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Identification of a Novel Endothelial-Derived Gene EG-1

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The identification of novel endothelial-derived genes is important in the study of angiogenesis, and may have potential uses in cancer diagnosis and treatment. We performed SSH (suppression subtractive hybridization) on control HUVECs (human umbilical vein endothelial cells) versus HUVECs exposed to tumor-conditioned media. We found that a novel cDNA (GenBank Accession No. AF358829) is differentially expressed in endothelial cells on Northern analysis, and named it endothelial-derived gene-1 (EG-1). This gene product is predicted to encode a 178-aa, 19.5-kDa protein, and is localized to chromosome 4. It has some homology to a mouse cDNA (94%) and a *Drosophila* cDNA (31%). On Northern analysis, endothelial cells express two EG-1 RNA species (1.2 and 2.4 kb). The expression of either transcripts is upregulated by endothelial cells when exposed to tumor conditioned media. This phenomenon is observed only under sparse conditions (50% confluency). Transcripts are present abundantly in highly vascular tissues such as placenta, testis, and liver. Interestingly, both Northern analysis and *in situ* hybridization studies show that this gene is expressed in other cell types as well, predominantly the epithelial type. Breast cancer, prostate cancer, and colon cancer cells show elevated expression of the higher 2.4-kb RNA form. Our data suggest that EG-1 is associated with a stimulated state in endothelial and epithelial cells, and may have a role in tumor angiogenesis.

The growth and metastasis of solid tumors is dependent on their ability to initiate and sustain new capillary growth, i.e., angiogenesis (1). Angiogenesis is a complex multistep process which includes endothelial cell proliferation, migration and differentiation into tube-like structures. These steps involve changes in the expression of multiple growth factors, proteases and adhesion molecules in endothelial cells, as well as in supporting cells. Researchers have shown that endothelial cells lining established blood vessels have a very slow turnover time, whereas those lining tumor capillaries undergo rapid proliferation and differentiation. Although much has been discovered about adult angiogenesis, it is unclear whether abnormal angiogenesis such as that occuring in solid tumor growth involves different mechanisms from desirable angiogenesis which occurs in endometrial proliferation or in wound healing (2).

In the past, efforts to identify the differences between the proliferating tumor endothelium and the normal quiescent endothelium have included antibody targeting (3), immunohistochemical analysis of known endothelial adhesion molecules (4), and phage display peptide libraries (5). Differential RNA expression cloning has also been pursued in endothelial cells treated with TPA (6) and in endothelial cells derived from colorectal cancer (7). In order to closely mimic a tumor environment, we have attempted to identify endothelial gene products expressed in response to a mixture of tumor derived growth factors found in tumor conditioned media. Toward this goal, we used a subtraction hybridization method called SSH (suppression subtractive hybridization, 8). In HUVEC (human umbilical vein endothelial cell) populations exposed to tumor conditioned media for 4 h, we have isolated approximately 300 up-regulated and another 300 down-regulated clones (9). We named one of these differentially expressed genes EG-1 (endothelial-derived gene-1). In the present report, we show that EG-1 expression is seen in endothelial cells in several tissues and that its expression can be upregulated by growth stimulation induced by tumor conditioned medium as well as specific angiogenic factors. These results suggest that EG-1 may play a role in tumor angiogenesis.

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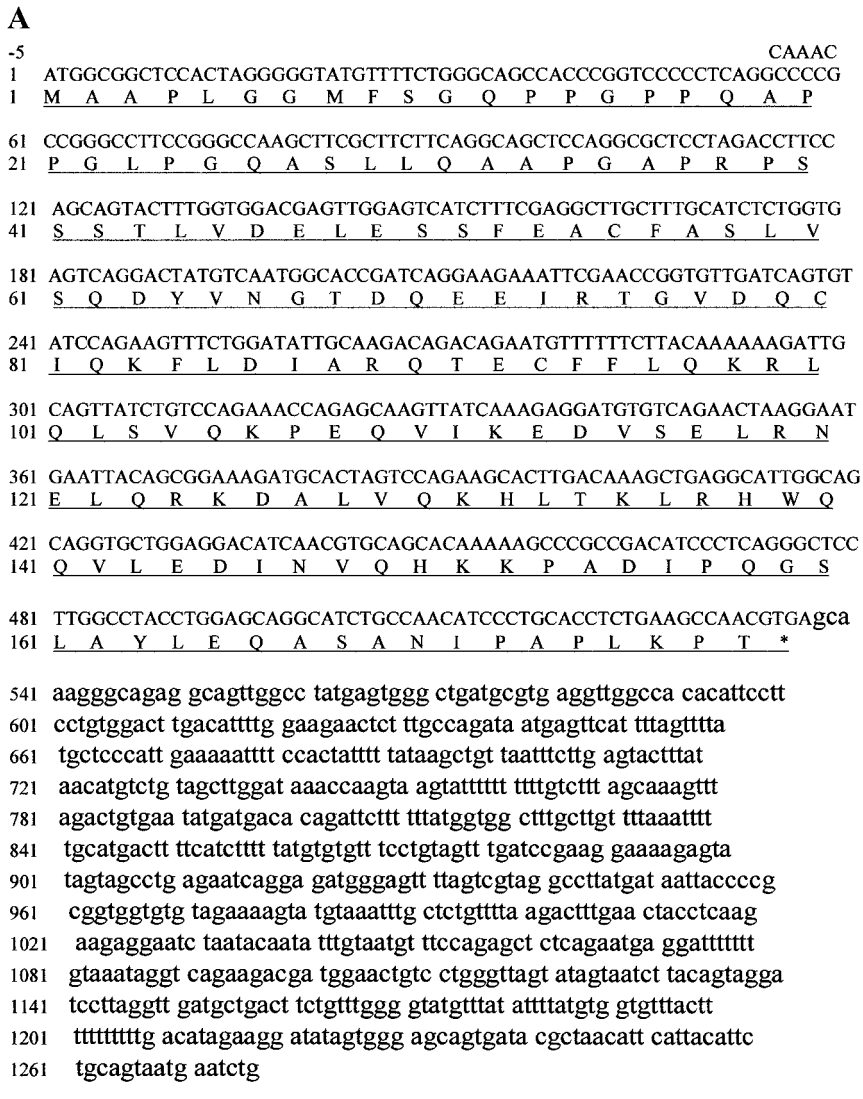

FIG. 1. (A) Alignment of deduced amino-acid sequence of endothelial-derived gene-1 (EG-1) with its nucleotide sequence. (B) Alignment of deduced amino-acid sequence of endothelial-derived gene-1 (EG-1) with its murine and *Drosophila* counterparts.

