The American Journal of Pathology, Vol. 172, No. 5, May 2008

DOI: 10.2353/ajpath.2008.070988

Tumorigenesis and Neoplastic Progression

Molecular Characterization of Human Breast Tumor Vascular Cells

Rajendra Bhati,* Cam Patterson,† Chad A. Livasy,‡§ Cheng Fan,‡ David Ketelsen,‡ Zhiyuan Hu,‡ Evangeline Reynolds,‡ Catherine Tanner,* Dominic T. Moore,‡ Franco Gabrielli,¶ Charles M. Perou,‡§‖ and Nancy Klauber-DeMore*†‡

From the Department of Surgery, Carolina Cardiovascular Biology Center,† Lineberger Comprehensive Cancer Center,‡ Department of Pathology and Laboratory Medicine,§ and Department of Genetics,‖ University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; and Department of Experimental Pathology and Medical Biotechnology,¶ University of Pisa, Pisa, Italy*

A detailed understanding of the assortment of genes that are expressed in breast tumor vessels is needed to facilitate the development of novel, molecularly targeted anti-angiogenic agents for breast cancer therapies. Rapid immunohistochemistry using factor VIII-related antibodies was performed on sections of frozen human luminal-A breast tumors ($n = 5$) and normal breast ($n = 5$), followed by laser capture microdissection of vascular cells. RNA was extracted and amplified, and fluorescently labeled cDNA was synthesized and hybridized to 44,000-element long-oligonucleotide DNA microarrays. Statistical analysis of microarray was used to compare differences in gene expression between tumor and normal vascular cells, and Expression Analysis Systematic Explorer was used to determine enrichment of gene ontology categories. Protein expression of select genes was confirmed using immunohistochemistry. Of the 1176 genes that were differentially expressed between tumor and normal vascular cells, 55 had a greater than fourfold increase in expression level. The extracellular matrix gene ontology category was increased while the ribosome gene ontology category was decreased. Fibroblast activation protein, secreted frizzled-related protein 2, Janus kinase 3, and neutral sphingomyelinase 2 proteins localized to breast tumor endothelium as assessed by immunohistochemistry, showing significantly greater staining compared with normal tissue. These tumor endothelial marker proteins also exhibited increased expression in breast tumor vessels compared with that in normal tissues. Therefore, these genetic markers may serve as potential targets for the development of angiogenesis inhibitors. ***(Am J Pathol 2008, 172:1381–1390; DOI: 10.2353/ajpath.2008.070988)***

Angiogenesis is the growth of new capillary blood vessels, and is a critical component of solid tumor growth.[1] Targeted anti-angiogenic therapy for metastatic breast cancer with bevacizumab, a monoclonal antibody to vascular endothelial growth factor, has shown efficacy in patients with metastatic breast cancer[2] and validated the approach of anti-angiogenesis therapy for this disease. Although vascular endothelial growth factor is one critical growth factor involved in breast cancer angiogenesis,[3] a more detailed understanding of the assortment of genes that are expressed in breast tumor vessels may facilitate the development of novel molecularly targeted anti-angiogenic agents.

Several studies have established evidence to suggest that blood vessels supplying tumors express genes not shared by blood vessels that reside in normal tissues.[4–7] St. Croix et al[7] used a tissue dissociation and cell immunopurification approach to isolate tumor and normal endothelial cells, and then compared gene expression patterns of endothelial cells derived from colorectal cancer tissue and normal colonic mucosa from the same patient. Using serial analysis of gene expression, this analysis identified 46 transcripts, named tumor endothelial mark-

Supported by an award to the University of North Carolina for a Breast Cancer Specialized Program of Research Excellence (SPORE) from the National Cancer Institute (NIH/NCI P50-CA58223), Department of Defense Physician Scientist Training Award for Breast Cancer Research (W23RYX-3340-N609), National Cancer Institute (1 K08 CA098034-01A2), American Society of Clinical Oncology/ Breast Cancer Research Foundation Career Development Award, and The Sidney Kimmel Foundation Translational Science Award to N.K-D.; and a Surgical Oncology Research Training Program Grant from the National Cancer Institute (NCI CA009688) to R.B.

Accepted for publication January 23, 2008.

The authors disclose Report of Invention: "Discovery of Novel Target for Angiogenesis Inhibition" Reference #OTD07-0008, Patent Pending (N.K.-D., C.P., R.B.).

Supplemental material for this article can be found at *http://ajp.amjpathol.org*.

Address reprint requests to Nancy Klauber-DeMore, M.D., 3010 Old Clinic Building, CB #7213, Chapel Hill, NC 27599. E-mail: nancy_demore@med.unc.edu.

ers (TEMs), which were significantly up-regulated in tumor relative to normal endothelium. Using a similar method, Parker et al[6] isolated endothelial cells from two human breast tumors and one normal reduction mammoplasty and identified genes that were differentially expressed between breast tumor and normal breast tissues. This study identified 30 breast tumor vascular genes, of which *hairy*-related basic helix-loop-helix and phosphatase of regenerating liver 3, were confirmed to be localized in endothelium by *in situ* hybridization. These studies have also shown tumor-specific differences in tumor endothelial markers among colon, breast, and brain tumors.[6] Buckanovich et al[4] subsequently used laser capture microdissection (LCM) of vessel cells from ovarian cancer and normal ovaries and identified 70 differentially expressed TEMs.

Given that TEMs differ *between* tumor types,[6] and that breast cancers are molecularly heterogeneous, we sought to determine whether TEMs differ *within* the different molecular subtypes of breast cancer. Gene expression studies using DNA microarrays have identified several distinct breast cancer subtypes[8] that differentiate breast cancers into separate groups that differ markedly in prognosis.[9] The intrinsic subtypes include two main subtypes of estrogen receptor negative (ER−) tumors: Basal subtype (ER− and Her2/neu negative [Her2/neu−]) and Her2/neu subtype (Her2/neu+ and ER−); and an ER+ (luminal subtype).[9] Our goal was to identify TEMs overexpressed in human breast cancer and elucidate their subtype specificity, which may be important in patient selection for vascular targeting agents.[10] In this study, we obtained molecular profiles of human luminal A breast tumor vascular cells and compared the gene expression patterns to normal breast vasculature. Protein expression was then evaluated with immunohistochemistry (IHC) among luminal A, basal, and Her2/neu breast tumor subtypes.

Materials and Methods

Breast Tissue Source

The frozen tissues and tumors used in this study were obtained from the Lineberger Comprehensive Cancer Center Tissue Procurement and Analysis Core and have been procured from patients who were appropriately informed and who have consented to having their tissue procured for research. The tissue was obtained from primary breast tumors in patients who were not treated with neoadjuvant chemotherapy, or from patients without cancer undergoing reduction mammoplasty. The breast tumors used for microdissection were ER+, Her2/neu− (luminal A) immunophenotype.

