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Expression of genes involved in vascular development and angiogenesis in endothelial cells freshly isolated from adult lungs

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ABSTRACT

Profiling gene expression in endothelial cells provides a strategy for developing a molecular understanding of normal vascular physiology and disease processes involving angiogenesis and vascular remodeling. However, the purification of endothelial cells for this purpose has been challenging because of the difficulty of isolating the cells and their low abundance in many organs and tumors. Here we describe an approach that exploits the propensity for certain types of endothelial cells to take up fluorescently labeled cationic liposomes from the bloodstream and use this property to isolate the cells from lung capillaries by FACS rapidly and with high purity. Out of some 39,000 genes and expressed sequence tags (ESTs) evaluated on custom oligonucleotide arrays, the expression of 234 genes and 321 ESTs was enriched in the endothelial cell fraction. These included familiar endothelial cell-associated genes such as VEGF, VEGFR-1, VEGFR-2, angiopoietin-2, Tie1, Tie2, Edg1 receptor, VE-cadherin, claudin 5, connexin 37, CD31, and CD34. Also enriched were genes in the semaphorin/neuropilin (Sema3c, Nrp1), ephrin/Eph (ephrin A1, B1, B2, EphB4), delta/notch (Hey1, Jagged 2, Notch 1, Notch 4, Numb, Siah1b), and Wingless (Frizzled-4, Tle1) signaling pathways that are expressed in vascular development and angiogenesis. The expression of representative genes in alveolar capillary endothelial cells was verified by immunohistochemistry. Such expression reflects features that endothelial cells of normal lung capillaries have in common with embryonic and growing blood vessels. About half of the enriched genes, including exostosin 2, lipocalin 7, phospholipid scramblase 2, pleckstrin 2, protocadherin 1, Ryk, scube1, serpinh1, SNF related kinase, and several tetraspanins, had little or no previous association with endothelial cells. This approach can also be used to profile genes expressed in angiogenic blood vessels in tumors, chronic inflammation, and other sites where endothelial cells avidly take up cationic liposomes.

Key words: blood vessels, cationic liposomes, immunohistochemistry, gene profiling, oligonucleotide arrays

INTRODUCTION

Microvascular endothelial cells are structurally and functionally heterogeneous. Organ-specific differences in endothelial cells result from the unique environment of each organ, as determined by exposure to soluble and physical factors, cell associations, and extracellular matrix (5, 30, 97). The characterization of normal endothelial cells is a necessary step in understanding changes that occur in cancer, chronic inflammation, and other diseases. Toward this end, the molecular features of endothelial cells are beginning to be identified, in part, through the use of high-throughput methods for profiling gene expression (16, 31, 51, 57, 58, 106). However, only a handful of endothelial cell-specific genes have been identified.

One major challenge in gene profiling of endothelial cells in organs or tumors is separating the cells from other cell types. Selective culture conditions (68, 70) and lectins or antibodies attached to magnetic beads (20, 22, 43, 44, 51, 74, 106) have proven useful in this regard. However, incomplete specificity for endothelial cells and removal of cell surface epitopes during dissociation are limitations of this approach. Another challenge is to minimize changes in expression that occur during the isolation process. Here, time is an important factor. Methods using multiple levels of selection with antibodies conjugated to magnetic beads involve multiple steps that may last 12 hours or longer (22, 106). Cell culture requires even more time and imposes in vitro conditions that increase the likelihood of changes in gene expression. Another factor is the number of cells needed for gene expression measurements, as dictated by the sensitivity of the assay. When only a small number of cells are available, methods used for gene expression profiling may not be sufficiently sensitive to detect basal levels of expression.

The purpose of the present study was to identify genes that are expressed in normal microvascular endothelial cells of the lung. To deal with the issues of specific cell labeling, speed of isolation, and number of cells isolated, we developed a method based on the natural capacity of the endothelial cells of lung capillaries to bind and internalize fluorescent cationic liposomes from the bloodstream (76). Because the liposomes do not cross the endothelium, cells outside the vasculature are not labeled (76, 114). In the past, this property has been used to target substances selectively to

certain types of endothelial cells, while sparing extravascular cells (76, 114). However, we reasoned that the same property could be used to label endothelial cells in mouse lungs for subsequent isolation and purification. After dissociation of the tissue, the fluorescent endothelial cells could be purified using fluorescence activated cell sorting (FACS) to yield a large number of endothelial cells rapidly and without the need for cell culture. Although intravascular leukocytes may also take up cationic liposomes (76), surface markers could be used to remove these cells by negative selection during FACS purification.

RNA from freshly isolated endothelial cells can be analyzed on oligonucleotide microarrays covering tens of thousands of genes or expressed sequence tags (ESTs) (64). In the present study, performed through a collaboration with Eos Biotechnology, Inc. (South San Francisco, CA), we used Affymetrix oligonucleotide microarrays covering 39,000 genes and ESTs in the mouse genome to investigate gene expression in endothelial cells isolated from normal lung capillaries. Genes with greater expression in endothelial cells were identified by relating expression in purified endothelial cells to the expression in unpurified lung cells. Expression of a representative selection of genes in endothelial cells was validated by immunohistochemistry on lung sections. Based on stringent selection criteria, the study revealed a profile of 234 known genes and 321 ESTs enriched in lung endothelial cells that contribute to their distinctive functions.

MATERIAL AND METHODS

Animals. Pathogen-free male C57BL/6 mice, weighing 24 to 28 g, from Charles River Laboratories (Hollister, CA) were used for gene profiling and most other experiments. Pathogen-free male C3H mice from the same source were used for some morphological studies of liposome uptake. All mice were housed in microisolation cages under barrier conditions. Experimental procedures were approved by the Committee of Animal Research at the University of California, San Francisco.

Preparation and injection of fluorescent cationic liposomes. Fluorescent cationic liposomes were prepared by mixing 1,2-dioleoyl-3 trimethylammonium-propane (DOTAP) with cholesterol (Avanti Polar Lipids, Alabaster, AL) and 1,2 dipalmitoyl-n-glycero-3-phosphoethanol amine-Lissamine-

rhodamine-B-sulfonyl chloride (Molecular Probes, Eugene, OR) in chloroform at a molar ratio of 55:44:1. Small unilamellar vesicles containing 10 mM total lipid in 5% glucose were prepared by sonication, filtered to sterility, and stored under argon as previously described (76, 114). Rhodamine-labeled liposomes (~1 mM total lipid in 100 µl) were injected via a femoral vein into anesthetized mice (sodium pentobarbital, 25 mg/kg ip, Nembutal, Abbott Laboratories, North Chicago, IL) and circulated for 20 min (76).

Verification of labeling of pulmonary endothelial cells. In initial validation studies, mice received an iv injection of rhodamine-labeled liposomes followed 18 min later by fluorescein isothiocyanate (FITC)-labeled *Lycopersicon esculentum* lectin (5 mg/kg iv, Vector, Burlingame, CA) to mark pulmonary vascular endothelial cells where liposomes were taken up (76). Two minutes later the lungs were fixed by vascular perfusion of 1% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, for 1 min at a pressure of 30 mm Hg via a cannula in the right ventricle (76). The lungs were inflated through the trachea with 3% SeaPlaque warm low melting point agarose (BioWhittaker Molecular Applications, Rockland, ME), excised, and cut into 100-µm sections with a Vibratome (The Vibratome Company, St. Louis, MO). Tissue sections were mounted in anti-fading mounting medium (Vectashield, Vector, Burlingame, CA) on glass microscope slides and examined with a Zeiss Axiophot fluorescence microscope or Zeiss LSM-410 confocal microscope. Although effects of liposome uptake on gene expression in endothelial cells cannot be excluded, no consistent differences in expression were found in pilot experiments using whole lung RNA on 8000-oligonucleotide arrays to compare mRNA from mice that received an iv injection of liposomes 20 min earlier with mRNA from mice that had not received liposomes (data not shown).

Endothelial cell isolation. In mice used for gene expression studies, fluorescent cationic liposomes were injected iv and 20 min later the lung vasculature was perfused in situ for 2 min with 1% collagenase D (Boehringer, Mannheim) in Hanks' buffer containing 2 mM Ca^{2+} and 2 mM Mn^{2+}, supplemented with 0.5% bovine serum albumin at 37˚C at a pressure of 30 mm Hg, to begin the enzymatic digestion *in situ*. In each of three identical experiments, the lungs of two mice were excised,

minced, and digested further in collagenase (1%, RNase-free, Boehringer, Mannheim) and DNase (1 U/ml, Boehringer, Mannheim) for 20 min at 37˚C. Cell clumps, debris, and undigested pieces of lung were removed by filtration through a 40-μm mesh. The cells were washed twice with cold Hanks' buffer and then incubated in FITC-conjugated leukocyte specific anti-mouse CD18 (LFA-1/beta2 integrin) antibody (Pharmingen, San Diego, CA) for 15 min at 4˚C, and washed twice. The CD18 antibody was used for negative selection by FACS to remove intravascular leukocytes that took up liposomes.

Cells were sorted on a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA). FITC was detected in the FL1 channel and rhodamine in the FL2 channel. Forward scatter (FSC) and side scatter (SSC) were measured using logarithmic and linear amplifiers, respectively. Initially, 50,000 events were collected and analyzed using CellQuest Software (Becton Dickinson). Two identical regions, R1 and R2, were set to delimit two cell populations (Fig. 1). Gate R1 defined the entire population of rhodamine-liposome labeled cells. Gate R2 defined the subset of population R1 that did not bind the CD18 antibody. Both gates were set to include cells with red fluorescence values > 10 intensity units and green fluorescence < 25 units. Most events with green fluorescence values greater than this threshold, regardless of their red fluorescence, were CD18-immunoreactive cells, autofluorescent cells, or debris.

Rhodamine-positive/FITC negative cells gated in region R2, which represented one population in the FSC/SSC profile, was the putative endothelial cell population. In each experiment, this population consisted of approximately 700,000 cells, which had an estimated diameter of 4 to 14 μm, as measured with calibrated microspheres. The cells were isolated for gene expression profiling by sorting directly into TRIzol (Life Technologies, Rockville, MD) over a 1-hr period. Subsequent samples were obtained for morphological analysis. The average preparation time from perfusion of the lungs until the isolated cells were in Trizol was 3.5 hr.

Viability, number, and morphology of isolated cells. At the end of the sort, a sample of cells in population R2 (~ 10,000 cells) was sorted into PBS and then diluted 1:40 to assess cell viability by

trypan blue exclusion. On average 62 ± 5% of the cells excluded trypan blue. Based on this value, an average of 440,000 ± 30,000 viable cells (700,000 total cells) were obtained from the lungs of each pair of mice (n = 3 groups of two mice each, mean ± S.E.). Another sample was saved for staining with Diff-Quik (Dade Behring Inc., Deerfield, IL) for examination of cell morphology. Sorted cell populations R1 and R2 were deposited onto glass poly-L-lysine-coated glass microscope slides with a Cytospin centrifuge (Shandon Inc., Pittsburgh, PA) and stained with Diff-Quik to show the cytoplasm and nucleus. Neutrophils were identified by their polymorphic nucleus, and monocytes were identified by their large size and kidney-shaped nucleus.

Identification of isolated cells by immunohistochemistry. To assess the FACS parameters, samples of isolated cells, gated for regions R1 or R2, were allowed to adhere to poly-L-lysine-coated glass microscope slides, fixed with 1% paraformaldehyde in PBS, and examined for liposome fluorescence or immunohistochemical markers by fluorescence and confocal microscopy. Uptake of fluorescent cationic liposomes was examined in specimens mounted in aqueous Gel/Mount (Biomeda Corp., Foster City, CA) without further staining. In immunohistochemical studies, endothelial cells were identified using a rat monoclonal anti-mouse CD31 antibody (platelet endothelial adhesion molecule, PECAM, Clone MEC 13.3, Pharmingen, San Diego, CA) (62) and rabbit polyclonal VE-cadherin antibody (Dr. Elisabetta Dejana, Institute Negri, Milan, Italy) (17). Prior to VE-cadherin immunohistochemistry, cells were permeabilized with 0.1% Triton X100 in HEPES-buffered saline to expose the cytoplasmic tail of the molecule (17). Leukocytes were identified using a rat monoclonal anti-mouse CD45.2 antibody (Clone 104, Pharmingen, San Diego, CA). Slides were incubated in primary antibodies (1:200 dilution) at room temperature for 15 min, washed 3 times with HEPES-buffered saline, incubated in Cy3-goat anti-rabbit IgG or Cy2-goat anti-rat IgG secondary antibody (1:800 dilution, Amersham Life Science, Pittsburgh, PA) at room temperature for 60 min, and mounted in Vectashield (Vector Laboratories, Burlingame, CA).

Uptake of acetylated low density lipoprotein (acetyl-LDL). As a functional assay, samples of population R2 were tested for uptake of fluorescent acetyl-LDL. Sorted cells were incubated for 3 hr at

room temperature in the presence of 10 mg/ml BODIPY-labeled acetyl-LDL (Molecular Probes, Eugene, OR) in Hanks' buffer supplemented with 10% fetal bovine serum. After incubation, the cells were washed twice, allowed to adhere to poly-L-lysine coated slides, fixed with 1% paraformaldehyde in PBS, mounted, and examined by fluorescence microscopy.

RNA purification, cDNA synthesis, in vitro *transcription and array hybridization.* TRIzol lysates of cells in FACS population R2 (endothelial cell fraction) and the corresponding dissociated but unpurified lung cell fraction were treated with phenol, and total RNA was precipitated with isopropanol. The integrity of the RNA was confirmed by running on an agarose gel 1 μg of total RNA extracted from the endothelial cell fraction. To construct 3'-directed cDNA libraries, cDNA was synthesized with a SuperScriptII cDNA synthesis kit (Life Technologies, Carlsbad, CA), using a T7:(dT)24 oligonucleotide to prime the first strand, and purified by phenol extraction and ethanol precipitation. The cDNA was used as a template for in vitro transcription using the MEGAscript system (Ambion, Austin, TX) with the inclusion of biotinylated CTP and UTP. The in vitro transcription product was separated from free nucleotides using a RNeasy column (Qiagen, Inc., Valencia, CA) and was fragmented with 150 mM magnesium chloride at 95˚C for 35 min. Fragmented cRNA was hybridized in 0.2 ml of a mixture of 120 mM sodium chloride, 10 mM Tris buffer (pH 7.4), 0.005% Triton X100, 1 mg/ml bovine serum albumin, 0.1 mg/ml herring sperm DNA, and bacterial transcripts spiked at known concentrations. For each of the three experiments, 10-15 μg of biotinylated cRNA was hybridized on an oligonucleotide array (see *Oligonucleotide array*) for 12-16 hr at 40˚C with rotation. The arrays were incubated at 50˚C for 1 h, washed at room temperature in PBS containing 2.5 mM EDTA diluted 1:1 with water, and stained with streptavidin-phycoerythrin, followed by amplification with biotinylated anti-streptavidin antibody, wash, and a second round of streptavidin-phycoerythrin. The arrays were scanned with a Hewlett Packard/Agilent GeneArray Scanner (Agilent Technology, Palo Alto, CA).