MATERIALS AND METHODS

Sequence analysis. The sequence of all clones was determined in both directions by automated cycle-sequencing by the UCLA Jonsson Comprehensive Cancer Center sequencing facility. Sequence analysis was performed with the Lasergene Navigator (DNASTAR, Inc., Madison, WI) software package and with searches of the GenBank database using BLASTN. For motif analysis, the following internet websites were used: http://pfam.wustl.edu/hmmsearch.shtml, and http://www.isrec.isb-sib.ch/software/PFSCAN_form.html.

Cloning of EG-1. The full-length cDNA sequence was obtained by standard molecular methods (10) using a HUVEC cDNA library. Briefly, the library was screened for desired clones using the partial fragment derived from SSH (GenBank Accession No. AW735731). The identity of the clones was validated by sequencing.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA). The cells were plated on tissue culture flasks coated with 1.5% gelatin (Difco, Detroit, MI) and were maintained in endothelial growth media (EGM: endothelial cell growth medium completed with 10 ng/ml hEGF (human epithelial growth factor), 2% fetal calf serum (FCS, Gemini, Calabasas, CA), 1.0 μg/ml hydrocortisone, gentamicin and amphotericin-B (Clonetics). Human aortic endothelial cells (HAECs) and human microvascular endothelial cells (HMVECs) were purchased from Cascade (Portland, OR). For some experiments, cells were rendered quiescent by "starving" in culture in Dulbecco's minimal essential medium (DMEM, Life Technologies, Carlsbad, CA) lacking additional supplements. For experiments with specific angiogenic factors, the endothelial cells were grown in DMEM with either bFGF (basic fibroblast growth factor, Chemicon International Inc., Temecula, CA) at 5 ng/ml or TNF-α (tumor necrosis factor alpha, Alexis Corp., San Diego, CA) at 200 units/ml.

The human melanoma line C8161 was obtained from Dr. Barsky (Los Angeles, CA), and the human breast cancer cell line Mda-Mb-231 from American Tissue Type Culture Collection (Rockville, MD) and maintained on nongelatinized flasks with DMEM and 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). The tumor conditioned media was prepared with confluent cultures of either C8161 or Mda-Mb-231 as previously described (4). Briefly, the serum-free DMEM media bathing the tumor cells over 48 h was collected, spun, and the supernatant

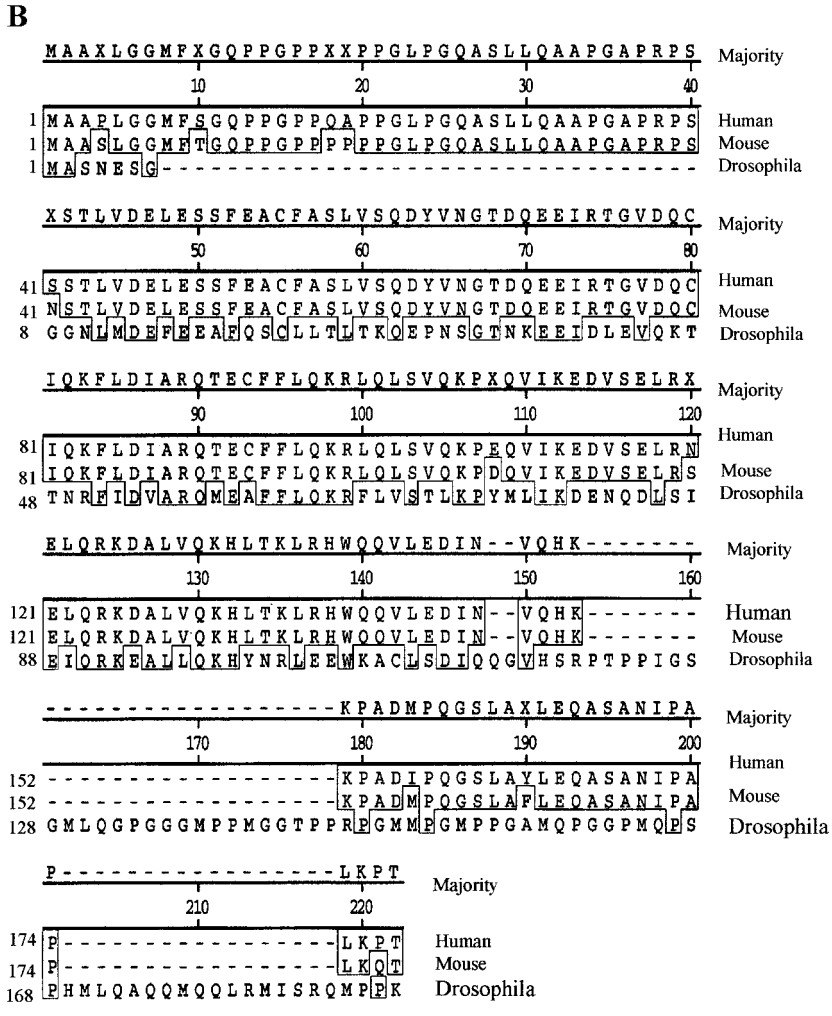

FIG. 1—*Continued*

concentrated approximately 5- to 10-fold with Centripreps with a 3000 MW cutoff.

Other cells used in this study included benign human fibroblast Ccd-sk-27, benign human liver, benign human lung, human breast cancer Mcf-7 and T47D, human colon cancer Colo-205 and Ls-174t, and human prostate cancer LnCap from ATCC. Human myoepithelial HMS cells were obtained from Dr. Barsky. These cells were all grown in DMEM with 10% FCS, with the exception of HMS which was grown in keratinocyte serum-free medium (K-SFM) supplemented with 50 μg/ml bovine pituitary extract and 5 ng/ml recombinant human epidermal growth factor (GIBCO/BRL, Carlsbad, CA).

Northern analysis. The multitissue mRNA blots were purchased from Origene (Rockville, MD). For other blots, total RNA was extracted from cell lines using Trizol (GIBCO/BRL). Twenty micrograms of total RNA was loaded per lane and resolved on 1.2% agarose gels prior to transfer to nitrocellulose membranes, as previously described (9). The EG-1 cDNA probe was labeled by the random primer method (11). All blots were also reprobed for β-actin (GIBCO/BRL) content to verify RNA quantity. Bands for Northern blots were quantitated using a Molecular Dynamic Laser Densitometer (Model PSD1) and an Image Quant Version. 1 software program.

Human tissue. Human tissue samples were obtained from the UCLA Human Tissue Research Center. Only archival tissue was used, and the identity of the human subjects was removed so as to make the samples untracable. As for all studies involving human tissue, this study was conducted in compliance with the rules of the UCLA Human Subject Protection Committee.