IHC for LCM

Portions of snap frozen breast tissue are fixed in optimal cutting temperature compound and sectioned at −35°C on a cryostat at 8 μm onto polyethylene naphthalate membrane glass slides (Arcturus Bioscience, Mt View, CA). RNase-free technique is used throughout the procedure and buffers and alcohol solutions are used fresh each time. Slides are fixed in acetone for 2 minutes at 4°C and rinsed in Hanks' balanced salt solution (Gibco, Grand Island, NY). The slides are incubated with a mouse-anti-human antibody to factor VIII-related antigen (BioGenex, San Ramon, CA) at a 1:6 dilution for 7 minutes at 4°C. The IHC is performed with the DakoCytomation LSAB horseradish peroxidase 2 system (DakoCytomation, Carpinteria, CA), a three-step streptavidin-biotin system with the following modifications: After washing in Hanks' balanced salt solution, the biotinylated link is incubated for 5 minutes at room temperature. The alkaline phosphatase developer 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Vector Labs, Burlingame, CA) is used at a very high concentration (3 drops/300 μl buffer) and incubated for 10 to 15 minutes at 4°C. Slides are dehydrated in 75% ethanol for 30 seconds, 95% ethanol for 30 seconds, and 100% ethanol for 2 minutes (Arcturus Bioscience, Mountain View, CA). Protector RNase Inhibitor (Roche, Indianapolis, IN) is added at a 1:10 dilution to all buffers used in the staining process. The slides are placed on dry ice until microdissection, which occurs the same day as the IHC.

LCM

Microdissection immediately follows tissue preparation. LCM is performed on a Leica Laser Microdissection System (Leica Microsystems Inc., Bannockburn, IL). Tissues to be microdissected are viewed through a video microscope and the position of the slide is adjusted so that the desired cells are under the targeting light. Activation of the UV laser cuts the tissue around the groups of cells of interest. The cut tissue is then transported by gravity to an Eppendorf tube that contains 25 μl of RNA extraction buffer from the Picopure RNA Extraction Kit (Arcturus). To maintain RNA integrity, slides are kept on dry ice until microdissection, and microdissection is performed for no longer than 15 minutes per slide. Fifteen slides are microdissected per sample. RNA is then extracted with the Arcturus Picopure RNA Extraction Kit as described in the manufacturer's instructions and DNase I-treated.

Amplification of RNA

RNA amplification is performed using a two-round amplification system. The first round employs the RiboAmp HS RNA Amplification Kit (Arcturus). Five hundred ng from the first round of amplification is then put into the Agilent Low-Input Fluorescent Linear RNA Amplification Kit (Agilent, Palo Alto, CA). This second round employs a T7 polymerase amplification that incorporates the fluorescent probe in preparation for microarray analyses.

Analyses of RNA Integrity

RNA integrity is checked after the first round of amplification before each microarray experiment, using RT-PCR

detection of genes of different abundance levels and demonstration of intact, full-length cDNA preparations with the cDNA Integrity Kit (KPL, Gaithersburg, MD). This system utilizes primer sets and target genes that allow evaluation of in-process or double-stranded cDNA for the presence of full-length and extended cDNA transcripts. Primer sets amplify regions of the 3′ and 5′ ends of the housekeeping genes GAPDH and the low-expressed ADP ribosylation factor I gene. Generation of product using the 3′ primer sets indicate that the gene is expressed in the system, and amplimer production using the 5′ primer sets indicate full-length, intact cDNA.

Measurement of Amplification Bias

MDA-MB-435 breast cancer cells were plated (2.5×10^6 cells) in 75-ml flasks or 100-mm plates in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 100 U of penicillin-streptomycin (Gibco). After 48 hours, total RNA was extracted using the Qiagen RNeasy Kit and purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Samples underwent only one round of amplification (Group A) or two rounds of amplification (Group B). Correlation coefficients among arrays were compared with interclass correlation.[11]

Microarray Experiments

Synthesis of labeled cDNA was performed as described previously with reference cDNA, that is the Stratagene (La Jolla, CA) Human Universal Reference[11] labeled with Cy3-dUTP and sample cDNAs labeled with Cy5-dUTP. Microarray hybridizations were performed using Agilent Human oligonucleotide (custom-designed Agilent 1Av1-based for cell lines and Agilent 44k for vessel-dissected specimens) microarrays as previously described.[11] Technical replicates (which refer to using the same RNA from one tumor on two microarrays) were performed for all vessel-dissected specimens.

Data Normalization, Preprocessing, and Statistics

Gene expression values were quantified using the $\log_2$ ratio of the Lowess normalized red channel intensity versus green channel intensity.[12] The UNC Microarray database (*https://genome.unc.edu/*) was used to perform the filtering and preprocessing. All data have been deposited into the Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo*) under the accession number GSE7413. A two-class SAM (Significance Analysis of Microarrays, *http://www-stat.stanford.edu/~tibs/SAM/*)[13,14] was performed to identify significantly differentially expressed genes between all five tumor vascular samples versus all five normal vascular samples. Each sample had a technical replicate array, thus there were 10 arrays in each group that were used for the SAM. To identify differentially expressed genes that encode potential membrane or secreted proteins, we searched Gene Cards (*http://www.genecards.org/index.shtml*) to identify the potential subcellular location for genes with >fourfold increased expression.

To interpret the gene lists derived from the results of SAM, and convert the gene list into biological themes, we applied the Expression Analysis Systematic Explorer analysis, available from the Database for Annotation, Visualization, and Integrated Discovery (*http://david.abcc.ncifcrf.gov/*). If genes in a biological category are significantly enriched in the SAM-derived gene list, then that biological category may be involved in the biological system.

Identity of Cell Types in Microdissected Vessel Cells

We identified the cell types comprising the microdissected vessels by analyzing gene expression for genes known to be selectively expressed in specific populations of cells (endothelial, hematopoietic, pericytes, and epithelial) and compared gene expression profiles from our vascular cell specimens to endothelial cell cultures *in vitro* and breast tumor-derived cell cultures *in vitro*. Human endothelial cell total RNAs were purchased from Cell Application Incorporation (San Diego, CA). Total RNA was purified from breast cancer cell lines using the Qiagen RNAeasy Kit. RNA integrity was determined using the RNA 6000 Nano LabChip Kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Genes specific for endothelium,[6,7,15] previously characterized TEMs,[6,7] hematopoietic markers, pericyte markers, and luminal epithelium[8] were analyzed and the data displayed using Java Treeview.[16]

Confirmation of Vascular Origin of Vascular Marker Genes

To validate the vascular origin of the genes associated with tumor endothelium obtained by immuno-LCM, we performed IHC with antibodies to select gene transcripts and compared staining on subsequent sections stained with antibodies to factor VIII-related antigen on paraffin-embedded ER+, Her2/neu− breast tumors.

Commercially Available Antibodies

Rabbit polyclonal antibody to secreted frizzled-related protein 2 (SFRP-2; H-140; Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:150 dilution. Rabbit polyclonal antibody to fibroblast activation protein (FAP), α-Stalk region (Abcam, Cambridge, MA) was used at 1:600 dilution. Mouse monoclonal antibody to Janus kinase 3 (JAK3, Genetex Inc., San Antonio, TX) was used at 1:100 dilution. Mouse anti-Hep27[17] (also known as dehydrogenase/reductase [SDR family] member 2; DHRS2) antibody, a gift from Dr. Franco Gabrielli (Università di Pisa, Pisa, Italy), was used at 1:1000 dilution. Mouse anti-human antibody to factor VIII-related antigen (BioGenex), was used at 1:100 dilution. Mouse monoclo-

nal anti-human CD-19 antibody (AbD Serotec, Raleigh, NC) was used at 1:200 dilution.