Oligonucleotide array. Gene expression was measured using an oligonucleotide microarray manufactured by Affymetrix (Santa Clara, CA) to a custom design specified by Eos Biotechnology.

The array design was based on the five commercial Affymetrix mouse gene arrays Mu11K A and B and Mu19K A, B and C. A subset of perfect-match probes was selected that had a high degree of co-correlation as gene expression levels change over diverse samples based on a large quantity of experimental data. With this approach, 8 oligonucleotides for each of approximately 39,000 potentially expressed sequences were tiled in a single array of more than 300,000 oligonucleotides. After hybridization, gene intensities were represented as the Tukey's trimean of the 8 oligonucleotide intensities. Oligonucleotide intensities were normalized across samples by fitting the intensities onto a distribution derived from a large set of experimental data. The assumption of this normalization scheme developed by Eos is that the distribution of all RNA species changes little across matched samples, and the scheme attempts to ascertain changes in the position within this distribution of individual RNA species from sample to sample. The normalization scheme is more robust to subtle alterations in hybridization conditions, array manufacture, and other variables than normalization schemes that match only a single statistic (for example, the 70th percentile) using a linear scaling factor.

Analysis of gene expression. Gene expression data from the oligonucleotide microarrays were mined and analyzed using bioinformatics approaches and tools developed and implemented at Eos Biotechnology. The fluorescence intensity for each gene was calculated based on the trimean of the hybridization intensity of each probe. Hybridization intensities correspond only approximately to absolute expression levels because the protocol relies on an amplification scheme that may not be strictly quantitative for all transcripts. However, relative expression levels of the same transcript across different samples are preserved. The resulting average differences in intensity were used to represent expression levels and to calculate the ratio of the value for the endothelial cell fraction to the value for unpurified lung cells.

For each gene tiled on the microarray, the statistical significance of observed differences in the expression level between purified endothelial cells and unpurified lung cells was evaluated using a three-factor fully crossed analysis of variance design. The factors specified for each gene were: (i) purification (purified cells in population R2 versus unpurified lung cells); (ii) replication of the experiment (replication 1, 2, or 3), and (iii) interrogating chip oligonucleotide (8 oligonucleotides tiled

per gene). Thus, each data point represented one of two purification conditions, one of three replications of the experiment, and the fluorescence intensity (expression) value for one of eight oligonucleotides representing a specific gene or EST. F statistics and the corresponding *P*-values were calculated for each of the three main factor effects. The three-way interaction term (between purification, replication, and oligonucleotide) was used as the error term, because, for this design, there was a single entry per cell.

Candidates for genes/ESTs enriched in the endothelial cell fraction were selected by three criteria. First, the ratio of expression in the endothelial cell fraction to expression in the unpurified lung cell fraction (*R* = enrichment ratio) was ≥ 1.5 (geometric mean) for the three experiments. Of 39,000 genes/ESTs represented on the microarrays, 1623 met this criterion. Second, the difference in fluorescence intensity between the two fractions (*D* = expression difference) was ≥ 50 units. A total of 3388 genes/ESTs met this criterion, but only 868 had an enrichment ratio ≥ 1.5 and an intensity difference ≥ 50. Third, the *P*-value (*P* = probability of no difference) was < 0.05 for the three-factor analysis of variance (ANOVA) test between the two fractions[1]. Only 555 of the 868 genes/ESTs met the third criterion was well as the other two.

Despite the large number of genes/ESTs examined, most were not relevant to the statistical analysis because the first two selection criteria (ratio ≥ 1.5 and expression difference ≥ 50) excluded all

[1] ANOVA was used as a statistical test based on the experimental design and consideration of several assumptions. Independence among samples is a potential issue for oligonucleotides, but the 8 oligonucleotides tiled per gene were designed to be non-overlapping so they would give independent measures of gene expression. Outliers in the data would decrease the power of the statistical test, giving false negatives rather than over-reporting. This was addressed in the design of the custom arrays by eliminating oligonucleotides that gave data inconsistent with their counterparts on commercial arrays. Outlier oligonucleotides were rare and usually showed promiscuous binding, giving saturated signals no matter what RNA was put on the array. In terms of the distribution of the data, ANOVA is quite robust with non-normality when the design is balanced, as was the case in the present study. Although we did not test for non-normality, the problem is more likely to produce loss of power than false positives. The issue of unequal variances was not readily addressed as there was only one value per cell.

but 868 of the original 39,000 genes/ESTs, thereby controlling multiple comparisons (10). By applying the statistical test to 868 genes/ESTs at a per comparison error rate of 0.05, 43 genes/ESTs would by chance alone be expected to be false positives. Because a total of 555 genes/ESTs met all three criteria, the estimated false discovery rate would be 43/555. Thus, an estimated 8% of the 555 genes/ESTs designated as endothelial cell-associated could be false positives.

The accession number of each of the 555 genes/ESTs was evaluated using standard nucleotide-nucleotide BLAST (Basic Logic Alignment Search Tool, http://www.ncbi.nlm.nih.gov:80/blast), and the bit score *S* and *E*-value of the best match were identified. The 234 transcripts considered genes matched named genes with an *E*-value < e-100; the remaining 321 were designated ESTs.

Validation of gene expression by immunohistochemistry. Endothelial cell expression of representative genes identified on microarrays was validated in sections of mouse lungs stained by immunohistochemistry. Genes were selected by their novelty and availability of antibodies for immunohistochemical staining[2]. The antibodies tested included as positive controls two well established endothelial cell markers (CD31, VE-cadherin). Other controls included E-cadherin, which is expressed by lung epithelial cells but not endothelial cells, and omission of the primary antibody.

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[2] Primary antibodies used for validation of gene expression in lung endothelial cells in situ included: (1) Alk-1 (goat polyclonal, 1:1000, R&D systems, Minneapolis, MN); (2) bone morphogenic protein receptor type II (goat polyclonal, undiluted, R&D systems, Minneapolis, MN); (3) CD31 (PECAM-1, rat monoclonal, clone MEC 13.3, 1:500, BD Pharmingen, San Diego, CA); (4) dipeptidylpeptidase IV (CD26, rat monoclonal, clone H194-112, 1:1000, BD BioSciences, San Diego, CA); (5) integrin alpha6 (CD49f, rat monoclonal, clone GoH3, 1:1000, BD Pharmingen, San Diego, CA); (6) LYVE-1 (rabbit polyclonal, 1:1000, from David Jackson, Oxford University, UK); (7) neuropilin-1 (rabbit polyclonal, 1:1000, Oncogene Research, Cambridge, MA); (8) phospholipid scramblase 2 (rabbit polyclonal, 1:1000, Oncogene Research, Cambridge, MA); (9) VE-cadherin (rabbit polyclonal, 1:1000, from Elisabetta Dejana, FIRC Institute of Molecular Oncology, Milan, Italy); (10) VEGFR-2 (rabbit polyclonal, 1:2000, from Rolf Brekken and Philip Thorpe, University of Texas Southwestern Medical Center, Dallas, TX); and (11) E-cadherin (goat polyclonal, 1:500, R&D systems, Minneapolis, MN). Three other antibodies that were tested gave faint staining of alveolar endothelial cells and epithelial cells (Notch4) or no staining of lung (ADAMTS-1, Notch1).

Mice anesthetized with ketamine (100 mg/kg ip) and xylazine (5 mg/kg ip) were perfused with PBS through the aorta for 1 min at 120 mmHg, perfused with PBS through the pulmonary artery for 20 sec at 20 mmHg, and then fixed by perfusion with 1% paraformaldehyde in PBS through the aorta for 2 min at 120 mmHg. Alveoli were expanded by infusion of warm 2% SeaPlaque agarose in PBS into the lungs via the trachea with a 20-gauge needle and allowed to solidify. Inflated lungs were removed, fixed in 1% paraformaldehyde for 1 hr at 4°C, rinsed with PBS, infiltrated with 30% sucrose for 12-15 hr, and frozen. Cryostat sections 80 μm in thickness were dried on slides for 12-15 hr, permeabilized with 0.3% Triton X100 in PBS, and incubated in 5% normal goat, hamster, or mouse serum (Jackson ImmunoResearch, Inc., West Grove, PA) in PBS+ (PBS containing 0.3% Triton X100, 0.2% bovine serum albumin, Sigma, and 0.01% thimerosal, Sigma) for 1 hr to block nonspecific antibody binding. Sections were double-labeled by incubation for 12-15 hr in humidified chambers with CD31 antibody and one of nine other primary antibodies[2] diluted in 5% normal goat, hamster, or mouse serum in PBS+. After rinsing with PBS containing 0.3% Triton X100, slides were incubated for 5 hr with fluorophore (FITC or Cy3)-conjugated secondary antibodies (goat anti-rat, goat anti-rabbit, donkey anti-goat, or mouse anti-goat, 1:400, Jackson ImmunoResearch, Inc., West Grove, PA) diluted in 5% normal goat, hamster, or mouse serum in PBS+. All incubations were at room temperature. Slides were rinsed with PBS containing 0.3% Triton X100, fixed briefly in 4% paraformaldehyde, rinsed with PBS, mounted in Vectashield (Vector Laboratories, Burlingame, CA) and imaged with a Zeiss LSM 510 confocal microscope (40x oil Plan Apochromat objective, 2x zoom). Two-dimensional projections were made from stacks of 0.7-μm confocal optical slices of 80-μm physical sections of lung alveoli.

RESULTS

Endothelial cells of pulmonary capillaries were doubly labeled after iv injection of rhodamine-labeled cationic liposomes and FITC-labeled lectin. The luminal surface of the vessels had a uniform coating of green fluorescent lectin, and the endothelial cells had punctate red fluorescence from liposomes internalized into endosomes (Fig. 1A, B). Occasional leukocytes within pulmonary

capillaries were the only other cells found to contain liposomes. These features matched the pattern reported previously for the distribution of cationic liposomes in the lung after intravenous injection (76).

Isolation of Endothelial Cell Fraction by FACS

For the gene expression profiling experiments, where fluorescent liposomes were injected but lectin was not, lung cells were dissociated and then divided into two fractions: one was used to isolate cells by FACS (Fig. 1C-F); the other served as the unpurified cell reference for gene profiling.

Cell population R1 isolated by FACS had red fluorescence from internalized cationic liposomes (Fig. 1C). Cell population R2, a subset of population R1, lacked green fluorescence from FITC-conjugated CD18 antibody (Fig. 1D). The remaining cells either lacked rhodamine fluorescence or had FITC fluorescence from CD18 immunoreactivity or autofluorescence. Forward and side scatter plots showed the subpopulations of R1 (Fig. 1E) and the endothelial cell population, R2 (Fig. 1F).

Characterization of Endothelial Cell Fraction

Several steps were taken to confirm the identity and assess the homogeneity of cell populations isolated by FACS (Table 1). After Diff-Quik staining, population R1 was found to consist of abundant small, round cells with little cytoplasm (Fig. 2A), interspersed by scattered larger cells, some of which had abundant cytoplasm and a large round nucleus typical of monocyte/macrophages (Fig. 2B). When viewed by confocal microscopy, population R2 cells had punctate rhodamine fluorescence, were uniform in size, and had a diameter averaging ~ 7 μm (Fig. 2C).

Immunohistochemical staining for endothelial cell and leukocyte markers revealed that 96% of population R2 cells had VE-cadherin immunoreactivity (Fig. 3A, B), but only 74% were CD31-positive (Table 2). Fewer than 1% of population R2 cells had CD45.2 immunoreactivity (Fig. 3C, D and Table 2). Approximately 80% of population R2 cells took up BODIPY-acetyl-LDL (Table 2), which appeared as a punctate cytoplasmic staining (Fig. 3E, F). By comparison, 49% of population R1 cells had VE-cadherin immunoreactivity, 82% had CD31 immunoreactivity, and 47% were CD45.2-positive (Table 2).

Evaluation of Selection Criteria for Genes Enriched in the Cell Fractions

Gene expression profiles for the three replicates of the endothelial cell fraction were compared to their counterpart for the unpurified fraction of lung cells. The approach of comparing the two fractions was used to minimize the effect of the isolation process on the selection of candidate endothelial cell genes, because the fractions were exposed to similar conditions (liposomes, collagenase, and mechanical separation). A total of 555 genes/ESTs met the three criteria for endothelial cell enrichment. These genes/ESTs, which are described in the next section, represented 1.4% of the 39,000 transcripts on the microarrays.

As one test of the selection criteria, we examined 270 genes/ESTs (0.7% of the transcripts on the array) that were at the opposite end of the spectrum from the endothelial cell candidates. This group had more than twofold greater expression in the unpurified lung cells fraction ($R < 0.5$, $D < -250$, $P < 0.05$). Consistent with expression in non-endothelial cells, the group included many epithelial cell genes, including cytokeratin 19, E-cadherin, and surfactant proteins A, B, C, and D, or leukocyte/macrophage genes, including C-fms proto-oncogene (colony stimulating factor receptor), f-MLP receptor, interleukin-1β, lymph node homing receptor MEL-14, macrophage inflammatory proteins (MIP-1, MIP-2), mast cell high affinity IgE receptor, P-selectin ligand, and tumor necrosis factor (Table 3).

As another test of the isolation process and selection criteria, we assessed the expression of genes that have little or no known association with endothelial cells or other lung cells. No significant expression (intensity value < 50) was detected in either fraction for the mammary gland-specific genes alpha-lactalbumin or gamma-casein, gamma-D-crystallin gene of the lens, green cone pigment gene of the retina, neuronal Clock gene involved in circadian rhythms, odorant-binding protein gene OBP-I of olfactory cells, preproinsulin I and II genes of pancreatic β-cells, or the gene for tyrosinase, an enzyme involved in melanin synthesis in melanocytes of skin, retina, and tumors.

