers to supplementary materials, which are designated by Tables W1, W2, and W3 and Figure W1 and are available online at www.neoplasia.com.

Received 26 July 2007; Revised 14 September 2007; Accepted 17 September 2007.

DOI 10.1593/neo.07643

collecting lymph and returning to the blood. These secondary forms of lymphedema are commonly caused by filarial infections or lymphatic vessel transection during surgery [17,18].

Research on lymphatic vessels and LECs has lagged behind that of BECs and blood vessels. With the identification of molecules that can distinguish LECs from BECs, the field has grown tremendously. Yong et al. [19] used a spotted oligonucleotide array to determine the gene expression changes that occurred when LECs were subjected to VEGF-C. In contrast to this study, previous comparisons of the transcriptional expression of LECs and BECs [20–22] did not have a full genome array available and did not make the full data sets accessible. One recent study compared cultured and uncultured LECs and BECs and found that differences in the handling of the cells can change the differential expression of the LECs and BECs [23].

Here we compare LECs and BECs with the largest possible gene array to identify differentially expressed genes that will strengthen our understanding of lymphatic and blood vascular biology and potentially uncover targets for therapeutic intervention in lymphedema and lymphatic metastasis. We used the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. Our analysis yields targets that may enhance our understanding of lymphatic and blood vascular biology.

Materials and Methods

Cell Maintenance and Separation

Three independent lots of $CD31^+$ human dermal microvascular endothelial cells (HDMECs; PromoCell, Heidelberg, Germany) isolated from different male individuals were maintained on fibronectin-coated tissue culture plates in endothelial cell growth medium MV low serum (PromoCell) according to the manufacturer's instructions. The isolation of cells from separate individuals allowed us to compare LECs and BECs from the same person, reducing the intrasample variability. The cells were immunomagnetically separated with mouse anti–human podoplanin IgG (a generous gift of Dr. Dontscho Kerjaschki [24]). Cells were washed with PBS/2% BSA solution and incubated in a magnetic particle concentrator (MPC-1; Invitrogen, Carlsbad, CA). Bead-bound cells (LECs) were washed five times to remove all unbound cells. Unbound cells in the wash buffer were collected and centrifuged to pellets (900*g* for 3 minutes at room temperature). Unbound cells (BECs) were resuspended in wash buffer and placed on the magnetic particle concentrator to remove bead-bound cells; the process was repeated five times. Pure populations of LECs ($CD31^+$/podoplanin$^+$) and BECs ($CD31^+$/podoplanin$^-$) were cultured in HDMEC media and grown for two to four passages. Cells were used in passage four to nine for microarray analysis and harvested at approximately the same confluence.

RNA Isolation, Complementary RNA Preparation, and Microarray Experiment

Total RNA was isolated from subconfluent LECs and BECs with TRIzol reagent followed by a Qiagen RNA cleanup procedure (RNeasy; Qiagen, Valencia, CA). Five micrograms of total RNA was used from each of the three independent LEC and BEC preparations from cells isolated from three separate individuals. Samples were submitted to the Biopolymer Institute, a microarray core facility (Harvard Medical School, Boston, MA). Total RNA (2.5–5 μg) was used in the generation of the biotinylated complementary RNA as recommended by the manufacturer using the One-Cycle Eukaryotic Target Labeling Assay (Affymetrix, Santa Clara, CA). Biotinylated targets were hybridized to an Affymetrix GeneChip Human Genome U133 Plus 2.0 Array with the Gene Chip Hybridization Oven 640 (Affymetrix) and the Gene Chip Fluidics Station 450 (Affymetrix). The hybridized array was scanned with the Gene Chip Scanner 3000 7G (Affymetrix). Affymetrix GeneChip Operating Software (GCOS) performed a post run normalization by scaling the data to the global mean of 500.

Quantitative Reverse Transcription–Polymerase Chain Reaction (qRT-PCR)

Single-strand cDNA synthesis from total RNA was prepared using *Taq*Man Reverse Transcription Reagents as recommended by the manufacturer (Applied Biosystems, Foster City, CA). *Taq*Man Assays-On-Demand were purchased from Applied Biosystems for *HoxA5* (Hs00430330_m1), *HoxA10* (Hs00538183_m1), *Podoplanin* (Hs00366764_m1), and *Prox1* (Hs00160463_m1). The genes *ESX1* and *BEX1* were assayed using Power SYBR Green-I (Applied Biosystems). Primers for the assay are as follows: *ESX1*, forward primer 5′–CTCAATATCCCGACGTTGTGG–3′ and reverse primer 5′–CCAAACCTGCACTCTGTCTTCA–3′; *BEX1*, forward primer 5′–GATAGGCCCAGGAGTAATGGAG–3′ and reverse primer 5′–CTTGGTTGGCATTTTCCATG–3′; and human glyceraldehyde-3-phosphate dehydrogenase, forward primer 5′–CATGAGAAGTATGACAACAGCCT–3′ and reverse primer 5′–AGTCCTTCCACGATACCAAAGT–3′. Each qRT-PCR performed was normalized to the endogenous glyceraldehyde-3-phosphate dehydrogenase levels and calibrated to the relative BEC signal for each gene using the $\Delta\Delta C_T$ method as previously described [25]. All qRT-PCR analyses were performed in triplicate with ABI PRISM 7300 (Applied Biosystems).