Antibody Generation Methods

Peptides to the SLITRK6 (Cys-SRPRKVLVEQTKNEYFEL-KANLHAEPDYLEVLEQQT) and neutral sphingomyelinase 2 (SMPD3 [TSKSSGQKGRKELLKGNGRRIDYMLHC]) proteins were synthesized and conjugated to keyhole limpet hemocyanin for the immunizations of rabbits. New Zealand White rabbits (5 to 6 lbs) were immunized three times with 200 μg of the peptide conjugate mixed with Freund's Complete Adjuvant for the primary immunization. Freund's Incomplete Adjuvant was used for all booster immunizations. The routes of injection were subcutaneous and intramuscular at multiple sites. Sera were collected from blood sampling after the third immunization. SLITRK6 antibody was used at 1:5000 dilution and SMPD3 antibody was used at 1:1000 dilution.

IHC on Paraffin-Embedded Breast Tumor and Normal Samples

The tissue was sectioned at 8 μmol/L onto Superfrost plus slides. Slides were dewaxed by immersing in xylene for 5 minutes twice. Slides were hydrated in 100% ethanol and 95% ethanol for 3 minutes each. Slides were quenched in 3% H2O2 for 10 minutes, and rinsed in 70% ethanol for 3 minutes, and then phosphate-buffered saline for 3 minutes. Citra buffer (BioGenex) was warmed in a 60°C oven and slides were immersed in citra buffer at 100°C in a rice steamer for 30 minutes. Slides were rinsed in phosphate-buffered saline for 3 minutes and then marked with a PAP pen. Primary antibody (100 μl to 200 μl) was applied and slides were placed in a sealed box in a 4°C cold room overnight. Slides were then rinsed in phosphate-buffered saline for 3 minutes, and 1 to 2 drops of biotinylated secondary antibody was added to each slide for 20 minutes. Slides were rinsed in phosphate-buffered saline for 3 minutes, and 1 to 2 drops of streptavidin-horseradish peroxidase was applied for 20 minutes. One to two drops of diaminobenzidine complex was applied and slides were placed in a dark drawer for approximately 10 minutes. Slides were rinsed in distilled water for 3 minutes and counterstained with trypan blue (Sigma, St Louis, MO) for 30 to 45 seconds. Slides were rinsed in phosphate-buffered saline, dehydrated through graded alcohol and xylene, and Cytoseal XYL (Richard-Allan, Kalamazoo, MI) and cover slides were applied. A negative control without primary antibody was performed for all experiments, and the positive control was factor VIII-related antigen.

Evaluation of Differential Protein Expression of Vascular Genes between Breast Tumor and Normal Breast Tissue

Once the vascular genes were confirmed to localize to endothelium, we next evaluated whether differential mRNA expression correlated with differential protein expression using IHC on paraffin-embedded breast tumors and normal breast tissue.

Selection of Breast Tumors

Three groups of formalin-fixed paraffin-embedded breast tumors were used, designated as luminal A, basal, or Her2/neu based on their immunophenotypes[18] ("luminal A" ER+, Her2/neu−; "basal" ER−, PR−, HER2/neu−; ck5/6+ or epidermal growth factor receptor positive; and "Her2/neu" ER−, PR−, Her2/neu+), as well as normal breast tissue from reduction mammoplasty. Normal breast tissues were first stained with antibody to factor VIII-related antigen, and only tissue that had vessels in the sample were used. ER−, PR−, Her2/neu− tumors were stained for CK5/6 antibody (clone 05/16B4 1:10 dilution, Boehringer Mannheim, Indianapolis, IN) as previously described[18] and epidermal growth factor receptor antibody (clone pharmDx, DakoCytomation) per manufacturer's instructions to further define the basal phenotype.

IHC Scoring

A single board-certified pathologist (C.A.L.) scored each tissue section for FAP, SFRP2, JAK3, SMPD3, SLITRK6, DHRS2, and CD19 expression based on a scoring system that measured intensity of stain in endothelium as: (Vessel Intensity Score) 0 = none; 1 = borderline; 2 = weak; 3 = moderate/strong; and percent positive endothelial cells staining as: 0 = none; 1 = 1 to 24%; 2 = 25 to 49%; 3 = 50 to 74%; and 4 = 75 to 100%. We then dichotomized and evaluated differences in the Vessel Intensity Score between tumors and normal, where a "high" score was 3 and a low score was 0 to 2. To further define angiogenesis expression, we dichotomized expression as high (3+ intensity and $\geq$75% positive cells) and not high (0, 1, or 2 intensity and/or $<$75% positive cells), and designated this as the Angiogenesis Score. Fisher's exact test was used to test for possible differences in proportions (or percentages) of expression, categorized as either 'high' or 'low' for both Angiogenesis Score and Vessel Intensity Score between luminal A versus normal, Her2neu versus normal, and basal versus normal tissue. Statistical analyses were performed using SAS statistical software, Versions 9.1 (SAS Institute Inc., Cary, NC).

Results

Vessel Isolation and Microarray Analysis

To study differences in gene expression between tumor and normal vessels, we performed rapid IHC with antibodies to factor VIII-related antigen, followed by LCM of vascular cells from five luminal A breast tumors and five normal breast tissue specimens from reduction mammoplasty. Immunostaining according to our protocol requires only 30 to 35 minutes from fixation to LCM. The

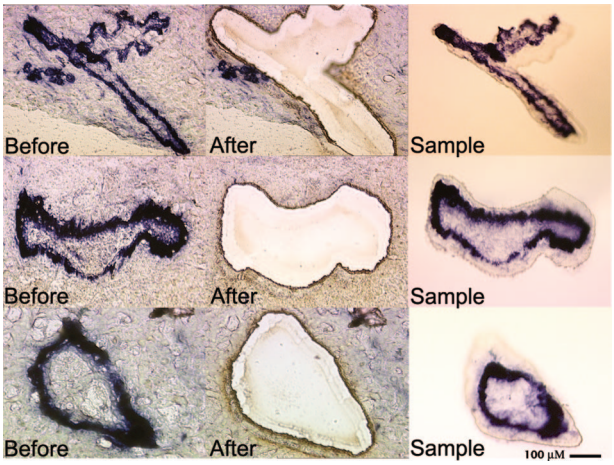

Figure 1. LCM of human breast vascular cells. Rapid IHC for factor VIII-related antigen was followed by LCM of vascular cells. Shown are representative breast tissue specimens before and after LCM along with the collected sample containing vessels (400×).

quality of staining was excellent, the vascular cells were easily identified, and LCM was performed successfully (Figure 1).

