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14. ABSTRACT

We report that EG-1 can stimulate cellular proliferation. Transfection experiments which overexpressed the full length EG-1 gene in human embryonic kidney HEK-293 cells or human breast cancer cell lines resulted in significantly increased in vitro proliferation, in comparison to transfection with empty vectors. On the other hand, siRNA co-transfection resulted in inhibition of proliferation. A subcutaneous xenograft assay was carried out in a SCID (severe combined immunodeficient) mouse model. We found that injection of high EG-1 expressing HEK-293 clones resulted in significantly larger tumors, in comparison with clones carrying the empty vectors. To further clarify the function of this gene, we investigated its interaction with Src and members of the MAPK (mitogen activated protein kinase) family. Immunoprecipitation with anti-Src antibody, followed by immunoblotting with anti-EG-1 antibody demonstrated an association between these two molecules. Over-expression of EG-1 was correlated with activation of the following kinases: ERK-1 and -2 (extracellular signal-regulated), JNK (Jun-terminal), and p38. These observations collectively support the hypothesis that the novel gene EG-1 is a positive stimulator of cellular proliferation, and may possibly be involved in signaling pathways involving Src and MAPK activation.

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Angiogenesis, endothelial gene, tumor marker

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DOD Grant "Endothelial Genes"

Introduction

Recently, our laboratory has utilized a subtraction hybridization technique to identify two novel genes: endomucin and EG-1 (endothelial-derived gene-1). Much of the work during the past year concentrated on the novel gene EG-1.

Body

We have made progress in all four tasks outlined in the grant Statement of Work. This progress is detailed as follows:

Task 1: to generate antibody-based assays for endomucin and EG-1. Months 1-18

a. Production of polyclonal antibodies: We have generated five different antibodies against EG-1 and five others against endomucin. These were made in rabbits, that were injected with peptide fragments of the above gene products.

b. Western analysis and immunoprecipitation studies of antibodies: We have succesfully carried out Western analysis and immunoprecipitation of in vitro translated products of genes EG-1 and endomucin. This data was included in the first annual report.

c. ELISA: We have made recombinant EG-1 peptide via transfection and FLAG-tag column purification. Work is underway to produce monoclonal antibodies.

Task 2: to perform studies to characterize the functions of endomucin and EG-1. Months 18-48

a. Antibody studies in assays of angiogenesis and immunohistochemical studies: We have successfully performed immunohistochemistry of human tissue specimens with the polyclonal antibodies against EG-1. This data is included in our paper published in Clinical Cancer Research (1).

We have carried out other assay studies to further understand the functions of the gene EG-1. We transfected a full length cDNA of EG-1 into HEK293 (human embryonic kidney) cells. Succesfully stable clones of transfected cells have increased EG-1 expression by Northern analyses. Subsequent experiments showed that the transfected cells have increased proliferation in comparison to the ones transfected with empty vectors. Subsequent experiments with the malignant breast cancer cell lines MCF-7 and MDA-MB-231 also showed that the transfected cells have increased proliferation in comparison to the ones transfected with empty vectors. This data is included in our paper to be published in Cancer Research (2).

b. Adenoviral vectors and the effect on HUVEC functions: to be accomplished.

Task 3: to conduct a clinical trial to assess whether angiogenesis markers correlate with the surgical removal of early stage breast cancer. We will measure the levels of multiple known angiogenic factors and inhibitors as well as E-selectin, endomucin, and EG-1 in the serum of early stage breast cancer patients. Months 1-36

a. Collect serum from surgical patients preop and postop: We have received permission from the UCLA (IRB) for this protocol, and are starting to enroll patients.

b. Measure the levels of angiogenesis markers and perform statistical comparisons between preop and postop values: to be accomplished.

Task 4: to conduct a clinical trial to assess whether angiogenesis markers correlate with the anti-angiogenic therapy of metastatic stage breast cancer. We will measure the levels of multiple known angiogenic factors and inhibitors as well as E-selectin, endomucin, and EDG-1 in the serum of metastatic breast cancer patients. Months 1-36

a. Collect serum from metastatic cancer patients prior and during/after anti-angiogenic therapy: We have received permission from the UCLA (IRB) Institutional Review Board and ISPRC (Internal Scientific Peer Review Committee) for this protocol, and are starting to enroll patients.

b. Measure the levels of angiogenesis markers and perform statistical comparisons between pre-treatment and post-treatment values: to be accomplished.

Key Research Accomplishments

1) The novel gene EG-1 stimulates cellular proliferation in vitro and in vivo.

2) EG-1 interacts with the oncogene c-Src.

Reportable Outcomes

1) A paper published in Clinical Cancer Research

2) A paper in press in Cancer Research

3) A patent application on EG-1

4) Two abstracts:

Zhang L., Lu M., Sartippour M.R., Norris A., Eilber F., and **Brooks M.N.** (2005). The effect of the gene EG-1 on proliferation. Proc. Am. Assoc. Cancer Res. 46:1293

Lu M., Zhang L., Sartippour M., Norris A., and **Brooks M.N.** (2005). The novel gene EG-1 interacts with c-Src. Proc. Am. Assoc. Cancer Res. 46:1095

Conclusions

Data thus far support that EG-1 may be relevant to the malignant process of tumorigenesis. Its expression is elevated in human cancer. It stimulates cellular proliferation in vitro and in vivo. These studies suggest possible important roles for our novel gene EG-1 in cancer detection and/or treatment.

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1) Zhang L, Maul RS, Rao J, Apple S, Seligson D, Sartippour M, Rubio R, **Brooks MN** (2004). Expression pattern of the novel gene EG-1 in cancer. Clin Cancer Res 10:3504-8

2) Lu M, Zhang L, Maul RS, Sartippour MR, Norris A, Whitelegge J, Rao J, **Brooks MN** (2005). The novel gene EG-1 stimulates cellular proliferation. Cancer Res, in press

Appendices

1) Zhang L, Maul RS, Rao J, Apple S, Seligson D, Sartippour M, Rubio R, **Brooks MN** (2004). Expression pattern of the novel gene EG-1 in cancer. Clin Cancer Res 10:3504-8

2) Lu M, Zhang L, Maul RS, Sartippour MR, Norris A, Whitelegge J, Rao J, **Brooks MN** (2005). The novel gene EG-1 stimulates cellular proliferation. Cancer Res, in press

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Expression Pattern of the Novel Gene EG-1 in Cancer

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ABSTRACT

Purpose: **We recently discovered a novel gene responsive to tumor-conditioned media: endothelial-derived gene 1 (EG-1). Its transcript has been shown to be present in epithelial cells, as well as in endothelial cells. In this study, we examined the levels of EG-1 protein expression in breast, colon, prostate, and lung cancers, which constitute the four most common solid malignancies in the United States.**

Experimental Design: **Polyclonal antibodies were generated that recognize the EG-1 peptide. These antibodies were used in immunoblot analysis, as well as immunohistochemistry of multiple human clinical specimens of cancer.**

Results: **In immunoblots of whole cell lysates, EG-1 antibodies revealed the presence of a 22-kDa peptide. Immunohistochemistry of breast, colon, and prostate specimens showed higher levels of EG-1 peptides in cancer tissues, in comparison with their benign counterparts. However, EG-1 expression was minimal in both benign and malignant lung tissues.**

Conclusions: **Here, we demonstrated that the expression of EG-1 is elevated in cancerous in comparison to benign epithelial cells, as seen in immunohistochemistry of human pathological specimens. These observations collectively support the hypothesis that the novel gene EG-1 is associated with the malignant phenotype of the common epithelial-derived cancers of the breast, colon, and prostate.**

INTRODUCTION

Cancer is a major cause of morbidity and the second leading cause of death in the American population. Overall, cancer incidence and mortality began to stabilize in the mid to late 1990s but have not improved significantly in recent years (1). Several major oncogenes and tumor suppressor genes have been identified to contribute to the neoplastic transformation of epithelial cells. These include p53, c-myc, ras, retinoblastoma, BRCA-1 and BRCA-2 (breast cancer susceptibility genes), Her-2, cyclin D1, and phosphatase and tensin homologue (2). Other alterations in the cell such as DNA methylation contribute to the overall genetic instability, whereas abnormal maintenance of telomerases results in replicative immortality (3).

Another important biological phenomenon in the tumorigenic and metastatic phenotype involves the process of angiogenesis. Three decades of experimental evidence has demonstrated that the growth and metastasis of solid tumors is dependent on their ability to initiate and sustain new capillary growth, *i.e.,* angiogenesis (4). Angiogenesis is a complex multistep process, which includes endothelial cell proliferation, migration, and differentiation into tube-like structures. These steps involve multiple growth factors, proteases, and adhesion molecules among endothelial cells, as well as those with other supporting cells (5). In the healthy human adult, the endothelium is generally quiescent, and turnover of endothelial cells is extremely slow. An exception to this is the angiogenesis that occurs during wound healing and endometrial proliferation. Abnormal angiogenesis occurs in rheumatoid arthritis, diabetic retinopathy, and in cancer growth and metastasis.

Multiple clinical observations in human cancer have added support to the hypothesis that tumors are angiogenesis dependent. The number of vessels in a tumor specimen correlates with the disease stage and can add prognostic value independent of other routinely used markers (6). Furthermore, the levels of various angiogenic factors in bodily fluids have been demonstrated to correlate with prognosis in cancer patients (7–9). Many agents have been developed to inhibit tumor angiogenesis, and there have been reports of some encouraging results (10, 11).