In situ hybridization. Formalin-fixed, paraffin-embedded tissues were sectioned, placed on 3-aminopropyltriethoxysilane-treated slides (GIBCO/BRL), then baked at 60°C for 1 h. The paraffin was removed by incubation in xylene, followed by 100% ethanol. The sections were digested with 40 μg/ml of proteinase K (GIBCO/BRL) for 10 min at 37°C, then washed with PBS. All samples were then fixed for 1 min in 10% buffered formalin, washed with PBS, dehydrated through graded alcohols, and air dried in preparation for hybridization. The probe was labeled with biotin by nick translation according to the manufacturers' instructions (BioPRIME DNA Labeling System, Life Technologies). Unincorporated nucleotides were removed by column chromatography using BioGel P-60 gels (Bio-Rad, Hercules, CA). Double strand probes were heat denatured for 5 min at 100°C prior to hybridization. Hybridization was conducted using the GIBCO BRL In Situ Hybridization and Detection System. Slides were hybridized for overnight at 42°C. After hybridization, the slides were washed in 0.2× SSC. The signal was detected using streptavidin alkaline phosphatase conjugate and NBT-BCIP (nitroblue tetrazolium, 4-bromo-5-chloro-3-indolylphosphate) substrates. The slides were counterstained with methyl green (Sigma, St. Louis, MO), dehydrated through graded alcohols, and mounted

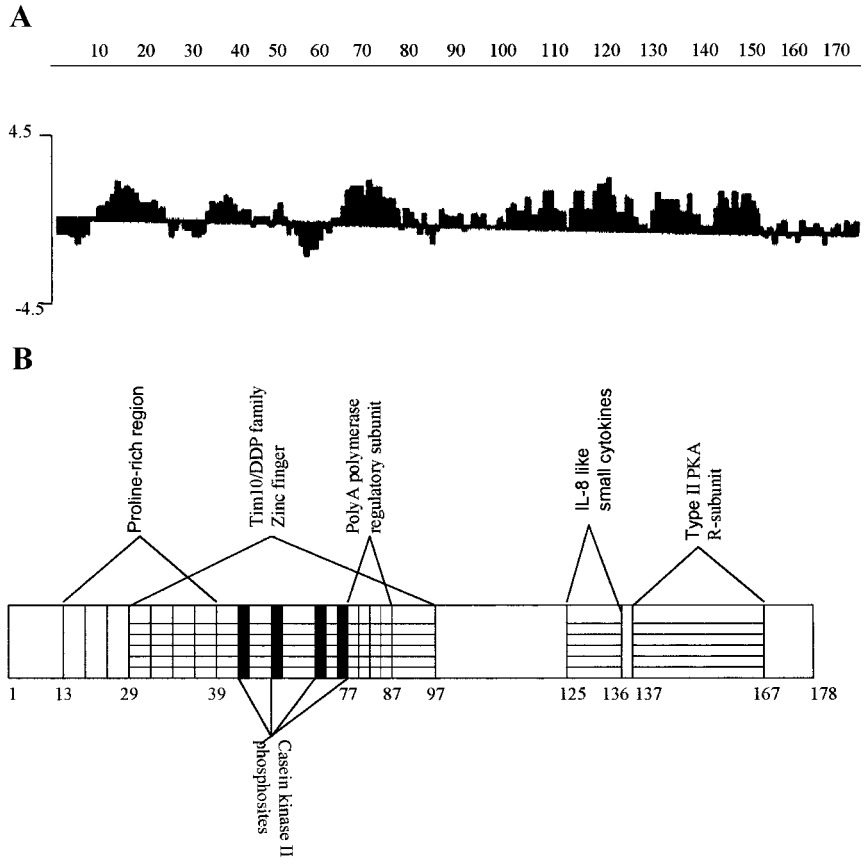

FIG. 2. (A) Hydrophilicity plot of endothelial-derived gene-1 (EG-1). (B) Structural features of endothelial-derived gene-1 (EG-1). Motif analysis reveals a proline-rich region (13–39), one N-glycosylation site (66–69), four casein kinase II phosphorylation sites (43–46, 50–53, 68–71, 75–78), and two N-myristoylation sites (6–11, 76–81). There is some alignment with the following: Tim 10/DDP (deafness dystonia protein) family zinc finger (aa 29–97), poly(A) polymerase regulatory subunit (aa 77–87), interleukin-8 like small cytokines (intecrine/chemokine) (aa 125–136), and regulatory subunit of type II PKA (cAMP-dependent protein kinase) R-subunit (aa 137–167).

with Permount solution (Fisher Scientific, Tustin, CA). Photography was carried out with a Leica DMLS microscope (McBain Instruments, Chatsworth, CA) and a Nikon N6006 camera (Tokyo, Japan).

Furthermore, for a standard fee, *in situ* hybridization was performed independently by the Dana Farber Cancer Institute In Situ Core Facility (Boston, MA). The Facility uses its own human tissue bank for this work. The plasmid was linearized with appropriate restriction enzymes and transcribed with T7 or T3 RNA polymerase (Promega, Madison, WI) and ^{35}S-labeled UTP (New England Nuclear) to generate antisense and sense radiolabeled-RNA probes. Tissue sections were deparaffinized, fixed in 4% paraformaldehyde in PBS, and treated with proteinase K. After washing in 0.5× SSC, the sections were covered with hybridization solution (50% deionized formamide, 0.3 M NaCl, 20 mM Tris (pH 8.0), 5 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate, and 10 mM dithiothreitol) and prehybridized for 2 h at 55°C. ^{35}S-labeled antisense and sense RNA probes (3×10^5 cpm/slide) were added to the hybridization solution, and the incubation continued for 12–18 h at 55°C. After hybridization, the sections were washed for 20 min in 2× SSC, 10 mM β-mercaptoethanol, and 1 mM EDTA, treated with RNase A (10 μg/ml) for 30 min at room temperature, and washed at high stringency (0.1× SSC, 10 mM β-mercaptoethanol, and 1 mM EDTA) for 2 h at 60°C. The sections were dehydrated, dipped in photographic emulsion NTB2 (Kodak), and stored at 4°C. After 2 weeks of exposure, the sections were developed and counterstained with hematoxylin and eosin.

RESULTS

Analysis of Predicted Sequence

A BLASTN search in the GenBank database reveals that EG-1 (GenBank Accession No. AF358829) is on chromosome 4. It spans four exons (8–169, 170–237, 238–349, and 350–1288) and three introns (5087, 1619, and 1901 bp). From the nucleotide sequence, the predicted peptide has 178 amino acids, and weighs 19.5 kDa (Fig. 1A). There are 17 strongly basic amino acids, 21 strongly acidic amino acids, 60 hydrophobic amino acids, and 48 polar amino acids. The peptide has significant homology to a murine cDNA (94%, GenBank Accession No. NP_080171, 12) and a *Drosophila* cDNA (31%, GenBank Accession No. AAF56470, 13) (Fig. 1B). It has no signal peptide nor transmembrane sequences (Fig. 2A). The isolectric point is 5.393, and the peptide has a −3.660 charge at pH 7.0. The melting temperature is 85°C.