RNA amplification was performed using a two-round amplification system. RNA integrity was evaluated after the first round of amplification. The extracted RNA maintained its integrity as shown by RT-PCR detection of genes of different abundance levels (Figure 2). No signals were observed after amplification of the negative control (RNA extraction buffer without the microdissected sample, data not shown). RNA integrity was checked on all samples before microarray hybridization and only samples that maintained RNA integrity were used for microarray analyses.

To estimate our amplification bias, we compared one round of amplified RNA to two rounds of amplification of RNA extracted from human MDA-MB-435 breast cancer cells grown *in vitro*. When both amplified and unamplified RNA were hybridized to 44,000-element Agilent long-oligonucleotide DNA microarrays, we found correlation coefficients ranged from 0.95 to 0.97 among technical replicates.

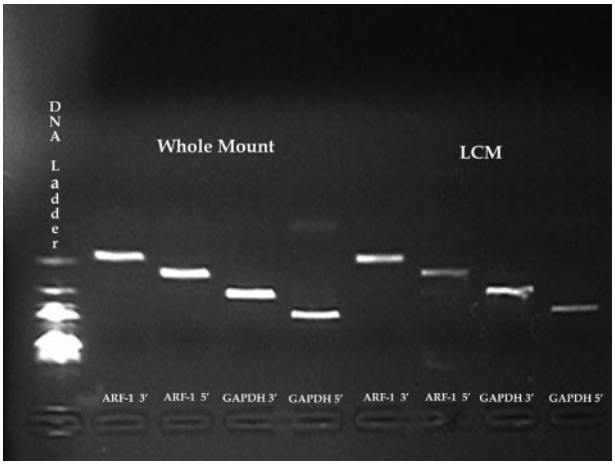

Figure 2. RNA integrity analyses. High-quality RNA isolated from breast tumor vessel cells. RT-PCR primers for genes of low and high abundance levels were used on cDNA from **Whole Mount**, which refers to a frozen section of the whole tumor before microdissection, and **LCM**, which refers to the sample of vessels microdissected from a frozen section of a human breast tumor. Lane 1, DNA ladder; lane 2, 3′ end of the low expressed ADP ribosylation factor I gene (ARF F1) from the Whole Mount (239 bp); lane 3, 5′ end of ARF F1 from the Whole Mount (336 bp); lane 4, 3′ end of the housekeeping gene GAPDH from the Whole Mount (540 bp); lane 5, 5′ end of GAPDH from the Whole Mount (887 bp); lane 6, 3′ end of ARF F1 from the microdissected vessel cells; lane 7, 5′ end of ARF F1 from the microdissected vessel cells; lane 8, 3′ end of GAPDH from the microdissected vessel cells; and lane 9, 5′ end of GAPDH from the microdissected vessel cells.

Confirmation of Vascular Cell Identity and Purity

Genes specific to endothelium were uniformly and highly expressed in the vascular cell specimens and endothelial cell lines with significantly lower expression seen in the breast tumor cell lines, confirming that our vascular cell samples were highly enriched for endothelium (Figure 3).

Tumor endothelial markers 1, 2, 4, 5, 6, 7, 7R, 8 (previously reported to be differentially expressed between colon tumor and normal endothelium)[7] were highly expressed in *both* the tumor and normal vascular cells relative to the low expression seen in the breast tumor cell lines (Figure 3). Previously reported breast specific tumor vascular genes (*hairy*-related basic helix-loop-helix, collagen, type IV, alpha 2, complement component 4A, secreted protein, acidic, cysteine-rich like 1, SNAIL1)[6] were also similarly highly expressed in our samples by *both* tumor and normal vascular cells, with low expression in the breast tumor cell lines (Figure 3). These results suggest that these are markers of breast endothelium, but their expression was not consistently higher in tumor versus normal vascular cells.

Platelet derived growth factor receptor β, a pericyte marker, was highly expressed in the vascular cell samples, which confirmed the presence of pericytes (Figure 3). There was high expression of genes specific to luminal breast tumor epithelium in the breast cancer cell lines, with low expression in the vascular cell samples and endothelial cell lines (Figure 3). This confirmed enrichment for endothelial cells and pericytes without high levels of expression of epithelial-associated genes.

Expression of hematopoietic markers in the vascular cell samples was similar to the expression in endothelial cell lines *in vitro* (Figure 3). CD45 (leukocytes) and CD22 (B cells) had low expression in LCM vessels and endothelial cell lines. CD14 (macrophages) and CD5 (T cells) were increased in both the vascular cell samples and the endothelial cell lines. This could be explained by the presence of RNA from macrophages and T cells in the vascular cell samples. Alternatively, it is possible that CD14 and CD5 were expressed on endothelial cells, as there is previous evidence for monocyte origin of vascular cell precursors[19] and expression of CD14 in endothelial cells[20]; CD14 was also elevated in a previous report of microdissected ovarian tumor endothelium,[21] and CD5 has also previously been reported to be present on vascular endothelium.[22]