Microarray Data Analysis

The unfiltered microarray data were subjected to *X*–*Y* plot analysis using GCOS. Three independently obtained LEC–BEC data pairs were superimposed on a single, log-scale *X*–*Y* plot (*X*-axis: BEC mRNA expression; *Y*-axis: LEC mRNA expression). Gene expression calls (Present/Absent) were calculated using the default setting of GCOS and indicated by color of each datum point: *red*, present in both BEC and LEC; *blue*, absent in either BEC or LEC; *yellow*, absent in both BEC and LEC. Datum points for representative BEC- and LEC-specific genes were also indicated on the plot.

For gene ontology analysis, unfiltered microarray data were uploaded into GeneSifter (www.genesifter.net), a web-based analysis program with BEC data sets selected as control. Normalized data was $\log_2$-transformed followed by

applying a fold change filter of ≥ 2.0 and a statistical *t* test with a $P < .05$ cutoff (two-tailed unpaired Student's *t* test assuming equal variance). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed on the resultant probe sets and scored for significance. A *z*-score of absolute value greater than 2 indicated whether a pathway occurs more or less frequently than expected. Extreme positive numbers (greater than 2) indicated that the term occurred more frequently than expected, whereas an extreme negative number (less than −2) indicated that the term occurred less frequently than expected.

The data were additionally filtered by expression level, selecting probe sets whose ratios were the result of having at least one of the two comparisons (LECs or BECs) having a $\log_2$-transformed expression level greater than 6.644 (this number equals a scaled raw expression value of > 100) in the numerator, i.e., LECs/BECs or BECs/LECs.

Two-dimensional hierarchical clustering analysis of 312 informative genes was performed using Cluster software and visualized with TreeView software (Lawrence Berkeley National Laboratories, Berkeley, CA) [26]. Complete linkage clustering analysis was performed using an uncentered correlation as a similarity metric for both genes and array data sets.

Results

Analysis of Microarray Data

We analyzed six individual Affymetrix gene arrays, three from $CD31^+$/podoplanin− cell types and three from $CD31^+$/$podoplanin^+$ cell types, hereafter referred to as BECs and LECs, respectively. Each BEC and LEC cell line was isolated from a mixed population of $CD31^+$ HDMECs taken from the same individual person, so each of the three sets was genetically identical. The mixed HDMEC population was immunomagnetically sorted into podoplanin-positive and -negative cells with thorough washing. The cultures were compared for podoplanin gene expression by measuring the amount of podoplanin mRNA in each cell type by qRT-PCR. LECs had podoplanin mRNA expression values ranging from 100- to 1500-fold higher than cultured BECs (data not shown).

To examine microarray data quality, scaled data were subjected to scatterplot analysis of LECs *versus* BECs (Figure 1). The overall shape of the plotted data showed a narrow, symmetrical triangular contour pointing to the right upper corner, which is typically observed when significantly similar data sets are compared by this method. On the *X*–*Y* plot for three superimposed BEC–LEC pair data sets, reproducible detection of differential expression of several known LEC-specific marker genes (i.e., podoplanin [*PDPN*], *Prox-1*, and mannose receptor C, type I [*MRC1*]) and BEC-specific marker genes (i.e., interferon, alpha-inducible protein 27 [*IFI27*], *STAT6*, selectin E [*SELE*], and von Willebrand Factor [*vWF*]) are demonstrated. These results strongly support the notion that the LEC and BEC cell preparations used in the present study appropriately represented gene expression characteristics for these cell types and that the microarray analysis of differential gene expression was properly performed.

To further validate our gene array data, we choose representative genes with altered gene expression in LECs and BECs. Podoplanin and *Prox-1* gene expression were measured because they are recognized as typical LEC-associated molecules and were differentially expressed in LECs. These genes were shown to have more abundant expression in LECs than BECs using *Taq*Man and SYBR Green qRT-PCR (Figure 2). Four other genes, *ESX1*, *BEX1*, *HOXA5*, and *HOXA10*, were chosen for verification because they comprised a range of expression levels and they had not been previously been associated with LECs. *ESX1* and *BEX1* exhibited high levels of gene expression. *ESX1* was more abundant than podoplanin using qRT-PCR (Figure 2). *HOXA5* and *HOXA10* had similar relevant expression to *Prox-1* (Figure 2). This small subset of genes helps verify the original array data and gives more confidence in the validity of the data.

To obtain objective evidence, we performed hierarchical clustering analysis of genes differentially expressed between LECs and BECs (Figure W1). The clustering algorithm clearly separated the triplicate LEC and BEC data sets (Figure W1*A*), providing preliminary lists of LEC/BEC cell type–discriminating marker genes. Because the number of genes that were expressed stronger in BECs (Figure W1*B*) and in LECs (Figure W1C) were comparable, the clustering analysis was unlikely to reflect nonspecific differences in cell viability or lower RNA quality for one of these cell types.

