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7. The plasmid GBT9-APC, which encodes the GAL4 DNA-binding domain fused to the armadillo repeat domain of human APC (amino acids 446 to 880), was used as target in two-hybrid screens of a human fetal brain cDNA library (Clontech). Seven clones containing a fragment of the Asef cDNA were obtained from 1.1×10^7 transformants. The remaining 5′- and 3′-end regions were obtained by the 5′ and 3′ rapid amplification of cDNA ends (RACE) systems, respectively, with the "Marathon"-ready human brain cDNA (Clontech).
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9. Y. Kawasaki *et al.*, unpublished data.
10. The ^{35}S-methionine–labeled armadillo repeat domain of APC and Asef−M were synthesized by use of the coupled transcription-translation TNT system (Promega). GST and GST-fusion proteins (2 μg) immobilized to glutathione-Sepharose beads were mixed with in vitro–translated proteins in binding buffer [20 mM tris-HCl (pH 8.0), 140 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol (DTT), 10% glycerol, 1% Triton X-100, aprotinin (10 μg/ml), leupeptin and pepstatin] for 1 hour at 4°C. After five washes with binding buffer, bound proteins were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) followed by autoradiography.
11. Antibodies to Asef were prepared by immunizing rabbits with a peptide containing amino acids 73 to 126 of Asef. Antibodies to APC were prepared by immunizing rabbits with a peptide containing amino acids 1122 to 1729 of APC. Mouse monoclonal antibody (mAb) to APC was raised against the COOH-terminal 15 amino acids of APC. Antibodies were purified by affinity chromatography with columns to which the antigens used for immunization had been linked. Mouse mAbs to β-catenin and the hemagglutinin (HA) tag (12CA5) were from Transduction Laboratories and Boehringer, respectively. Rabbit polyclonal antibody to the Myc tag (polyclonal version of 9E10) was from MBL. Immunoprecipitation was performed as in (*6*). Immunoblotting analysis was performed with alkaline phosphatase–conjugated mouse anti-rabbit immunoglobulin G (IgG) or goat anti-mouse IgG (Promega) as a second antibody. On the basis of the immunoblotting and immunoprecipitation experiments, we estimate that about 10 to 20% of Asef and APC are included in the Asef-APC complex (*9*).
12. Supplemental Web material is available at *Science* Online at www.sciencemag.org/feature/data/1049013.shl.
13. Serial frozen sections of mouse colon and brain were stained with anti-APC or anti-Asef and visualized by incubation with avidin-biotin-peroxidase complex (*14*).
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16. Double-labeling immunoelectron microscopy was performed with rabbit anti-Asef and mouse mAb to APC as in (*6*).
17. His_6-AsefΔAPC bound to ProBond Resin (Invitrogen) was mixed with RhoA, Rac1, and GST-Cdc42 in buffer A [50 mM Hepes (pH 7.0), 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM DTT, phenylmethylsulphonyl fluoride (50 μg/ml), leupeptin (1 μg/ml), and aprotinin (1 μg/ml)] containing 0.1% NP-40 for 1 hour at 4°C and then washed extensively with buffer A. Proteins adhering to the resin were analyzed by 13% SDS-PAGE and subjected to immunoblotting. Mouse mAb to Rac1 was from Transduction Laboratories. Rabbit polyclonal antibodies to Cdc42Hs and mouse mAb to RhoA were from Santa Cruz.
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19. The [^{3}H]GDP-bound form of Rac1 was obtained by incubating 2 pmol of Rac1 with 0.2 μM [^{3}H]GDP for 20 min at 30°C in 20 mM tris-HCl (pH 8.0), 3 mM $MgCl_2$, 10 mM EDTA, and 1 mM DTT. To prevent dissociation of [^{3}H]GDP from Rac1, we added 375 mM $MgCl_2$ to a final concentration of 18 mM, and the mixtures were immediately cooled on ice. Dissociation of [^{3}H]GDP was measured at 25°C in 20 mM tris-HCl (pH 8.0), 6 mM $MgCl_2$, 3.5 mM EDTA, and 1 mM DTT by addition of a 250-fold excess of unlabeled GDP, GTP, and the indicated amounts of Asef. The reaction was stopped by addition of 1 ml of ice-cold stop buffer [20 mM tris-HCl (pH 8.0), 25 mM $MgCl_2$, and 100 mM NaCl]. The diluted mixtures were passed through nitrocellulose filters, the filters were washed several times with stop buffer, and the trapped radioactivity was counted.
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21. Canine kidney epithelial MDCK cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Plasmids encoding HA-tagged Asef, HA-tagged AsefΔAPC, Myc-tagged APC-arm, green fluorescent protein (GFP), and GFP-ABR were subcloned into pcDNA3.1(+) and transfected into MDCK cells with LipofectAMINE (Life Technologies). Cells were double-stained with antibodies against HA, Myc, and APC. Staining patterns obtained with anti-HA were visualized with fluorescein isothiocyanate–labeled anti-rabbit IgG; those obtained with anti-Myc and anti-APC were visualized with rhodamine-labeled anti-mouse IgG. The cells were photographed with a Carl Zeiss LSM510 laser-scanning microscope.
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25. We thank Y. Takai for recombinant Rac1, Rho, and Cdc42; K. Kohno and M. Kurimoto for mouse mAbs to APC; and M. Lamphier for reading the manuscript. Supported by Grants-in-Aid for Scientific Research on Priority Areas and the Organization for Pharmaceutical Safety and Research.