Several researchers, including our laboratory, have investigated the difference between molecules of the proliferating tumor endothelium from those in the normal quiescent endothelium (12, 13). To closely mimic a tumor environment, we have attempted to identify endothelial gene products expressed in response to a mixture of growth factors found in tumor conditioned media. Toward this goal, we used a subtraction hybridization method called suppression subtractive hybridization (14). In human umbilical vein endothelial cell (HUVEC) populations exposed to conditioned media from human cancer cells (15) for 4 h, we have isolated ~300 up-regulated and another 300 down-regulated clones (16, 17). One of these differentially expressed genes is endothelial-derived gene 1 (EG-1; Ref. 18). In the present study, we show that EG-1 expression is elevated in several cancer cell types. These results suggest that EG-1 may be a novel marker of the malignant phenotype of common epithelial-derived cancers, including breast, colon, and prostate.

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Note: L. Zhang and R. Maul contributed equally to this article. Research was performed at the University of California at Los Angeles Medical Center, Los Angeles, CA.
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MATERIALS AND METHODS

Cell Culture. Human embryonic kidney cells, HEK-293 and HEK-293T, and the human breast cancer cell, MDA-MB-231, were purchased from American Tissue Type Culture Collection (Manassas, VA), and maintained in DMEM (Life Technologies, Inc., Grand Island, NY) with 10% heat-inactivated FCS, 100,000 units/liter penicillin, and 100 mg/liter streptomycin, at 37°C in 5% CO_2. HUVECs were obtained from Cascade Biologics (Portland, OR). The cells were plated on tissue culture flasks coated with 1.5% gelatin (Difco, Detroit, MI) in PBS. They were maintained in endothelial growth media completed with low serum growth supplement (Cascade Biologics), penicillin, and streptomycin.

Transfection. We used the pcDNA3.1D/V5-His-TOPO (Invitrogen, Carlsbad, CA) and pShuttle-IRES-hrGFP-1 (Stratagene, La Jolla, CA) vectors to carry the full-length EG-1 gene. Empty vectors were used as negative controls. Liposomal reagents were used to transfect the pcDNA3.1D/V5-His-TOPO vectors into cells (19). pShuttle-IRES-hrGFP-1 vector with a 3xFLAG tag was transfected into HEK-293 or HEK-293T cells using the MBS Mammalian Transfection kit according to the manufacturer's protocol (Stratagene).

Generation of Antibodies. Polyclonal antibodies that recognize five different epitopes on human EG-1 were generated by Washington Biotechnology (Baltimore, MD). Briefly, different antigenic peptide fragments of human EG-1 were synthesized and used to immunize the rabbits. Preimmune and immune sera were harvested. Polyclonal antibodies were also affinity purified. For Western analysis, the secondary antibody used was horseradish peroxidase-conjugated goat antirabbit IgG from Jackson ImmunoResearch (West Grove, PA). The anti-FLAG M2 antibodies were obtained from Sigma (St. Louis, MO).

Western Analysis. Cell pellets were lysed in preheated 0.025 M Tris (pH 7.4), 0.001 M EDTA, and 0.3% SDS and then boiled for 5 min. The cell lysate was centrifuged at 12,000 × *g* for 10 min, and the supernatant was saved. Protein concentration was measured by the Bradford assay (Bio-Rad, Hercules, CA).

For Western analysis, ~40 μg of protein were separated by a 10% Tris-HCl Ready Gel (Bio-Rad) and transferred to a nitrocellulose membrane by electrophoretic blotting. The membrane was blocked overnight (4°C) with 5% nonfat dry milk in TBST (Tris-buffered saline, 0.1% Tween 20) and then incubated with a 1:500 dilution of EG-1 antiserum for 2 h. The blots were then washed three times over 30 min in TBST and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody goat antirabbit IgG (1:10,000) and then washed in PBS-Tween as before. The membranes were then developed using the Supersignal West Pico Chemiluminescent Western blotting detection system according to the manufacturer's instructions (Pierce, Arlington Heights, IL).

Human Tissue. Human archival tissue samples were obtained from the University of California at Los Angeles Department of Pathology. As for all studies involving human tissue, this study was conducted in compliance with the rules and regulations of the University of California at Los Angeles Institutional Review Board.

Immunohistochemistry. Immunohistochemical procedures were performed similarly to previously described methods (13, 20). Briefly, paraffin-embedded specimens were cut into 5-μm sections, then baked at 65°C for 30 min. H&E preparations of each specimen were performed to confirm the presence of nonnecrotic tumor. The paraffin was removed by incubation in xylene, followed by graded alcohols.

Immunostaining was performed with the DAKO Envision peroxidase rabbit ready-to-use system. The slides were sequentially incubated at room temperature as follows: (*a*) in DAKO antigen block reagent to block nonspecific antibody binding; (*b*) with the specific primary antibody for 1 h; (*c*) with the DAKO secondary antibody to rabbit for 30 min; and (*d*) developed with DAKO 3,3′-diaminobenzidine solution. The tissues were then stained with Gill's hematoxylin, dehydrated through graded alcohols, and mounted. For EG-1 studies, we used antigen retrieval with 0.01 M sodium citrate (pH 6.0) in a 95°C water bath for 20 min. The EG-1 antiserum was used at 1:400 dilution and EG-1 affinity-purified polyclonal antibodies at 1:2000. The negative control was preimmune rabbit serum at 1:400 dilution.

The histological slides were reviewed and scored by three pathologists (J. Rao, S. Apple, and D. Seligson). Both the staining intensity and percentage of staining were taken into consideration. The intensity of staining was graded from − to +++. Because the percentage of tumor cells staining correlated strongly with the staining intensity, the staining intensity was used as an indicator for EG-1 expression. Photography was carried out with a Leica DMLS microscope (McBain Instruments, Chatsworth, CA) and a Nikon CoolPix 995 digital camera (Tokyo, Japan).

Confocal Microscopy. Immunofluorescence labeling was performed in a Lab-Tek chamber slide (Nalge Nunc, Naperville, IL). Cells were fixed in 4% formalin, permeabilized in acetone, and washed in 1× PBS. Cells were placed in 75% ethanol for 5 min, 3% H_2O_2 for 20 min, and washed in 1× PBS. Cells were blocked in 5% goat serum in PBS for 30 min and incubated with EG-1 antiserum at a 1:400 dilution. Secondary antibodies, biotinylated antirabbit IgG (DAKO), were used at 1:200 dilution and Streptavidin-conjugated Texas Red (DAKO) as the final reporter. Confocal microscopy was performed with an Olympus AX 70 Confocal Microscope (Melville, NY) and the same Nikon digital camera.

RESULTS

The EG-1 Antibodies Recognized a 22-kDa Peptide. We generated five sets of rabbit antiserum against different antigenic synthetic peptide fragments of EG-1. Two of these five sets detected EG-1 bands on Western analysis and EG-1 signals in immunohistology studies. Western analysis of cell lysates demonstrated the presence of a 22-kDa peptide in MDA-MB-231 cells and two bands (28 and 30 kDa) in the HEK-293 cells transfected with the full-length EG-1 cDNA carrying the 3xFLAG signal (Fig. 1). The blots were also probed with anti-FLAG antibodies for confirmation. In previous analysis, the negative control using preimmune rabbit serum did not detect any EG-1 bands. The signal was slightly larger in the lysates from transfected cells because of the additional weight of the three FLAG proteins (6–8 kDa). In other studies, *in vitro* transcription and translation was carried out with the full-length

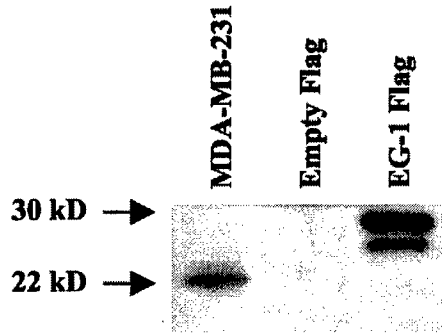

Fig. 1 Western analysis of endothelial-derived gene 1 (EG-1) expression in cell lysates. Forty μg of protein was loaded/lane. *Lane 1* represents cell lysates from the human breast cancer cells MDA-MB-231. *Lanes 2* and *3* contain cells lysates from HEK-293 cells transfected with empty vector and with EG-1 and 3xFLAG tag vector, respectively.

EG-1 cDNA without FLAG and yielded a single protein product at 20 kDa (data not shown).

Immunohistochemistry Revealed Increased Expression of EG-1 in Human Cancer. To examine the involvement of EG-1 in the malignant progression of human epithelial-derived cancers, the expression of EG-1 in multiple clinical samples was analyzed by immunohistochemistry. The histological slides were reviewed independently by three pathologists. The staining intensity of the slides was scored from − to +++ (Table 1). The archival pathological specimens were obtained from surgical resection of invasive breast, colon, prostate, and lung cancer cases. Corresponding benign areas from the same patient specimens were available for analysis in almost all cases. Fig. 2*A–H* shows representative sections of breast, colon, and prostate tissues, which demonstrate higher expression of the EG-1 protein in the cancer cells, in comparison to the benign epithelial cells from the same surgical specimens. The first specimen was obtained from a 1.5-cm invasive ductal breast carcinoma case, poorly differentiated with high nuclear grade, extensive comedo ductal carcinoma *in situ* with estrogen receptor positive, progesterone receptor positive, Her2 positive, and negative axillary lymph nodes. The second specimen was derived from a colon adenocarcinoma case, 7 cm in length, moderately differentiated, with lymphovascular invasion, extending to the serosa, 4/12 positive lymph nodes, and liver metastasis. The third specimen was obtained from a 3.5-cm prostate adenocarcinoma case, Gleason grade 4 + 3 = 7, extending into but not through the capsule, with perineural invasion, and negative nodes. Table 1 summarizes the characteristics of these cancer cases and their observed staining intensities for the EG-1 peptide. Cancer stage was assigned by the standard Tumor-Node-Metastasis classification of malignant tumors. We observed minimal expression of EG-1 in seven lung cancer cases (four squamous and three adenocarcinoma), both in the malignant and corresponding normal epithelial cells.