A Profile Scan search reveals a long proline-rich region spanning from amino acids 13 to 39. There are one N-glycosylation site (aa 66–69), four casein kinase

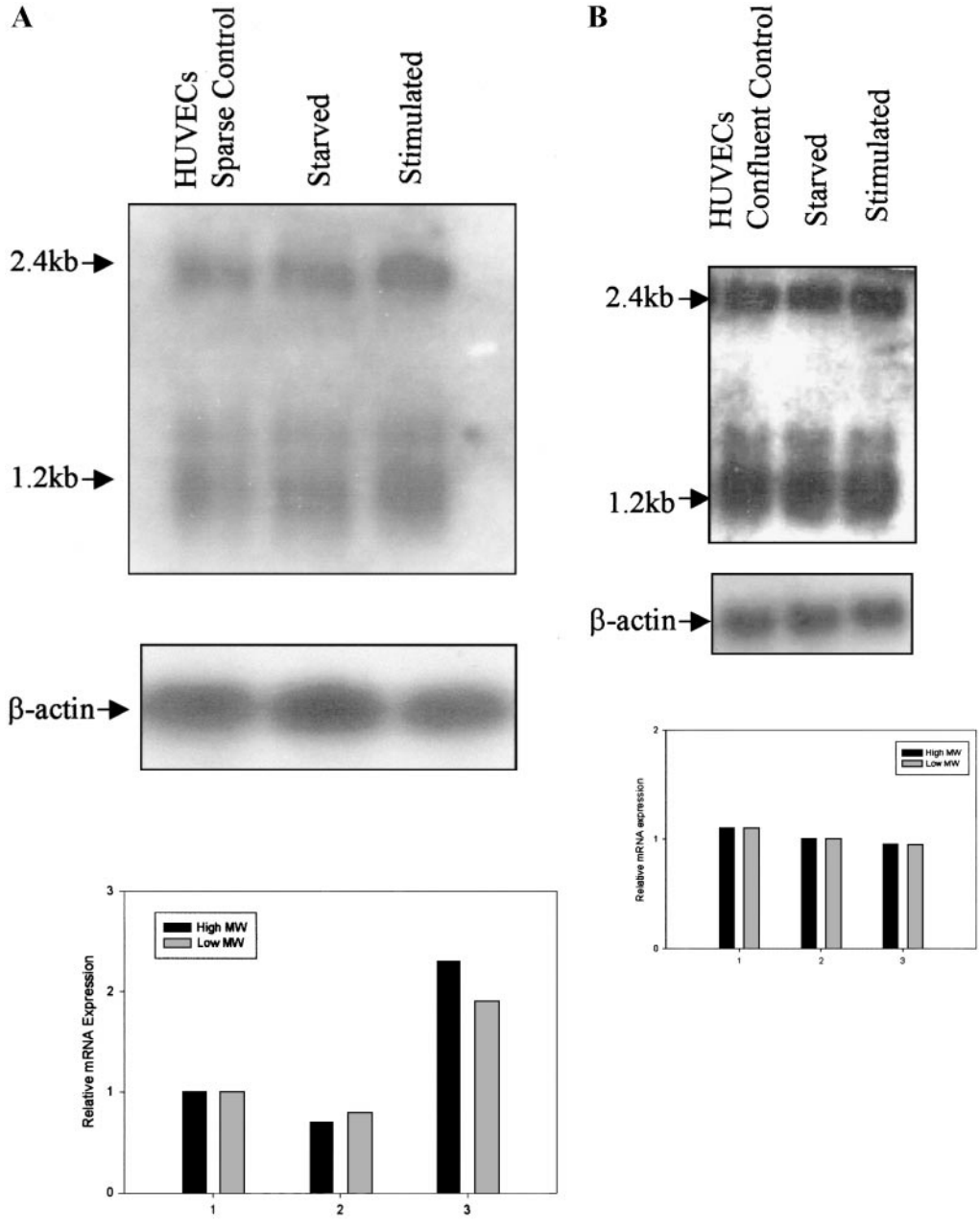

FIG. 3. (A) Induction of endothelial-derived gene EG-1 expression by tumor conditioned media in sparse conditions (50% confluency). Control HUVECs (human umbilical vein endothelial cells) were cultured in growth media (lane 1), starved in plain DMEM (lane 2), and stimulated HUVECs in tumor conditioned media (lane 3). 20 μg of RNA was hybridized with EG-1 and β-actin cDNA probes. (B) Induction of endothelial-derived gene-1 (EG-1) expression by tumor conditioned media under confluent conditions (90–100% confluency). Control HUVECs (human umbilical vein endothelial cells) were cultured in growth media (lane 1), starved in plain DMEM (lane 2), and stimulated HUVECs in tumor conditioned media (lane 3). 20 μg of RNA was hybridized with EG-1 and β-actin cDNA probes.

II phosphorylation sites (aa 43–46, 50–53, 68–71, 75–78), and two N-myristoylation sites (aa 6–11, 76–81). A Pfam search looking for motif match show some alignment with the following: Tim 10/DDP (deafness dystonia protein) family zinc finger (aa 29–97, E value 9.3), poly(A) polymerase regulatory subunit (aa 77–87, E value 8.8), interleukin-8 like small cytokines (intecrine/chemokine) (aa 125–136, E value 1.5), and regulatory subunit of type II PKA (cAMP-dependent protein kinase) R-subunit (aa 137–167, E value 1.4) (Fig. 2B).

Northern Analysis of EG-1

SSH revealed an RNA sequence (GenBank Accession No. AW735731), whose expression is increased in