A further test of the effect of the isolation process and efficacy of the selection criteria involved a comparison of the expression profile of a group of housekeeping genes[3] that would be expected to be expressed in both cell fractions. This comparison showed expression of these genes in both cell fractions and no significant difference in amount of expression between the fractions.

The question of whether the isolation process itself perturbed gene expression in the endothelial cell fraction was also addressed by examining expression of immediate early genes that respond rapidly after activation. None of the genes for heat shock proteins or fos-related antigens (Fra-1, Fra-2) examined showed a significant difference in expression in the two fractions. Expression of c-Jun was higher in the endothelial cell fraction (Table 4.3), but c-Fos, FosB, Krox-20, and mitogen-activated protein (MAP, p38) kinase were significantly enriched in the unpurified cell fraction.

Overall, by the selection criteria used, only 2.1% of genes/ESTs were found to be enriched in endothelial cells or non-endothelial cells. Most (97.9%) of the transcripts tiled on the microarrays did not meet the selection criteria for enrichment in either population because they were expressed relatively equally in both fractions or did not have significant expression in either fraction.

Genes Expressed in Endothelial Cell Fraction

Many of the 234 genes that met the three selection criteria for endothelial cell enrichment have a well documented association with endothelial cells (Tables 4.1 – 4.4). Among the familiar genes enriched in this fraction were key growth factors and their receptors: angiopoietin-2 and Tie1 and Tie2 receptors, vascular endothelial growth factor (VEGF) and two of its receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk1, KDR) (Table 4.1). Well documented endothelial cell junctional molecules included VE-cadherin, CD31, claudin 5, ZO-1, and gap junction protein connexin 37 (Table 4.2). Endothelin-1,

[3] Housekeeping genes included alcohol dehydrogenase, asparagine synthetase, ATP synthase beta-subunit, beta 2-microglobulin, beta-glucoronidase, cytoplasmic beta actin, DNA polymerase gamma, elongation factor 1-alpha, glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, isocitrate dehydrogenase, lactate dehydrogenase, mitochondrial ribosomal protein S12, ornithine decarboxylase, RNA polymerase I subunit, RNA polymerase II subunit, and ubiquitin.

endothelin-B receptor, endothelin-converting enzyme, leptin receptor, endomucin, and L-selectin-binding sialomucin CD34 were among the other familiar enriched genes (Table 4.1, 4.2).

Two integrin subunits, alpha6 and beta1, which are known to be expressed in endothelial cells (59), were enriched in the endothelial cell fraction (Table 4.2). Alpha6beta1 integrin, which is a laminin receptor, is associated with the attachment of endothelial cells and other cells to their basement membrane (45). Expression of alpha4 and beta7 integrin subunits, which are associated with leukocytes (25, 90), were enriched in the unpurified cell fraction. Other integrin subunits were expressed about equally in both fractions.

Also among the genes enriched in the endothelial cell fraction were the lymphangiogenic growth factor VEGF-C (98), LYVE-1, a hyaluronan receptor associated with lymphatic endothelial cells but also expressed in some types of vascular endothelial cells (7, 85), and stabilin-1, a fasciclin-like hyaluronan receptor present on sinusoidal endothelial cells (94) (Table 4.1). VEGFR-3 (Flt-4), the receptor for VEGF-C on lymphatic and vascular endothelial cells, had roughly equal expression in both fractions.

Genes involved in development and angiogenesis but less commonly associated with quiescent endothelial cells in the adult included members of the semaphorin/neuropilin (semaphorin 3C, Nrp1), Delta/Notch (Hey1, Jagged 2, Notch1, Notch4, Numb), and wingless/Wnt (Frizzled 4, Tle1) signaling pathways (Table 4.1). Also enriched were Edg1 (endothelial differentiation gene 1), a sphingosine 1-phosphate receptor involved in platelet-derived growth factor signaling and essential for vascular maturation and smooth muscle cell/pericyte envelopment during development (66), four members of the ephrin/Eph family (ephrin A1, B1, B2, EphB4), Sdf1-α (stromal cell derived factor 1-α), a chemotactic and antiviral CXC chemokine and ligand for CXCR-4 receptors that is expressed by endothelial cells and is required for cardiovascular development (100), Alk-1 (activin receptor-like kinase-1), a member of the TGF-β receptor family and the site of missense mutations that cause hereditary hemorrhagic telangiectasia type 2 (1), and bone morphogenic protein receptor type II, another TGF-β receptor family member and the site of mutations that cause primary pulmonary hypertension (4) (Table 4.1).

Proteases enriched in the endothelial cell fraction included ADAMTS-1, a disintegrin and metalloproteinase with thrombospondin motifs (117), carboxypeptidase D, a duck hepatitis B virus receptor homologue previously identified on liver sinusoidal endothelial cells (13), dipeptidylpeptidase IV (CD26) (36), and neurotrypsin (Table 4.4). Two protease inhibitors, Timp3 and serpinh1, were also enriched in the endothelial cell fraction (Table 4.4).

Genes that have limited or no previous association with endothelial cells included five members of the tetraspanin family (Table 4.2) and many of the 15 transcription factors that were found to be enriched (Table 4.3). Other genes not usually linked to endothelial cells included Ryk, a receptor protein tyrosine kinase essential for normal craniofacial development (Table 4.1), three G-protein coupled receptors (CD97, Etl1, Rdc1; Table 4.1), three ion channels (Kcnb1, Kcnn4, Trpm7; Table 4.3), four cell junction-related proteins (afadin, nectin-3, protocadherin 1, sorbs1; Table 4.2), multiple cytoskeletal proteins (dynein Dnahc8, kinesin Kif1b, midline 2, palmdelphin, pleckstrin 2, septin 2; Table 4.2), as well as exostosin 2, lipocalin 7, phospholipid scramblase 2, scube1 growth factor, and SNF related kinase (Tables 4.1, 4.3, 4.4).

Because of the conservative design of the three selection criteria, not all potentially important genes expressed by endothelial cells were detected. For example, von Willebrand factor (factor VIII-related antigen), a glycoprotein in endothelial cell Weibel-Palade bodies involved in hemostasis, slightly missed meeting all three criteria ($R = 1.61$, $D = 501$, $P = 0.055$). Other genes that are known to be expressed in endothelial cells but did not meet the three criteria included angiotensin-converting enzyme (ACE, $R = 1.6$, $D = 413$, $P = 0.08$), endothelial constitutive nitric oxide synthase (ecNOS, $R = 1.3$, $D = 58$, $P = 0.3$), endoglin (CD105, $R = 1.2$, $D = 333$, $P = 0.1$), and thrombomodulin ($R = 1.3$, $D = 204$, $P = 0.065$).

Few of the 321 ESTs that met the criteria for enrichment in the endothelial cell fraction had previously been studied in relation to their cell associations. Table 5 shows 20 ESTs that had the greatest enrichment in this fraction ($R \geq 2$, $D \geq 50$, $P < 0.01$).

Immunohistochemical validation of endothelial cell gene expression

Immunohistochemical staining verified the expression in lung capillaries of 10 representative genes enriched in the endothelial cell fraction. Confocal microscopic imaging of CD31 immunoreactivity, a standard marker of endothelial cells, showed the distinctive pattern of alveolar capillaries in 80-μm tissue sections (Fig. 4A). Consistent with an endothelial cell association, LYVE-1 immunoreactivity had a pattern matching that of CD31 (Fig. 4A, B). Similarly, as expected, VE-cadherin colocalized with CD31 on lung capillaries (Fig. 4C, D). No staining was present when the primary antibody was omitted (data not shown). E-cadherin, an epithelial cell marker, had a conspicuously different distribution from that of CD31 (Fig. 4E, F). These contrasting results made it possible to distinguish pulmonary endothelial cells from alveolar epithelial cells and confirmed that visualization of the capillary pattern required staining of a protein expressed by endothelial cells. Using these staining patterns as a reference, we compared the distributions of Alk-1, bone morphogenic protein receptor type II, CD31, dipeptidylpeptidase IV, integrin alpha6, neuropilin-1, phospholipid scramblase 2, and VEGFR-2 (Fig. 5). All had generally similar patterns that fit with endothelial cells of pulmonary capillaries. The staining of LYVE-1 and Alk-1 was homogeneous, consistent with a uniform plasma membrane distribution (Figs. 4B, 5A), but dipeptidylpeptidase IV (Fig. 5D) and VEGFR-2 (Fig. 5H) immunoreactivities were granular. Antibodies to bone morphogenic protein receptor type II (Fig. 5B) and dipeptidylpeptidase (Fig. 5D) stained endothelial cells and a second cell type, possible type II alveolar epithelial cells. None of the antibodies tested other than E-cadherin[2] stained predominately non-endothelial cells in lung.

DISCUSSION

The goal of this study was to profile gene expression in endothelial cells freshly isolated from normal lung capillaries. Toward this end we developed a new method for isolating endothelial cells from mouse lungs based on their propensity to bind and internalize fluorescent cationic liposomes (76, 114). After in vivo labeling with liposomes, endothelial cells were dissociated and then purified by FACS. Leukocytes were eliminated by negative sorting for the surface marker CD18. Purity of the

endothelial cell fraction was assessed by using VE-cadherin and other immunohistochemical markers. From the RNA isolated from these cells, and cRNA prepared therefrom, genes were profiled on oligonucleotide microarrays configured to measure the expression of 39,000 genes and ESTs. Stringent analysis of the expression values revealed that 234 genes and 321 ESTs in the endothelial cell fraction were enriched in comparison to unpurified lung cells. Published evidence of an endothelial cell association was found for only half of the 234 enriched genes.

Isolation of Endothelial Cells

It has been challenging to isolate a sufficient number of endothelial cells from whole organs to permit studies of gene expression. In some previous studies, endothelial cells have been isolated from the lung or other organs and then grown in vitro to increase the cell number (22, 31, 57, 68, 70, 96). Comparisons of gene expression profiles in endothelial cells grown as capillary-like tubes, as a model for angiogenesis, to cells grown in monolayer culture have shown differences in expression of matrix metalloproteinases, integrins, and extracellular matrix proteins (33, 52). There is a question, however, of how closely such in vitro models reflect the normal spectrum of endothelial cell functions that depend on interactions of endothelial cells, pericytes, extracellular matrix, and surrounding cells as well as blood pressure and flow.

To avoid changes that occur when cells are grown in vitro, endothelial cells have been isolated freshly from organs (51, 106). Profiling of gene expression in endothelial cells isolated from high endothelial venules in mice led to the identification of genes involved in the distinctive role of these venules in lymphocyte homing to lymph nodes (51). Gene expression patterns have also been compared in endothelial cells isolated from normal colon and colon cancer using Serial Analysis of Gene Expression (106). This approach led to the identification of multiple genes that have higher expression in endothelial cells of tumor vessels than in normal vessels (16, 106).

The purity of the endothelial cell fraction is obviously a key factor in gene profiling studies. Based on current methods, this purity depends on the selective labeling of the cells during the purification process. Usually endothelial cells are labeled with a specific lectin or antibody after enzymatic digestion of the tissue. The selectivity of the labeling is determined by the specificity of the

label, presence of other cell types, and loss of cell-specific antigens during digestion. Another factor is the time required for cell isolation. The more prolonged the isolation procedure, the greater the likelihood of changes in gene expression occurring after the cells are removed from their normal environment. Cessation of blood pressure and flow, separation of intercellular junctions, detachment of pericytes, removal from extracellular matrix attachment, exposure to hypoxia, and changes in chemical environment are among the conditions that could trigger changes in gene expression.

The relatively simple isolation procedure used in the present studies made it possible to obtain within three and a half hours more than 100,000 viable endothelial cells from each mouse lung. By labeling endothelial cells with fluorescent cationic liposomes (76, 114), the cells could be isolated by FACS. Because the liposomes do not extravasate, cells outside the vasculature were not labeled. Pericytes or smooth muscle cells do not come into contact with the intravascular liposomes even though the cells are intimately associated with endothelial cells. Although intravascular macrophages and neutrophils do have access to and may take up cationic liposomes (76), these cells were removed by negative selection by using an anti-CD18 antibody (39). After purification, fewer than 1% of the cells in the endothelial cell fraction expressed the leukocyte marker CD45.2.

Several endothelial cell surface markers, CD31 being the most common (44) and VE-cadherin being the most specific (17), are routinely used to confirm the identity of established endothelial cell lines. The CD31 antibody we used uniformly labels intact endothelial cells of the pulmonary vasculature but labeled only 74% of the cells in the endothelial cell fraction. One explanation for the incomplete labeling is that the collagenase digestion reduced the amount of CD31 on the cell surface. Because of possible loss of such surface epitopes, we used for the main test of cell purity a VE-cadherin antibody that targets an intracellular epitope on the molecule (17). Based on VE-cadherin immunoreactivity, the population of isolated cells was at least 96% pure. The 4% that were VE-cadherin-negative may have been endothelial cells with subthreshold amounts of the marker. However, we cannot exclude that the fraction contained some liposome-containing leukocytes that did not express sufficient CD18 to be removed by negative selection or lacked CD45.2 immunoreactivity and were thus not detected in the validation experiments.

As part of the cell isolation procedure, vascular perfusion of collagenase expedited the enzymatic digestion and made it possible to limit exposure to the enzyme at 37ºC to only 20 min. The cells were kept on ice thereafter. This approach coupled with the use of a high-speed cell sorter shortened the isolation time. The endothelial cell fraction from the lungs of two mice provided sufficient RNA to prepare cRNA (10-15 μg) for the oligonucleotide arrays without the need to expand the cell population in vitro.

Evaluation of Gene Expression

Multiple levels of comparison were included in the experimental design to identify candidate genes/ESTs: (i) expression in the endothelial cell fraction was compared to that in unpurified lung cells; (ii) endothelial cell isolation experiments, each involving both lungs of two mice, were replicated on three separate days; and (iii) gene expression measurements using RNA from the isolated cells were made on microarrays that contained 8 oligonucleotides for each of approximately 39,000 genes/ESTs. The evaluation of differences in gene expression took all three of these factors into consideration.