We also observed minimal EG-1 staining in inflammation or wound healing situations. Fig. 2*I–J* shows no staining in specimens from inflamed breast tissue and granulated healing breast tissue.

Observations from several immunohistochemical specimens showed that the EG-1 protein appeared to be localized mostly in the cytoplasm of the cells and partially in the nucleus. Confocal microscopy performed on HUVECs in culture also confirmed this observation (Fig. 2, *K* and *L*).

Table 1 Immunohistochemistry of EG-1 in human cancerous tissues and their benign counterparts (in the same specimens): breast; colon; and prostate

Specimen no.	Histology	Stage	EG in cancer	EG in benign
Breast				
1	Invasive ductal	1	+++	−
2	Invasive	3	+++	−
3	Inv ductal/lobular	2	+++	−
4	Invasive ductal	3	+++	+
5	Invasive ductal	2	+++	+
6	Invasive ductal	2	+++	+
7	Invasive ductal	1	+++	+
8	Invasive ductal	2	+++	+
9	Invasive lobular	2	+++	+
10	Invasive ductal	2	+++	+
11	Invasive ductal	3	+++	+
12	Invasive ductal	2	+++	++
13	Invasive ductal	1	+++	+++
14	Invasive ductal	2	++	−
15	Invasive ductal	2	++	−
16	Invasive ductal	2	++	−
17	Invasive ductal	2	++	+
18	Invasive ductal	2	++	+
19	Invasive ductal	1	++	+
20	Invasive lobular	1	++	+
21	Inflammatory	3	++	+
22	Invasive ductal	1	++	++
23	Invasive tubular	1	++	++
24	Invasive ductal	1	++	+++
25	Invasive ductal	2	++	+++
26	Invasive ductal	2	++	N/A[a]
27	Squamous	3	++	N/A
28	Invasive ductal	2	+	−
29	Invasive ductal	1	+	+
30	Invasive ductal	2	+	++
31	Invasive ductal	3	+	+++
32	Invasive ductal	1	±	−
Colon				
1	Adenocarcinoma	4	+++	−
2	Adenocarcinoma	3	+++	−
3	Adenocarcinoma	3	+++	−
4	Adenocarcinoma	4	+++	+
5	Adenocarcinoma	1	+++	+
6	Adenocarcinoma	4	+++	+
7	Adenocarcinoma	3	+++	+
8	Adenocarcinoma	3	+++	+
9	Adenocarcinoma	2	++	+
Prostate				
1	Adenocarcinoma	2	++	+
2	Adenocarcinoma	2	+	−
3	Adenocarcinoma	2	+	−
4	Adenocarcinoma	3	+	−
5	Adenocarcinoma	2	+	−
6	Adenocarcinoma	3	+	N/A
7	Adenocarcinoma	2	−	−
8	Adenocarcinoma	2	−	−
9	Adenocarcinoma	2	−	−
10	Adenocarcinoma	2	−	−
11	Adenocarcinoma	2	−	−

[a] N/A, not available.

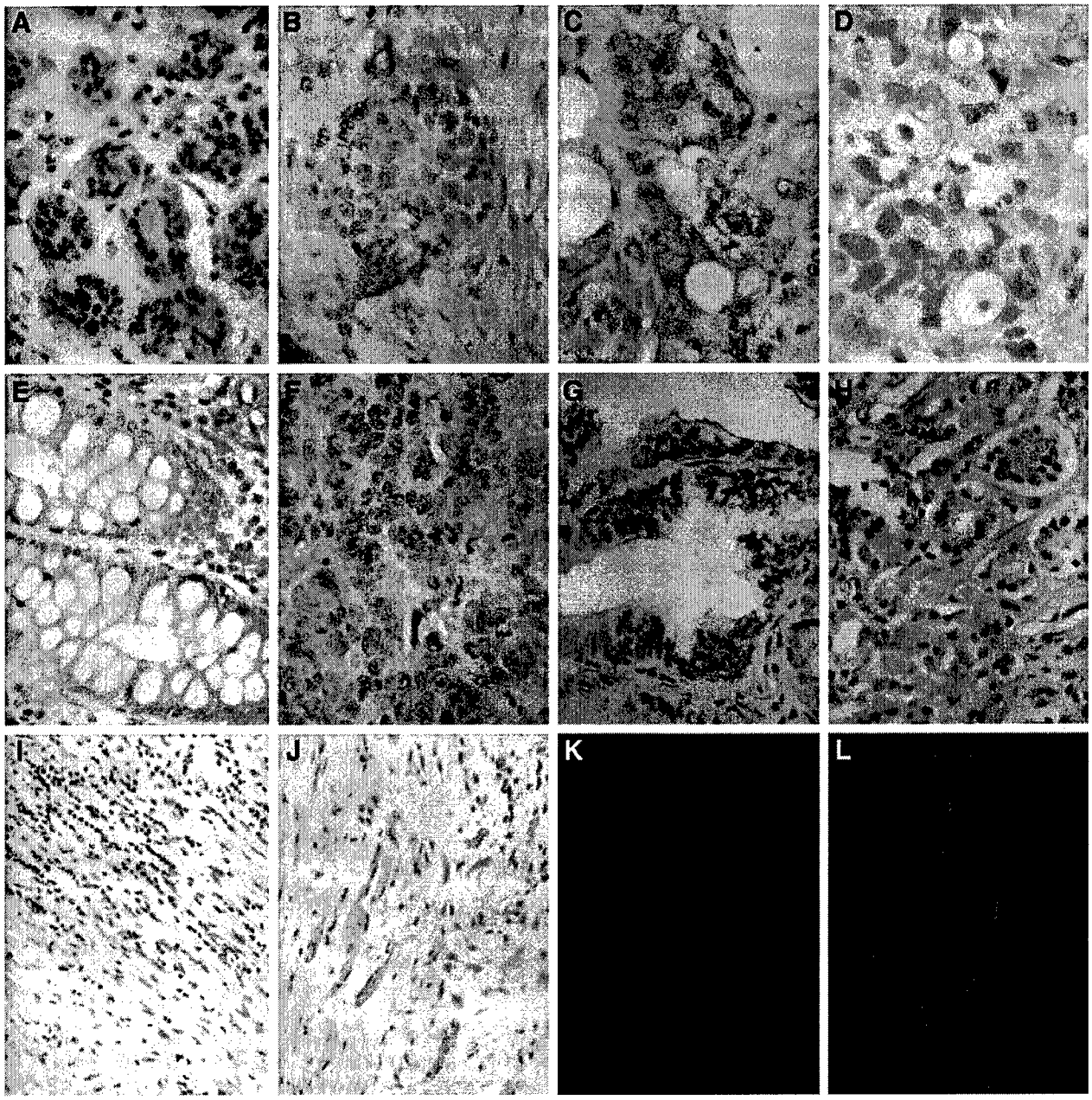

Fig. 2 *A–J*, immunohistochemistry of human specimens, with positive staining in *brown: A*, benign breast, endothelial-derived gene 1 (EG-1) antibody; *B*, breast ductal carcinoma *in situ*, EG-1 antibody; *C*, breast invasive cancer, EG-1 antibody; *D*, breast invasive cancer, control preimmune serum; *E*, benign colon, EG-1 antibody; *F*, colon adenocarcinoma, EG-1 antibody; *G*, benign prostate, EG-1 antibody; *H*, prostate adenocarcinoma, EG-1 antibody; *I*, inflamed breast, EG-1 antibody; *J*, granulated healing breast, EG-1 antibody. *K* and *L*, confocal immunofluorescence of human umbilical vein endothelial cells, with positive staining in *red: K*, control preimmune serum and (*L*) EG-1 antiserum.

DISCUSSION

We show here that the expression of EG-1 is elevated in cancerous in comparison to benign epithelial cells, as seen in immunohistochemistry of several human pathological specimens. These observations collectively support the hypothesis that the novel gene EG-1 is associated with the malignant phenotype of the common epithelial-derived cancers of the breast, colon, and prostate. In this small sample size, colon cancer seems to consistently have elevated EG-1 signals, whereas the increased staining pattern is more variable in breast and prostate cancer types. Lung cancer does not appear to express much EG-1, as detected by our first generation of polyclonal antibodies. It is possible that this staining pattern may change with future new and improved antibodies against EG-1, as well as a larger sample size.

The first and only publication to date on EG-1 came from

our laboratory (18). Suppression subtractive hybridization revealed an RNA sequence (GenBank accession no. AW735731), the expression of which is increased in HUVECs treated with tumor conditioned media derived from human cancer cells. Subsequent cloning of the full-length cDNA from a HUVEC library (AF358829), and a Basic Local Alignment Search Tool for Nucleotide search in the GenBank database shows that EG-1 is on chromosome no. 4. It spans four exons and three introns. The human EG-1 sequence has significant homology to a murine cDNA (94%) and a Drosophila cDNA (31%). From the nucleotide sequence, the predicted peptide has 178 amino acids and weighs 19.5 kDa. This is consistent with our Western analysis results which reveal a protein at slightly higher weight than that predicted above, suggesting some degree of posttranslational modifications.