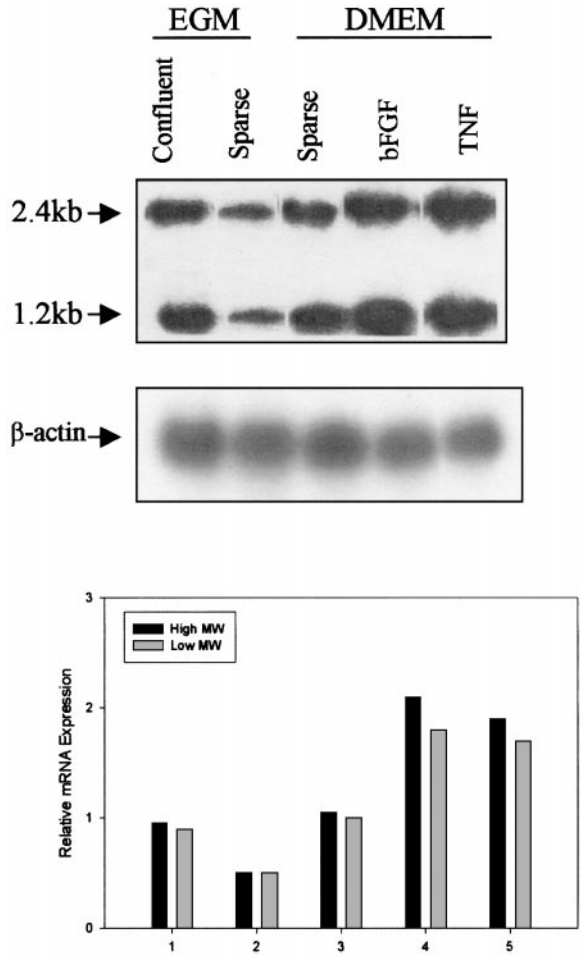

FIG. 4. Induction of endothelial-derived gene-1 (EG-1) expression by bFGF (basic fibroblast growth factor) and TNFα (tumor necrosis factor α). HUVECs (human umbilical vein endothelial cells) were cultured in EGM (endothelial growth media) or plain DMEM. Confluent condition is 80% confluency (lane 1), and sparse condition is $<$50% confluency (lanes 2–3). Sparse HUVECs were exposed to 5 ng/ml bFGF (lane 4) or 200 units/ml TNFα (lane 5). 20 μg of RNA was hybridized with EG-1 and β-actin cDNA probes.

HUVECs treated with tumor conditioned media derived from either melanoma (C8161) or breast cancer (MDA-MB231). Subsequent cloning of the full length cDNA (GenBank Accession No. AF358829), and a BLASTN search for sequence homology performed in the GenBank database reveals that EG-1 has no significant homology to any gene with a known function. Northern analysis confirms that EG-1 expression is upregulated approximately two-fold in HUVECs exposed to tumor conditioned media (Fig. 3A). Two signals corresponding to a 2.4 kb and a second 1.2 kb are observed to both increase in intensity. The expression of EG-1 is unchanged when HUVECS are approaching confluency (90–100% confluency) in culture (Fig. 3B). We then treated HUVECs to specific angiogenic factors. Stimulation with bFGF increases the expression of EG-1 by approximately two to three-fold, and TNFα by approximately twofold (Fig. 4). When HUVECs are starved, the EG-1 transcript level decreases slightly (Fig. 5). The above observations are also seen in other types of endothelial cells including HAECs and HMVECs (Fig. 5). The increase in signal intensity due to exposure to tumor conditioned media is also observed in HAECs and HMVECs.

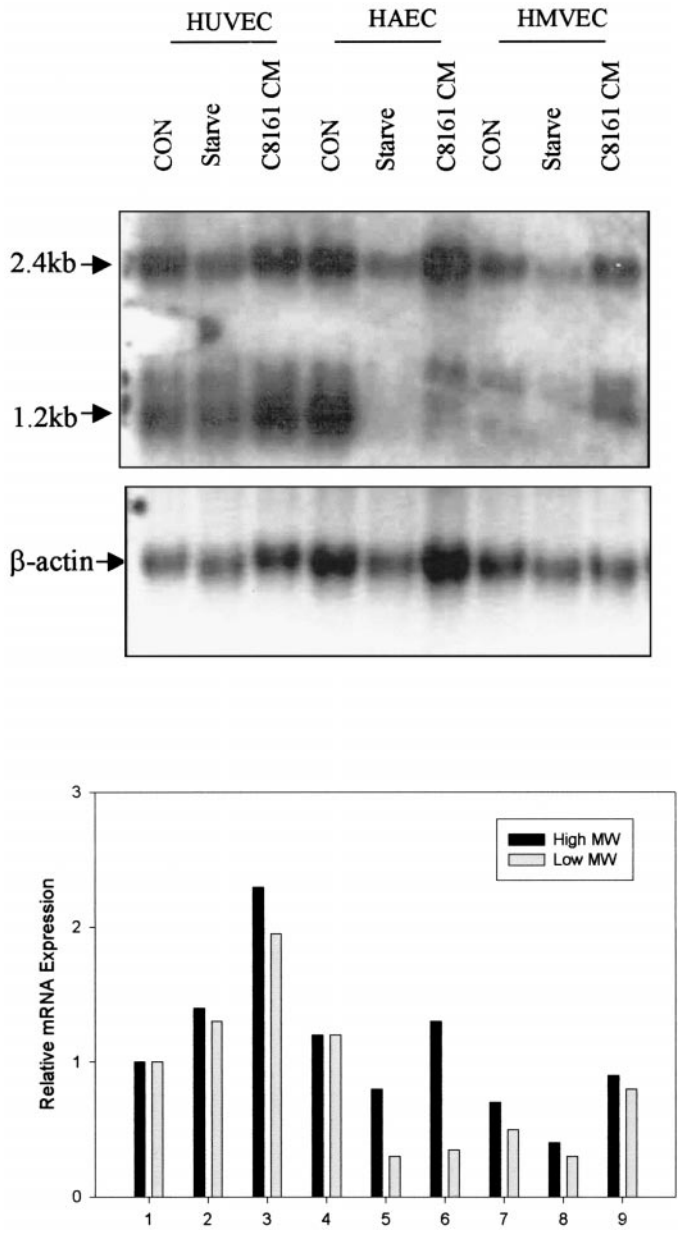

FIG. 5. Presence of endothelial-derived gene-1 (EG-1) in different types of endothelial cells. 20 μg of RNA from HUVECs (human umbilical vein endothelial cells), human aortic endothelial cells (HAECs), and human microvascular endothelial cells (HMVECs) were hybridized with EG-1 and β-actin cDNA probes. Control cells were cultured in EGM (endothelial growth media) (lanes 1, 4, and 7). Starved cells were cultured in plain DMEM (lanes 2, 5, and 8). Conditioned media from the malignant melanoma C8161 was used to stimulate endothelial cells (lanes 3, 6, and 9).