Data from the scaled microarray results were subjected to selection criteria and used to create a list of differentially expressed genes. The initial selection criteria (1.5-fold differential expression; *t* test *P* value < .05) identified 2287 probe sets; 1197 probe sets had a higher expression in LECs and 1090 probe sets had higher expression in BECs. This data set was used for KEGG pathway analysis. We applied a more stringent selection criteria by removing all probe sets with both LEC and BEC $\log_2$-transformed intensity values less than 6.644

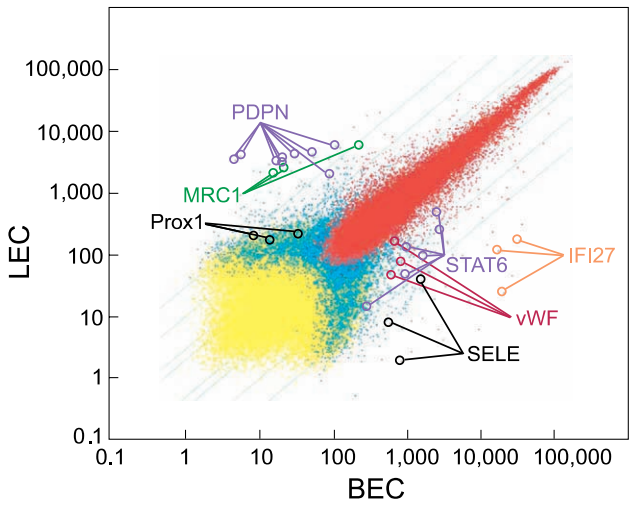

Figure 1. *Gene expression characteristics of LEC and BEC cell types. Microarray data were superimposed onto an* X–Y *plot and typical marker genes were highlighted for each cell type.* Prox1*, prospero-related homeobox 1;* MRC1*, mannose receptor C, type I;* PDPN*, podoplanin;* SELE*, selectin E;* vWF*, von Willebrand factor;* STAT6*, signal transducer and activator of transcription 6;* IFI27*, interferon, alpha-inducible protein 27.*

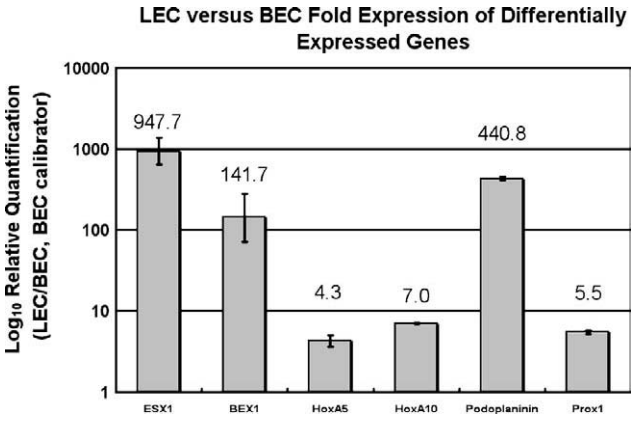

Figure 2. *qRT-PCR expression analysis. Error bars are the relative quantification minimum and maximum.*

(< 100 of a nontransformed scaled intensity value), which is generally not detectable by qRT-PCR. There were 1262 probe sets that passed this additional selection (see Table W1); 699 for LECs and 563 for BECs. This data set was used to generate the top 25 genes that demonstrated the highest expression differences between LECs and BECs (Table 1).

Podoplanin had the highest fold change overall suggesting it is an appropriate molecule to use to separate BECs and LECs. The next highest expressed gene in LECs was the hyaluronan and proteoglycan link protein 1 (*HAPLN1*), an ECM-linking protein that acts as a stabilizer of the interaction between versican and hyaluronan in various tissues [27]. Also included in the top 25 LEC genes were transcription factors (*BATF* and *ESX1*), receptors (*MRC1*, *SORL1*, *CNR1*, *PRLHR*, and *CHRND*), and several open reading frames of unknown function or lacking description.

BECs also had a wide variety of genes expressed at higher levels than in LECs. The interferon, alpha-inducible protein 27 (*IFI27*) was the highest differentially expressed gene for BECs. The only cell-surface receptor in the BEC top 25 list was EPH receptor A4 and was accompanied by the cell adhesion molecule (CAM), selectin E, which was expressed as expected [28]. Critical for extracellular signaling, the chemokine ligands *CXCL11* and *CX3CL1* were represented.

We employed KEGG pathways analysis to group the differentially expressed genes into known functional or biochemical pathways. Table 2 lists the pathways identified for LECs and BECs that were scored as significant (> |2| *z*-score). Each pathway listed in Table 2 has the associated genes listed in Tables W2 and W3. LECs had high *z*-scores for the cell cycle, pyrimidine metabolism, and one carbon pool by folate pathways, indicating optimal growth media used in culturing the LECs compared to BECs. The calcium signaling pathway also scored significantly for LECs.

Cell Adhesion Molecules

BECs scored high (> 4) for the carbazole degradation, 2,4-dichlorobenzoate degradation, and CAM pathways (Table 2). The carbazole degradation and 2,4-dichlorobenzoate degradation pathways had few members within the array, possibly because the pathway is new or still in development. Cell adhesion molecules are of great importance to the structure and function of blood and lymphatic vessels. Table 3 lists the members of the CAM pathway identified by KEGG analysis.

Claudin-5 [25], intercellular adhesion molecule 1 [29], inducible T-cell costimulator ligand (ICOSL) [30], CD58 [31], poliovirus receptor [32], protein tyrosine phosphatase, receptor type, M [33,34], selectin E [28], and selectin P [35] have been previously identified with vascular endothelial cell–cell adhesion and were expected. Surprisingly, several genes had not been reported as associated with BECs, such as claudin-9, the major histocompatibility complex class II DO alpha and DR alpha, neurexin-2, neurexin-3, and neuronal growth regulator-1. Nevertheless, all the CAM-associated genes in Table 3 were novel for LEC expression.