The 555 genes/ESTs that were considered significantly enriched in the endothelial cell fraction met three criteria. First, the geometric mean of the amount of enrichment, calculated from the ratio of expression in the endothelial cell fraction to that in the unpurified fraction, was at least 1.5 for the three replications. Second, the difference in the absolute expression of a given gene/EST in the two fractions was at least 50. Third, the analysis of variance test comparing expression values for each gene/EST in the two fractions had a P-value < 0.05. These criteria were determined by several factors. Because endothelial cells constitute about 40% of lung cells (37), the maximal enrichment would be expected to be 2.5, assuming that the isolated cells had the same proportions as the intact lung, and the purification procedure was completely efficient and preserved numerical relationships. An enrichment ratio of ≥ 1.5 took into account the presumption that endothelial cells would not be as efficiently isolated as some other lung cells. Genes/ESTs meeting the selection criteria had a mean enrichment ratio of 1.92. The difference of ≥ 50, which is equivalent to an estimated five copies per cell, is just above the threshold for detecting expression (fluorescence) on the microarray.

About half of the genes that met all three selection criteria are known to be expressed in endothelial cells. VE-cadherin, CD31, VEGFR-1, VEGFR-2, Tie1, Tie2, and angiopoietin-2 were among the familiar ones that had significantly greater expression in the endothelial cell fraction than in unpurified lung cells. Others included ZO-1, claudin 5, connexin 37, neuropilin-1, Edg-1, endothelin-1, endothelin converting enzyme, endothelin-B receptor, Notch4, and focal adhesion kinase. Expression of Alk-1 and bone morphogenic protein receptor type II (Bmpr-2) in lung endothelial cells is significant because of their roles in hereditary hemorrhagic telangiectasia and primary pulmonary hypertension respectively (1, 4). The consistency of finding such familiar examples in the endothelial cell fraction favors the likelihood that other genes meeting the same criteria – but not previously linked to endothelial cells – do indeed have greater expression in endothelial cells than in other lung cells.

The validity of the microarray findings was tested by immunohistochemical staining with antibodies against 13 proteins encoded by genes enriched in the endothelial cell fraction. Ten of the antibodies clearly labeled alveolar capillaries, and the other three gave little or no staining in lung. The complex geometry of alveolar capillaries and the proximity of endothelial cells to alveolar epithelial cells make it difficult to distinguish endothelial cells from other lung cells by conventional light microscopic immunohistochemistry. This problem was solved by using a confocal microscopic approach, whereby CD31 and VE-cadherin were used as standards and immunoreactivity was examined in three-dimensional 80-μm thick sections of mouse lung. These standards illustrated the pattern of endothelial cell staining. Staining for E-cadherin documented the contrasting appearance of alveolar epithelial cells. With this perspective, we confirmed the expression in endothelial cells of the remaining genes, including two (dipeptidylpeptidase IV, phospholipid scramblase 2) that have not to our knowledge been described as expressed in endothelial cells, as well as others (integrin alpha6, LYVE1, neuropilin-1) that have received little or no attention in adult lung capillaries.

Further validation of the effectiveness of the isolation procedure was found in the significant enrichment of markers of non-endothelial cells in the unpurified cell fraction. Genes of this type included markers of lung epithelial cells such as E-cadherin and cytokeratin 19, markers of Type II alveolar epithelial cells such as surfactant-associated proteins A, B, C and D, as well as multiple markers

of macrophages and leukocytes. These results indicate that the method used to isolate endothelial cells from lungs provided a relatively pure population that expressed many known genes in a predictable fashion.

Although the immunohistochemically validated genes enriched in the endothelial cell fraction were all expressed in normal lungs under baseline conditions, we cannot exclude that the expression of some genes in endothelial cells was turned on by binding or uptake of cationic liposomes, exposure to collagenase, detachment from the basement membrane, or other steps of the isolation procedure. Indeed, the transcription factor Jun was enriched in the endothelial cell fraction, and several immediate early genes were enriched in the other fraction. However, the two fractions had the same treatment except for the FACS isolation step, and the test of enrichment involved the comparison of one fraction with the other. Any effects on gene expression should be reflected in both fractions, unless genes in certain cells are particularly sensitive to one of the steps. Changes that occur early might indicate a potential for rapid involvement in the injury/repair process in the lung.

Some genes (ACE, ecNOS, endoglin, and thrombomodulin, for example) that are known to be present in endothelial cells did not meet the selection criteria for the endothelial cell fraction. There are at least four reasons for not detecting these or other genes by the approach we used. First, of particular relevance to the present study where unpurified lung cells were used as a reference for gene enrichment in the endothelial cell fraction, genes that were expressed roughly equally in both fractions did not meet the selection criteria. This applied to 97.9% of the transcripts on the oligonucleotide arrays. In the case of ACE, ecNOS, endoglin, and thrombomodulin, expression tended to be higher in the endothelial cell fraction, but one or more of the other criteria for inclusion were not met. Endoglin and thrombomodulin are expressed by monocyte-macrophages as well as by endothelial cells (75, 88). Second, the appropriate oligonucleotide for detecting a gene might not be present on the oligonucleotide arrays. For example, oligonucleotides for genes of endothelial cell junction adhesion molecules (JAM family (19)) and tumor endothelial markers (TEMs (16)) were not in the microarrays. Third, gene expression cannot be measured when the amount of mRNA is below the limit of detection, is unstable, or is degraded by exonuclease during the isolation procedure. And fourth, detection would be impaired

by low specificity of the oligonucleotides for a particular gene because the sensitivity of the detection depends on the specificity of the oligonucleotides on the microarray. Indeed, absolute expression values for different genes/ESTs were not used as selection criteria because they are influenced by the affinity of the oligonucleotides for the corresponding transcripts as well as by the amount of RNA. For these reasons, lack of detection does not mean lack of expression, and the endothelial cell genes identified here are likely to represent only a small proportion of all genes expressed in normal endothelial cells.

Diverse Genes Expressed by Pulmonary Endothelial Cells

Several genes found in the endothelial cell fraction are expressed in the vascular and nervous systems during embryogenesis, consistent with parallels that have been identified between these systems in development (104). Lung endothelial cells expressed semaphorin 3C and its receptor neuropilin-1 as well as Notch4, Jagged2, ephrin B2, and EphB4. Neuropilin-1 is not only involved in axonal guidance but also potentiates signaling of vascular endothelial growth factor-165 (VEGF165) in endothelial cells by forming a complex with VEGF165 and VEGFR-2 (27, 34). As possible evidence of its role in angiogenesis, neuropilin-1 has been implicated in rheumatoid arthritis (50) and tumorigenesis (78). Notch4 receptors are involved in branching morphogenesis of the vasculature and other systems (116). Jagged is upregulated by factors that stimulate endothelial cell migration in vitro (125). Ephrin B2, the transmembrane ligand for EphB4, is expressed by arterial endothelial cells in the embryo and is thought to participate in the definition of boundaries between arteries and veins in the formation of the vasculature (2, 119). In the adult, ephrin B2 continues to be expressed mainly on the arterial side of the microcirculation, including the arterial end of some capillaries (29). Deletions or mutations of about a quarter of the genes lead to recognized embryonic defects or pathological conditions in the adult.[4]

[4] Enriched genes associated with developmental defects or diseases included activin receptor IIB (left-right axis malformations); Alk-1 (hereditary hemorrhagic telangiectasia type 2); ATPase, Ca^{++} transporting 2 (Darier-White disease); bone morphogenic protein receptor type II (familial primary pulmonary hypertension); claudin 5 (DiGeorge syndrome); endothelin-converting enzyme 1

Five members of the tetraspanin superfamily (TM4SF) of proteins, which have four transmembrane domains and form macromolecular complexes with other transmembrane proteins, were expressed in endothelial cells. Tetraspanins may act as linkers between extracellular domains of integrins and intracellular signaling molecules, and some are implicated in integrin-mediated endothelial cell migration in wound healing and angiogenesis (11, 21).

The expression of genes involved in vascular development, angiogenesis, axonal guidance, cell boundaries, and lateral inhibition suggests that endothelial cells in the adult lung undergo continuous remodeling or are predisposed to do so. However, the doubling time for endothelial cells in the normal lung appears to be long, estimated at 327 days in mice (47). Therefore, these genes may have different functions in quiescent cells. Also, gene expression gauged by amount of mRNA is not necessarily indicative of amount of protein synthesis.

VEGF and its receptors VEGFR-1 and VEGFR-2 were among the genes expressed in the endothelial cell fraction. The adult lung is a site of high VEGF expression, which may function as a survival factor for alveolar endothelial cells (56, 118). Clues that VEGF expression changes in lung injury, and may decrease under conditions leading to endothelial cell apoptosis, are beginning to emerge (56, 72, 73, 122).

(Hirschsprung disease); frizzled-4 (exudative vitreoretinopathy); galactosamine (N-acetyl)-6-sulfate sulfatase (Morquio syndrome); Gata2 transcription factor (Mobius syndrome); growth hormone receptor (Laron's syndrome); hyaluronidase 1 (mucopolysaccharidosis IX); integrin alpha 6 subunit (epidermolysis bullosa); kinesin heavy chain member 1B (Charcot-Marie-Tooth disease type 2A); Kit ligand (Steel-Dickie mutation); laminin beta 2 (Walker-Warburg syndrome); lectin, mannose-binding 1 (combined factor V-factor VIII deficiency); leptin receptor (obesity); multiple exostosis protein 2 (hereditary multiple exostoses type 2); neurofibromatosis 1 (neurofibromatosis vasculopathy); oligophrenin 1 (X-linked mental retardation); peripheral myelin protein 22 (Dejerine-Sottas syndrome); potassium intermediate/small conductance calcium-activated channel Kcnn4 (Diamond-Blackfan anemia); Ryk receptor-like tyrosine kinase (cleft palate); sarcoglycan epsilon (myoclonus-dystonia syndrome); semaphorin 3C (congenital heart defects); serpinh1 (ruptured blood vessels); sorbs1 (insulin resistance); Tie2 (venous malformations); Timp3 (Sorsby fundus dystrophy); Tm4sf2 (X-linked mental retardation).

In conclusion, by using the capacity of pulmonary capillary endothelial cells to take up fluorescent cationic liposomes *in vivo*, we isolated the cells with FACS and measured gene expression on microarrays, revealing many familiar genes as well as others that contribute to the distinctive functional and morphological properties of these cells. Some genes expressed in normal lung endothelial cells are linked to angiogenesis, neuronal guidance, boundary formation, or branching morphogenesis during development. Still unresolved is how gene expression suggestive of proliferation and remodeling can be reconciled with the limited capacity of the adult lung to regenerate in response to injury. The present study provides an approach and baseline data to address this issue by examining functionally altered endothelial cells at sites of angiogenesis in tumors, inflammation, or other pathological conditions.

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Table 1. Criteria used to identify lung cells isolated by FACS

Criteria	Methods	Endothelial cells	Monocyte-macrophages	Other leukocytes
Cationic liposome uptake	FACS	+	+	+/-
CD18 immunoreactivity	FACS	-	+	+
Cellular structure	Diff-Quik staining	little cytoplasm, small cell size	distinct nucleus, large cell size	distinct nucleus, medium cell size
VE-cadherin immunoreactivity	IHC	+	-	-
CD31 immunoreactivity	IHC	+	+/-	+/-
CD45.2 immunoreactivity	IHC	-	+	+
Acetyl-LDL uptake	Functional assay	+	+	+/-

Summary of markers used to separate lung cells by FACS, confirm cell identity, and assess homogeneity of cell populations R1 and R2. + indicates presence of marker, - indicates absence of marker, and +/- indicates hint of marker. FACS, fluorescence activated cell sorting; IHC, immunohistochemistry

Table 2. Proportions of FACS-isolated lung cells labeled by cellular markers

Cellular markers	Population R1		Population R2	
	% labeled	Total cells counted	% labeled	Total cells counted
VE-cadherin immunoreactivity	49%	250	96%	221
CD31 immunoreactivity	82%	242	74%	130
CD45.2 immunoreactivity	47%	299	< 1%	162
Acetyl-LDL uptake	ND	ND	80%	195

Percent of cells in populations R1 and R2 that were immunoreactive for markers of endothelial cells (VE-cadherin, CD31) or leukocytes (CD45.2, CD31) or took up Acetyl-LDL in vitro. Experiments performed in triplicate for each assay. Total cells counted indicates number for three experiments. ND, not done.

Table 3. Enrichment of epithelial, macrophage, and leukocyte genes in mouse lung cells

Gene	Cell specificity	Ratio	Difference	*P*-value	Accession number
C-fms proto-oncogene (CSF-1 receptor)	Macrophages	0.29	-278	0.010	X06368
Cytokeratin 19	Epithelial cells	0.37	-472	0.002	M28698
E-cadherin	Epithelial cells	0.32	-250	0.001	X06115
F4/80	Macrophages	0.34	-290	< 0.001	X93328
Interleukin 1-beta (IL-1β)	Macrophages/Leukocytes	0.07	-655	0.006	M15131
Leukocyte adhesion protein (LFA-1, CD18)	Leukocytes	0.23	-1006	0.001	X14951
Lymph node-specific homing receptor (MEL-14)	Leukocytes	0.21	-277	0.011	M25324
Lysozyme M	Macrophages/Epithelial cells	0.39	-1408	0.006	M21050
Macrophage inflammatory protein (MIP, MIP-1α)	Macrophages/Epithelial cells	0.21	-380	0.008	X12531 {M73061}
Macrophage inflammatory protein-2 (MIP-2)	Macrophages/Epithelial cells	0.02	-600	0.002	X53798
Macrophage mannose receptor	Macrophages	0.09	-532	0.001	Z11974
Mast cell high affinity IgE receptor	Mast cells	0.35	-716	0.011	W41745
Mouse eosinophil-associated ribonuclease-2 (mEAR-2)	Leukocytes (Eosinophils)	0.26	-990	0.005	AA124831 {AF306665}
N-formyl peptide chemotactic receptor	Macrophages/Leukocytes	0.29	-461	0.002	L22181
P-selectin glycoprotein ligand 1 (PSGL-1)	Leukocytes	0.43	-454	0.001	X91144
Pulmonary surfactant protein A (SP-A)	Epithelial cells	0.33	-362	0.006	AA097686 {AK004788}
Pulmonary surfactant protein B (SP-B)	Epithelial cells	0.12	-362	0.014	AA061213 {S78114}
Pulmonary surfactant protein C (SP-C)	Epithelial cells	0.38	-1504	0.006	M38314
Pulmonary surfactant protein D (SP-D)	Epithelial cells	0.42	-494	0.005	L40156 {NM_009160}
T-cell receptor	Leukocytes	0.38	-571	0.013	X00619
Tumor necrosis factor	Macrophages	0.46	-272	0.003	X02611
Thy-1 antigen	Leukocytes/Other cells	0.31	-295	0.012	X03151

Genes found to be enriched in non-endothelial cells based on three criteria: (1) ratio < 0.5 for expression in endothelial cell fraction to expression in unpurified lung cell fraction; (2) expression difference < -250 between the two fractions; and (3) $P < 0.05$ for the probability of no difference in expression between the two fractions. Lower ratios mean higher expression in unpurified lung cells relative to endothelial cell fraction. Accession number for BLAST best match in curly brackets.