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Genes Expressed in Human Tumor Endothelium

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To gain a molecular understanding of tumor angiogenesis, we compared gene expression patterns of endothelial cells derived from blood vessels of normal and malignant colorectal tissues. Of over 170 transcripts predominantly expressed in the endothelium, 79 were differentially expressed, including 46 that were specifically elevated in tumor-associated endothelium. Several of these genes encode extracellular matrix proteins, but most are of unknown function. Most of these tumor endothelial markers were expressed in a wide range of tumor types, as well as in normal vessels associated with wound healing and corpus luteum formation. These studies demonstrate that tumor and normal endothelium are distinct at the molecular level, a finding that may have significant implications for the development of anti-angiogenic therapies.

Tumors require a blood supply for expansive growth (*1–3*), an observation that has stimulated a profusion of research on tumor angiogenesis. However, several basic questions about tumor vessels remain unanswered. For example, is endothelium that lines blood vessels in tumors qualitatively different from endothelium in vessels of normal tissue? What is the relation of tumor angiogenesis to angiogenesis associated with wound healing or other physiological processes? The answers to these questions critically impact the potential for new therapeutic approaches to inhibit angiogenesis in a tumor-specific manner.

To determine if tumor-specific endothelial markers exist, we compared gene expression profiles in endothelium derived from normal and tumor tissue. Human colorectal cancer was chosen for these studies because it has a high incidence, tends to grow slowly, and is often resistant to chemotherapeutic drugs. Importantly, the progressive growth of this tumor type appears to be angiogenesis-dependent (*4*).

Global analysis of gene expression in tumor and normal endothelium is difficult be-

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cause (i) the endothelium is enmeshed in a complex tissue consisting of vessel wall components, stromal cells, and epithelial cells, and (ii) only a small fraction of the cells within these tissues are endothelial. Thus, we needed to develop methods for isolating highly purified endothelial cells (ECs) and for evaluating gene expression profiles from relatively few cells.

To overcome the first obstacle, we attempted to purify ECs from dispersed human colorectal tissue using CD31, an endothelial marker commonly used for this purpose (*5–8*). This resulted in a substantial enrichment of ECs but also in contamination of the preparations by hematopoietic cells, most likely due to expression of CD31 by macrophages (*9*). We therefore purified ECs from human tissues using P1H12, a recently described marker for ECs (*10*). Unlike CD31, CD34, and VE-cadherin, P1H12 specifically localized to ECs of all vessels including microvessels of normal and cancerous colorectal tissue (Fig. 1A). Our purification protocol also optimized the detachment of ECs from neighboring cells, leaving cell surface proteins intact, and included positive and negative affinity purifications using a mixture of antibodies (Fig. 1B). The ECs purified from normal colorectal mucosa and colorectal cancers were essentially free of epithelial and hematopoietic cells as judged by reverse transcription–polymerase chain reaction (RT-PCR) (Fig. 1C) and subsequent gene expression analysis (see below).

To evaluate gene expression, we used a modification of the serial analysis of gene expression (SAGE) technique (*11*). SAGE associates individual mRNA transcripts with 14–base pair (bp) tags derived from a specific position near their 3′ termini (*12*). The abundance of each tag provides a quantitative measure of the transcript level present within the mRNA population studied. SAGE is not dependent on preexisting databases of expressed genes and therefore provides an unbiased view of gene expression profiles. This feature is particularly important in the analysis of cells that constitute only a small fraction of the tissue under study, as transcripts from these cells are unlikely to be well represented in extant expressed sequence tag (EST) databases.