A Basic Local Alignment Search Tool for Nucleotide search for sequence homology performed in the GenBank database reveals that EG-1 has no significant homology to any gene with a known function. A Profile Scan search reveals a long proline-rich region, one *N*-glycosylation site, two *O*-glycosylation sites, four casein kinase II phosphorylation sites, and two *N*-myristoylation sites. A search looking for motif match shows some alignment with the following: disheveled specific domain; Wilms' tumor protein signature; phosphoinositide 3-kinase family; ras-binding domain; C2 domain; p85-binding domain; breast cancer type I susceptibility protein signature and BRCA2 repeat; C-C chemokine receptor type 9 signature; cadherin-2; xeroderma pigmentosum group B protein signature; and SKI/SNO proto-oncogene. Although the function of EG-1 is to be determined, its sequence alignment with multiple oncogenes and cancer-related genes is consistent with our hypothesis that it may be involved in tumorigenesis.

In summary, based on its expression profile in human tissues, EG-1 appears to be particularly relevant to those cancer types of ductal epithelial origin such as breast, colon, and prostate. These results will form the basis for additional studies of this interesting gene and the possible translation of the discovery of this molecule into potential use in cancer diagnosis and/or treatment.

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Research Article

The Novel Gene *EG-1* Stimulates Cellular Proliferation

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Abstract

We recently discovered a novel gene and named it endothelial-derived gene 1 (*EG-1*). Previously, we have shown that the expression of EG-1 is significantly elevated in the epithelial cells of breast cancer, colorectal cancer, and prostate cancer. Here, we report that EG-1 can stimulate cellular proliferation. Transfection experiments which overexpressed the full-length *EG-1* gene in human embryonic kidney HEK-293 cells or human breast cancer cell lines resulted in significantly increased *in vitro* proliferation, in comparison with transfection with empty vectors. On the other hand, small interfering RNA cotransfection resulted in inhibition of proliferation. S.c. xenograft assays were carried out in a severe combined immunodeficient mouse model. We found that injection of high EG-1 expressing HEK-293 clones resulted in significantly larger tumors, in comparison with clones carrying the empty vectors. To further clarify the function of this gene, we investigated its interaction with Src and members of the mitogen-activated protein kinase (MAPK) family. Immunoprecipitation with anti-Src antibody, followed by immunoblotting with anti–EG-1 antibody, showed an association between these two molecules. Overexpression of EG-1 was correlated with activation of the following kinases: extracellular signal-regulated kinases 1 and 2, c-jun-NH_2-kinase, and p38. These observations collectively support the hypothesis that the novel gene *EG-1* is a positive stimulator of cellular proliferation, and may possibly be involved in signaling pathways involving Src and MAPK activation. (Cancer Res 2005; 65(14): 1-8)

Introduction

Cancer is a major cause of morbidity and the second leading cause of death in the American population. Several major oncogenes and tumor suppressor genes have been identified to contribute to the neoplastic transformation of epithelial cells. These include *src*, *p53*, c-*myc*, *ras*, *Rb* (retinoblastoma), *BRCA-1* and *BRCA-2* (breast cancer susceptibility genes), *Her-2*, *cyclin D1*, and *PTEN* (phosphatase and tensin homologue; ref. 1). Other alterations in the cell, such as DNA methylation, contribute to the overall genetic instability, and abnormal maintenance of telomerases results in replicative immortality (2).

Another important biological phenomenon in the tumorigenic and metastatic phenotype involves the process of angiogenesis. Three decades of experimental evidence has shown that the growth and metastasis of solid tumors is dependent on their ability to initiate and sustain new capillary growth (i.e., angiogenesis; ref. 3). Multiple clinical observations in human cancer have added support to the hypothesis that tumors are angiogenesis dependent. The number of vessels in a tumor specimen correlates with the disease stage and can add prognostic value independent of other routinely used markers (4). Furthermore, the levels of various angiogenic factors in bodily fluids have been shown to correlate with prognosis in cancer patients (5–7). Many agents have been developed to inhibit tumor angiogenesis, and there have been reports of some encouraging results (8, 9).

Several researchers, including our laboratory, have investigated the difference between molecules of the proliferating tumor endothelium and those of the normal quiescent endothelium (10, 11). To closely mimic a tumor environment, we have attempted to identify endothelial gene products expressed in response to a mixture of growth factors found in tumor-conditioned media. Toward this goal, we used a subtraction hybridization method called suppression subtractive hybridization (12). In human umbilical vein endothelial cell populations exposed to conditioned media from human cancer cells (13) for 4 hours, we have isolated multiple clones (14, 15). One of these differentially expressed genes is endothelial-derived gene 1 (*EG-1*; ref. 16). In addition to its expected presence in blood vessels, EG-1 expression is significantly elevated in the epithelial cells of several cancers including breast, colorectal, and prostate cancer (17). In this article, we present *in vitro* and *in vivo* data which suggest that EG-1 stimulates cellular proliferation and may be involved in signaling pathways involving Src and mitogen-activated protein (MAP) kinase (MAPK) activation.

Materials and Methods

Cell culture. Human embryonic kidney HEK-293 cells and the human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from American Tissue Type Culture Collection (Rockville, MD). The cells were maintained in DMEM (Invitrogen, Carlsbad, CA) with 10% heat-inactivated FCS, 100,000 units/L penicillin, and 100 mg/L streptomycin at 37°C in 5% CO_2.

Proliferation assay. The cells were plated onto 48-well culture plates at 10,000 cells/well and incubated at 37°C in 5% CO_2 for 48 hours in DMEM with 10% FCS. On the third day, 1 μCi of [methyl-^{3}H]thymidine (Amersham, Piscataway, NJ) was added to each well. Approximately 15 hours later, the plates were washed with PBS. The cells were fixed with trichloroacetic acid, washed with ethyl alcohol, and lysed with sodium hydroxide. After adding glacial acetic acid, the radioactivity of the cell lysates was counted in scintillation solution (ScintiVerse, Fisher, Pittsburgh, PA). The *in vitro* assays were done in triplicates. Certain experiments were carried out with MAPK inhibitors (PD98059, SB203580, and U0126) purchased from Calbiochem (La Jolla, CA). The cells were treated with 10 mmol/L of one of the above inhibitors for 24 hours before harvest.

Transfection. We used the pcDNA3.1D/V5-His-TOPO vectors (Invitrogen) to carry the full-length human *EG-1* gene according to the instructions of the manufacturer. Empty vectors were used as negative controls. Specifically, standard calcium-phosphate DNA coprecipitation was used for

Note: M. Lu and L. Zhang contributed equally to the work.

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obtaining stable transfectants. Individual clones were selected for Geneticin (Invitrogen) resistance over a period of several weeks. Expression of the *EG-1* gene by individual clones was confirmed by Northern and Western blot analyses.

For transient transfection, we used the pcDNA3.1D/V5-His-TOPO and pShuttle-IRES-hrGFP-1 (Stratagene, La Jolla, CA) vectors to carry the full-length *EG-1* gene. Empty vectors were used as negative controls. Liposomal reagents were used to transfect the pcDNA3.1D/V5-His-TOPO vectors into cells using the MBS Mammalian Transfection Kit according to the protocol of the manufacturer (Stratagene).

Small interfering RNA. EG-1 expression knockdown was achieved by transfecting a lentivirus vector expressing a small interfering RNA (siRNA) against EG-1, *cis*-linked with a green fluorescent protein expression cassette, into HEK-293 cells or breast cancer cells. The pCSUECG plasmid (U6-shRNA-EG1-CMV-GFP) was constructed by ligating the Bam*HI*/Eco*RI* digests of pCSCG and the U6-shRNA-EG1 PCR product. The U6-shRNA-EG1 PCR was done using a hU6-containing plasmid at an annealing temperature of 60°C with the primers 5′-GGGGGATCCCAAGGTCGGGCAGGAAGAGGGCCTATTTCC-3′; for siRNA1, 5′-GGGGAATTCAAAAAGAAATTCGAACCGGTGTTGTCTCTTGAACAACACCGGTTCGAATTTCGGTGTTTCGTCCTTTCCACAAGATATATAAA-3′; for siRNA2, 5′-GGGGAATTCAAAAAGTTTCTGGATATTGCAAGATCTCTTGAATCTTGCAATATCCAGAAACGGTGTTTCGTCCTTTCCACAAGATATATAAA-3′; and for siRNA3, 5′-GGGGAATTCAAAAAGAGGATGTGTCAGAACTAATCTCTTGAATTAGTTCTGACACATCCTCGGTGTTTCGTCCTTTCCACAAGATATATAAA-3′.

Mouse tumor model. Severe combined immunodeficient (SCID) mice were bred in a pathogen-free colony at the University of California at Los Angeles School of Medicine. Eight- to ten-week-old female mice were housed four per cage, and fed *ad libitum* with sterilized food pellets and sterile water. We injected cells s.c. into the flank of the mice. We examined the effect of EG-1 on the tumorigenicity of HEK-293 cells. Wild-type, stably transfected empty vector or EG-1–overexpressing clones (4×10^7-5×10^7 cells) were injected s.c. into the flank of SCID mice. The tumor size was measured in three dimensions with calipers starting at day 7. The mice were observed for any change in behavior, appearance, or weight. At the end of the experiment, the mice were sacrificed by nitrogen gas environment. The primary tumor tissues were fixed in 10% formalin and converted into paraffin blocks.

Generation of antibodies. Polyclonal antibodies that recognize five different epitopes on human EG-1 were generated by Washington Biotechnology (Baltimore, MD). Briefly, different antigenic peptide fragments of human EG-1 were synthesized and used to immunize the rabbits. Preimmune and immune sera were harvested. Polyclonal antibodies were also affinity purified. For Western blot analysis, the secondary antibody used was horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (IgG) from Jackson ImmunoResearch (West Grove, PA). The anti-FLAG M2 antibodies were obtained from Sigma (St. Louis, MO). The antibodies against MAPK and phospho-MAPK, c-jun-NH_2-kinase (JNK) and phospho-JNK, p38 and phospho-p38, and Src were purchased from Cell Signaling (Beverly, MA).