Cytokines and Cytokine Receptors

Cytokines and their receptors are important features of endothelial cells. BECs in this study express the following genes that have not been previously reported for this endothelial cell type. Chemokine (C–C motif) ligand 15 was expressed nine-fold higher in BECs than in LECs. Chemokine (C–C motif) ligand 19 was expressed five-fold higher in BECs. Chemokine (C–X–C motif) ligand 11, expressed in endothelial cells and hepatocytes [36,37], was expressed in BECs at high levels (> 48-fold) compared to LECs in this study.

NOTCH2, a member of the Notch transmembrane protein family and usually expressed in BECs [38], was expressed 3.32-fold higher in LECs, as was *HEY2* (9.9-fold), a transcriptional product of downstream Notch signaling [39].

Discussion

Our data show that LECs have a transcriptional program distinct from that of blood vascular endothelial cells. LECs and BECs perform similar functions but in a different microenvironment. Blood vascular endothelial cells line the lumen of capillary tubular structures assisted by perivascular cells and continuous basement membrane. Blood cells and fluid pass through these tubes at high velocity and under considerable pressure, which changes depending on the current state of the physiology of the organism. Protein and fluid are lost from the blood vascular system, bathing the surrounding cells with extracellular fluid. Protein- and lipid-rich fluid enters the initial lymphatic vessels along with immune cells *en route* to the lymph nodes and eventually reenter the circulatory system. LECs, the major component of the initial lymphatic system, in contrast to BECs, have much slower fluid dynamics. Lymph enters the lymphatic system through pressure-induced fluid transport. The two endothelial cell types are part of a vascular system, but with widely divergent physiological needs and requirements.

The transcriptome snapshot of LECs and BECs helps us to understand the differences and the similarities each cell type has. Each is tethered to the ECM. Many more ECM proteins are specifically required for proper BEC functioning. Endothelial cells of blood vessels need to actively interact with molecules displayed on other circulating cells, such as

Table 1. List of 25 Highly Expressed Genes in LECs and BECs.

Probe Set ID	Gene	Description	Ratio	Gene Identifier	Chromosome
LECs					
204879_at	*PDPN*	Podoplanin*	428.1	NM_006474	1
205523_at	*HAPLN1*	Hyaluronan and proteoglycan link protein 1*	148.4	U43328	5
204438_at	*MRC1*	Mannose receptor, C type 1	71.0	NM_002438	10
212560_at	*SORL1*	Sortilin-related receptor, L(DLR class) A repeats-containing	20.3	AV728268	11
218332_at	*BEX1*	Brain-expressed, X-linked 1	18.3	NM_018476	X
1552445_a_at	*ESX1*	Extraembryonic, spermatogenesis, homeobox 1 homolog (mouse)	17.9	NM_153448	X
205015_s_at	*TGFA*	Transforming growth factor, alpha	17.4	NM_003236	2
229029_at	*CAMK4*	Calcium/calmodulin–dependent protein kinase IV	17.3	AI745230	5
213436_at	*CNR1*	Cannabinoid receptor 1 (brain)	16.8	U73304	6
204301_at	*KBTBD11*	Kelch repeat and BTB (POZ) domain containing 11	15.1	NM_014867	8
1559975_at	*BTG1*	B-cell translocation gene 1, antiproliferative	14.8	BC009050	12
213974_at	*ADAMTSL3*	ADAMTS-like 3	14.4	AB033059	15
205965_at	*BATF*	Basic leucine zipper transcription factor, ATF-like	13.3	NM_006399	14
218741_at	*CENPM*	Centromere protein M	13.1	NM_024053	22
223631_s_at	*C19orf33*	Chromosome 19 open reading frame 33	12.7	AF213678	19
211959_at	*IGFBP5*	Insulin-like growth factor binding protein 5	12.6	AW007532	2
220318_at	*EPN3*	Epsin 3	12.6	NM_017957	17
1557196_a_at	–	cDNA DKFZp547B198 (from clone DKFZp547B198)	12.4	AL831886	X
229081_at	*SLC25A13*	Solute carrier family 25, member 13 (citrin)	12.2	AW268880	7
208161_s_at	*ABCC3*	ATP-binding cassette, subfamily C (CFTR/MRP), member 3	12.0	NM_020037	17
204393_s_at	*ACPP*	Acid phosphatase, prostate	11.5	NM_001099	3
231805_at	*PRLHR*	Prolactin releasing hormone receptor	11.5	AL563031	10
207024_at	*CHRND*	Cholinergic receptor, nicotinic, delta	11.5	NM_000751	2
238529_at	–	CDNA clone IMAGE:6342029	10.3	AA573088	1
236817_at	*DEADC1*	Deaminase domain containing 1	10.3	AI336346	6
BECs					
202411_at	*IFI27*	Interferon, alpha-inducible protein 27	274.53	NM_005532	14
206211_at	*SELE*	Selectin E (endothelial adhesion molecule 1)	104.42	NM_000450	1
207173_x_at	*CDH11*	Cadherin 11, type 2, OB-cadherin (osteoblast)	76.01	D21254	16
209994_s_at	*ABCB1*	ATP-binding cassette, subfamily B (MDR/TAP), member 1	51.57	AF016535	7
210163_at	*CXCL11*	Chemokine (C–X–C motif) ligand 11	48.73	AF030514	4
212554_at	*CAP2*	CAP, adenylate cyclase–associated protein, 2 (yeast)	34.81	N90755	6
203881_s_at	*DMD*	Dystrophin (muscular dystrophy, Duchenne and Becker types)	33.64	NM_004010	X
244204_at	*PRR3*	Proline-rich 3	19.89	W87300	6
209074_s_at	*FAM107A*	Family with sequence similarity 107, member A	18.51	AF089853	3
228617_at	*BIRC4BP*	XIAP-associated factor-1	17.7	AA142842	17
205174_s_at	*QPCT*	Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	16.07	NM_012413	2
201332_s_at	*STAT6*	Signal transducer and activator of transcription 6, interleukin-4 induced	15.37	BC004973	12
1552902_a_at	*FOXP2*	Forkhead box P2	15.32	NM_148898	7
209474_s_at	*ENTPD1*	Ectonucleoside triphosphate diphosphohydrolase	15.16	AV717590	10
230109_at	*PDE7B*	Phosphodiesterase 7B	14.99	AI638433	6
226281_at	*DNER*	Delta/notch–like EGF repeat containing	14.38	BF059512	2
1566161_at	*MEIS2*	Meis1, myeloid ecotropic viral integration site 1 homolog 2 (mouse)	14.33	AA340499	15
230741_at	–	Full-length insert cDNA clone YX74D05	13.76	AI655467	12
210882_s_at	*TRO*	Trophinin	13.6	U04811	X
226992_at	*NOSTRIN*	Nitric oxide synthase trafficker	13.58	AK002203	2
228948_at	*EPHA4*	EPH receptor A4	13.07	T15545	2
203687_at	*CX3CL1*	Chemokine (C–X3–C motif) ligand 1	13.03	NM_002996	16
202718_at	*IGFBP2*	Insulin-like growth factor–binding protein 2, 36 kDa	12.72	NM_000597	2
203153_at	*IFIT1*	Interferon-induced protein with tetratricopeptide repeats 1	12.66	NM_001548	10
239669_at	*HIST1H3D*	Histone cluster 1, H3d	12.54	AW006409	6