We generated a SAGE library of ~96,000

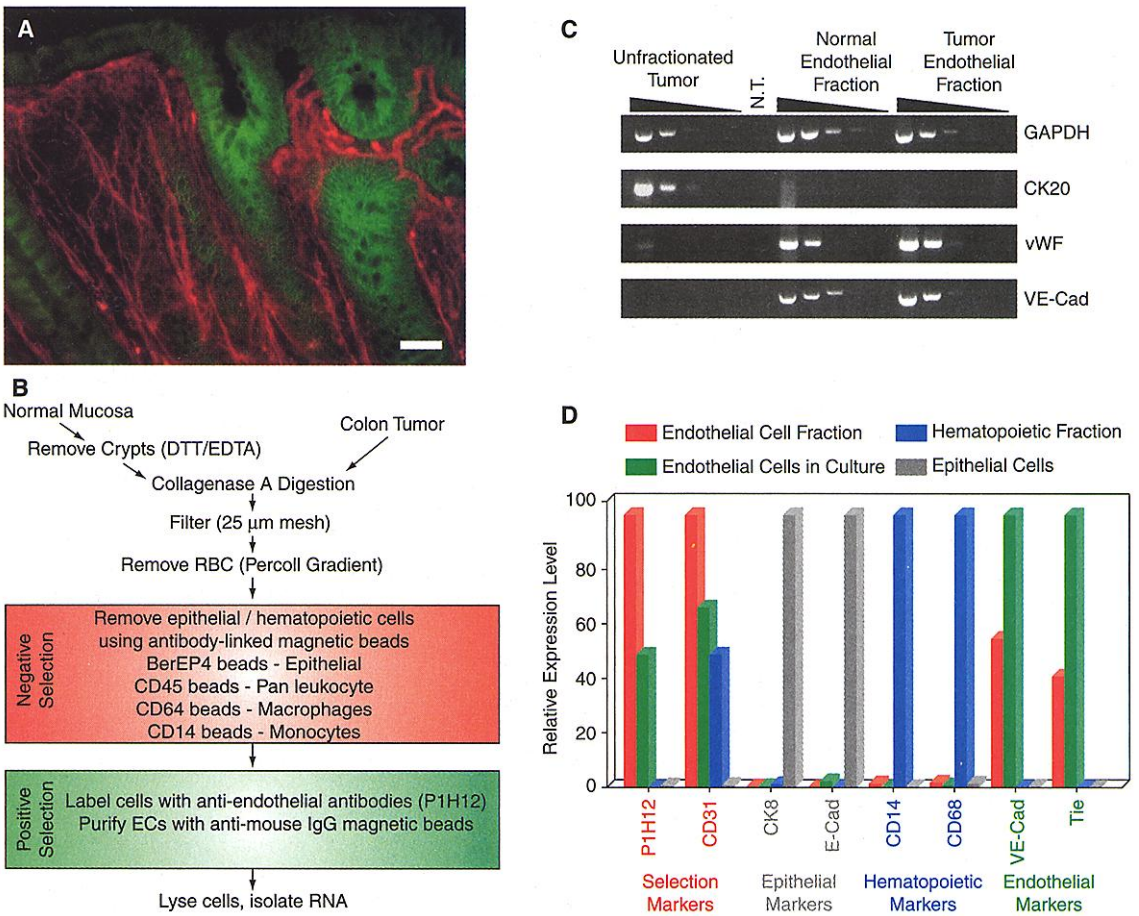

Fig. 1. Purification of ECs from human normal and malignant tissue. **(A)** Vessels (red) of frozen sections were stained by immunofluorescence with anti-P1H12 (Chemicon, Temecula, California) and detected with a biotinylated goat anti-mouse IgG secondary antibody followed by rhodamine-linked strepavidin. The vessels stained are from within the lamina propria of normal colonic mucosa. E-cadherin–positive epithelial cells (green) at the edge of the crypt were simultaneously visualized with a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California), followed by a goat anti-rabbit IgG secondary antibody labeled with Alexa (Molecular Probes, Eugene, Oregon). Bar, 50 μM. **(B)** For isolation of pure EC populations from collagenase-dispersed tissues, the epithelial and hematopoietic cell fractions were sequentially removed by negative selection with magnetic beads. The remaining cells were stained with P1H12, and ECs were isolated by positive selection with magnetic beads (*39*). **(C)** RT-PCR analysis used to assess the purity of the EC preparations. Semiquantitative PCR analysis was performed on cDNA generated directly from colorectal cancer tissue (Unfractionated Tumor) or from purified ECs isolated from normal colonic mucosa (Normal Endothelial Fraction) or colorectal cancer (Tumor Endothelial Fraction). Expression of the epithelial-specific transcript cytokeratin 20 (CK20) was limited to the unfractionated tumor. Two endothelial-specific transcripts, vWF and VE-cadherin (VE-Cad), showed robust amplification only in the endothelial fractions, whereas transcripts corresponding to the ubiquitous housekeeping enzyme glyceraldehyde phosphate dehydrogenase (GAPDH) were amplified in all samples. No signal was detected in the no-template (N.T.) control. cDNA templates were diluted 1:10, 1:100, 1:1000, 1:4000, and 1:40,000, as indicated by the declining wedge. **(D)** The relative expression level of select genes was determined by measurement of the tag abundance from several SAGE libraries combined into four groups. The first was composed of ~193,000 tags from the two in vivo–derived EC preparations (Endothelial Cell Fraction), whereas the second contained a single library of ~57,000 tags containing macrophages and other leukocytes derived from the negative selection (Hematopoietic Fraction). The third group contained ~401,000 tags from cultured HUVEC and HMVEC (Endothelial Cells in Culture), and the fourth consisted of ~748,000 tags from six colon cancer cell lines in culture (Epithelial Cells). After normalization, the library with the highest tag number for each marker was given a value of 100%, and the corresponding relative expression levels of the remaining three libraries were plotted on the ordinate. A high level of CD31 is apparent on hematopoietic cells, the likely cause of the impurity of the initial endothelial selection, compared with the selectivity of P1H12.

tags from the purified ECs of a colorectal cancer and a similar library from the ECs of normal colonic mucosa from the same patient. These ~193,000 tags corresponded to over 32,500 unique transcripts (*13*). The expression pattern of hematopoietic, epithelial, and endothelial markers confirmed the purity of the preparations (Fig. 1D).