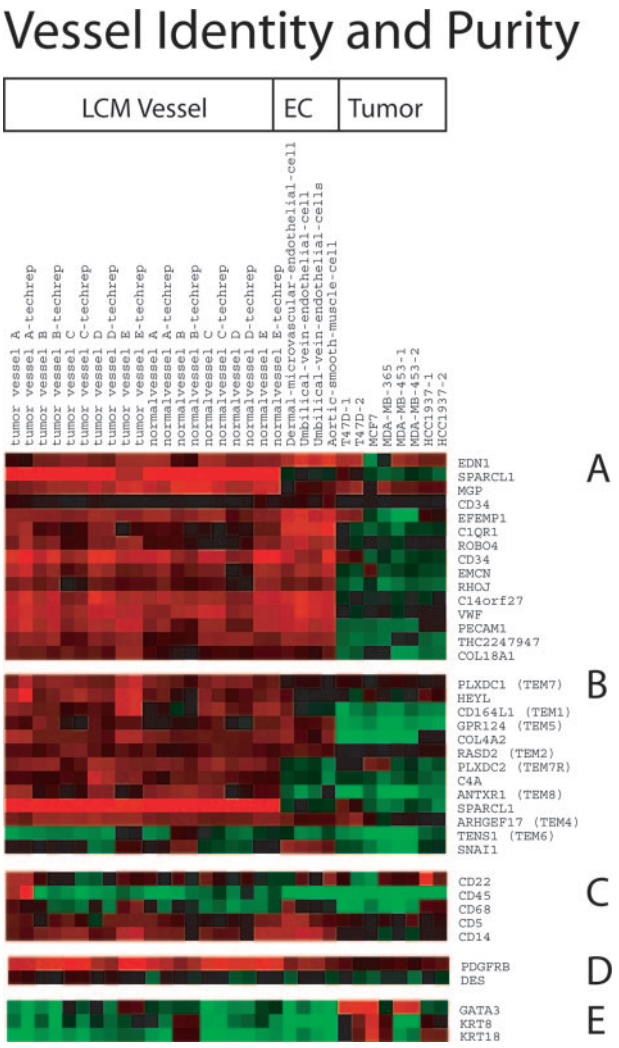

Figure 3. Gene expression analysis confirming vascular identity. The 44,000-feature long-oligo arrays were performed with RNA from vessel cells microdissected from five human luminal A breast tumors and five normal breast tissue samples from reduction mammoplasty, and compared to RNA from endothelial cells and breast tumor-derived cell lines cultured *in vitro*. Arrays for LCM vessel cells, endothelial cell lines, and breast tumor-derived cell lines were ordered from left to right. The arrays for LCM vessels were performed in duplicate, thus the designation technical replicate (**techrep**). Arrays from endothelial cells cultured *in vitro* are labeled: **Dermal-microvascular-endothelial-cell**, **Umbilical-vein-endothelial-cell**, **Umbilical-vein-endothelial-cells**, **Aortic-smooth-muscle-cell**. Arrays from breast tumor-derived cell lines *in vitro* are labeled: **T47D-1**, **T47D-2**, **MCF7**, **MDA-MB-365**, **MDA-MB-453**, **HCC1937-1**, and **HCC1937-2**. The data for different gene sets were identified, and clustered within each relevant category, in descending order: **A**, endothelial genes, **B**, TEMs, **C**, hematopoietic genes, **D**, pericyte genes, and **E**, epithelial genes.

Supervised Analysis of Tumor Versus Normal Vessels

Using SAM, we identified differentially expressed genes between tumor and normal vascular cells and found 1176 genes differentially expressed with a median number of false significant = 7.76 (Supplemental Table S1 available at *http://ajp.amjpathol.org*), of which 368 were increased. To interpret the gene list derived from SAM and convert the gene list into biological themes, we applied the Expression Analysis Systematic Explorer. When examining Bonferonni adjusted results, we found that the extracellular matrix ontology category was increased in tumor vascular cells, while the ribosome ontology category was decreased (Supplemental Table S2 available at *http://ajp.amjpathol.org*), demonstrating a separate biological response.

Confirmation of Vascular Origin of Vascular Marker Genes

To validate the vascular origin of the genes associated with tumor endothelium obtained by immuno-LCM we performed IHC on paraffin-embedded luminal A human breast tumors. Because our goal was to identify highly differentially expressed genes, we focused first on the 55 genes that had >fourfold increased expression in tumor vessel cells (Table 1). From this list, we searched Gene Cards to identify the potential subcellular location for genes with >fourfold increased expression, and focused on some of the genes that potentially encode membrane proteins (FAP, JAK3, SMPD3, SLITRK6, CD19), and a secreted protein (SFRP2). These would offer particularly good drug targets due to their accessibility. We also chose a gene that has recently been described to be expressed in endothelium *in vitro* (DHRS2).[23]

We used with antibodies to factor VIII-related antigen for a positive control to identify endothelium, and on subsequent sections, we performed IHC with antibodies to FAPα, SFRP2, JAK3, SMPD3, SLITRK6, DHRS2, and CD19.

Antibodies to FAP, SFRP2, JAK3, SMPD3, SLITRK6, and DHRS2 all showed staining with cellular localization in endothelium (Figure 4), as well as tumor stroma and tumor epithelium. CD19, a B-cell marker, did not localize to endothelium. Therefore, 6/7 vascular marker genes identified by immuno-LCM that we studied appear to be validated of vascular origin.

Evaluation of Differential Protein Expression of Vascular Genes between Breast Tumor Vessels and Normal Breast Vessels

For the six genes validated to be of vascular origin, we next evaluated whether differential mRNA expression correlated with differential protein expression using IHC on paraffin-embedded normal, luminal A, Her2/neu, and basal tumors. We failed to detect significant differential protein expression for SLTRK6 and DHRS2, possibly because there was very high staining in both the tumor endothelium and normal endothelium (data not shown). For SMPD3 there was no difference in the Angiogenesis Score for luminal A versus normal, but there was an increase in the Vessel Intensity Score comparing luminal A versus normal (15/16 (94%) vs. 6/10 (60%) $P = 0.05$). JAK3 had higher staining in luminal A and Her2/neu tumors compared to normal ($P = 0.01$ and $P = 0.006$, respectively, Figure 5D) and was nearly statistically significant for the Angiogenesis score ($P = 0.11$, Figure 5C). Basal tumors had very low expression of JAK3 (Figure 5 C, D). For FAP, the Angiogenesis Scores were signifi-

Table 1. Upregulated Genes in Tumor Vessel Cells with Greater than Fourfold Change

Gene symbol	Gene name	Fold change
NAT1	*N*-Acetyltransferase 1 (arylamine *N*-acetyltransferase)	17.6
DHRS2	Dehydrogenase/reductase (SDR family) member 2	11.9
IF127	Interferon, alpha-inducible protein 27	11.7
S100A8	S100A8 S100, calcium-binding protein A8 (calgranulin A)	11.7
MTL5	MTL5, metallothionein-like 5, testis-specific (tesmin)	10.9
FAP	FAP, fibroblast activation protein, alpha	10.7
IFI27	Interferon, alpha-inducible protein 27	10.1
UNG2	Uracil-DNA glycosylase 2	9.0
	THC1546313	8.9
APXL2	Apical protein 2	8.8
MGC16121	Hypothetical protein MGC16121	8.7
MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	8.1
MMP11	Matrix metalloproteinase 11 (stromelysin 3)	8.1
SULF1	Sulfatase 1	7.9
SLITRK6	SLIT and NTRK-like family, member 6	7.6
LTB	Lymphotoxin beta (TNF superfamily, member 3)	7.3
INHBA	Inhibin, beta A (activin A, activin AB alpha polypeptide)	7.2
	THC1598071	6.6
PREX1	Phosphatidylinositol 3,4,5-trisphosphate-dependent RAC exchanger 1	6.4
CHST8	Carbohydrate (*N*-acetylgalactosamine 4-0) sulfotransferase 8	6.4
SFRP2	Secreted frizzled-related protein 2	6.3
SMPD3	Sphingomyelin phosphodiesterase 3, neutral membrane	6.3
KAZALD1	Kazal-type serine peptidase inhibitor domain 1	6.2
FGFR3	Fibroblast growth factor receptor 3	6.2
SPOCD1	SPOC domain containing 1	6.1
IRF7	Interferon regulatory factor 7	5.9
COL1A2	Collagen, type I, alpha 2	5.8
CD19	CD19 antigen	5.7
BF	B-factor, properdin	5.6
SQLE	Squalene epoxidase	5.6
HOXB6	Homeo box B6	5.6
MLPH	Melanophilin	5.2
DKFZp434E2321	Hypothetical protein DKFZp434E2321	5.2
HTRA3	HtrA serine peptidase 3	5.1
T3JAM	TRAF3-interacting Jun N-terminal kinase (JNK)-activating modulator	4.9
ASCL2	Achaete-scute complex-like 2 (Drosophila)	4.9
	I_960623	4.7
HSPB1	Heat shock 27-kDa protein 1	4.6
COL12A1	Collagen, type XII, alpha 1	4.6
HOXB2	Homeo box B2	4.6
HIG2	Hypoxia-inducible protein 2	4.6
FLJ00332	FLJ00332 protein	4.6
JAK3	Janus kinase 3 (a protein tyrosine kinase, leukocyte)	4.5
S100P	S100 calcium binding protein P	4.5
RAMP1	Receptor (calcitonin) activity-modifying protein 1	4.4
COL5A1	Collagen, type V, alpha 1	4.4
CENPF	Centromere protein F, 350/400ka (mitosin)	4.3
DOK3	Docking protein 3	4.2
	AA516420	4.2
NID2	Nidogen 2 (osteonidogen)	4.2
	I_1000437	4.1
FGD3	FGD1 family, member 3	4.1
	Hypothetical gene supported by AK098833	4.1
AEBP1	AE binding protein 1	4.0
	A_23_BS21882	4.0