*This is the highest representative of multiple probe sets.

immune surveillance cells. It is not unusual to discover that some genes expressed in BECs are associated with cell-to-cell junction communication, such as claudin-9, which appears to be a developmentally expressed claudin. Before this report, only expression of claudin-9 in neonatal proximal tubule endothelial cells forming tight junctions within the mouse kidney was published [40]. The BECs isolated from neonatal HDMECs taken from infant foreskin for this study express claudin-9, but may not be expressed in adult BECs.

There is mounting evidence that the vascular and nervous systems use common protein machinery to establish the placement of vessels and nerves [41,42]. Neurexin-1, neurexin-2, and the neuronal growth factor regulator-1 were differentially expressed in BECs compared to LECs, providing another example of common usage in nerve and blood vessels patterning.

LECs expressed several genes that have not been previously associated with the lymphatic system. CD58 costimulates CD4(+) T-cells through the CD2–CD58 system by promoting lipid raft formation [31]. The expression of *CD58* by LECs may indicate another location previously unknown that T-cell activation can occur, although this study does not indicate whether the expression of *CD58* occurs on the luminal side or on the abluminal side of the initial lymphatic vessels.

The role of programmed cell death 1 ligand 2 (PDL2) was examined when Zhang et al. [43] generated knockout

Table 2. KEGG Pathway Summary for LECs and BECs.

	Total				*z* Score	
	List	LEC	BEC	Array	LEC	BEC
(A) LEC KEGG Pathways						
Cell cycle	26	22	4	112	7.98	−0.26
Pyrimidine metabolism	13	10	3	90	3.14	−0.35
One carbon pool by folate	4	3	1	16	2.80	0.45
Calcium signaling pathway	26	15	11	174	2.77	1.56
Circadian rhythm	3	3	0	18	2.54	−0.87
Glycosphingolipid biosynthesis – *neo*-lactoseries	4	3	1	21	2.21	0.17
Insulin signaling pathway	2	1	1	134	−2.10	−1.97
Diterpenoid biosynthesis	1	1	0	4	2.01	−0.41
Styrene degradation	2	1	1	4	2.01	2.13
(B) BEC KEGG Pathways						
Carbazole degradation	1	0	1	1	−0.21	4.87
2,4-Dichlorobenzoate degradation	2	0	2	5	−0.48	4.08
Cell adhesion molecules	19	5	14	127	−0.26	4.06
Fluorene degradation	1	0	1	2	−0.30	3.30
Histidine metabolism	6	1	5	38	−0.53	2.86
Glycosaminoglycan degradation	4	1	3	17	0.30	2.85
Parkinson's disease	4	1	3	18	0.24	2.72
Glycan structures – degradation	5	1	4	29	−0.25	2.67
1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane degradation	1	0	1	3	−0.37	2.58
Oxidative phosphorylation	1	1	0	121	−1.95	−2.29
Styrene degradation	2	1	1	4	2.01	2.13
Prion disease	3	1	2	13	0.58	2.08

mice. The heterozygous −/− animals were healthy; antigen-presenting cells from *Pdl2*$^{-/-}$ mice induced stronger T-cell proliferation than was expected compared to wild-type cells *in vitro* and *in vivo*. Zhang et al. also concluded that PDL2 negatively regulated T-cells and played an essential role in immune tolerance.