We next identified pan endothelial markers (PEMs), that is, transcripts that were expressed at substantially higher levels in both normal and tumor-associated endothelium compared with other tissues. Tags expressed at similar levels in both tumor and normal ECs were compared with ~1.8 million tags from a variety of cell lines derived from tumors of nonendothelial origin. This simple comparison identified 93 transcripts that were expressed at levels at least 20-fold higher in ECs in vivo compared with nonendothelial cells in culture (*14*). Among the most abundant transcripts, there were 15 tags corresponding to characterized genes, 12 of which had been previously shown to be preferentially expressed in endothelium (*10*, *15–26*), and the other 3 genes not previously associated with endothelium (Table 1). These data also revealed many novel PEMs, which became increasingly prevalent as tag expression levels decreased (Table 1). We validated the expression of selected PEMs in vivo using a highly sensitive nonradioactive in situ hybridization method that allowed the detection of relatively rare transcripts from frozen sections of human tissues (*27*). Expression of PEM3 and PEM6 was limited to vascular ECs in both normal and neoplastic tissues (Fig. 2, A and B). For other PEMs, their endothelial origin was confirmed by SAGE analysis of ~401,000 transcripts derived from primary cultures of human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HMVEC) (Table 1). These data also suggest that ECs maintained in culture do not completely recapitulate expression patterns observed in vivo. For example, Hevin and several other PEMs were expressed at high levels in both tumor and normal ECs in vivo, but few or no transcripts were detected in cultured HUVEC or HMVEC (Table 1). The source of the Hevin transcripts

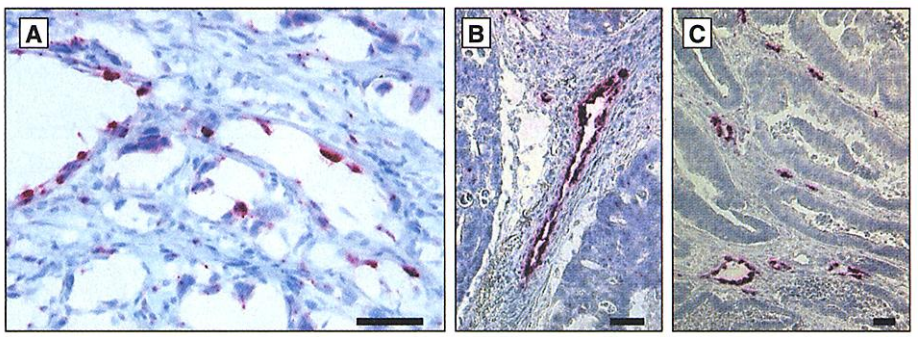

Fig. 2. Expression of PEMs is limited to ECs. (**A** to **C**) The endothelial origin of PEMs identified by SAGE was confirmed by a highly sensitive in situ hybridization assay (*27*). Localization of novel PEMs to the ECs (red stain) was demonstrated by examining two representative PEMs, PEM3 (A) and PEM6 (B), in lung cancer and colon cancer, respectively. Hevin expression was readily detected in the ECs of a colon tumor (C) despite its low level of expression in cultured ECs. Bars, 50 μM.

Table 1. SAGE analysis reveals previously characterized and novel pan endothelial markers. The most abundant characterized or novel tags derived by summing the tags from normal EC (N-ECs) and tumor EC (T-ECs) SAGE libraries are listed in descending order. For comparison, the corresponding number of SAGE tags found in HUVEC and HMVEC endothelial cell cultures, and several nonendothelial cell lines (*14*), are shown. Tag numbers for each group were normalized to 100,000 transcripts. A description of the gene product corresponding to each tag is given, followed by alternative names in parentheses. Some uncharacterized genes have predicted full-length coding sequence. The sequence CATG precedes all tags, and the 15th base (11th shown) was determined as described (*38*).