cantly higher in the luminal A, Her2/neu and basal tumors compared to normal ($P = 0.04$, $P = 0.03$, and $P = 0.03$, respectively, see Figure 5 A). For SFRP2, the Angiogenesis Score was significantly higher in luminal A tumors and basal tumors compared to normal, (Figure 5 B; $P = 0.03$ and $P = 0.02$, respectively) with near significance in Her2/neu tumors ($P = 0.10$). This appears to validate the original discovery of differential gene expression in luminal A versus normal vessel cells on a second sample using a different platform (IHC).

Discussion

An established vascular supply is critical for the continued growth of solid tumors and plays a major role in metastatic spread.[1] This importance has led to the concept of targeting the tumor vasculature as a form of cancer therapy. Previous studies have described tumor endothelial markers using a tissue dissociation and cell immunopurification approach to isolate tumor and normal endothelial cells, and have shown tumor specific differ-

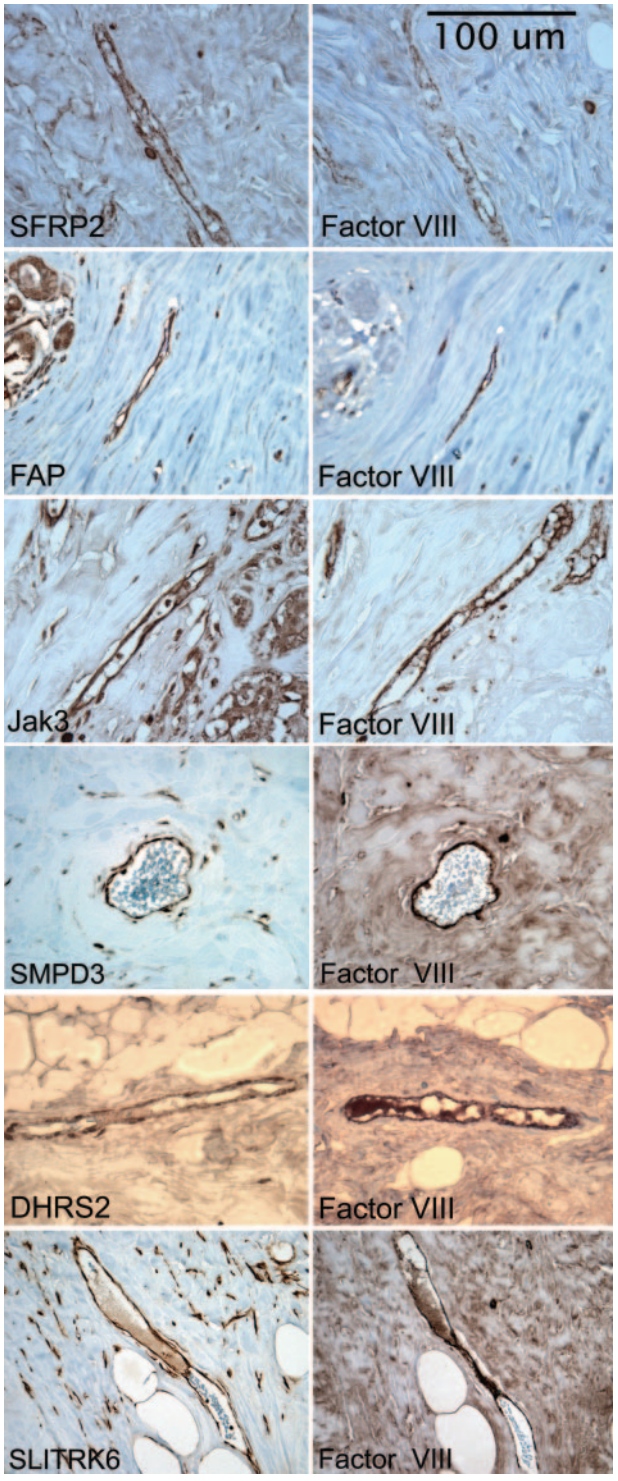

Figure 4. Confirmation of vascular origin of vascular marker genes. Parrafin-embedded human breast tumors were stained with antibodies to factor VIII-related antigen to identify vessels, and on the next section, an antibody to SFRP2, FAP, JAK3, SMPD3, DHRS2, or SLITRK6. Photographs were taken at ×600 magnification. Negative controls were performed with each experiment and showed no background staining (data not shown).

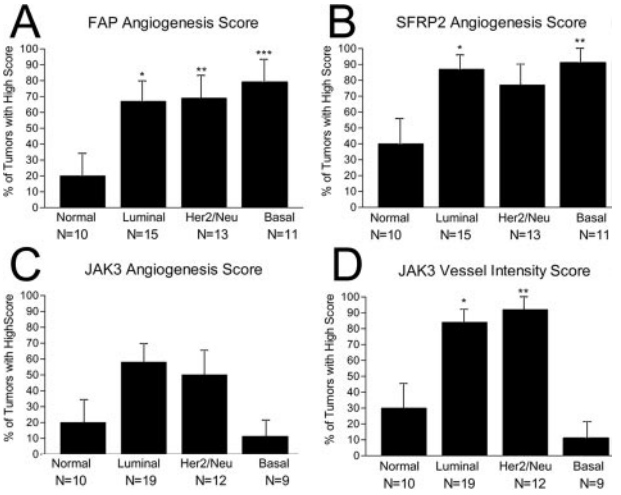

Figure 5. IHC with antibodies to FAP, SFRP2, and JAK3 on paraffin-embedded breast tumors and normal breast tissue. A high Angiogenesis Score refers to an intensity score of 3 (moderate to high staining) and percentage of enothelium staining >75%. A high Intensity Score refers to an intensity score of 3 (moderate to high staining). **A:** FAP had significantly higher Angiogenesis Score in luminal A, Her2/neu, and basal tumors compared to control ($*P = 0.04$, $**P = 0.03$, $***P = 0.03$). **B:** SFRP2 had significantly higher Angiogenesis Score in luminal A and basal tumors compared to normal ($*P = 0.03$, $**P = 0.02$), with near significance in Her2/neu tumors ($P = 0.10$). **C:** For JAK3, there was not enough evidence to show that the Angiogenesis Score was significantly higher in the tumors than the normal, however, in **D**, the Intensity Score for JAK3 was statistically significantly higher in luminal and Her2/neu tumors versus normal ($*P = 0.01$, $**P = 0.006$). Basal tumors had low expression of JAK3.

ences in tumor endothelial markers between colon, breast, and brain tumors.[6] Breast tumors are heterogeneous, and whether luminal, Her2/neu, and basal tumors share the same tumor endothelial markers is unknown. To realize fully the potential of targeting endothelial cells in breast cancer, it may be necessary to exploit new or yet to be identified targets of the tumor vasculature, which may be achieved by extracting vessels from a larger number of tumors. Therefore, our objective was to characterize molecular changes in breast tumor vessels from a larger sample size between luminal A breast tumor vessels and normal vessels, and then evaluate for protein expression of select genes in luminal A, Her2/neu, and basal tumors.