Of interest, the poliovirus receptor–related 3 protein may have cell-adhesive functions, but this has not been extensively studied. However, poliovirus receptor–related 2 is thought to participate in the regulation of hematopoietic/endothelial homophilic cellular functions and act as an intercellular junction protein [44]. Thus, poliovirus receptor–related 3 protein may be a new CAM expressed on LECs that helps LECs form cell–cell junctions conducive to lymph formation and immune cell intravasation. In the same class of proteins, LECs express claudin-7, which is associated with tight junctions, which exclude water and solutes across endothelial cells. Claudin-7 also excludes the lateral transfer of lipids and membrane proteins in epithelial cells that defines their apical and basolateral compartments. Claudin-7 may perform the same function in LECs and maintain their polarity.

Cell signaling is an essential characteristic of BECs. Many of the chemokine and chemokine receptors that were identified in this study have been associated with the blood vascular system. These molecules may be significant as many tumor cells express receptors for chemokines and home to a metastatic niche. We have identified several chemokines that have not previously been associated with BECs. For example, chemokine (C–C) ligand 15 (CCL15) [45] can act as a signal for endothelial cell mobilization and, according to this study, BECs can also produce it, which may be context-dependent.

Dendritic cells (DCs) express chemokine (C–C) ligand 19 (CCL19), which regulates DC trafficking and the recruiting of naïve T-cells to the area of DC activity [46]. Expression of CCL19 in BECs indicates that the blood vascular endothelium may take an active role in the recruiting of DCs to areas of activity.

CXCL11 is known to be involved in the negative regulation of angiogenesis [47] through the CXCR3 receptors during interferon-γ induction. Activated T-cells, usually of the Th_1 subtype, internalize CXCR3 when bound to their cognate ligand CXCL11 [48]. The expression of CXCL11 on LECs indicates another possible mechanism to attract activated T-cells to lymphatic vessels. Tumor cells may take advantage of this attracting mechanism to actively home the lymphatic network.

Table 3. Cell Adhesion Molecules' KEGG Pathway–Associated Genes.

BECs	LECs
Claudin-5	CD58 molecule
Claudin-9	Claudin-7
Inducible T-cell costimulator ligand (ICOSL)	Major histocompatibility complex, class I, C
Intercellular adhesion molecule 1 (CD54)	Major histocompatibility complex, class II, DP alpha 1
Major histocompatibility complex, class II, DO alpha	Poliovirus receptor–related 3
Major histocompatibility complex, class II, DR alpha	
Neurexin-2	
Neurexin-3	
Neuronal growth regulator 1	
Poliovirus receptor	
Programmed cell death 1 ligand 2	
Protein tyrosine phosphatase, receptor type, M	
Selectin E	
Selectin P	

The expression of *NOTCH2*, a transcription factor, and its downstream transcriptional product, *HEY2* indicates active Notch signaling in LECs. Notch signaling molecules are known to determine the cell fate of many cell types and developmental processes of several organs, including the nervous system. Notch may act as one of the cell-fate determinates for LECs.

In conclusion, this report confirms previous reports of molecules that are important determinants for cellular function of LECs and BECs. We also report several molecules that are newly associated with either LECs or BECs. Cell signaling is very important for BECs, as they express many more chemokine ligands and receptors, which may not be as important for the LECs due to their different microenvironment and need for cellular recruitment. The molecules identified may also be tested as possible ligands that attract tumor cells as they metastasize.

Extracellular adhesion molecules are more necessary for the BECs to handle the stress and strain of rapid fluid flow and ever changing blood vessel pressure. However, several molecules were discovered that are expressed by LECs that may lead to further characterization of LEC function, especially CD58 and the poliovirus receptor–related 3 molecules. This study reveals additional molecular markers that may be used as targets to modify either the lymphatic or the blood vascular endothelium in pathologic states.

Acknowledgements

We thank Angera Kuo and Johanna Lahdenranta for critical reading of the manuscript. We appreciate the superb technical skill and assistance from Trent Rector and Sarah Annis of the Biopolymers Institute for microarray processing. Special thanks to Dontscho Kerjaschki for the gift of the podoplanin antibody.

References

[1] Schmid-Schonbein GW (1990). Microlymphatics and lymph flow. *Physiol Rev* **70** (4), 987–1028.

[2] Alitalo K and Carmeliet P (2002). Molecular mechanisms of lymphangiogenesis in health and disease. *Cancer Cell* **1** (3), 219–227.

[3] Hoshida T, Isaka N, Hagendoorn J, di Tomaso E, Chen YL, Pytowski B, Fukumura D, Padera TP, and Jain RK (2006). Imaging steps of lymphatic metastasis reveals that vascular endothelial growth factor-C increases metastasis by increasing delivery of cancer cells to lymph nodes: therapeutic implications. *Cancer Res* **66** (16), 8065–8075.

[4] Veronesi U, Paganelli G, Viale G, Luini A, Zurrida S, Galimberti V, Intra M, Veronesi P, Robertson C, Maisonneuve P, et al. (2003). A randomized comparison of sentinel-node biopsy with routine axillary dissection in breast cancer. *N Engl J Med* **349** (6), 546–553.