Genes determined by SAGE analysis to be potential pan endothelial markers							
Tag sequence	N-ECs	T-ECs	HUVEC	HMVEC	Cell lines	Description	
Known genes							
CATATCATTAA	247	501	130	87	2	Angiomodulin (ANG, IGFBP-7, IGFBP-rP1, Mac25, TAF)*	
TGCACTTCAAG	328	141	0	0	0	Hevin*	
TTTGCACCTTT	165	84	191	115	4	Connective tissue growth factor (CTGF, IGFBP-rP2)*	
TTGCTGACTTT	73	131	2	14	1	Collagen, type VI, alpha 1*	
ACCATTGGATT	102	67	0	0	2	Interferon induced transmembrane protein 1 (9-27, Leu 13)*	
ACACTTCTTTC	104	44	60	62	2	Guanine nucleotide binding protein 11	
TTCTGCTCTTG	71	67	118	72	0	Von Willebrand factor*	
TCCCTGGCAGA	66	68	3	13	3	Cysteine-rich protein 2 (CRP-2, ESP-1, SmLIM)	
TAATCCTCAAG	26	106	34	16	1	Collagen, type XVIII, alpha 1*	
ATGTCTTTTCT	58	65	17	17	3	Insulin-like growth factor-binding protein 4*	
GGGATTAAAGC	40	67	30	14	2	CD146 (S-Endo 1, P1H12, Muc18, MCAM, Mel-CAM)*	
TTAGTGTCGTA	38	69	9	13	0	SPARC (osteonectin, BM-40)*	
TTCTCCCAAAT	20	86	16	64	2	Collagen, type IV, apha 2*	
GTGCTAAGCGG	24	74	0	10	2	Collagen, type VI, alpha 2*	
GTTTATGGATA	35	56	11	11	1	Matrix Gla protein (MGP)	
Novel genes							
CCCTTGTCCGA	131	104	1	1	0	PEM1	ESTs
CCCTTTCACAC	52	33	0	0	0	PEM2	ESTs
CAACAATAATA	42	25	13	6	0	PEM3	ESTs
GGCCCTACAGT	26	13	2	3	0	PEM4	ESTs/KIAA0821 protein
GCTAACCCCTG	7	31	0	1	0	PEM5	ESTs
GGCACTCCTGT	22	13	19	12	0	PEM6	ESTs
TCACAGCCCCC	20	15	8	5	0	PEM7	ESTs
TTTCATCCACT	20	13	0	2	0	PEM8	ESTs, KIAA0362 protein
ATACTATAATT	25	6	2	0	0	PEM9	ESTs
AATAGGGGAAA	13	19	4	1	0	PEM10	KIAA1075 protein

*Characterized genes that have previously been shown to be expressed predominantly in endothelium (*10*, *15–26*).