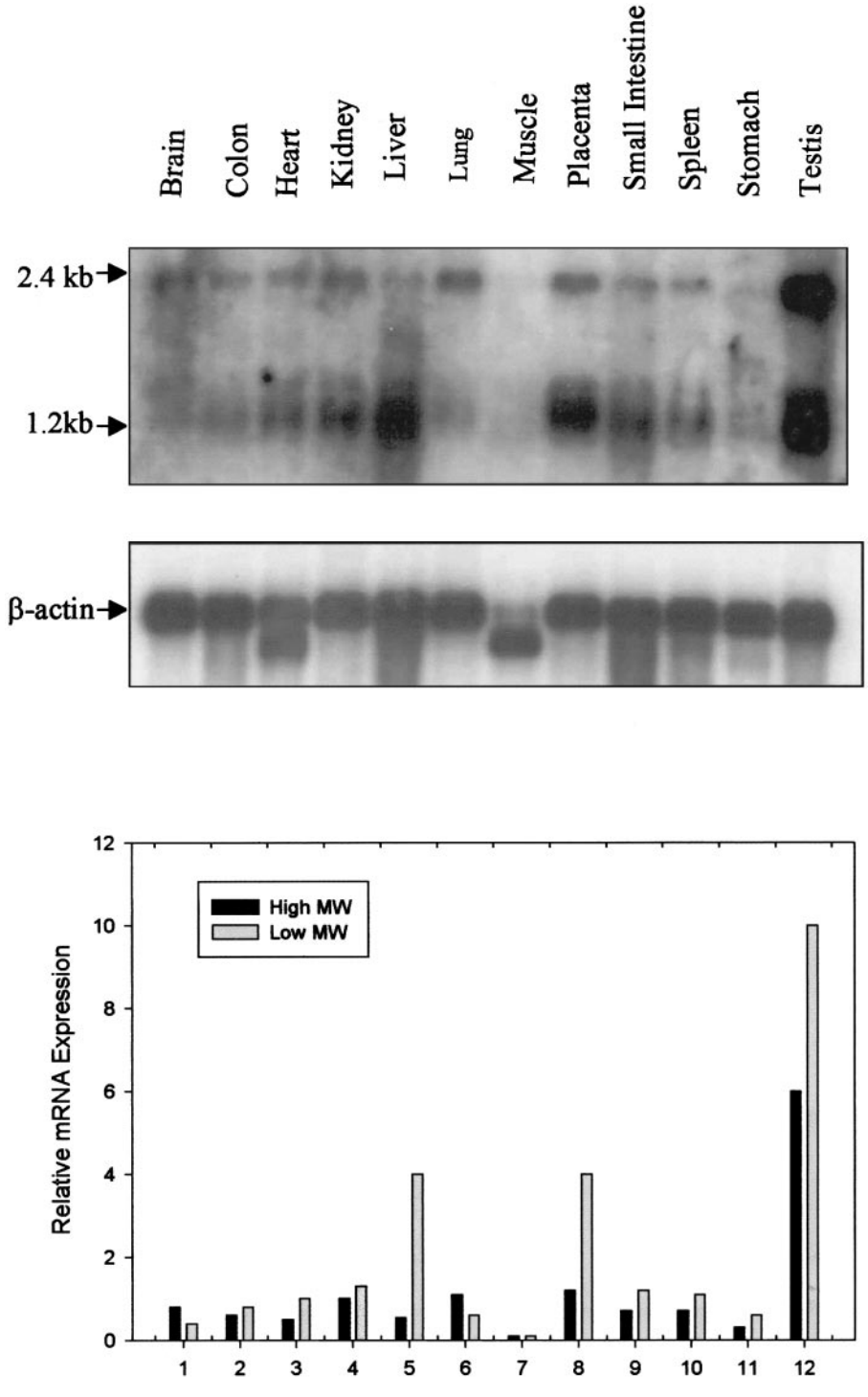

FIG. 6. Presence of endothelial-derived gene-1 (EG-1) in different types of human tissues. mRNA multi-tissue blots from Origene were hybridized with EG-1 and β-actin cDNA probes.

Further Northern studies of EG-1 show that it is highly expressed in liver, placenta, and testis (Fig. 6). The high expression is seen in both 2.4 kb and 1.2 kb forms in testis, but only in the lower MW 1.2 kb form in liver and placenta. When Northern analysis is performed with many different cells types, both m.w. forms can be detected (Fig. 7). These cell lines include benign types (fibroblast, myoepithelium, liver, and lung) as well as cancer cell lines derived from breast, colon, prostate, and melanoma. Interestingly, the higher MW 2.4 kb form is elevated in the breast cancer, colon cancer and prostate cancer cell lines.

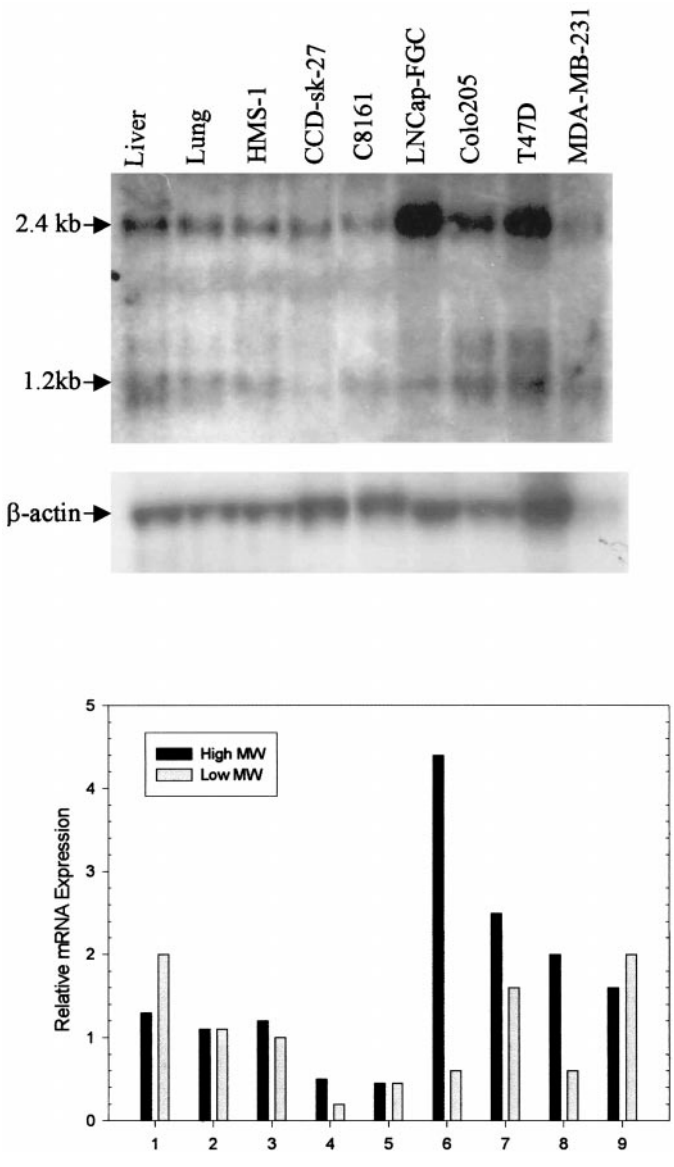

FIG. 7. Expression of endothelial-derived gene-1 (EG-1) in non-endothelial cell types. 20 μg of RNA from HUVECs (human umbilical vein endothelial cells) and other cell lines were hybridized with EG-1 and β-actin cDNA probes. Benign human cells include liver, lung, myoepithelial HMS, and fibroblast Ccd-sk-27. Malignant human cells include melanoma C8161, prostate cancer LnCap, colon cancer Colo-205, and breast cancer T47D and Mda-Mb-231.