[5] Morton DL, Thompson JF, Cochran AJ, Mozzillo N, Elashoff R, Essner R, Nieweg OE, Roses DF, Hoekstra HJ, Karakousis CP, et al. (2006). Sentinel-node biopsy or nodal observation in melanoma. *N Engl J Med* **355** (13), 1307–1317.

[6] Kukk E, Lymboussaki A, Taira S, Kaipainen A, Jeltsch M, Joukov V, and Alitalo K (1996). VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Development* **122** (12), 3829–3837.

[7] Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, and Alitalo K (1996). A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* **15** (2), 290–298.

[8] Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, and Stacker SA (1998). Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci USA* **95** (2), 548–553.

[9] Nagy JA, Vasile E, Feng D, Sundberg C, Brown LF, Detmar MJ, Lawitts JA, Benjamin L, Tan X, Manseau EJ, et al. (2002). Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. *J Exp Med* **196** (11), 1497–1506.

[10] Hirakawa S, Kodama S, Kunstfeld R, Kajiya K, Brown LF, and Detmar M (2005). VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. *J Exp Med* **201** (7), 1089–1099.

[11] Kajiya K, Hirakawa S, Ma B, Drinnenberg I, and Detmar M (2005). Hepatocyte growth factor promotes lymphatic vessel formation and function. *EMBO J* **24** (16), 2885–2895.

[12] Cao R, Bjorndahl MA, Gallego MI, Chen S, Religa P, Hansen AJ, and Cao Y (2006). Hepatocyte growth factor is a lymphangiogenic factor with an indirect mechanism of action. *Blood* **107** (9), 3531–3536.

[13] Cao Y (2005). Direct role of PDGF-BB in lymphangiogenesis and lymphatic metastasis. *Cell Cycle* **4** (2), 228–230.

[14] Isaka N, Padera TP, Hagendoorn J, Fukumura D, and Jain RK (2004). Peritumor lymphatics induced by vascular endothelial growth factor-C exhibit abnormal function. *Cancer Res* **64** (13), 4400–4404.

[15] Padera TP, Kadambi A, di Tomaso E, Carreira CM, Brown EB, Boucher Y, Choi NC, Mathisen D, Wain J, Mark EJ, et al. (2002). Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science* **296** (5574), 1883–1886.

[16] Karkkainen MJ, Ferrell RE, Lawrence EC, Kimak MA, Levinson KL, McTigue MA, Alitalo K, and Finegold DN (2000). Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. *Nat Genet* **25** (2), 153–159.

[17] Golshan M and Smith B (2006). Prevention and management of arm lymphedema in the patient with breast cancer. *J Support Oncol* **4** (8), 381–386.

[18] Ottesen EA (2006). Lymphatic filariasis: treatment, control and elimination. *Adv Parasitol* **61**, 395–441.

[19] Yong C, Bridenbaugh EA, Zawieja DC, and Swartz MA (2005). Microarray analysis of VEGF-C responsive genes in human lymphatic endothelial cells. *Lymphat Res Biol* **3** (4), 183–207.

[20] Podgrabinska S, Braun P, Velasco P, Kloos B, Pepper MS, and Skobe M (2002). Molecular characterization of lymphatic endothelial cells. *Proc Natl Acad Sci USA* **99** (25), 16069–16074.

[21] Petrova TV, Makinen T, Makela TP, Saarela J, Virtanen I, Ferrell RE, Finegold DN, Kerjaschki D, Yla-Herttuala S, and Alitalo K (2002). Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *EMBO J* **21** (17), 4593–4599.

[22] Hirakawa S, Hong YK, Harvey N, Schacht V, Matsuda K, Libermann T, and Detmar M (2003). Identification of vascular lineage-specific genes by transcriptional profiling of isolated blood vascular and lymphatic endothelial cells. *Am J Pathol* **162** (2), 575–586.

[23] Wick N, Saharinen P, Saharinen J, Gurnhofer E, Steiner CW, Raab I, Stokic D, Giovanoli P, Buchsbaum S, Burchard A, et al. (2007). Transcriptomal comparison of human dermal lymphatic endothelial cells *ex vivo* and *in vitro*. *Physiol Genomics* **28** (2), 179–192.

[24] Breiteneder-Geleff S, Soleiman A, Kowalski H, Horvat R, Amann G, Kriehuber E, Diem K, Weninger W, Tschachler E, Alitalo K, et al. (1999). Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. *Am J Pathol* **154** (2), 385–394.

[25] Morita K, Sasaki H, Furuse K, Furuse M, Tsukita S, and Miyachi Y (2003). Expression of claudin-5 in dermal vascular endothelia. *Exp Dermatol* **12** (3), 289–295.

[26] Eisen MB, Spellman PT, Brown PO, and Botstein D (1998). Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* **95** (25), 14863–14868.

[27] Shi S, Grothe S, Zhang Y, O'Connor-McCourt MD, Poole AR, Roughley PJ, and Mort JS (2004). Link protein has greater affinity for versican than aggrecan. *J Biol Chem* **279** (13), 12060–12066.

[28] Silverman MD, Babra B, Pan Y, Planck SR, and Rosenbaum JT (2005). Differential E-selectin expression by iris *versus* retina microvascular endothelial cells cultured from the same individuals. *Microvasc Res* **70** (1–2), 32–42.