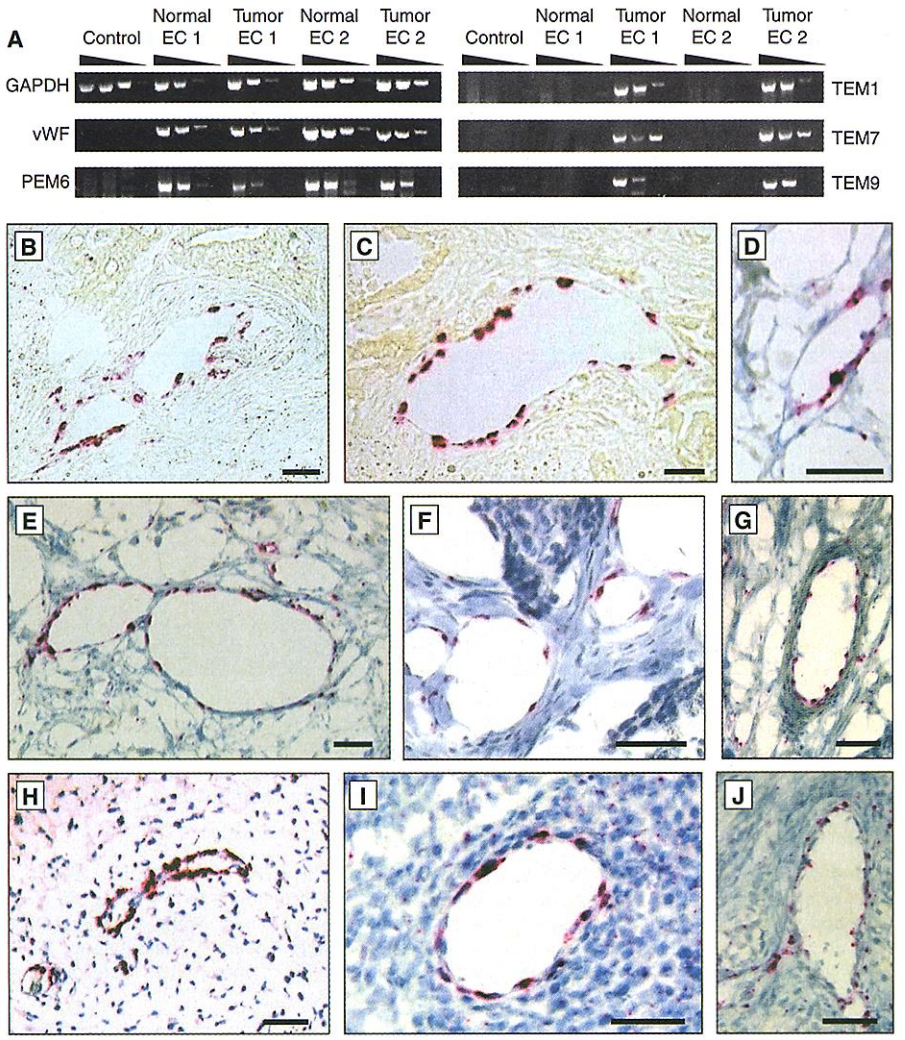

Fig. 3. Expression of TEMs. (**A**) RT-PCR analysis confirmed the tumor-specific expression of novel TEMs. Semiquantitative PCR analysis was performed on cDNA generated from purified tumor epithelial cells as a negative control (Control) or from purified ECs isolated from normal colonic mucosa (Normal ECs) or colorectal cancer (Tumor ECs) from two different patients. Two endothelial-specific markers, vWF and PEM6, showed robust amplification only in the endothelial fractions, whereas GAPDH was observed in all samples. TEM1, TEM7, and TEM9 were specifically expressed in tumor ECs. The cDNA template was diluted 1:10, 1:100, 1:1000, and 1:10,000, as indicated by the declining wedge. (**B** to **J**) The endothelial origin of TEMs identified by SAGE was confirmed by in situ hybridization, as in Fig 2. Expression of TEM1 (B) and TEM7 (C) was highly specific to the ECs in colorectal cancers; sections were imaged in the absence of a counterstain to show the lack of detectable expression in the nonendothelial cells of the tumor. TEM7 was also expressed in ECs from a metastastic liver lesion (D) arising from a primary colorectal cancer, and primary tumors derived from lung (E), breast (F), pancreatic (G), and brain cancer (H), as well as in a sarcoma (I). TEM7 expression was also localized to vessels during normal angiogenesis of human corpus luteum (J). Bars, 50 μM.

was confirmed to be endothelium by in situ hybridization in normal and malignant colorectal tissue (Fig. 2C).

We next identified transcripts that were differentially expressed in endothelium derived from normal or neoplastic tissues. This comparison revealed 33 tags that were elevated at least 10-fold in normal endothelium and 46 tags that were elevated 10-fold or more in tumor endothelium (*28*). Because transcripts expressed at higher levels in tumor endothelium are most likely to be useful for diagnostic and therapeutic purposes, our subsequent studies focused on this class. Of the top 25 tags most differentially expressed, 12 tags corresponded to 11 previously identified genes, 1 containing alternative polyadenylation sites (Table 2). Of these 11 genes, 6 were previously recognized as markers of angiogenic vessels (*16*, *22*, *29*–*33*), and at least 7 encode proteins involved in extracellular matrix formation or remodeling. These matrix-related processes are likely to be critical to the growth of new vessels. The remaining 14 tags corresponded to uncharacterized genes, most of which have been deposited as ESTs (Table 2).

To validate the expression patterns of these genes, we focused on nine tumor endothelial markers (TEM1 through TEM9) for which EST sequences but no other information was available (Table 2). RT-PCR analysis was used to evaluate the expression of the corresponding transcripts in purified ECs derived from normal and tumor tissues of two patients different from the original one used to construct the SAGE libraries. As expected on the basis of the SAGE data, von Willebrand Factor (vWF) and PEM6 were expressed at similar levels in normal and tumor ECs from both patients. These controls were not detected in purified tumor epithelial cells (Fig. 3A). In contrast, all nine TEMs chosen for this analysis were prominently expressed only in tumor ECs, but were absent or barely detectable in normal ECs (Fig. 3A). These RT-PCR assays were sensitive indicators of expression, and the absence of detectable transcripts in the normal endothelium, combined with their presence in tumor endothelial RNAs even when diluted 100-fold, provides important confirmation of their differential expression. These results also show that these transcripts were not simply expressed differentially in the ECs of the original patient, but were characteristic of colorectal cancer endothelium in general.

To exclude the possibility that the differentially expressed transcripts were derived from contaminating nonendothelial cells, we performed in situ hybridization on normal and neoplastic colon tissue. In every case where transcripts could be detected (TEM 1, 3, 4, 5, 7, 8, and 9), they were specifically localized to ECs (*34*) (Fig. 3, B and C). Although caution must be used when interpreting negative in situ hybridization results, none of the TEMs were expressed in vascular ECs associated with normal colorectal tissue even though vWF and Hevin were clearly expressed (*34*).