Table 4.1. Growth factor and signaling genes enriched in endothelial cell fraction of mouse lung cells

Category	Name	Ratio	Difference	*P*-value	Accession number	BLAST best match {bit score *S*, *E*-value} *ec reference
Angiopoietin/Tie	Angiopoietin-2	2.0	59	0.019	AF004326	*AF004326 {4799, 0} (71)
Angiopoietin/Tie	Tie1 (Tie receptor tyrosine kinase)	1.5	431	0.014	X73960	*X73960 {7069, 0} (102)
Angiopoietin/Tie	Tie2 receptor tyrosine kinase (Tek)	1.9	289	0.015	AA017847	*NM_013690 {950, 0} (109)
Cytokine receptor superfamily	Growth hormone receptor (Ghr)	2.0	160	0.001	AA124236	*BC024375 {1102, 0} (67)
Cytokine receptor superfamily	Leptin receptor (Lepr, Obr)	1.9	107	0.024	AA060276	*AK009569 {854, 0} (93)
Endothelins	Endothelin receptor type B (Ednrb)	2.1	277	0.001	U32329	*U32329 {3790, 0} (101)
Endothelins	Endothelin-1 (Et-1, Edn1)	2.0	160	0.009	D43775	*D43775 {2210, 0} (82)
Endothelins	Endothelin-converting enzyme 1 (Ece1)	2.4	289	0.005	AA155143	*XM_131743 {1231, 0} (111)
Ephrin/Eph	Eph receptor B4 (EphB4)	1.8	262	0.012	U06834	*U06834 {7091, 0} (105)
Ephrin/Eph	Ephrin A1 (Efna1, Eplg1, Ligand of Eph-related kinase 1, Lerk1)	3.0	252	0.030	AA033136	*BC002046 {995, 0} (89)
Ephrin/Eph	Ephrin B1 (Efnb1, Eplg2, Ligand of Eph-related kinase 2, Lerk2, Stra1)	1.8	277	0.021	Z48781	*Z48781 {4248, 0} (2)
Ephrin/Eph	Ephrin B2 (Efnb2, Eplg5, Ligand of Eph-related kinase 5, Lerk5, Htkl)	1.9	600	0.003	L38847	*L38847 {3152, 0} (29)
G-protein coupled receptors	Calcitonin receptor-like receptor (Calcrl)	2.3	118	0.021	AA178183	*NM_018782 {751, 0} (55)
G-protein coupled receptors	CD97 antigen (Cd97)	1.6	521	0.003	AA204482	XM_134459 {878, 0}
G-protein coupled receptors	Edg1 (Endothelial differentiation gene 1)	2.8	68	0.009	U40811	*U40811 {3348, 0} (66)
G-protein coupled receptors	Etl1 (EGF-TM7-latrophilin-related protein)	1.5	375	0.004	AI594845	NM_133222 {823, 0}
G-protein coupled receptors	Ramp2 receptor (calcitonin) activity modifying protein 2	2.0	675	0.014	AA000051	*XM_122291 {609, e-172} (55)
G-protein coupled receptors	RDC1 orphan chemokine receptor 1 (Cmkor1)	2.1	164	0.005	AF000236	AF000236 {2767, 0}
IL-1R superfamily	Sigirr (Single Ig IL-1 receptor-related protein)	2.0	133	0.026	AA261584	XM_133951 {860, 0}
Immunoglobulin superfamily receptors	Magic roundabout (Drosophila Roundabout homologue 4, Robo4)	2.9	295	0.018	AA553155	*NM_028783 {587, e-165} (49)
Jagged/Notch	Hey1 (Hairy/enhancer-of-split related-1, Hesr1)	1.9	460	0.008	AA269753	*NM_010423 {373, e-101} (42)
Jagged/Notch	Jagged 2 (Jag2)	1.5	133	0.006	Y14331	*Y14331 {1294, 0} (115)
Jagged/Notch	Notch1	1.5	303	0.011	AA271199	*AF508809 {535, e-149} (60)
Jagged/Notch	Notch4	2.5	103	0.001	U43691	*U43691 {13170, 0} (63)
Jagged/Notch	Numb	2.2	83	0.002	AI605646	AK004553 {434, e-119}
Jagged/Notch	Siah1b (Seven in absentia 1B, siah-1B)	2.0	119	0.039	Z19580	Z19580 {2621, 0}
Other growth factors	Kit ligand (c-kit ligand, mast cell growth factor, stem cell factor, steel)	2.0	436	0.001	S40536	*S40536 {2317, 0} (79)
Other growth factors	Scube1 (signal peptide, CUB domain, EGF-like 1)	2.0	110	0.039	AA096908	AF276425 {615, e-173}
Other growth factors	Sdf1-alpha (Stromal cell derived factor 1-alpha)	2.7	329	0.006	AA103556	*NM_021704 {898, 0} (100)
Protein serine/threonine kinase receptors	Activin receptor IIB (ActR IIB, Acvr2b)	2.0	114	0.003	M84120	M84120 {3364, 0}
Protein serine/threonine kinase receptors	Activin receptor-like kinase-1 (Alk-1)	2.2	567	0.014	L48015	*L48015 {3854, 0} (1)
Protein serine/threonine kinase receptors	Bone morphogenic protein receptor, type II (Bmpr2)	2.2	143	0.021	U78048	*U78048 {6415, 0} (4)
Protein serine/threonine kinases	SNF related kinase (Snrk)	1.7	246	0.006	AA444931	NM_133741 {492, e-136}
Protein tyrosine kinase receptors	Ryk receptor-like tyrosine kinase	1.6	73	0.003	L02210	L02210 {4324, 0}
Protein tyrosine phosphatase receptors	Protein tyrosine phosphatase receptor type B (Ptprb, Ptpr-beta)	1.8	332	< 0.001	X58289	*X58289 {3473, 0} (28)
Protein tyrosine phosphatase receptors	Protein tyrosine phosphatase receptor type G (Ptprg, Ptpr-gamma)	5.1	157	0.006	L09562	*L09562 {5077, 0} (28)
Protein tyrosine/threonine kinases	Tyrosine- and threonine-specific cdc2-inhibitory kinase (Pkmyt1, Myt1)	1.6	137	0.006	AA183223	BC025061 {456, e-126}
Secreted proteins	Lipocalin 7 (Lcn7)	1.7	145	0.003	AI316647	XM_124342 {856, 0}
Secreted proteins	Selenoprotein P (Selp)	2.1	53	0.013	AA690760	*AF021345 {391, e-106} (15)
Semaphorin/neuropilin	Neuropilin 1 (Nrp1)	1.6	215	< 0.001	D50086	*D50086 {7170, 0} (86)
Semaphorin/neuropilin	Semaphorin 3C (Sema3c, Semaphorin E, Collapsin-3)	1.8	156	0.010	X85994	*X85994 {4910, 0} (26)
VEGF	VEGF-A (Vascular endothelial growth factor A, VEGF)	2.6	440	0.014	M95200	*M95200 {1943, 0} (48)
VEGF	VEGF-C (Vascular endothelial growth factor C, Flt4 ligand)	2.5	148	0.003	U73620	*U73620 {3424, 0} (98)
VEGF, Protein tyrosine kinase receptors	VEGFR1 (FMS-like tyrosine kinase 1, Flt-1)	2.3	274	0.003	D88690	*D88690 {4954, 0} (53)
VEGF, Protein tyrosine kinase receptors	VEGFR2 (Kdr, Flk1)	1.9	530	0.013	X59397	*X59397 {10690, 0} (80)
Wnt (Wingless-type)	Frizzled-4 (Fzd4, Fz4, mFz4)	2.1	300	0.002	AA467487	*BC015256 {1072, 0} (123)
Wnt (Wingless-type)	Tle1 (Transducin-like enhancer of split 1, Groucho-related protein 1, Grg1)	1.6	66	0.009	U61362	U61362 {2441, 0}

Table 4.2. Cell junction, adhesion, cytoskeleton, and matrix genes enriched in endothelial cell fraction of mouse lung cells

Category	Name	Ratio	Difference	*P*-value	Accession number	BLAST best match {bit score *S*, *E*-value} *ec reference
Cell junctions	Afadin (Af6, Mllt4)	2.8	115	0.026	AI155181	AF172447 {789, 0}
Cell junctions	Catenin alpha 1 (Catna1)	1.8	145	0.025	AI194532	*NM_009818 {547, e-153} (61)
Cell junctions	Claudin 5 (Cldn5)	2.2	632	0.016	AA275227	*XM_147222 {902, 0} (84)
Cell junctions	Connexin 37 (Cx37, Gap junction protein alpha-4, Gja4)	2.2	279	0.029	X57971	*X57971 {3184, 0} (95)
Cell junctions	Nectin-3 (Poliovirus receptor-related 3, Pvrl3)	1.8	92	0.030	AA497887	AF195835 {618, e-174}
Cell junctions	Pecam-1 (platelet-endothelial cell adhesion molecule, CD31)	2.3	235	0.001	L06039	*L06039 {5023, 0} (108)
Cell junctions	Protocadherin 1 (Pcdh1, cadherin-like 1, protocadherin 42) {similar to}	1.7	242	0.024	AI613756	XM_128995 {831, 0}
Cell junctions	Sorbs1 (Sorbin and SH3 domain containing 1, ponsin, c-Cbl associated protein, CAP)	2.2	232	0.010	U58883	U58883 {5301, 0}
Cell junctions	VE-Cadherin (Vascular-endothelial cadherin, Cadherin 5, Cdh5)	2.8	335	0.002	D63942	*D63942 {5396, 0} (17)
Cell junctions	ZO-1 (Zona occludens 1, tight junction protein 1, Tjp1)	1.7	478	0.011	D14340	*D14340 {11670, 0} (6)
Cell adhesion	AA4 cell surface antigen (AA4, homologue of human C1q receptor)	1.5	554	0.008	AA185911	*AF081789 {591, e-166} (18)
Cell adhesion	Endomucin (Emcn)	3.1	322	0.008	AA208581	*NM_016885 {892, 0} (83)
Cell adhesion	Endothelial cell-selective adhesion molecule (Esam)	1.6	544	0.005	W62991	*XM_134643 {757, 0} (46)
Cell adhesion	Focal adhesion kinase (Fak, protein tyrosine kinase 2, Ptk2)	1.7	100	0.022	M95408	*M95408 {8348, 0} (77)
Cell adhesion	Integrin alpha 6 subunit (Itga6, CD49f)	1.6	162	0.001	X69902	*X69902 {7315, 0} (59)
Cell adhesion	Integrin beta 1 subunit (Itgb1)	1.5	498	0.012	U47283	*U47283 {1790, 0} (59)
Cell adhesion, hyaluronan receptors	Lyve1 (lymphatic vessel endothelial hyaluronan receptor-1, Xlkd1)	2.3	437	0.005	W29485	*AJ311501 {706, 0} (7, 85)
Cell adhesion, hyaluronan receptors	Stabilin 1 (Stab1, Ms-1)	2.2	393	0.006	AA286479	*NM_138672 {559, e-157} (94)
Cell adhesion, leukocyte adhesion	CD34 antigen (Cd34, Hematopoietic progenitor cell antigen, Hpca1)	5.9	304	0.010	W65699	*NM_133654 {749, 0} (9)
Tetraspanin superfamily	Peripheral myelin protein 22 (Pmp-22, Growth-arrest-specific protein 3, Gas3)	1.5	275	0.001	M32240	M32240 {1917, 0}
Tetraspanin superfamily	Transmembrane 4 superfamily member 1 (Tm4sf1, L6 antigen)	1.5	588	0.002	L15443	L15443 {2018, 0}
Tetraspanin superfamily	Transmembrane 4 superfamily member 2 (Tm4sf2, Tspan-2, A15, PE31, TALLA)	2.1	371	0.001	AA500156	NM_019634 {1033, 0}
Tetraspanin superfamily	Transmembrane 4 superfamily member 6 (Tm4sf6, Tetraspanin 6, Tspan-6)	1.7	116	0.002	AA003056	NM_019656 {819, 0}
Tetraspanin superfamily	Transmembrane 4 superfamily member 7 (Tm4sf7, Nag2, Tspan-4)	1.6	204	0.029	AA044511	*NM_053082 {916, 0} (110)
Cytoskeleton	Actin, beta (Actb)	1.7	265	0.002	AA536630	*NM_007393 {795, 0} (3)
Cytoskeleton	Calponin 3, acidic (Cnn3)	1.5	306	0.006	AA123989	*NM_028044 {783, 0} (99)
Cytoskeleton	Dynein, axonemal heavy chain 8 (Dnahc8, Dnah8)	1.8	58	0.008	AA089110	AF356520 {1215, 0}
Cytoskeleton	Kinesin heavy chain member 1B (Kif1b, D4Mil1e)	1.5	93	0.032	W14775	XM_131849 {720, 0}
Cytoskeleton	Midline 2 (Mid2)	1.5	189	0.021	AI463163	NM_011845 {555, e-155}
Cytoskeleton	Myosin alkali light chain, atrial/fetal isoform (Myla, MLC1a, MLC1emb)	1.8	142	< 0.001	M19436	M19436 {1108, 0}
Cytoskeleton	Palmdelphin (Palmd, paralemmin-like, Palml)	1.8	134	0.012	AI115758	AF263246 {696, 0}
Cytoskeleton	Pleckstrin 2 (Plek2)	1.7	85	0.002	AA403397	BC028902 {989, 0}
Cytoskeleton	Sarcoglycan epsilon (Sgce)	1.6	270	< 0.001	AA203787	NM_011360 {801, 0}
Cytoskeleton	Septin 2 (Sept2, Nedd5)	1.6	81	0.021	W51490	NM_010891 {745, 0}
Cytoskeleton	Spectrin, beta, non-erythrocytic 2 (Spnb2, Sptbn2, beta-III spectrin)	2.3	368	0.008	AF017112	*NM_009260 {16120, 0} (41)
Cytoskeleton	Tubulin, beta 5 (Tubb5)	1.5	389	0.001	AA030813	NM_011655 {579, e-162}
Cytoskeleton	Villin-like protein (Villp)	1.5	72	0.034	AA109911	XM_135210 {765, 0}
Basement membrane/extracellular matrix	Agrin	1.8	253	0.021	AA273938	*M92658 {396, e-108} (8)
Basement membrane/extracellular matrix	Collagen type IV alpha-1 chain (Col4a1)	1.6	601	0.012	J04694	*J04694 {11370, 0} (124)
Basement membrane/extracellular matrix	Heparan sulfate D-glucosaminyl 3-O-sulfotransferase 1 (Hs3st1, 3Ost1)	2.5	173	0.029	AF019385	AF019385 {3239, 0}
Basement membrane/extracellular matrix	Hyaluronidase 1 (Hyal1)	1.7	122	0.010	AA051686	AF417498 {557, e-156}
Basement membrane/extracellular matrix	Laminin, beta 2 (Lamb2, Lams)	2.1	180	0.046	AA030963	*BC026051 {928, 0} (107)