In Situ Hybridization of EG-1

In situ hybridization of human tissues was performed independently in two separate laboratories (ours and the Dana Farber Cancer Institute In Situ Core Facility). These studies reveal staining of EG-1 in the endothelial cells of blood vessels. This is seen in arteries (Fig. 8A), veins (Fig. 8B), and capillaries (Fig. 8C). The signal is also detected in spleen endotheliocytes (Fig. 8D) and the placental Hoffbauer cells (Fig. 8E), which are presumed to be the precursor cells for endothelial cells, as well as in hemangioma blood vessels (Fig. 8F). We see the EG-1 signal in the epithelial cells of many organs, and this signal appears to be more intense with malignant transformation. Examples include breast cancer (Figs. 9A and 9B), colon cancer (Figs. 9C and 9D), prostate cancer (Figs. 9E and 9F), and lung cancer (Figs. 9G and 9H). No EG-1 signal is detected in lymphoid tissues (tonsils, thymus, lymph nodes, splenic lymphocytes), muscle (skeletal, smooth, cardiac, uterine), or fat (data not shown).

DISCUSSION

Endothelial-derived gene EG-1 seems to be a human gene, which has homology to both murine and *Drosophila* forms. From our Northern and *in situ* hybridization studies, it appears that EG-1 is expressed in endothelial cells. The expression of EG-1 seems to correlate with cellular proliferation or stimulation, as it is upregulated by tumor conditioned media. Previously, we have seen that tumor conditioned media from C8161 and/or Mda-Mb-231 is rich with multiple angiogenic growth factors (14). In this study, we further see that EG-1 expression is increased with exposure to two angiogenic factors bFGF and TNF-α.

Several researchers, including our laboratory, have investigated the difference between molecules of the proliferating tumor endothelium from those in the normal quiescent endothelium. One approach toward studying the tumor endothelium involves immunohistochemical analysis of known endothelial adhesion molecules using tumor specimens. These studies have shown that multiple surface molecules are significantly increased in the tumor vasculature. These molecules include E-selectin (4), the $\alpha_v\beta_3$ integrin, VCAM-1 (vascular cellular adhesion molecule), ICAM-1 and -2 (intercellular adhesion molecule), CD 31, CD 34, CD 36, and CD 44 (15). Other investigators have used the antibody targeting approach. This approach has produced multiple candidate markers of the tumor vasculature. These include endoglin which is recognized by the TEC-11 antibody, endosialin which is recognized by the FB5 antibody, the antigen recognized by the EN7/44 antibody, the antigen recognized by the E-9 antibody (16), a truncated form of tissue factor (3), and the fibronectin B-FN isoform (17). Phage display peptide libraries have also been used successfully to characterize tumor blood vessels (5). Differential RNA expression cloning has also been successfully pursued in endothelial cells treated with TPA (6) and in endothelial cells derived from colorectal cancer (7).

Recent reports of the effect of known angiogenic growth factors on the endothelium have advanced our understanding of the mechanisms of tumor angiogenesis at a molecular level. The best studied angiogenic growth factor is VEGF (vascular endothelial cell growth factor, 18). Other growth factors have been

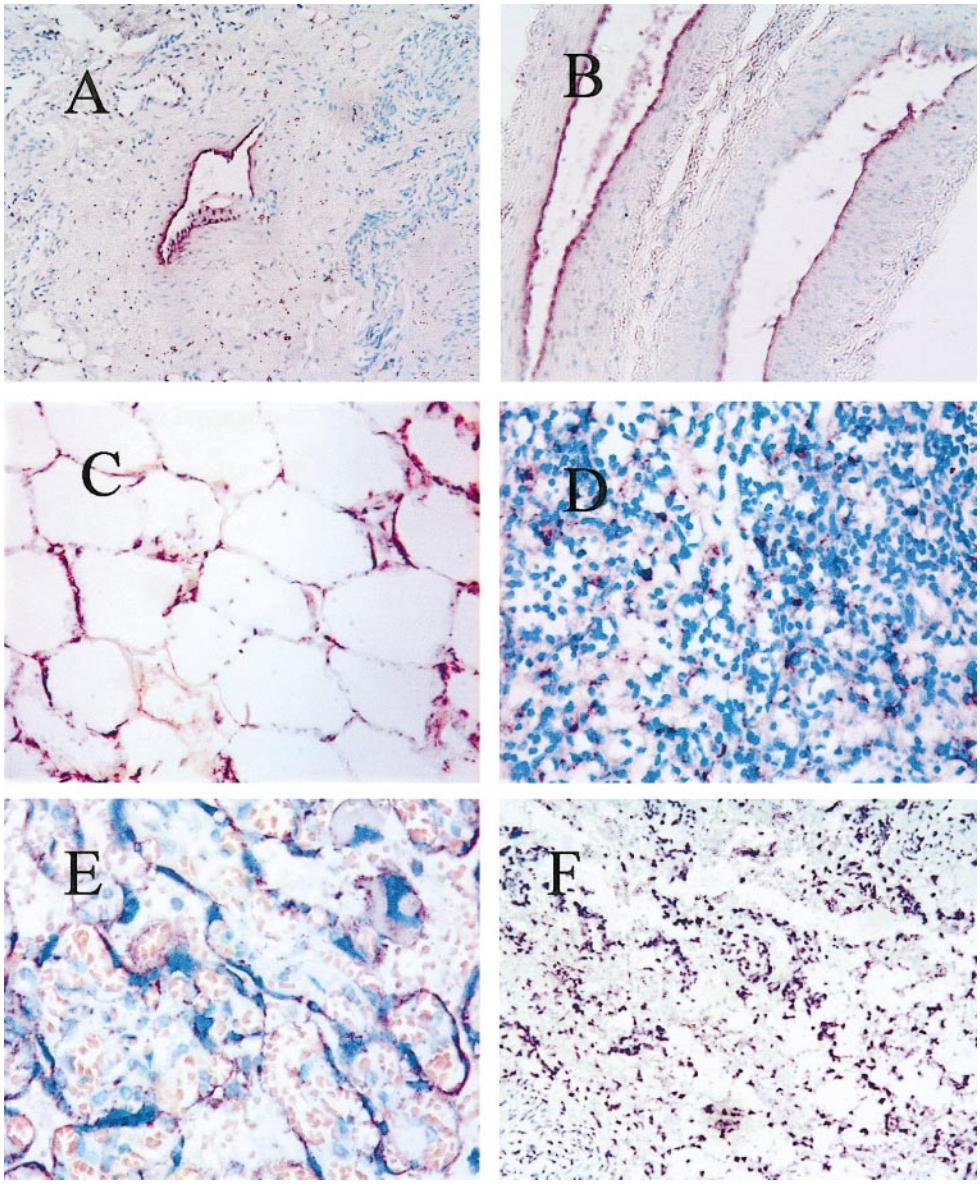

FIG. 8. Presence of endothelial-derived gene-1 (EG-1) in the endothelial cells of capillaries (A), arteries (B), and veins (C). EG-1 is also present in spleen endotheliocytes (D), placenta Hoffbauer cells (E), and hemangioma blood vessels (F). *In situ* hybridization was performed as detailed under Materials and Methods.