To determine whether TEMs were specifically expressed in the endothelium from primary colorectal cancers, or whether they were characteristic of tumor endothelium in general, we studied the expression of a representative TEM (TEM7) in a liver metastasis from a colorectal cancer, a primary sarcoma, and in primary cancers of the lung, pancreas, breast, and brain. As shown in Fig. 3, the TEM7 transcript was expressed specifically in the endothelium of each of these cancers, whether metastatic (Fig. 3D) or primary (Fig. 3, E to I). Analysis of the other six TEMs (TEM 1, 3, 4, 5, 8, and 9) revealed a similar pattern in lung tumors, brain tumors, and metastatic lesions of the liver (*34*).

Finally, we investigated whether these transcripts were expressed in angiogenic states other than that associated with tumorigenesis. As assessed by in situ hybridizations, these transcripts were generally expressed both in the corpus luteum and in

Table 2. SAGE tags elevated in tumor endothelium. The top 25 tags with the highest tumor EC (T-ECs) to normal EC (N-ECs) tag ratios are listed in descending order. To calculate tag ratios, we assigned a value of 0.5 in cases where zero tags were observed. The SAGE libraries are the same as those listed in Table 1. Tag numbers for each group were normalized to 100,000 transcripts. A description of the gene product corresponding to each tag is given, followed by alternative names in parentheses. TEM9 was uncharacterized at the outset of these studies but was recently characterized as a lectin present on ECs in culture and on microvascular vessels of human placenta in vivo (*41*).

Tag sequence	Name	Acc. #	N-ECs	T-ECs	HUVEC	HMVEC	Cell lines	Description
GGGGCTGCCCA	TEM1	AF279142	0	28	0	2	0	ESTs, similarity to thrombomodulin
GATCTCCGTGT	TEM2	AF279143	0	25	0	0	0	ESTs, similarity to rat Rhes ras-related protein
CATTTTTATCT	TEM3	AF070526	0	23	0	0	0	ESTs
CTTTCTTTGAG			0	22	6	20	1	Regulated in glioma-like 7-1 (Dkk-3/REIC)
TATTAACTCTC	TEM4	AB002335	0	21	1	3	1	ESTs, similarity to JNK interacting protein-3a
CAGGAGACCCC			0	16	2	0	0	MMP-11 (stromelysin 3)
GGAAATGTCAA			1	31	53	22	1	MMP-2 (gelatinase A, 72-kD type IV collagenase)*
CCTGGTTCAGT			0	15	0	0	0	ESTs
TTTTTAAGAAC	TEM5	AB040964	0	14	1	4	0	ESTs
TTTGGTTTTCC			5	139	0	16	0	Collagen, type 1, alpha 2, transcript A*†
ATTTTGTATGA			0	13	4	8	0	Nidogen (enactin)*
ACTTTAGATGG			1	23	0	15	0	Collagen, type VI, alpha 3*
GAGTGAGACCC			3	63	0	0	1	Thy-1 cell surface antigen*
GTACACACACC			0	10	0	0	0	Cystatin S
CCACAGGGGAT			2	38	0	2	1	Collagen, type III, alpha 1
TTAAAAGTCAC	TEM6	AK001539	1	19	1	3	1	ESTs
ACAGACTGTTA	TEM7	AF279144	4	74	0	0	0	ESTs, similarity with sea squirt nidogen
CCACTGCAACC			1	18	0	1	0	
CTATAGGAGAC	TEM8	AF279145	1	18	1	1	0	ESTs, similarity with homeobox protein DLX-3
GTTCCACAGAA			0	9	0	3	0	Collagen, type I, alpha 2, transcript B*†
TACCACCTCCC			0	9	4	1	1	ESTs
GCCCTTTCTCT	TEM9	AB014609	1	17	3	1	2	Endo180 lectin (macrophage mannose receptor family)
TTAAATAGCAC			2	33	0	4	0	Collagen, type 1, alpha 1*
AGACATACTGA			1	16	1	0	0	ESTs, DKFZP434G162 protein
TCCCCCAGGAG			1	16	0	0	0	Bone morphogenetic protein 1 (metalloprotease)

*Genes previously shown to be up-regulated in angiogenic vessels (*16, 22, 29–33*). †Multiple tags for this gene are due to alternative polyadenylation sites.

the granulation tissue of healing wounds (*34*) (Fig. 3J). One possible exception is TEM8, which we failed to detect in corpus luteum. In all tissues examined, expression of the genes was either absent or confined to the EC compartment.

The studies described above provide a definitive molecular characterization of ECs in an unbiased and general manner. They lead to several important conclusions that have direct bearing on long-standing hypotheses about angiogenesis. First, normal and tumor endothelium are highly related, sharing many endothelial cell–specific markers. Second, the endothelium derived from tumors is qualitatively different from that derived from normal tissues of the same type and is also different from primary endothelial cultures. We identified 46 transcripts that were expressed at substantially higher levels (>10-fold) in tumor endothelium than in normal endothelium, and 33 transcripts that were expressed at substantially lower levels in tumor than in normal endothelium. Most of these genes were either not expressed or were expressed at relatively low levels in ECs maintained in culture. Third, these genes are characteristically expressed in tumors derived from several different tissue types, demonstrating that tumor endothelium, in general, is different from the endothelium in surrounding normal tissue. Fourth, most of the genes expressed differentially in tumor endothelium are also expressed during angiogenesis of corpus luteum formation and wound healing. This is consistent with the idea that tumors may recruit vasculature by means of the same signals elaborated during other physiological or pathological processes. Indeed, the notion that tumors represent "unhealed wounds" is one of the oldest ideas in cancer biology (*35*). However, the fact that TEM8 expression was not detectable in developing corpus luteum suggests that there may be discrete differences between tumor angiogenesis and normal angiogenesis.