[29] Clark PR, Manes TD, Pober JS, and Kluger MS (2007). Increased ICAM-1 expression causes endothelial cell leakiness, cytoskeletal reorganization and junctional alterations. *J Invest Dermatol* **127** (4), 762–774.

[30] Khayyamian S, Hutloff A, Buchner K, Grafe M, Henn V, Kroczek RA, and Mages HW (2002). ICOS-ligand, expressed on human endothelial

cells, costimulates Th_1 and Th_2 cytokine secretion by memory $CD4^+$ T cells. *Proc Natl Acad Sci USA* **99** (9), 6198–6203.

[31] Mestas J and Hughes CC (2001). Endothelial cell costimulation of T cell activation through CD58–CD2 interactions involves lipid raft aggregation. *J Immunol* **167** (8), 4378–4385.

[32] Reymond N, Imbert AM, Devilard E, Fabre S, Chabannon C, Xerri L, Farnarier C, Cantoni C, Bottino C, Moretta A, et al. (2004). DNAM-1 and PVR regulate monocyte migration through endothelial junctions. *J Exp Med* **199** (10), 1331–1341.

[33] Bianchi C, Sellke FW, Del Vecchio RL, Tonks NK, and Neel BG (1999). Receptor-type protein–tyrosine phosphatase mu is expressed in specific vascular endothelial beds *in vivo*. *Exp Cell Res* **248** (1), 329–338.

[34] Koop EA, Lopes SM, Feiken E, Bluyssen HA, van der Valk M, Voest EE, Mummery CL, Moolenaar WH, and Gebbink MF (2003). Receptor protein tyrosine phosphatase mu expression as a marker for endothelial cell heterogeneity; analysis of *RPTPmu* gene expression using LacZ knock-in mice. *Int J Dev Biol* **47** (5), 345–354.

[35] Miyazaki Y, Satoh T, Nishioka K, and Yokozeki H (2006). STAT-6–mediated control of P-selectin by substance P and interleukin-4 in human dermal endothelial cells. *Am J Pathol* **169** (2), 697–707.

[36] Sheikh AM, Ochi H, Manabe A, and Masuda J (2005). Lysophosphatidylcholine posttranscriptionally inhibits interferon-gamma–induced IP-10, Mig and I-Tac expression in endothelial cells. *Cardiovasc Res* **65** (1), 263–271.

[37] Helbig KJ, Ruszkiewicz A, Semendric L, Harley HA, McColl SR, and Beard MR (2004). Expression of the CXCR3 ligand I-TAC by hepatocytes in chronic hepatitis C and its correlation with hepatic inflammation. *Hepatology* **39** (5), 1220–1229.

[38] Siekmann AF and Lawson ND (2007). Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. *Nature* **445** (7129), 781–784.

[39] Fischer A, Schumacher N, Maier M, Sendtner M, and Gessler M (2004). The Notch target genes *Hey1* and *Hey2* are required for embryonic vascular development. *Genes Dev* **18** (8), 901–911.

[40] Abuazza G, Becker A, Williams SS, Chakravarty S, Truong HT, Lin F, and Baum M (2006). Claudins 6, 9, and 13 are developmentally expressed renal tight junction proteins. *Am J Physiol Renal Physiol* **291** (6), F1132–F1141.

[41] Carmeliet P and Tessier-Lavigne M (2005). Common mechanisms of nerve and blood vessel wiring. *Nature* **436** (7048), 193–200.

[42] Eichmann A, Makinen T, and Alitalo K (2005). Neural guidance molecules regulate vascular remodeling and vessel navigation. *Genes Dev* **19** (9), 1013–1021.

[43] Zhang Y, Chung Y, Bishop C, Daugherty B, Chute H, Holst P, Kurahara C, Lott F, Sun N, Welcher AA, et al. (2006). Regulation of T cell activation and tolerance by PDL2. *Proc Natl Acad Sci USA* **103** (31), 11695–11700.

[44] Lopez M, Aoubala M, Jordier F, Isnardon D, Gomez S, and Dubreuil P (1998). The human poliovirus receptor related 2 protein is a new hematopoietic/endothelial homophilic adhesion molecule. *Blood* **92** (12), 4602–4611.

[45] Hwang J, Kim CW, Son KN, Han KY, Lee KH, Kleinman HK, Ko J, Na DS, Kwon BS, Gho YS, et al. (2004). Angiogenic activity of human CC chemokine CCL15 *in vitro* and *in vivo*. *FEBS Lett* **570** (1–3), 47–51.

[46] Pietila TE, Veckman V, Lehtonen A, Lin R, Hiscott J, and Julkunen I (2007). Multiple NF-kappaB and IFN regulatory factor family transcription factors regulate *CCL19* gene expression in human monocyte–derived dendritic cells. *J Immunol* **178** (1), 253–261.

[47] Romagnani P, Lasagni L, Annunziato F, Serio M, and Romagnani S (2004). CXC chemokines: the regulatory link between inflammation and angiogenesis. *Trends Immunol* **25** (4), 201–209.

[48] Sauty A, Colvin RA, Wagner L, Rochat S, Spertini F, and Luster AD (2001). CXCR3 internalization following T cell–endothelial cell contact: preferential role of IFN-inducible T cell alpha chemoattractant (CXCL11). *J Immunol* **167** (12), 7084–7093.