shown to be also important including bFGF, aFGF (acidic fibroblast growth factor), angiogenin, TGF-α and β (transforming growth factor alpha and beta), TNF-α, PD-ECGF (platelet derived endothelial growth factor), G-CSF (granulocyte colony stimulating factor), PlGF (placental growth factor), interleukin-8, HGF (hepatocyte growth factor), proliferin (1), and angiopoietin (19). Endogenous angiogenic inhibitors such as angiostatin, endostatin (20), thrombospondin, METH (21) may also play an important role in this process. Proteases and cytokines secreted by tumor cells are also very important.

In our laboratory, we used SSH to further investigate the molecular mechanisms of tumor angiogenesis by identifying genes that become activated as well as those that become down-regulated when quiescent endothelial cells are exposed to a tumor environment. Although this project utilizes cells in tissue culture, we think that this *in vitro* model does provide an adequate simulation of the tumor environment. With this model, we have recently identified human endomucin (22). Other investigators have used similar methods of differential display to study non-cancer-related *in vitro* models of angiogenesis and have found increased expression of important angiogenesis-related genes such as endothelial differentiation gene (6) and COX-1 (cyclooxygenase, 23).

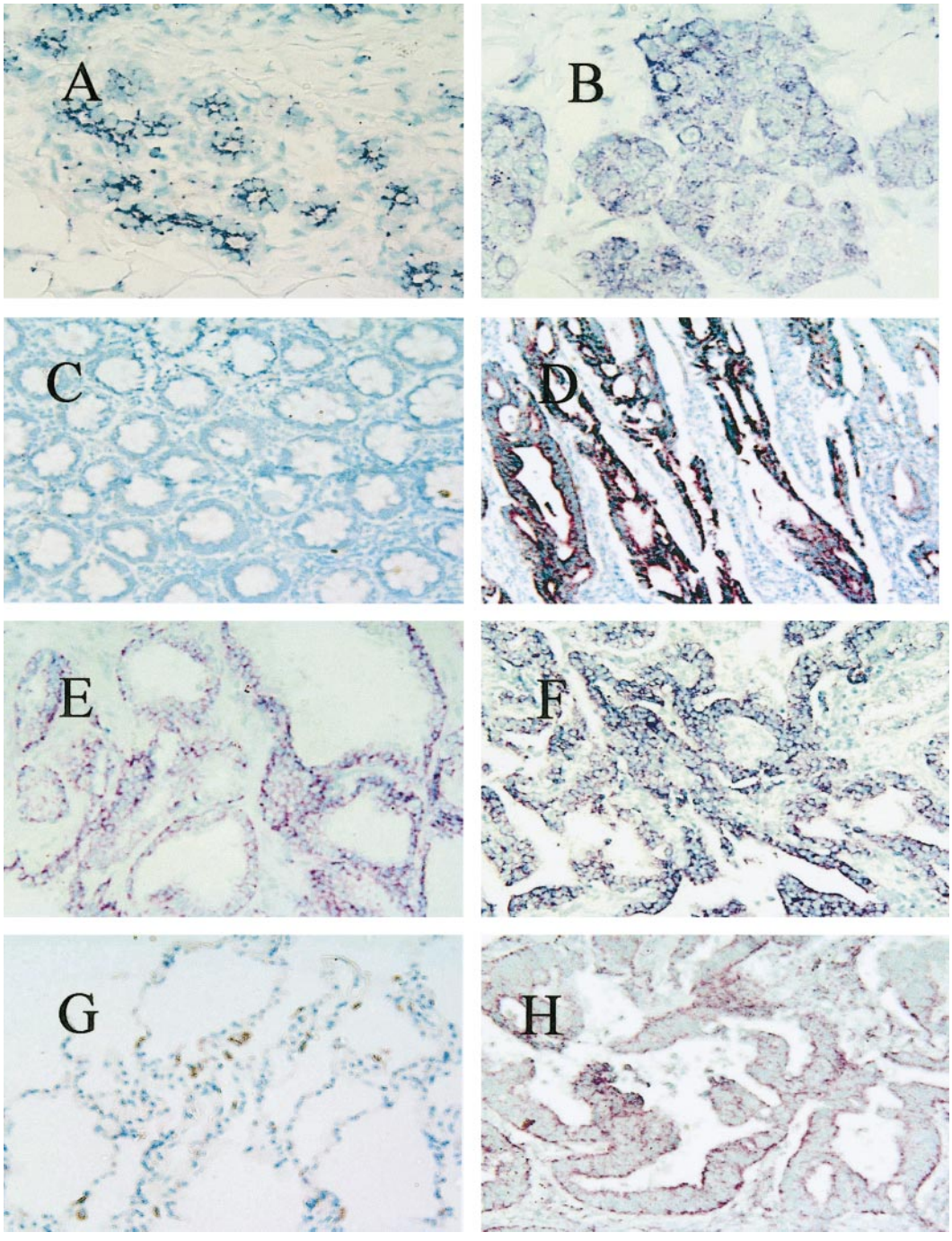

FIG. 9. Presence of endothelial-derived gene-1 (EG-1) in normal breast (A), breast cancer (B), normal colon (C), colon cancer (D), normal prostate (E), prostate cancer (F), normal lung (G), and lung cancer (H). *In situ* hybridization was performed as detailed under Materials and Methods.

The function of EG-1 is completely unknown. The molecule shows significant homology to a murine and a *Drosophila* form, whose functions are also unknown. Based on our own sequence analysis, EG-1 might be involved in signal transduction. The presence of four casein kinase II phosphorylation sites indicates that EG-1 might have the capacity to be a signaling molecule. EG-1 also shows some motif alignment with the Tim 10/DDP family zinc finger, the poly A polymerase regulatory subunit, the small IL-8-like cytokines, and the regulatory subunit of type II PKA R-subunit.

EG-1 may have a role in one or more steps of angiogenesis such as endothelial proliferation, migration or differentiation into tube-like structures. If this is true,

EG-1 can potentially be targeted in the treatment and diagnosis of human disease. Potential utility is seen in many angiogenesis-related diseases including heart disease and stroke, as well as in cancer.

ACKNOWLEDGMENT

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