Table 4.3. Transporters and signal transduction genes enriched in endothelial cell fraction of mouse lung cells

Category	Name	Ratio	Difference	*P*-value	Accession number	BLAST best match {bit score *S*, *E*-value} *ec reference
Transporters, ATPases, cation pumps	ATPase, Ca++ transporting 2 (Atp2a2, Sarcoplasmic/endoplasmic reticulum calcium ATPase 2, SERCA2)	1.7	104	0.006	AA033051	*NM_009722 {783, 0} (81)
Transporters, ATPases, cation pumps	ATPase, Ca++ transporting 3 (Atp2a3, Sarcoplasmic/endoplasmic reticulum calcium ATPase 3, SERCA3)	2.0	225	0.012	U49393	*U49393 {6556, 0} (65)
Transporters, drug transporters	ATP-binding cassette G2 transporter (Abcg2, Breast cancer resistance protein 1, Bcrp1)	2.2	104	0.026	AI226912	NM_011920 {835, 0}
Transporters, electron transporters	Cytochrome c oxidase subunit II (Cox2, COII, MTCO2)	4.9	211	0.018	AA462358	*AF378830 {664, 0} (40)
Transporters, ion channels	Potassium channel voltage-gated (Kcnb1, Shab)	1.5	59	0.015	M64228	M64228 {5103, 0}
Transporters, ion channels	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4 (Kcnn4, Kca4)	2.9	208	0.017	AA033013	BC010274 {997, 0}
Transporters, ion channels	Purinergic receptor P2X ligand-gated ion channel 4 (P2rx4, P2x4)	1.7	169	0.010	AA288288	*BC005597 {854, 0} (32)
Transporters, ion channels	Trpm7 (Transient receptor potential cation channel M7, Ltrpc7, Trp-Plik, ChaK)	1.5	139	0.002	AI647571	XM_123872 {1160, 0}
Transporters, phospholipid transporters	Phospholipid scramblase 2 (Plscr2)	1.7	53	0.007	AA080505	XM_135045 {505, e-141}
Transmembrane proteins	MHC class I heavy chain precursor (H-2D(d))	1.6	153	0.006	X00246	X00246 {2900, 0}
Transmembrane proteins	Proline-rich gamma-carboxyglutamic acid protein 2 (Prgp2, Prrg2, Tmg2)	1.7	85	0.041	AA240190	XM_133452 {618, e-175}
Transmembrane proteins	Tde1 (Tumor differentially expressed 1)	2.6	86	0.016	AA268985	XM_110390 {884, 0}
Transmembrane proteins	Transmembrane protein 2 (Tmem2)	2.2	297	0.006	AA032950	XM_129153 {730, 0}
Phospholipid binding proteins	Phosphatidylserine receptor (Ptdsr)	1.7	224	0.007	AU044558	NM_033398 {496, e-138}
Phospholipid binding proteins	Sdpr (Serum deprivation response phosphatidylserine-binding protein)	1.9	415	0.008	AA096657	XM_129762 {932, 0}
Signal transduction	Adenylate cyclase 4 (Adcy4)	2.7	405	0.012	AA397054	NM_080435 {835, 0}
Signal transduction	Go-alpha (Guanine nucleotide binding protein alpha o, Gnao, G(o)alpha)	2.2	69	0.014	M36777	*M36777 {2601, 0} (103)
Signal transduction, G protein regulation	Rgs12 regulator of G-protein signaling 12	1.6	259	0.017	AA000711	XM_132003 {767, 0}
Signal transduction, GTPase, Ras oncogene family	Rab-related GTP-binding protein (Rabj)	1.9	55	0.005	AA004102	AY035893 {547, e-153}
Signal transduction, GTPase, Ras oncogene family	RhoC GTPase (Ras homologue 9, Arh9)	1.8	99	0.049	AA261594	XM_124152 {605, e-170}
Signal transduction, GTPase, Ras oncogene family	Rab11a small GTPase (Rab11a)	1.7	149	0.005	AI132545	AF127669 {652, 0}
Signal transduction, membrane trafficking	Pikfyve (phosphatidylinositol-4-phosphate 5-kinase, type III, Pip5k3, PIP 5 kinase3)	2.2	121	0.015	AA412875	NM_011086 {583, e-164}
Signal transduction, negative regulator of Ras	Neurofibromatosis 1 (Nf1)	2.8	308	0.002	D30730	*D30730 {1249, 0} (87)
Signal transduction, Rho-GTPase-activating protein	Oligophrenin 1 (Ophn1)	1.8	62	0.034	AI594958	BC004845 {1033, 0}
Transcription factors	Af10 (mAF10, Mllt10)	1.8	63	0.016	AA270281	AF010135 {888, 0}
Transcription factors	E4f1 (E4F transcription factor 1, E4f)	1.8	66	0.015	X76858	X76858 {4446, 0}
Transcription factors	Elf2 (Ets family transcription factor Elf2B1)	1.8	219	0.013	AA166224	*AF256218 {795, 0} (24)
Transcription factors	Elk3 (Ets-domain protein) transcription factor	1.6	183	0.016	AA014346	BC005686 {704, 0}
Transcription factors	Forkhead (Foxf1a) transcription factor	1.8	232	0.014	AI639791	*AF346834 {597, e-168} (54)
Transcription factors	Gata2 transcription factor	1.5	105	0.012	AA073064	*NM_008090 {438, e-120} (23)
Transcription factors	Glial cells missing-1 (Gcm1, Gcma) transcription factor	1.8	158	0.010	D88612	D88612 {3727, 0}
Transcription factors	Gtf2ird1 (Williams-Beuren syndrome critical region 11, Wbscr11, Gtf2I, General transcription factor 3, Gtf3)	1.5	106	0.006	AA920317	AF325177 {868, 0}
Transcription factors	Heterogeneous nuclear ribonucleoprotein K (Hnrpk) transcription factor	1.8	176	0.002	AA027739	NM_025279 {1029, 0}
Transcription factors	Jun oncogene (c-Jun) transcription factor	1.5	271	0.039	X12761	*X12761 {2868, 0} (121)
Transcription factors	Laf4l (Lymphoid nuclear protein related to AF4-like, Alf4) transcription factor	2.3	88	0.016	AA183140	XM_122212 {825, 0}
Transcription factors	Meis1 (Myeloid ecotropic viral integration site 1) transcription factor	1.6	101	0.028	U33630	U33630 {3794, 0}
Transcription factors	Nfatc4 (Nuclear factor of activated T-cells, Nfat3, calcineurin-dependent 4, NF-ATc4)	1.6	81	0.035	AA052537	*AF309389 {791, 0} (35)
Transcription factors	Tal1 (T-cell acute lymphocytic leukemia 1, stem cell leukemia, Scl)	1.9	93	0.036	M59764	*M59764 {6264, 0} (112)
Transcription factors	Tcf11 (Transcription factor 11, Nrf1)	1.7	145	< 0.001	AI390838	AJ277444 {545, e-152}

Table 4.4. Enzymes and organelle-related genes enriched in endothelial cell fraction of mouse lung cells

Category	Name	Ratio	Difference	*P*-value	Accession number	BLAST best match {bit score *S*, *E*-value} *ec reference
Anhydrases	Carbonic anhydrase 14 (Car14, CA XIV)	2.2	131	0.017	AA237530	NM_011797 {950, 0}
Hydrolases	Acid phosphatase 1 (Acp1)	1.6	71	0.026	AA044510	*XM_126887 {981, 0} (92)
Hydrolases	Dimethylarginine dimethylaminohydrolase 1 (Ddah1)	1.6	108	0.036	AA222082	*BC034505 {385, e-104} (12)
Hydrolases	Galactosamine (N-acetyl)-6-sulfate sulfatase (Galns)	1.7	58	0.036	AA793622	NM_016722 {644, 0}
Hydrolases	Nudt7 (Coenzyme A diphosphatase, Nudix)	1.5	77	0.033	AA237808	NM_024437 {787, 0}
Hydrolases	Phosphodiesterase 9A (Pde9a)	2.5	215	0.002	AA108700	XM_128503 {494, e-137}
Hydrolases	Phospholipase C beta 1 (Plcb1)	2.4	112	0.008	X95344	X95344 {1076, 0}
Monooxygenases	Flavin-containing monooxygenase 1 (Fmo1)	1.6	422	0.008	U87456	*U87456 {4579, 0} (91)
Monooxygenases	Flavin-containing monooxygenase 2 (Fmo2, Pulmonary FMO)	1.7	224	0.027	AI314952	*BC031415 {922, 0} (91)
Proteases, cysteine proteases	Ctla2-alpha (Cytotoxic T lymphocyte-associated cysteine protease 2 alpha, Ctla-2a)	1.5	572	0.010	X15591	X15591 {1316, 0}
Proteases, metalloproteases	Adamts-1 (A disintegrin and metalloproteinase with thrombospondin motifs 1, Meth1)	2.9	140	0.012	D67076	*D67076 {8233, 0} (117)
Proteases, metalloproteases	Carboxypeptidase D (Cpd, duck hepatitis B virus receptor {gp180} homologue)	1.8	262	0.008	AA038455	*XM_122266 {646, 0} (13)
Proteases, serine proteases	Dipeptidylpeptidase IV (Dpp4, CD26)	1.6	137	0.041	AA168553	XM_123834 {823, 0}
Proteases, serine proteases	Neurotrypsin (motopsin, Prss12)	1.5	125	0.038	AA063841	NM_008939 {761, 0}
Proteases, serine proteases	Placental protein 11 related (Pp11r, T cell-specific protein, Tcl-30)	1.5	60	0.013	M95545	M95545 {4242, 0}
Protease inhibitors	Serpinh1 (Serine protease inhibitor, J6, colligin, hsp47, cbp1)	1.6	100	0.033	J05609	J05609 {3755, 0}
Protease inhibitors	Timp3 (Tissue inhibitor of metalloproteinase 3)	1.6	167	0.007	AA073910	*NM_011595 {1049, 0} (14)
Protease inhibitors	Tissue factor pathway inhibitor (Tfpi, Extrinsic pathway inhibitor, Epi)	1.7	125	0.022	AA111494	*AF004833 {458, e-126} (69)
Transferases, glycosyltransferases	Agl (Amylo-1,6-glucosidase, 4-alpha-glucanotransferase, glycogen debrancher)	1.7	180	0.036	C77182	XM_131166 {805, 0}
Transferases, glycosyltransferases	Ext2 (Multiple exostosis protein 2, Exostosin 2)	1.7	136	0.018	U67837	U67837 {5638, 0}
Transferases, glycosyltransferases	Sialyltransferase 7b (beta-galactoside alpha-2,6-sialyltransferase B, Siat7b)	1.7	160	0.010	X93999	*X93999 {3911, 0} (38)
Transferases, glycosyltransferases	Sialyltransferase 7c (beta-galactoside alpha-2,6-sialyltransferase C, Siat7c, ST6GalNAcIII)	2.1	94	0.007	AA175746	*Y11342 {676, 0} (38)
Transferases, methyltransferases	Glycine N-methyltransferase (Gnmt)	1.5	59	0.005	W14826	NM_010321 {920, 0}
Transferases, methyltransferases	Prenylcysteine carboxylmethyltransferase (PCCMT, PCMT, ICMT)	1.6	64	0.007	AA259443	AF209926 {468, e-129}
Transferases, sulfotransferases	Carbohydrate sulfotransferase 1 (Chst1, Keratan sulfate gal-6-sulfotransferase, Ksgal6st)	1.8	404	0.011	AI021619	*NM_023850 {819, 0} (113)
Apoptosis	TRAIL (TNF-related apoptosis inducing ligand, Tnfsf10, Apo2 ligand, Apo2L)	3.5	162	0.008	U37522	*U37522 {2629, 0} (57)
Centrosomes	Pericentriolar material 1 (Pcm1)	2.3	62	0.050	AA770808	NM_023662 {805, 0}
Endoplasmic reticulum	FK506 binding protein 10 (Fkbp10)	1.6	85	0.014	AA000160	XM_122325 {925, 0}
Endoplasmic reticulum	Tra1 (Tumor rejection antigen, gp96, ERp99, glucose-regulated protein, GRP94)	1.6	474	0.024	W55140	NM_011631 {624, e-176}
Golgi apparatus	Lectin, mannose-binding, 1 (Lman1, F5f8d, Ergic53, gp58)	1.6	296	0.008	AA265127	NM_027400 {930, 0}
Golgi apparatus	Neurobeachin (Nbea)	1.8	84	0.007	AI154580	Y18276 {761, 0}
Golgi apparatus	Nsg1 (Neuron specific gene family member 1, 21p)	1.6	82	0.040	AA008502	BC008272 {531, e-148}
Golgi apparatus	Tgn38 (Trans-Golgi network protein 2, Tgoln2, Tgn46)	3.2	203	0.009	D50032	*D50032 {4490, 0} (120)
Mitochondria	Mitochondrial ribosomal protein L55 (Mrpl55)	1.5	117	0.044	AA008472	XM_109885 {831, 0}
Nucleolus	Nol1r (Williams-Beuren syndrome critical region protein 20, Wbscr20)	1.6	52	0.032	AA021852	NM_145414 {846, 0}
Nucleolus	Nucleolin (Ncl, C23, Nucl)	1.5	172	0.022	AU017074	BC005460 {791, 0}
Nucleus	Matrin3 (Matr3)	1.6	52	0.004	AA987019	AB009275 {1108, 0}
Ribosomes	DEAD (Asp-Glu-Ala-Asp) box 5 (DEAD/H box 5, Ddx5, RNA Helicase nuclear 1)	1.7	207	0.043	AA009053	NM_007840 {924, 0}

Tables 4.1 - 4.4 list 166 of 234 named genes found to be enriched in endothelial cell fraction (FACS population R2) based on three criteria: (1) ratio ≥ 1.5 for expression in endothelial cell fraction to expression in unpurified lung cell fraction; (2) expression difference ≥ 50 between the two fractions; and (3) *P*-value < 0.05 for probability of no difference between the fractions. Higher ratios mean greater expression in endothelial cell fraction relative to that in unpurified lung cells. BLAST column gives the accession number of best match, with bits score *S* and *E*-value in curly brackets; asterisk (*ec) indicates published evidence of endothelial cell association accompanied by a reference.