Immunohistochemistry. Paraffin-embedded specimens were cut into 5 μm sections, then baked at 65°C for 30 minutes. H&E preparations of each specimen were done to confirm the presence of nonnecrotic tumor. The paraffin was removed by incubation in xylene, followed by graded alcohols. Immunostaining was done with the DAKO Envision peroxidase rabbit ready-to-use system. The slides were sequentially incubated at room temperature as follows: (*a*) in DAKO antigen block reagent to block nonspecific antibody binding; (*b*) with the specific primary antibody for 1 hour; (*c*) with the DAKO secondary antibody to rabbit for 30 minutes; and (*d*) developed with DAKO diaminobenzidine solution. The tissues were then stained with Gill's hematoxylin, dehydrated through graded alcohols, and mounted. For EG-1 studies, we used antigen retrieval with 0.01 mol/L sodium citrate (pH 6.0) in a 95°C water bath for 20 minutes. The EG-1 antiserum was used at 1:400 dilution, and EG-1 affinity-purified polyclonal antibodies at 1:2,000. The negative control was preimmune rabbit serum at 1:400 dilution. The histologic slides were reviewed by a Board-certified pathologist (J.Y.R.). Photography was carried out with a Leica DMLS microscope (McBain Instruments, Chatsworth, CA) and a Nikon CoolPix 995 digital camera (Tokyo, Japan).

Western blot analysis. Cell pellets were lysed in preheated 0.025 mol/L Tris (pH 7.4), 0.001 mol/L EDTA, and 0.3% SDS, and then boiled for 5 minutes. The cell lysate was centrifuged at 12,000 × *g* for 10 minutes, and the supernatant was saved. Protein concentration was measured by the Bradford assay (Bio-Rad, Hercules, CA). For Western blot analysis, ~40 μg of protein were separated by a 12% SDS-PAGE gel, and transferred to a nitrocellulose membrane by electrophoretic blotting. The membrane was blocked overnight (4°C) with 5% nonfat dry milk in TBS-0.1% Tween 20, and then incubated with a 1:500 dilution of EG-1 antiserum for 2 hours. The blots were then washed thrice over 30 minutes in TBS-Tween 20, and incubated for 1 hour with horseradish peroxidase–conjugated secondary antibody goat anti-rabbit IgG (1:10,000), and then washed in TBS-Tween 20 as before. The membranes were then developed using the Supersignal West Pico Chemiluminescent Western blotting detection system according to the instructions of the manufacturer (Pierce, Arlington Heights, IL).

Immunoprecipitation. Cell lysates were preincubated solely with protein A/G Plus-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 1 hour, and the mixture was centrifuged at 3,000 × *g* for 5 minutes to pellet these beads and any nonspecific interacting proteins. One milligram of supernatant protein was incubated with anti–EG-1 antibody and 30 μL of protein A/G Plus-Agarose overnight at 4°C under agitation, and 1 mg of proteins from the same source was incubated with normal rabbit IgG (Santa Cruz Biotechnology) and protein A/G Plus-Agarose (for negative controls). After incubation, immunocomplexes were pelleted by centrifugation at 3,000 × *g* for 5 minutes at 4°C. The pellets were then resuspended and washed three additional times with immunoprecipitation buffer to remove nonspecific interactions. Laemmli loading buffer was then added to the beads. After boiling, the proteins were separated by 12% SDS-PAGE and analyzed by Western blot.

Size exclusion chromatography-mass spectrometry. Anti-FLAG M2 affinity gel (Sigma) was used to purify recombinant FLAG-tagged EG-1 peptide from transiently transfected HEK-293 cells, following the protocol of the manufacturer. Briefly, cell lysates were collected and loaded onto the column under gravity flow. The column was washed and then eluted with seven 1-mL aliquots of 0.1 mol/L glycine-HCl at pH 3.5 into vials containing 25 μL of 1 mol/L Tris (pH 8.0). The vial with the highest concentration of the EG-1 protein (vial 2) was subjected to mass spectrometry (MS).

The protocol of Whitelegge et al. (18) was used to separate the EG-1 from salt by size exclusion chromatography before analysis in the mass spectrometer (size exclusion chromatography-MS). Approximately 10 μg of EG-1 suspended in 0.1 mol/L glycine-HCl at pH 7.0 were dried down in vacuo (SpeedVac) and then resuspended in 100 μL of 90% formic acid immediately before size exclusion chromatography-MS. The size exclusion chromatography was done using a mobile phase of $CHCl_3$/methanol/1% aqueous formic acid (4:4:1, v/v/v) and a Super SW 2000 column (4.6 × 300 mm, Tosoh Bioscience, Montgomeryville, PA) at 250 μL/min and 40°C. Before delivery to the electrospray-ionization source, the column effluent was monitored with a UV detector (280 nm). Electrospray ionization MS was done using a triple quadrupole instrument (API III, Applied Biosystems) tuned and calibrated as described (19). Data were processed using MacSpec 3.3, Hypermass, and BioMultiview 1.3.1 software (Applied Biosystems).

Microliquid chromatography with tandem mass spectrometry. After the EG-1 peptides were eluted from the FLAG column, we confirmed the presence and purity of EG-1 by Western blotting. Then the sample was subjected to 12% SDS-PAGE, and the gel was stained by SYPRO Ruby Protein Gel Stain (Molecular Probes, Eugene, OR) following the manual from the manufacturer. Subsequently, the EG-1 bands were excised and dehydrated in acetonitrile for 30 minutes, and dried completely by SpeedVac. DTT (10 mmol/L) dissolved in 100 mmol/L NH_4HCO_3 was added to the sample, which was then incubated for 1 hour at 56°C. The liquid was removed, and 55 mmol/L iodoacetamide dissolved in 100 mmol/L NH_4HCO_3 was added for 45 minutes at room temperature in the dark. The sample was washed in 100 mmol/L NH_4HCO_3 for 10 minutes, dehydrated in acetonitrile for 30 minutes, followed by swelling in 100 mmol/L NH_4HCO_3

for 30 minutes, dehydrated again in acetonitrile for 30 minutes, and finally dried completely by SpeedVac. Then, the sample was digested in trypsin solution (50 mmol/L NH_4HCO_3, 5 mmol/L $CaCl_2$, 12.5 ng/μL trypsin) on ice for 45 minutes. The liquid was then removed, and the sample incubated in the same solution without trypsin at 37°C overnight. On the following day, the sample was washed with 20 mmol/L NH_4HCO_3; then the extraction of the EG-1 peptides was carried out with 5% formic acid and 50% acetonitrile for 20 minutes, and repeated twice. The sample was extracted once with acetonitrile for 20 minutes. All the postdigestion extractions were pooled together and dried down by SpeedVac.

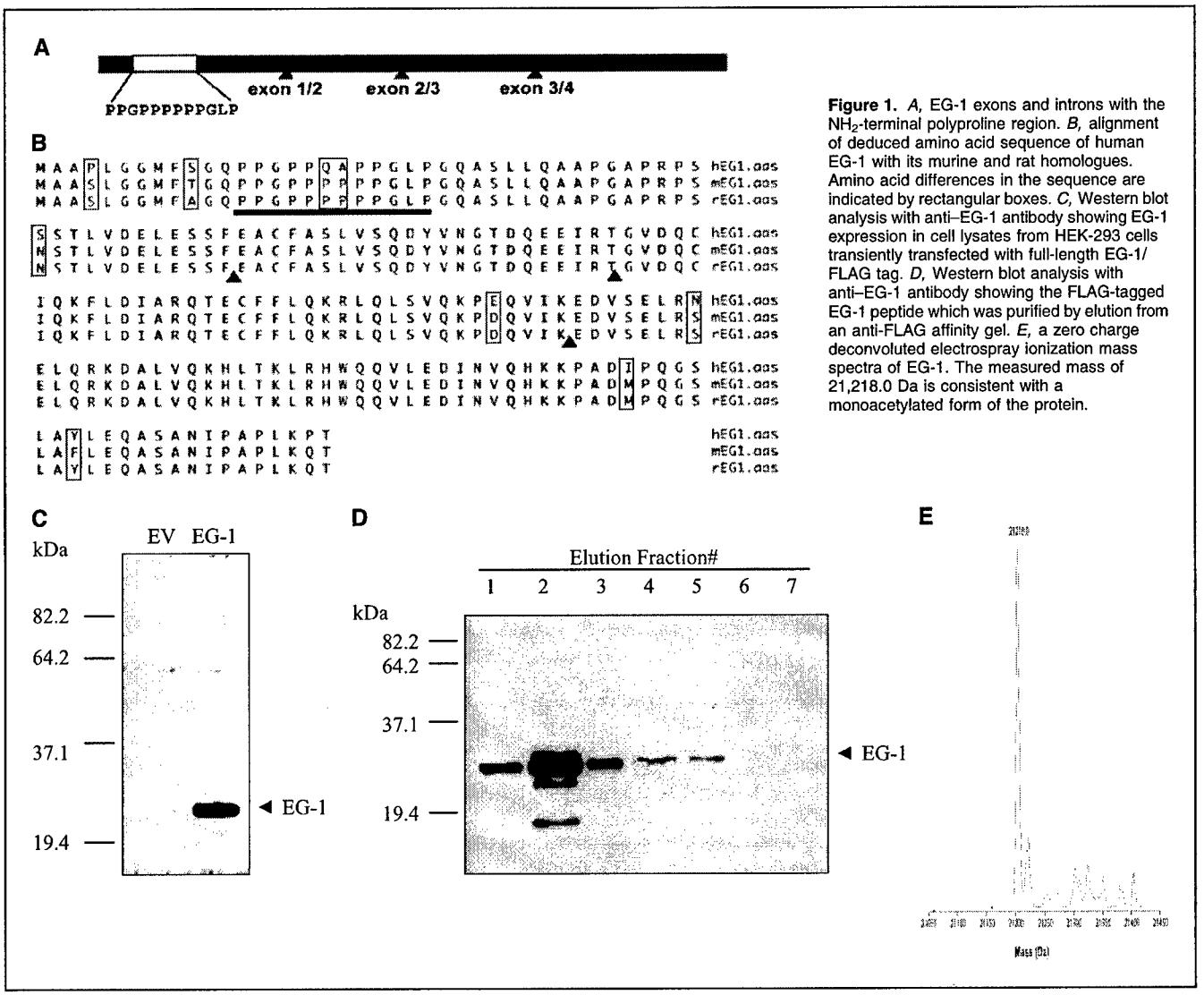
Q4 Samples were analyzed by microliquid chromatography with tandem mass spectrometry (micro-LC-MS/MS) with data-dependent acquisition (LCQ-DECA, ThermoFinnigan, San Jose, CA) after dissolution in 5 μL of 70% acetic acid (v/v). A reverse-phase column (200 μm × 10 cm; PLRP/S 5 μm, 300 Å; Michrom Biosciences, San Jose, CA) was equilibrated for 10 minutes at 1.5 μL/min with 95% A, 5% B (A, 0.1 % formic acid in water; B, 0.1% formic acid in acetonitrile) before sample injection. A linear gradient was initiated 10 minutes after sample injection ramping to 60% A, 40% B after 50 minutes and 20% A, 80% B after 65 minutes. Column eluent was directed to a coated glass electrospray emitter (TaperTip, TT150-50-50-CE-5, New Objective) at 3.3 kV for ionization without nebulizer gas. The mass spectrometer was operated in "triple-play" mode with a survey scan (*m/z* 400-1,500), data-dependent zoom scan and MS/MS. Individual sequencing experiments were matched to a custom protein sequence database using Sequest software (ThermoFinnigan).