For this purpose, we performed rapid IHC and LCM of vascular cells from frozen human breast tumors, where the RNA is of high quality and sufficient for genomic analysis. This method requires only fifteen 8-μm sections, making this technique applicable to large scale analyses of breast tumors. We confirmed enrichment for endothelial cells and pericytes in our microdissected vessel specimens by demonstrating overexpression of endothelial and pericyte specific genes and low expression of luminal epithelial genes. This method differs from a cell immunopurification approach that selects out only endothelial cells. LCM of vascular cells includes all components of the vessel wall such as endothelial cells, basement membrane, pericytes, and endothelial precursor cells all of which are potential targets for disruption of tumor angiogenesis.

The normal breast tissue used in this study was from reduction mammoplasty specimens. We chose reduction mammoplasty specimens for normal controls because they have been shown to have lower rates of genomic instability compared to histologically normal breast tissue from mastectomy specimens with invasive tumor. For

example, one study found the amount of genomic instability in normal tissue immediately adjacent to the primary carcinomas was 15.4%, and normal tissue from quadrants from breasts with synchronous invasive or *in situ* carcinomas was 2.7 to 6.2%; whereas normal tissue from reduction mammoplasty samples was only <0.8%.[24] Whether reduction mammoplasty specimens, which come from women with hyperplastic breast tissue, have differences in endothelial gene expression relative to women without hyperplastic tissue is not known.

We found 1176 genes differentially expressed between breast tumor and normal vessels, and identified biological differences with the gene ontology category extracellular matrix increased and ribosome decreased in tumor vessels. Although it is possible that genes involved in the extracellular matrix could be increased if our microdissected samples contained tumor stromal cells, we think this is a less likely explanation for this finding. Overexpression of genes involved in the extracellular matrix has been seen consistently in previous reports of tumor endothelial markers where the endothelial cells were isolated by immunomagnetic beads, which would not have possible contamination of surrounding tumor stroma. For tumor cells to invade the stroma and enter into the circulation, they have to cross the extracellular matrix. This process requires proteinases or the modification of the extracellular matrix architecture. St Croix et al[7] compared endothelium from colon cancer to normal endothelium and found that of the top 25 tags most differentially expressed, at least seven encode proteins involved in extracellular matrix formation or remodeling. Madden et al[5] isolated glioma endothelial cells and found among the gene products identified as glioma endothelial markers, several genes regulated tumor endothelium extracellular matrix architecture, including heparan sulfate proteoglycan 2 (perlecan), several type IV collagen transcript variants, and matrix metalloprotease 14. Parker et al[6] isolated breast cancer endothelial cells and also found several breast tumor vascular genes that regulate the extracellular matrix, including osteonectin, matrix metalloprotease 9, and tissue inhibitor of metalloproteinase 1. These findings suggest that changes in the extracellular matrix is a fundamental component of alterations in tumor endothelium. The significance of the decrease in ribosome gene ontology category is unclear. A decrease in ribosome gene expression was also seen by Parker et al,[6] where breast tumor endothelial cells had decreased expression of ribosomal protein S20, ribosomal protein S10, poly(A) polymerase α, and protein transport protein SEC61 α subunit. The biological significance of this finding requires further study.

Since our goal is to identify targets for the development of novel angiogenesis inhibitors, we focused on genes that had the highest degree of increased expression and found 55 genes that had >fourfold increased expression in tumor vessels. We further focused on secreted and membrane proteins, because these proteins have properties that lend themselves to be used as therapeutic agents or targets. Because they are present on the cell surface or within the extracellular space, they are directly accessible via the bloodstream, facilitating intervention with both macromolecular compounds and small molecules.

We first confirmed localization to vessels of the protein products of select gene transcripts using IHC with antibodies to FAP, SFRP2, JAK3, SMPD3, SLITRK6, and DHRS2. This appears to validate the use of immuno-LCM to identify genes localized to the endothelium. These proteins were not selectively expressed only in vessels, but were also expressed in the tumor epithelium and stroma. With the exception of FAP, these proteins have not previously been shown to be expressed at the protein level on blood vessels.

Although the measurement of transcribed mRNA has demonstrated to be very effective in the discovery of molecular markers and the elucidation of functional mechanisms, in many cases mRNA abundance is not a reliable indicator of corresponding protein abundance.[25] Therefore validation of differential protein expression of select tumor endothelial markers is critical to identifying targets for developing molecularly targeted angiogenesis inhibitors. For this reason we evaluated differential protein expression between luminal A breast tumors and normal breast tissue; and then evaluated protein expression in Her2/neu and basal breast tumors. The proteins that were statistically significantly expressed between tumor and normal vessels were FAP, SFRP2, JAK3, and SMPD3. Although there was significantly greater intensity of staining in luminal A tumors for SMPD3 compared to normal, the high level of staining in the normal vessels makes this a less attractive target. FAP and SFRP2 both had high staining in luminal A, Her2/neu, and basal tumors. In contrast, JAK3 had high staining for luminal A and Her2/neu tumors, with very low expression in basal tumors. Although tumor endothelial markers have previously been shown to be differentially expressed between tumor types (ie, colon, breast, and brain), to our knowledge JAK3 is the first tumor endothelial marker demonstrated to have differential protein expression based on breast tumor subtype.

SFRP2 belongs to a large family of SFRPs that are expressed in many cell types during embryogenesis and participate in Wnt-signaling and apoptosis,[26] and has shown to be expressed in canine breast cancer.[27] FAPα (also known as seprase) is a serine gelatinase expressed in different tumor types and plays a role in tumor cell invasion.[28–30] JAK3 is a tyrosine kinase involved in cytokine signaling and is predominantly expressed in lymphoid and myeloid lineages.[31] SMPD3, a sphingomyelinase, plays an important role in ceramide-mediated cell regulation and plays an important role in inducing cell growth, differentiation, and apoptosis.[32] Further studies are needed to elucidate the angiogenic properties of these tumor endothelial markers.

In summary, our studies demonstrate that gene expression patterns in breast tumor vessels differ greatly from normal vessels with biological differences in extacellular matrix and ribosomes, and differential expression of over 1000 genes. We found that FAP, SFRP2, JAK3, and SMPD3 are TEMs in human breast cancer. This suggests that these proteins may serve as potential tar-

gets for the development of novel angiogenesis inhibitors for breast cancer.