Table 5. ESTs with greatest enrichment in endothelial cell fraction

Accession number	Ratio	Difference	*P*-value	Closest homology by BLAST {bit score *S*, *E*-value}
AA000959	2.8	148	0.003	XM_130442 {884, 0}
AA048323	3.2	163	0.009	AC021701 {40, 9e-01}
AA065871	2.4	90	0.000	NM_016620 {101, 1e-19}
AA067861	3.4	149	0.007	AK009464 {153, 4e-35}
AA087828	2.8	257	0.004	XM_131069 {700, 0}
AA119828	2.3	275	0.002	NM_025809 {967, 0}
AA174997	2.0	76	0.006	XM_127757 {955, 0}
AA175599	2.3	127	< 0.001	AL445216 {180, 8e-43}
AA178106	2.8	114	0.007	BC029772 {833, 0}
AA183383	2.7	125	< 0.001	XM_127894 {837, 0}
AA216875	3.4	284	0.009	BC016539 {938, 0}
AA267417	2.2	199	0.008	AK013906 {906, 0}
AA276914	2.2	104	0.002	AL353713 {42, 6e-01}
AA277444	2.3	129	0.004	BC016539 {823, 0}
AA726916	2.0	56	0.002	XM_129161 {523, e-146}
AA873956	2.2	185	0.004	AC025253 {46, 4e-02}
AI481048	2.3	73	0.001	AF234887 {125, 4e-26}
AI552225	5.9	193	0.002	AC078927 {46, 4e-02}
AI553268	2.1	139	0.003	AC009964 {44, 1e-01}
C79308	4.4	324	0.006	AL354877 {92, 3e-16}

List showing 20 ESTs that had the greatest enrichment in the endothelial cell fraction as judged by three criteria: (1) ratio ≥ 2.0 for expression in endothelial cell fraction to expression in unpurified lung cell fraction; (2) expression difference between the two fractions ≥ 50; and (3) $P < 0.01$ for the probability of no difference between the two fractions. Higher ratios mean greater expression in endothelial cell fraction relative to that in the unpurified lung cells. Right hand column shows accession number for closest homology by BLAST, with bit score *S* and *E*-value for that match in curly brackets.

FIGURE LEGENDS

Fig. 1. ***A*** and ***B***: Fluorescence micrographs of a 150-μm section of mouse lung showing anastomotic alveolar capillaries at 2 min after iv injection of FITC-labeled *L. esculentum* lectin (***A***), and 20 min after injection of rhodamine-labeled cationic liposomes (***B***). Scale bar in ***B*** applies to ***A*** and ***B***; bar length represents 5 μm. ***C*** and ***D***: FACS analysis, using one- or two-color (red, green) fluorescence, of single-cell suspensions prepared by enzymatic digestion of lungs after endothelial cells were labeled in vivo with rhodamine-labeled cationic liposomes followed by ex vivo labeling of leukocytes with FITC-labeled anti-CD18 antibody. ***C***: Rhodamine fluorescence of unpurified lung cells. Region R1 denotes a population of cells that have taken up liposomes (rhodamine positive), which constitute 26.1 ± 4.3% (n = 7) of the total events. ***D***: After sorting for liposome fluorescence and CD18 surface staining, region R2 denotes a population of cells that are rhodamine-positive and CD18-negative and represent 17.8 ± 3.4% (n = 7) of the total events. Total events included cellular debris and electronic noise. ***E*** and ***F***: FACS analyses in ***C*** and ***D***, respectively, shown here as intensity of forward scattered (FSC) and side scattered (SSC) transmitted light. ***E***: Size profile of cells gated in region R1, indicating two populations of cells, one interpreted as putative endothelial cells and the other as CD18-positive leukocytes. ***F***: Size profile of cells gated in region R2 denoting the population of putative endothelial cells.

Fig. 2. Structural features of lung cells isolated by FACS. ***A***: Micrograph of cells from population R1 stained with Diff-Quik after deposition on glass slide with Cytospin centrifuge. The population consists mainly of small round cells (arrows). ***B***: Higher magnification showing three small round cells and a macrophage (arrow) from population R1. ***C***: Confocal microscope image of cells from population R2 showing six presumptive endothelial cells (arrows mark examples), all of which have punctate red fluorescence from rhodamine-labeled cationic liposomes in endosomes. Cells allowed to adhere to poly-L-lysine coated glass slides after FACS isolation. Scale bar in **C** applies to all figures; bar length represents 25 μm in ***A***, 10 μm in ***B*** and 5 μm in ***C***.

Fig. 3. Multiple approaches used to identify lung cells (arrows) in population R2 isolated by FACS after positive selection by rhodamine-labeled cationic liposomes and negative selection by CD18. Cells allowed to adhere to poly-L-lysine coated glass slides before immunohistochemical staining. ***A***: Brightfield micrograph of presumptive endothelial cells imaged by Nomarski illumination. ***B***: Fluorescence micrograph of same field showing that essentially all of the cells have VE-cadherin immunoreactivity (Cy3 fluorescence). ***C*** and ***D***: Fluorescence micrographs of a field of population R2 cells showing rhodamine-liposome fluorescence (***C***) and Cy2 fluorescence of the leukocyte marker CD45.2 (***D***). All of the cells have rhodamine-liposome fluorescence, but only one, probably a macrophage (arrow), is immunoreactive for CD45.2. ***E*** and ***F***: Fluorescence micrographs showing rhodamine-liposome fluorescence in all cells (***E***) and the same field showing the uptake of BODIPY-labeled acetyl-LDL by most cells (***F***). Scale bar in ***F*** applies to all figures; bar length represents 20 μm.

Fig. 4. Confocal microscopic images that distinguish the pattern of alveolar capillary endothelial cells (***A-D, F***) from that of alveolar epithelial cells (***E, F***) in 80-μm sections of mouse lung stained immunohistochemically for proteins expressed by the two cell types. ***A***: CD31 immunoreactivity of endothelial cells showing the characteristic anastomotic nature of alveolar capillaries (arrows). ***B***: LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1) immunoreactivity attributed to endothelial cells because the pattern matches CD31 staining of alveolar capillaries (arrows). ***C, D***: VE-cadherin immunoreactivity (***C***) outlines endothelial cell borders (arrows) and, when colocalized with CD31 immunoreactivity (***D***), shows the branching pattern of alveolar capillaries. ***E, F***: E-cadherin immunoreactivity (***E***) outlines the border of alveolar epithelial cells (arrows) and, when merged with CD31 immunoreactivity (***F***), highlights the contrasting patterns of epithelial cell staining (red) and endothelial cell staining (green). Scale bar in **F** applies to all figures; bar length represents 15 μm.

Fig. 5. Confocal microscopic images that verify endothelial cell localization of 8 proteins (***A-H***) identified on oligonucleotide microarrays as having enriched expression in the endothelial cell fraction. All show the characteristic branching pattern of alveolar capillaries in 80-μm sections of mouse lung stained by immunohistochemistry. ***A***: Alk-1 (Activin receptor-like kinase-1) immunoreactivity matches the pattern of alveolar capillaries. ***B***: Bone morphogenic protein receptor type II (Bmpr2) staining has a pattern that fits with alveolar capillaries plus a second cell type (arrows), which may be type II alveolar epithelial cells. ***C***: CD31 (PECAM-1) immunoreactivity serves as a reference for staining of alveolar capillary endothelial cells. ***D***: Dipeptidylpeptidase IV (CD26) immunoreactivity has a distribution that matches endothelial cells plus a second cell type (arrows), perhaps type II alveolar epithelial cells. ***E-H***: Integrin alpha6 (CD49f), neuropilin-1, phospholipid scramblase 2, and VEGFR-2 immunoreactivities all have patterns that match alveolar capillary endothelial cells. Some antibodies (***B, D, F-H***) produced granular staining, suggestive of a distribution in endosomes, lipid rafts, or other subcellular compartments. Scale bar in ***H*** applies to all figures; bar length represents 25 μm.

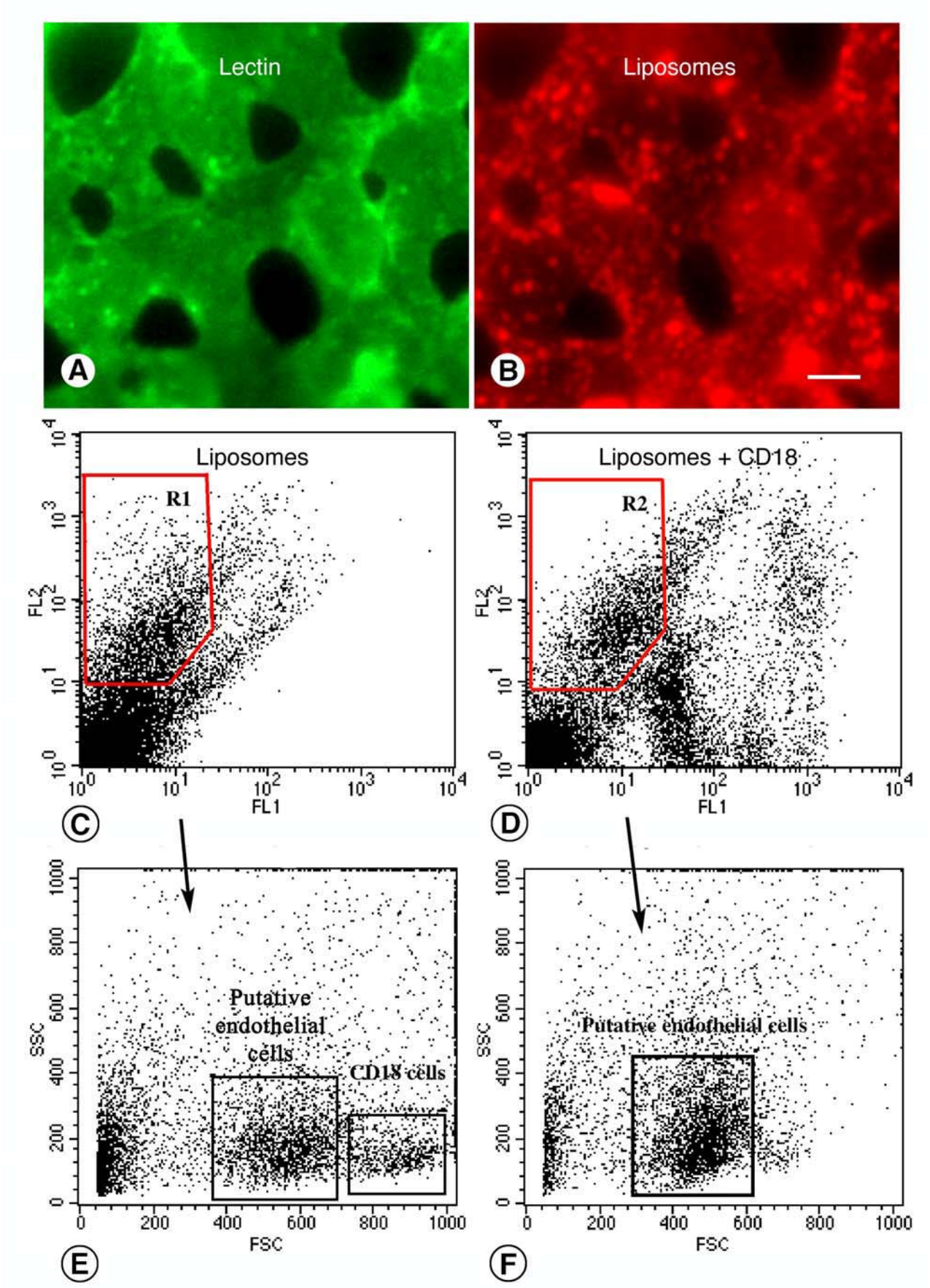
Lectin
Liposomes
A
B
Liposomes
R1
FL2
FL1
C
Liposomes + CD18
R2
FL2
FL1
D
Putative endothelial cells
CD18 cells
SSC
FSC
E
Putative endothelial cells
SSC
FSC
F