Statistical analysis. Descriptive statistics, such as mean and SE, were used to summarize the results. The ANOVA test was done for comparison among the various groups followed by the Bonferroni posttest. The Student's *t* test was used for comparison between only two groups. Statistical significance is defined by $P < 0.05$.

Results

The *EG-1* gene and peptide. A BLASTN search for sequence homology done in the GenBank database revealed that EG-1 has no significant homology to any gene with a known function. The homology of the human EG-1 peptide to its mouse counterpart is 94.9%, and 95.5% to its rat counterpart. The homology between mouse and rat EG-1 is 98.9%. A Profile Scan search revealed a proline-rich region in the NH_2 terminus (Fig. 1*A* and *B*). Of interest, F1 we have found similar sequences to EG-1 in several insects, but these seem to lack the NH_2-terminal polyproline region.

Western blot analysis showed that the transfected full-length EG-1/FLAG exists primarily as a 22 kDa protein, with possible degradation peptide products (Fig. 1*C* and *D*). It should be noted



Q5

Figure 1. *A*, EG-1 exons and introns with the NH_2-terminal polyproline region. *B*, alignment of deduced amino acid sequence of human EG-1 with its murine and rat homologues. Amino acid differences in the sequence are indicated by rectangular boxes. *C*, Western blot analysis with anti–EG-1 antibody showing EG-1 expression in cell lysates from HEK-293 cells transiently transfected with full-length EG-1/FLAG tag. *D*, Western blot analysis with anti–EG-1 antibody showing the FLAG-tagged EG-1 peptide which was purified by elution from an anti-FLAG affinity gel. *E*, a zero charge deconvoluted electrospray ionization mass spectra of EG-1. The measured mass of 21,218.0 Da is consistent with a monoacetylated form of the protein.

that our transfectant EG-1 product contains at the NH_2 terminus 15 additional amino acids containing the FLAG tag (MDYKDDDDKN-SAGSN). Based on amino acid sequence alone, the calculated masses of EG-1 alone and EG-1/FLAG tag transfectant are 19,520.2 and 21,176.8 Da, respectively.

To confirm the size of the EG-1/FLAG tag transfectant product, we did size exclusion chromatography-MS. Figure 1*D* shows the zero charge deconvoluted electrospray ionization mass spectra of EG-1. The measured mass of 21,218.0 Da is within 0.01% of that calculated for a monoacetylated (M ± 42 Da) form of EG-1 (21,218.8 Da; average mass) based on analysis using the ExPASy Proteomics Server (Swiss Institute of Bioinformatics).

Micro-LC-MS/MS was done so that peptides generated from a tryptic digest of the FLAG Tag EG-1 peptide could be searched against the protein sequence database using Sequest (ThermoFinnigan). T1 Peptides uniquely matching the EG-1 sequence are shown in Table 1. No significant matches with proteins other than EG-1 were found.

Overexpression of EG-1 stimulates HEK-293 proliferation. We transfected a full-length cDNA of EG-1 carrying a FLAG tag into HEK-293 cells (these cells do have a low level of endogenous EG-1). Successfully stable clones of transfected cells were confirmed to have increased EG-1 expression by Northern blot analyses (data not shown). Subsequent experiments showed that the EG-1–transfected cells have increased proliferation [28,092 ± 950 counts per minute (cpm)] in comparison with the ones transfected with empty vectors (16,546 ± 462 cpm, $P < 0.001$) as well as with the F2 wild-type cells (16,608 ± 627 cpm, $P < 0.001$; Fig. 2*A*). Figure 2*B* shows the corresponding levels of EG-1 peptide in these cells. One milligram of cell lysates was immunoprecipitated with anti–EG-1 antibody, and then immunoblotted also with anti–EG-1 antibody. EG-1 stably transfected cells contain ~3-fold as much of the 22 kDa EG-1/FLAG peptide, in comparison with empty vector–transfected cells.

Suppression of EG-1 inhibits cellular proliferation. We designed multiple plasmids carrying different siRNAs to the EG-1 sequence. These siRNAs were cotransfected with a full-length cDNA of EG-1/FLAG into HEK-293 cells. The proliferation results are as follows: 34,847 ± 2,060 cpm in vector-transfected cells; 26,892 ± 801 cpm in cells transfected with siRNA1; 25,785 ± 970 cpm in cells transfected with siRNA2; and 12,548 ± 12 cpm in cells transfected with siRNA3 (Fig. 2*C*). ANOVA analysis shows that the inhibitory effect exerted by siRNA3 is significant in comparison with that by vector alone ($P < 0.001$). Whole-cell lysates were collected 24 hours after transfection, subjected to SDS-PAGE, then immunoblotted with anti–EG-1 antibody. This analysis showed minimal levels of EG-1 peptide in cells cotransfected with siRNA3 (Fig. 2*D*).

Overexpression of EG-1 stimulates breast cancer cell proliferation. We transiently transfected a full-length cDNA of EG-1 carrying a FLAG tag into the human breast cancer cell lines MCF-7 and MDA-MB-231. Subsequent experiments showed that the EG-1–transfected MCF-7 cells have increased proliferation (9,613 ± 694 cpm) in comparison with the ones transfected with empty vectors (7,213 ± 252 cpm). Cotransfection with EG-1 and siRNA3 abrogated the increased proliferation (5,947 ± 264 cpm, $P = 0.0002$; Fig. 3*A*). The same phenomenon was observed with F3 MDA-MB-231 cells transfected with empty vector (34,651 ± 3,756 cpm), with EG-1 (61,605 ± 2,288 cpm), and with EG-1 and siRNA3 combination (34,197 ± 429 cpm, $P < 0.0001$; Fig. 3*C*). Figure 3*B* and *D* shows the corresponding levels of EG-1 peptide in these cells, as shown with Western blot analysis of cell lysates.

EG-1 overexpression increases the tumorigenicity of mouse xenografts. We next examined the effect of EG-1 on the tumorigenicity of permanently transfected and wild-type HEK-293 cells in SCID mice. One group of mice was injected s.c. in the

Table 1. EG-1 peptides identified by MS/MS

Sequence*	MH⁺	Charge	Sequence†	XC‡
K.KPADIPQGSLAYLEQASANIPAPLKPT.-	2,792.18	2	166-193	5.92
K.PADIPQGSLAYLEQASANIPAPLKPT.-	2,664.01	2	167-193	4.90
K.RLQLSVQKPEQVIK.E	1,666.99	3	113-127	4.17
R.LQLSVQKPEQVIKEDVSELR.N	2,339.68	2	114-134	3.98
K.KPADIPQGSLAYLEQASANIPAPLKPT.-	2,792.18	3	166-193	3.44
K.RLQLSVQKPEQVIKEDVSELR.N	2,495.86	3	114-134	3.41
K.RLQLSVQKPEQVIK.E	1,666.99	2	113-127	3.27
R.LQLSVQKPEQVIKEDVSELR.N	2,339.68	2	114-134	2.91
R.LQLSVQKPEQVIK.E	1,510.80	2	114-127	2.87
R.LQLSVQKPEQVIK.E	1,510.80	2	114-127	2.82
R.QTECFFLQK.R	1,201.33	2	104-113	2.40
K.EDVSELRNELQR.K	1,488.59	2	127-139	2.06
R.LQLSVQKPEQVIK.E	1,510.80	3	114-127	1.93
K.FLDIAR.Q	734.87	1	98-104	1.42

*Sequence of EG-1 peptide matched to tandem mass spectrum.