Acknowledgments

We acknowledge the technical assistance and support of the Tissue Procurement Core Facility, a core laboratory of the UNC Lineberger Comprehensive Cancer Center Specialized Program in Research Excellence (SPORE), and the Immunology Core Facility at the UNC Lineberger Comprehensive Cancer Center. Laser microdissection was performed in the Michael Hooker Microscopy Facility at University of North Carolina at Chapel Hill.

References

1. Folkman J: Tumor angiogenesis: therapeutic implications. N Engl J Med 1971, 285:1182–1186
2. Miller K: E2100 Study. Scientific session on monoclonal antibody therapy in breast cancer. Annual Meeting of the American Society of Clinical Oncology 2005, 8–29
3. Schneider BP, Sledge GW Jr: Drug insight: vEGF as a therapeutic target for breast cancer. Nat Clin Pract Oncol 2007, 4:181–189
4. Buckanovich RJ, Sasaroli D, O'brien-Jenkins A, Botbyl J, Hammond R, Katsaros D, Sandaltzopoulos R, Liotta LA, Gimotty PA, Coukos G: Tumor vascular proteins as biomarkers in ovarian cancer. J Clin Oncol 2007, 25:852–861
5. Madden SL, Cook BP, Nacht M, Weber WD, Callahan MR, Jiang Y, Dufault MR, Zhang X, Zhang W, Walter-Yohrling J, Rouleau C, Akmaev VR, Wang CJ, Cao X, St MT, Roberts BL, Teicher BA, Klinger KW, Stan RV, Lucey B, Carson-Walter EB, Laterra J, Walter KA: Vascular gene expression in nonneoplastic and malignant brain. Am J Pathol 2004, 165:601–608
6. Parker BS, Argani P, Cook BP, Liangfeng H, Chartrand SD, Zhang M, Saha S, Bardelli A, Jiang Y, St Martin TB, Nacht M, Teicher BA, Klinger KW, Sukumar S, Madden SL: Alterations in vascular gene expression in invasive breast carcinoma. Cancer Res 2004, 64:7857–7866
7. St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, Kinzler KW: Genes expressed in human tumor endothelium. Science 2000, 289:1197–1202
8. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D: Molecular portraits of human breast tumours. Nature 2000, 406:747–752
9. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein LP, Borresen-Dale AL: Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 2001, 98:10869–10874
10. Bagley RG, Weber W, Rouleau C, Teicher BA: Pericytes and endothelial precursor cells: cellular interactions and contributions to malignancy. Cancer Res 2005, 65:9741–9750
11. Hu Z, Troester M, Perou CM: High reproducibility using sodium hydroxide-stripped long oligonucleotide DNA microarrays. Biotechniques 2005, 38:121–124
12. Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, Speed TP: Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res 2002, 30:e15
13. Storey JD: A direct approach to false discovery rates. J Royal Stat Soc 2002, Series B:479–498
14. Tusher VG, Tibshirani R, Chu G: Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 2001, 98:5116–5121
15. Ho M, Yang E, Matcuk G, Deng D, Sampas N, Tsalenko A, Tabibiazar R, Zhang Y, Chen M, Talbi S, Ho YD, Wang J, Tsao PS, Ben-Dor A, Yakhini Z, Bruhn L, Quertermous T: Identification of endothelial cell genes by combined database mining and microarray analysis. Physiol Genomics 2003, 13:249–262
16. Saldanha AJ: Java Treeview–extensible visualization of microarray data. Bioinformatics 2004, 20:3246–3248
17. Pellegrini S, Censini S, Guidotti S, Iacopetti P, Rocchi M, Bianchi M, Covacci A, Gabrielli F: A human short-chain dehydrogenase/reductase gene: structure, chromosomal localization, tissue expression and subcellular localization of its product. Biochim Biophys Acta 2002, 1574:215–222
18. Livasy CA, Karaca G, Nanda R, Tretiakova MS, Olopade OI, Moore DT, Perou CM: Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. Mod Pathol 2006, 19:264–271
19. Coukos G, Benencia F, Buckanovich RJ, Conejo-Garcia JR: The role of dendritic cell precursors in tumour vasculogenesis. Br J Cancer 2005, 92:1182–1187
20. Jersmann HP: Time to abandon dogma: cD14 is expressed by non-myeloid lineage cells. Immunol Cell Biol 2005, 83:462–467
21. Buckanovich RJ, Sasaroli D, O'brien-Jenkins A, Botbyl J, Conejo-Garcia JR, Benencia F, Liotta LA, Gimotty PA, Coukos G: Use of immuno-LCM to identify the in situ expression profile of cellular constituents of the tumor microenvironment. Cancer Biol Ther 2006, 5:635–642
22. Gogolin-Ewens K, Meeusen E, Lee CS, Brandon M: Expression of CD5, a lymphocyte surface antigen on the endothelium of blood vessels. Eur J Immunol 1989, 19:935–938
23. Shafqat N, Shafqat J, Eissner G, Marschall HU, Tryggvason K, Eriksson U, Gabrielli F, Lardy H, Jornvall H, Oppermann U: Hep27, a member of the short-chain dehydrogenase/reductase family, is an NADPH-dependent dicarbonyl reductase expressed in vascular endothelial tissue. Cell Mol Life Sci 2006, 63:1205–1213
24. Ellsworth DL, Ellsworth RE, Liebman MN, Hooke JA, Shriver CD: Genomic instability in histologically normal breast tissues: implications for carcinogenesis. Lancet Oncol 2004, 5:753–758
25. Gygi SP, Rochon Y, Franza BR, Aebersold R: Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 1999, 19:1720–1730
26. Lee JL, Chang CJ, Chueh LL, Lin CT: Secreted frizzled related protein 2 (SFRP2) decreases susceptibility to UV-induced apoptosis in primary culture of canine mammary gland tumors by NF-kappaB activation or JNK suppression. Breast Cancer Res Treat 2006, 100:49–58
27. Lee JL, Chang CJ, Wu SY, Sargan DR, Lin CT: Secreted frizzled-related protein 2 (SFRP2) is highly expressed in canine mammary gland tumors but not in normal mammary glands. Breast Cancer Res Treat 2004, 84:139–149
28. Aoyama A, Chen WT: A 170-kDa membrane-bound protease is associated with the expression of invasiveness by human malignant melanoma cells. Proc Natl Acad Sci USA 1990, 87:8296–8300
29. Chen WT, Lee CC, Goldstein L, Bernier S, Liu CH, Lin CY, Yeh Y, Monsky WL, Kelly T, Dai M, Mueller SC: Membrane proteases as potential diagnostic and therapeutic targets for breast malignancy. Breast Cancer Res Treat 1994, 31:217–226
30. Monsky WL, Lin CY, Aoyama A, Kelly T, Akiyama SK, Mueller SC, Chen WT: A potential marker protease of invasiveness, seprase, is localized on invadopodia of human malignant melanoma cells. Cancer Res 1994, 54:5702–5710
31. Yamaoka K, Saharinen P, Pesu M, Holt VE III, Silvennoinen O, O'Shea JJ: The Janus kinases (Jaks). Genome Biol 2004, 5:253
32. Tani M, Hannun YA: Analysis of membrane topology of neutral sphingomyelinase 2. FEBS Lett 2007, 581:1323–1328