Figure 1

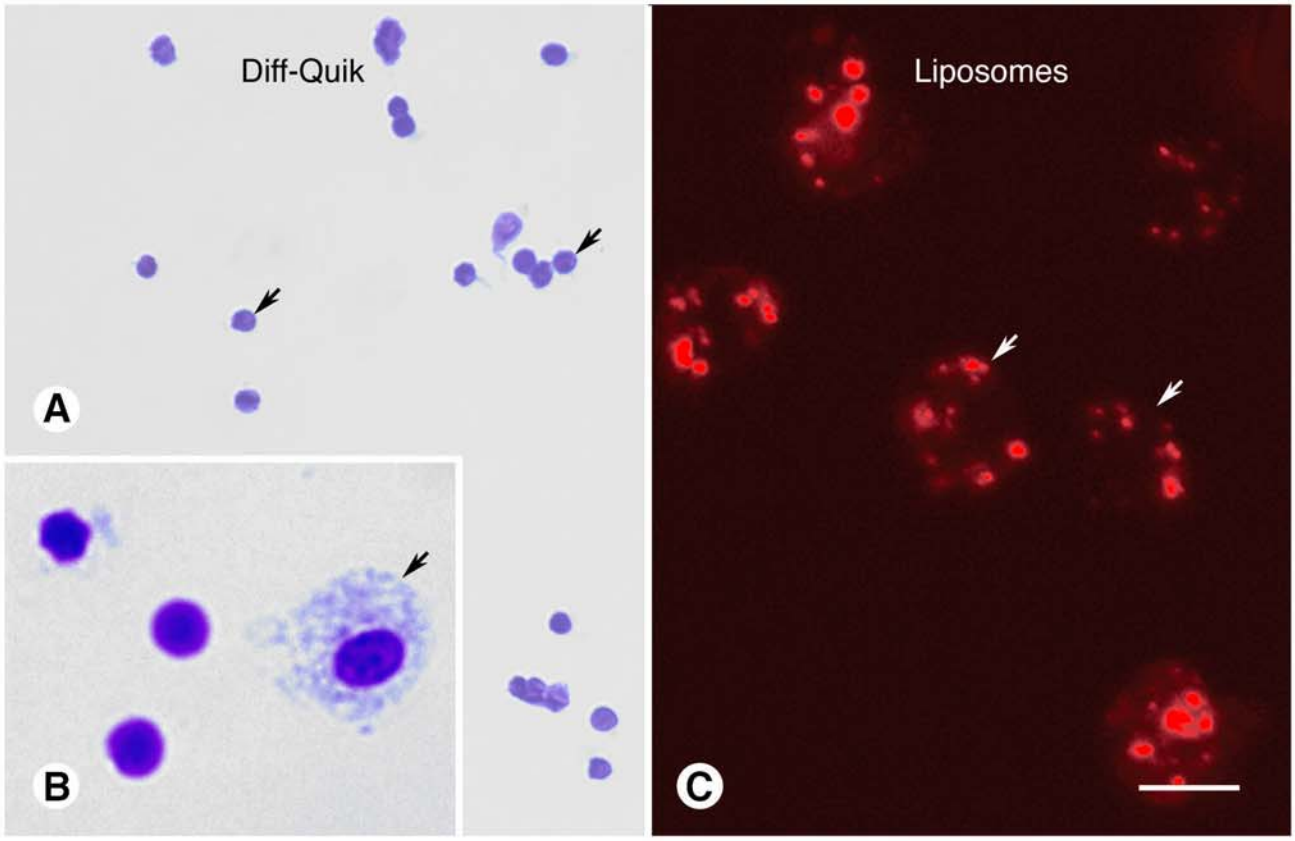

Figure 2

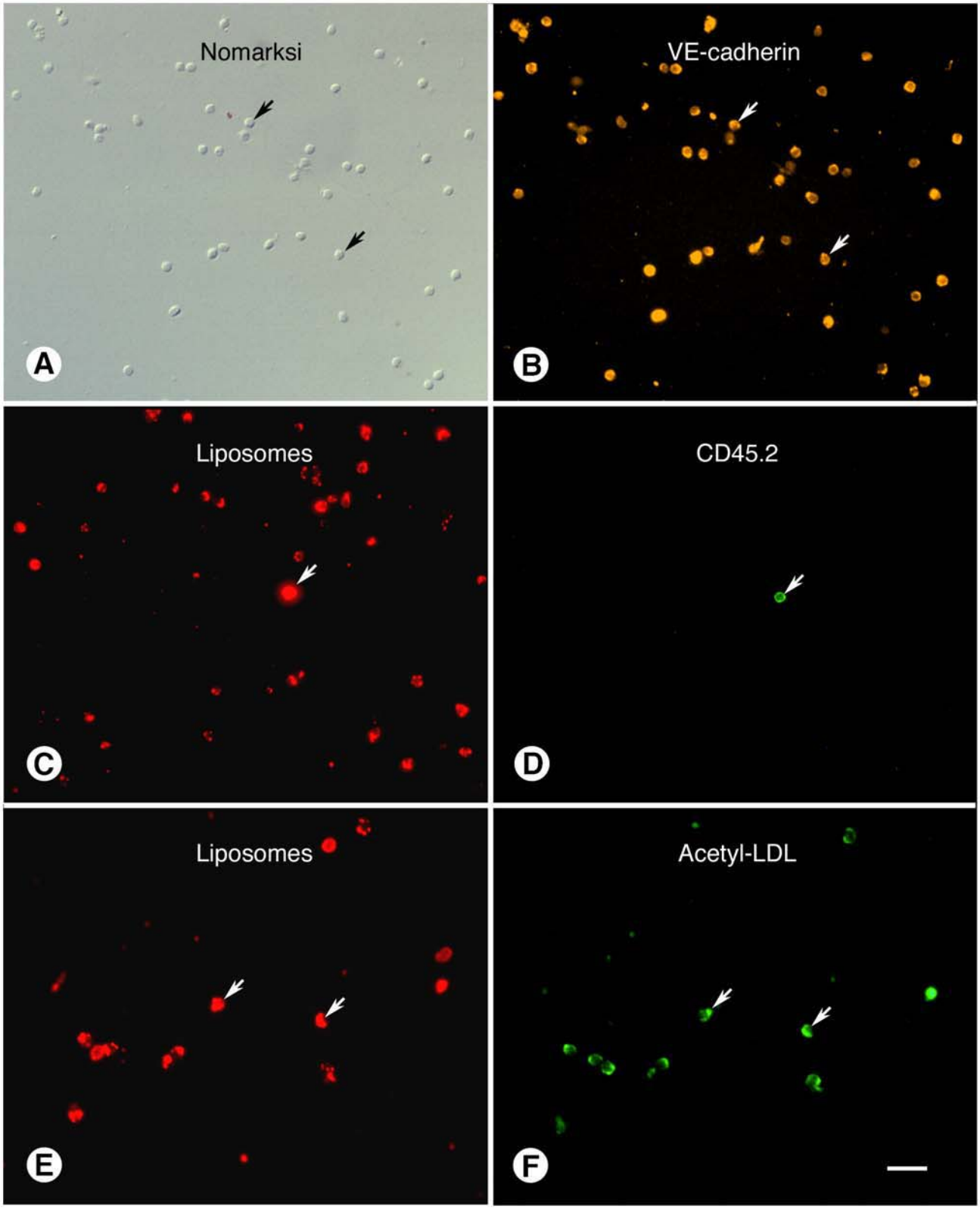
Nomarksi
A
VE-cadherin
B
Liposomes
C
CD45.2
D
Liposomes
E
Acetyl-LDL
F

Figure 3

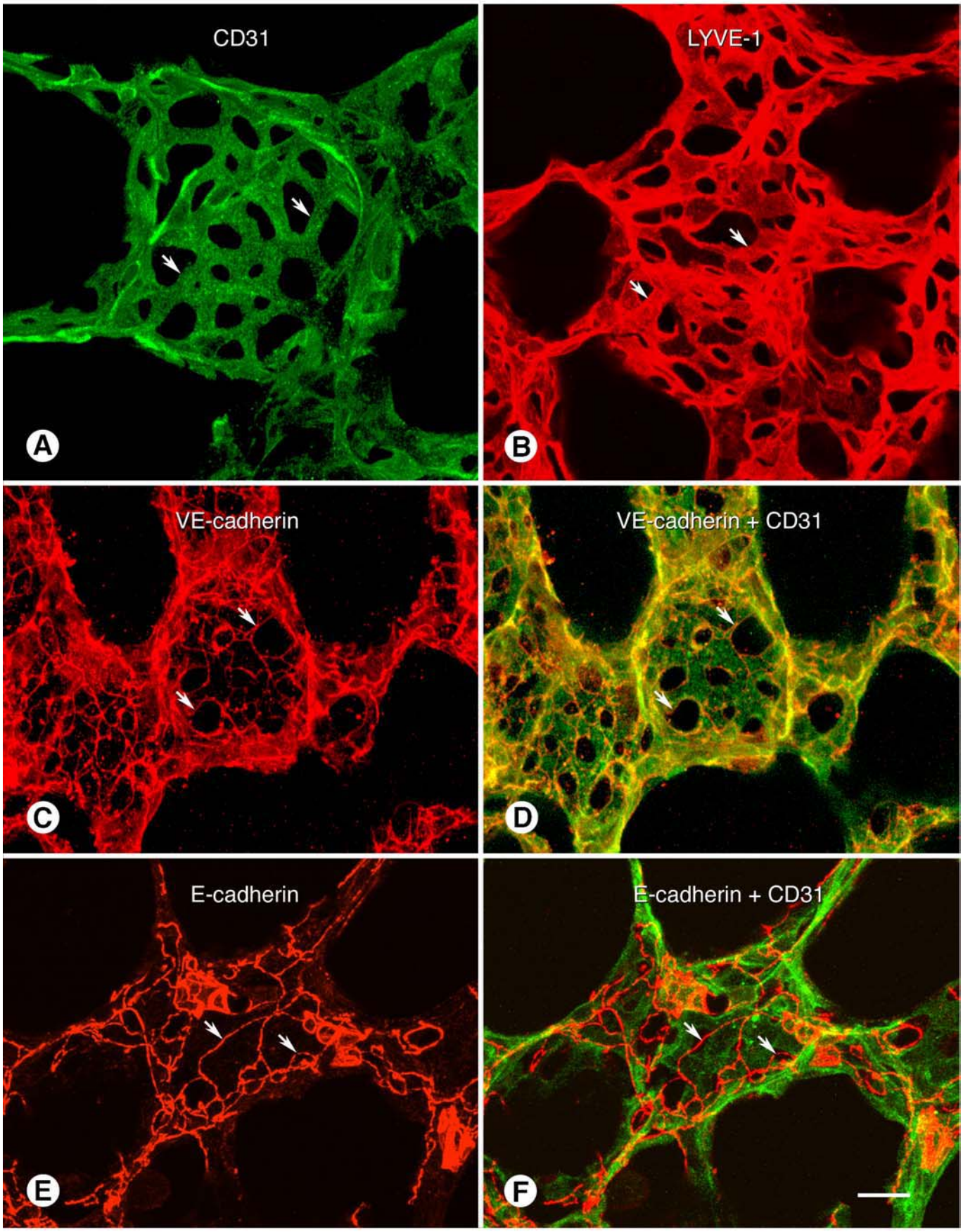
CD31
LYVE-1
A
B
VE-cadherin
VE-cadherin + CD31
C
D
E-cadherin
E-cadherin + CD31
E
F

Figure 4

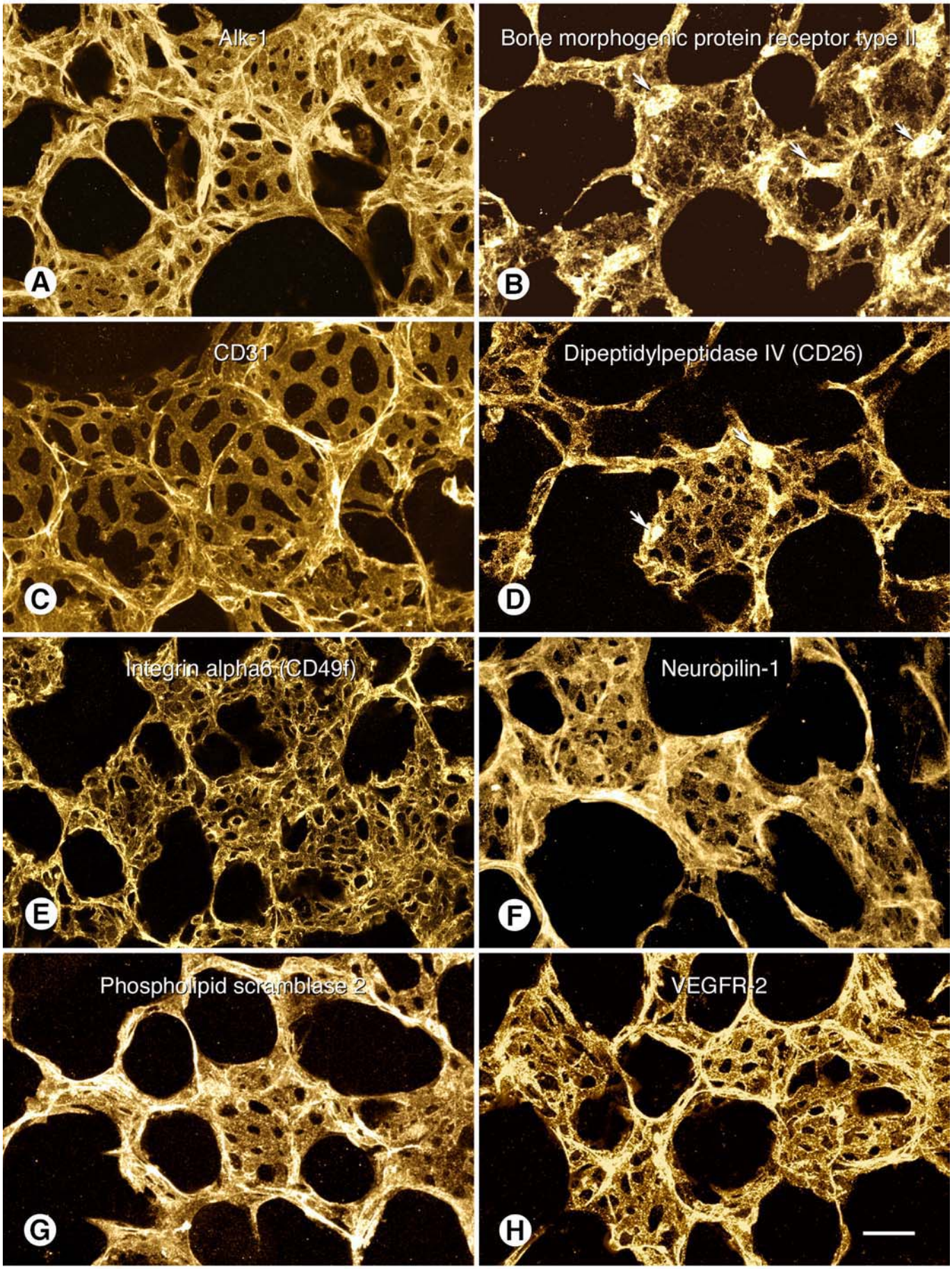
Alk-1
A
Bone morphogenic protein receptor type II
B
CD31
C
Dipeptidylpeptidase IV (CD26)
D
Integrin alpha6 (CD49f)
E
Neuropilin-1
F
Phospholipid scramblase 2
G
VEGFR-2
H

Figure 5