†Numbered according to complete FLAG-tagged sequence; MDYKDDDDKNSAGSNMAAPLGGMFSGQPPGPPQAPPGLPGQASLLQAAPGAPRPSSSTLV-DELESSFEACFASLVSQDYVNGTDQEEIRTGVDQCIQKFLDIARQTECFFLQKRLQLSVQKPEQVIKEDVSELRNELQRKDALVQKHLTKLRHWQ-QVLEDINVQHKKPADIPQGSLAYLEQASANIPAPLKPT.

‡ For the peptides highlighted in bold, a cross correlation coefficient (XC) of >2.55 for a 1+ charge state, 3.39 for a 2+ charge state, and 3.78 for a 3+ charge state or greater is considered a highly significant match. Lower scoring matches are included because they are tryptic peptides identified without specifying this option in the search.

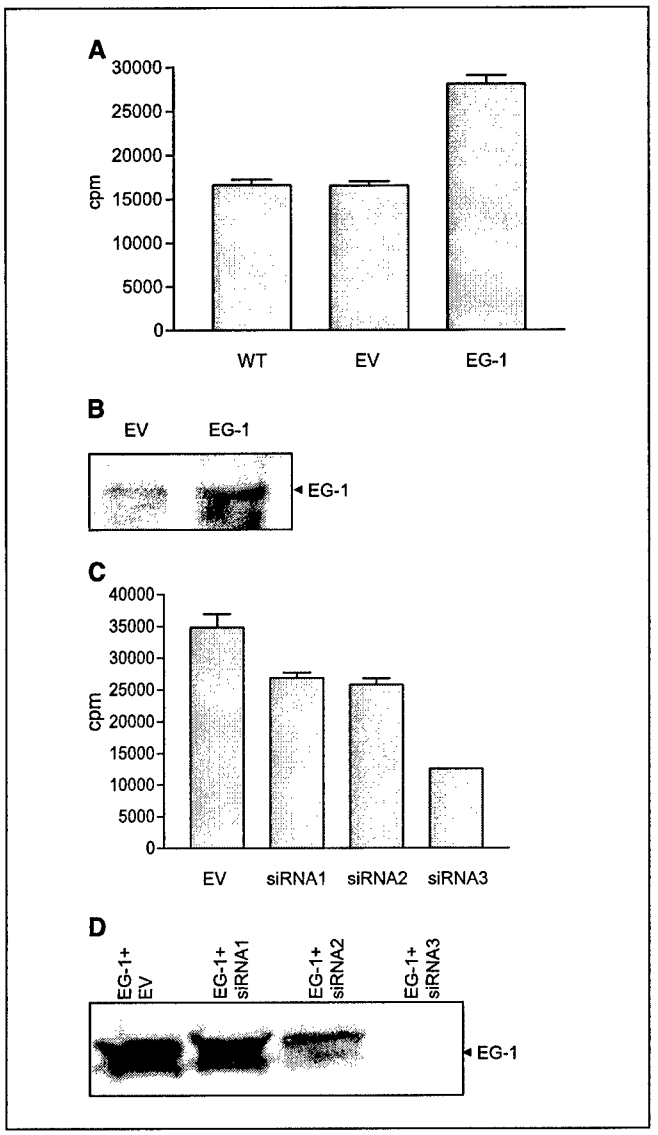


Figure 2. *A*, proliferation of HEK-293 cells. *Lane 1*, wild-type; *lane 2*, permanent clone of HEK-293 cells transfected with empty vector; *lane 3*, permanent clone of HEK-293 cells transfected with EG-1. *Columns*, mean expressed in counts per minute; *bars*, SE. $P < 0.001$. *B*, one milligram of the above cell lysate proteins was immunoprecipitated with anti–EG-1 antibody overnight, then immunoblotted with the same antibody. *C*, proliferation of HEK-293 cells transfected with EG-1. *Lane 1*, cotransfected with empty vector; *lane 2*, cotransfected with siRNA1; *lane 3*, cotransfected with siRNA2; *lane 4*, cotransfected with siRNA3. *Columns*, mean expressed in counts per minute; *bars*, SE. $P < 0.001$. *D*, Western blot analysis of EG-1 expression of the above cell lysates.

flank with wild-type HEK-293 cells, a second group with a stably transfected empty vector clone, and the third group with one EG-1–overexpressing clone. There were four mice per group. At day 30, the xenograft tumor sizes are as follows: 1,204 ± 384 mm^3 in the wild-type group, 899 ± 313 mm^3 in the empty vector group, and 1,956 ± 441 mm^3 in the EG-1 vector group (Fig. 4*A*). ANOVA analysis shows that the stimulatory effect exerted by EG-1 transfection in the xenografts is significant in comparison with that by vector alone ($P < 0.01$) as well as with that by wild-type ($P < 0.05$). This phenomenon was similarly observed in other xenograft experiments using other stable clones generated in our laboratory (Fig. 4*C*; $P < 0.0001$).

To examine the expression of the EG-1 product in mouse tumors, multiple xenograft samples were analyzed by immunohistochemistry using the anti–EG-1 antibody. The histologic slides were reviewed by a Board-certified pathologist (J.Y.R). Figure 4*B* shows a representative slide prepared from a vector-transfected tumor, and a slide from an EG-1–transfected tumor. The EG-1–transfected xenografts seem to express a higher amount of the EG-1 peptide, in comparison with those transfected with empty vector.

EG-1 overexpression influences certain kinase pathways. Western blot analysis of cell lysates showed that the phosphorylated p44/42 MAP kinase level is elevated in EG-1–transfected HEK-293 cells in comparison with those transfected with empty vector

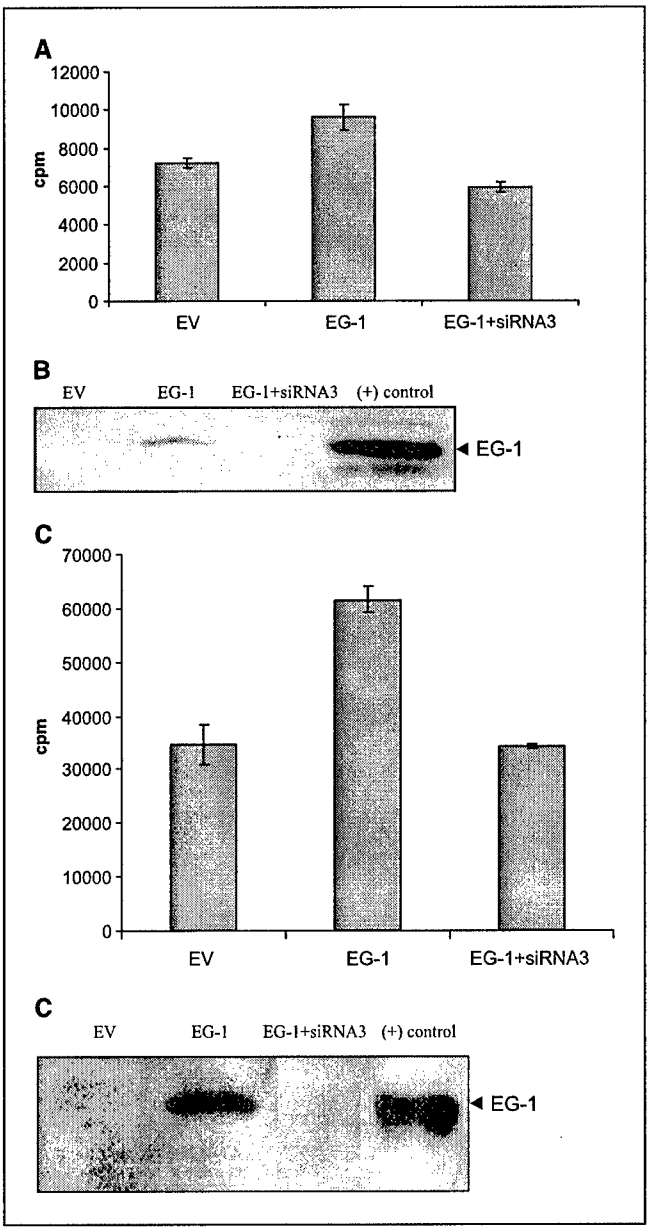


Figure 3. Proliferation of MCF-7 (*A*) and MDA-MB-231 (*C*) human breast cancer cells. *Lane 1*, cells transfected with empty vector; *lane 2*, cells transfected with EG-1; *lane 3*, cells transfected with both EG-1 and siRNA3. *Columns*, mean expressed in counts per minute; *bars*, SE. $P < 0.001$. Western blot analysis of EG-1 expression of the above cell lysates compared with purified EG-1 peptide positive control. *B*, MCF-7; *D*, MDA-MB-231.