Finally, it is perhaps not surprising that so many of the endothelial-specific transcripts identified in this study, whether expressed only in neovasculature or in endothelium in general, have not been previously characterized, and some are not even represented in EST databases. ECs represent only a minor fraction of the total cell population within normal or tumor tissues, and only those EC transcripts expressed at the highest levels would be expected to be represented in libraries constructed from unfractionated tissues. The genes described in the current study should therefore provide a valuable resource for basic and clinical studies of human angiogenesis in the future.

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14. To identify pan endothelial-specific transcripts, we normalized the number of tags analyzed in each group to 100,000 and limited our analysis to transcripts that were expressed at levels at least 20-fold higher in ECs than in nonendothelial cell lines in culture and present at fewer than five copies per 100,000 transcripts in nonendothelial cell lines and the hematopoietic fraction (~57,000 tags) (*39*). Nonendothelial cell lines consisted of 1.8×10^6 tags derived from a total of 14 different cancer cell lines including colon, breast, lung, and pancreatic cancers, as well as one nontransformed keratinocyte cell line, two kidney epithelial cell lines, and normal monocytes. A complete list of PEMs is available at www.sagenet.org/angio/table1.htm.

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28. Differentially expressed endothelial-specific transcripts were defined as those expressed at levels at least fivefold higher in ECs in vivo than in nonendothelial cell lines in culture (*14*) and present at no more than five copies per 100,000 transcripts in nonendothelial cell lines and the hematopoietic cell fraction (*39*). Transcripts showing statistically different levels of expression ($P < 0.05$) were then identified by Monte Carlo analysis (*40*). Transcripts preferentially expressed in normal endothelium were then defined as those expressed at levels at least 10-fold higher in normal endothelium than in tumor endothelium. Conversely, tumor endothelial transcripts were present at levels at least 10-fold higher in tumor versus normal endothelium. See www.sagenet.org/angio/table2.htm and www.sagenet.org/angio/table3.htm for a complete list of differentially expressed genes.
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Inflammation Dampened by Gelatinase A Cleavage of Monocyte Chemoattractant Protein–3

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Tissue degradation by the matrix metalloproteinase gelatinase A is pivotal to inflammation and metastases. Recognizing the catalytic importance of substrate-binding exosites outside the catalytic domain, we screened for extracellular substrates using the gelatinase A hemopexin domain as bait in the yeast two-hybrid system. Monocyte chemoattractant protein–3 (MCP-3) was identified as a physiological substrate of gelatinase A. Cleaved MCP-3 binds to CC-chemokine receptors–1, –2, and –3, but no longer induces calcium fluxes or promotes chemotaxis, and instead acts as a general chemokine antagonist that dampens inflammation. This suggests that matrix metalloproteinases are both effectors and regulators of the inflammatory response.

Degradomics, the identification of biologically relevant substrates for the increasing number of recognized proteinases, is a challenge of proteomics. Although yeast two-hybrid screening (*1*) has identified intracellular protein-protein interactions, a screening rationale with a proteinase catalytic domain as target is tenuous because cleavage of library-encoded substrate would preclude detection. Therefore, to identify potential matrix metalloproteinase (MMP) substrates, we used a substrate-binding exosite domain, the hemopexin COOH-terminal (C) domain (*2*, *3*), that is structurally and functionally distinct from the catalytic domain in a two-hybrid screen. Collagen binding by this domain of collagenolytic MMPs is a prerequisite for cleavage. Therefore, other proteins that bind to this domain may also be MMP substrates.

To determine the suitability of the two-hybrid system for extracellular proteins, we assessed the interaction between the single–disulfide-bonded gelatinase A (MMP-2) hemopexin C domain and the C domain of the tissue inhibitor of metalloproteinase–2 (TIMP-2), which contains three disulfide bonds. Deletion (*4*) and domain-swapping (*5*) studies indicate that these domains interact in the cellular activation and localization of gelatinase A to cell surface membrane type (MT)–MMPs (*6*). Association was detected (Fig. 1, A and B), indicating that in yeast at 30°C, a stable, functional protein fold in domains that normally contain disulfide bonds was achieved.

A cDNA library was constructed from human gingival fibroblasts treated with concanavalin A (Con A), a stimulant of extracelluar matrix degradation through the activation of gelatinase A (*7*). Using the human

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