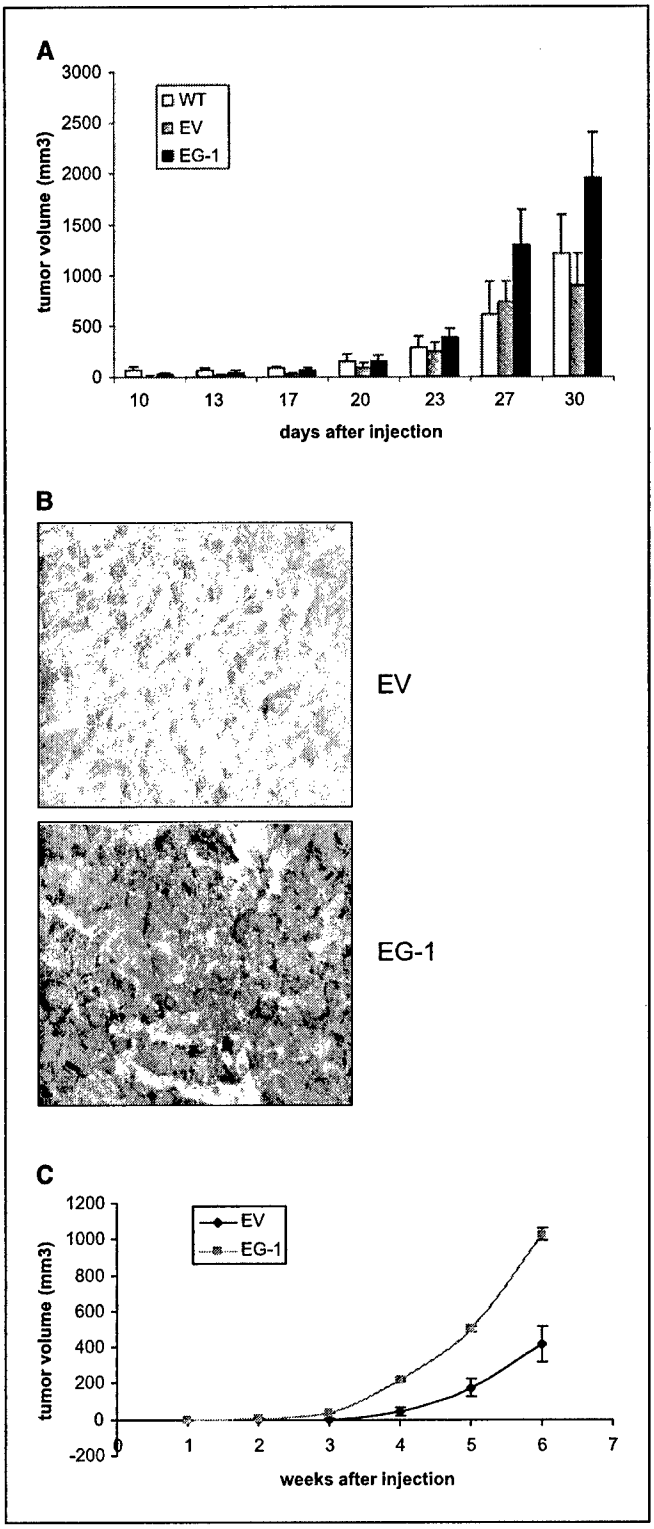


Figure 4. The tumorigenicity of different HEK-293 clones. Cells were injected s.c. into mice, and tumor size measured in three dimensions with calipers and expressed as volume. *Columns,* mean; *bars,* SE. *A,* 4×10^7 wild-type cells (*white columns*), and a clone of HEK-293 cells stably transfected with empty vector (*diagonal lines*) or with EG-1 (*black columns*); $n = 4$, $P < 0.01$. *B,* immunohistochemistry of mouse xenografts from empty vector versus EG-1 stable clones, with positive staining for EG-1 in brown. *C,* 5×10^7 cells of other clones of HEK-293 stably transfected with empty vector (♦) or with EG-1 (▨); $n = 8$, $P < 0.0001$.

(Fig. 5*A*). The phosphorylated forms of the JNK (Fig. 5*B*) and the p38 kinase (Fig. 5*C*) are similarly increased in EG-1–overexpressing cells. Of note, we also observed similar changes in phospho-JNK levels in MCF-7 cell experiments, and in p44/42 MAPK levels in MDA-MB-231 cell experiments (data not shown). We further investigated the proliferation of EG-1–transfected HEK-293 cells when treated with the following MAPK inhibitors: PD98059 (inhibits MAPK), SB203580 (inhibits p38), and U0126 (inhibits

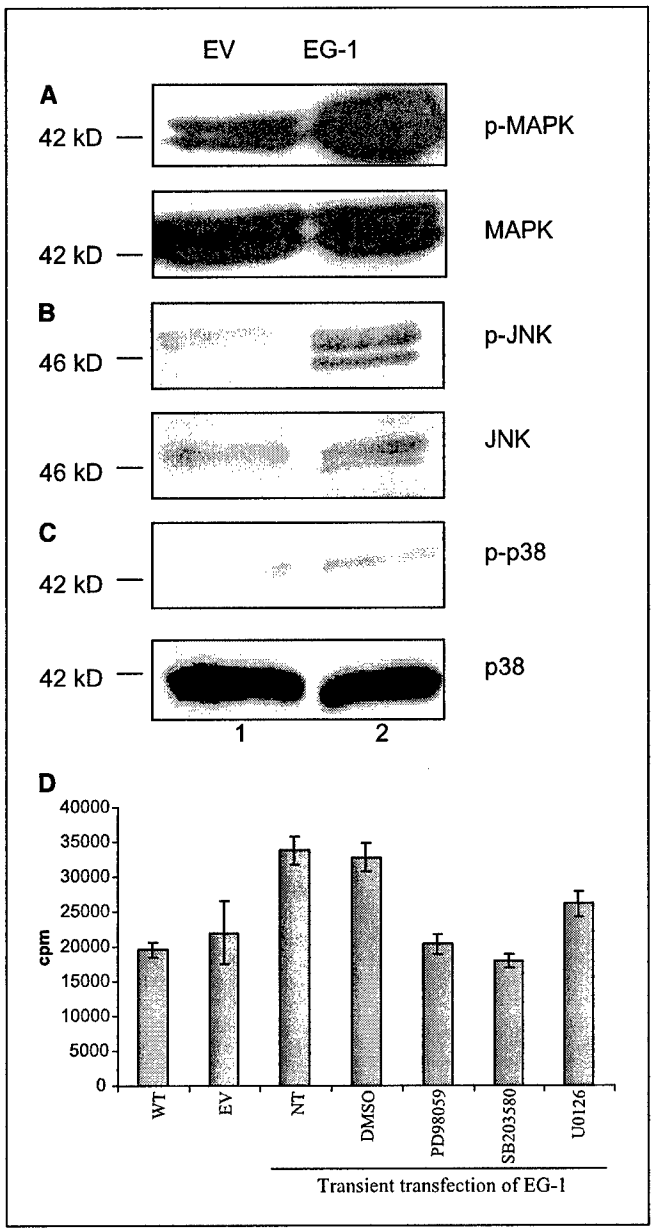


Figure 5. Western blot analysis of kinase expression in HEK-293 cell lysates. Equivalent amounts of protein were loaded per lane. *Lane 1,* permanent clone of HEK-293 cells transfected with empty vector; *lane 2,* permanent clone of HEK-293 cells transfected with EG-1. Expression of phosphorylated versus nonphosphorylated p44/42 MAP kinase (*A*), JNK (*B*), and p38 kinase (*C*). *D,* MAPK inhibitors block the increase of proliferation by EG-1 overexpression in HEK-293 cells. *Columns,* mean expressed in counts per minute; *bars,* SE. Cells were untreated (*WT*), transfected with empty vector (*EV*), or transfected with EG-1 (*NT*), $P = 0.015$. In the group with EG-1 transfection, cells were either not treated (*NT*), or treated with vehicle (*DMSO*) or MAPK inhibitors (PD98059, SB203580, and U0126; 10 mmol/L), $P < 0.05$.

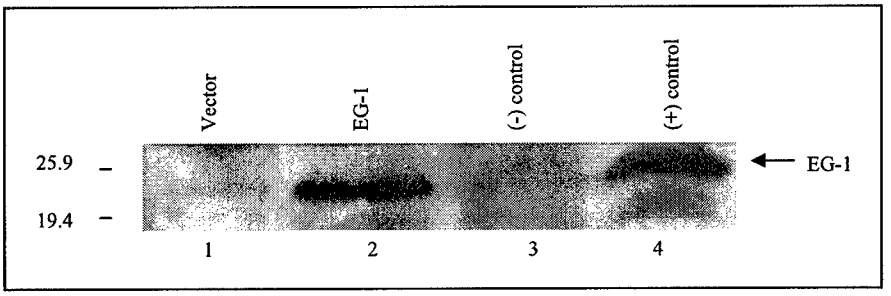


Figure 6. Western blot analysis of EG-1 expression in HEK-293 cell lysates. *Lane 1,* cells transiently transfected with empty vector; *lanes 2* to *4,* cells transiently transfected with EG-1. Equivalent amounts of cell lysate proteins were immunoprecipitated with an anti-Src antibody (*lanes 1 and 2*). *Lane 3,* negative control, in which normal mouse antibody was used to immunoprecipitate an equivalent amount of the same cell lysate of transiently EG-1–transfected HEK-293. *Lane 4,* positive control, in which an equivalent amount of the same cell lysate was immunoprecipitated with anti–EG-1 antibody. Subsequently, all lanes were blotted with anti–EG-1 antibody.

MAP/extracellular signal-regulated kinase (ERK) kinase 1 and 2). These inhibitors significantly block the increase of proliferation by EG-1 overexpression in HEK-293 cells (Fig. 5*D*; $P < 0.05$).

EG-1 and c-Src form a protein complex. In other experiments, we found that EG-1 recombinant peptides bind to the Src homology 3 domain of c-Src by Domain Array assays (data not shown). We thus asked if EG-1 and c-Src can also have protein-protein interaction inside living mammalian cells. We transiently transfected HEK-293 cells with either empty vector or with EG-1 plasmid, and prepared cell lysates. We then immunoprecipitated c-Src with anti–c-Src antibody, followed by Western blotting using the anti–EG-1 antibody, and discovered that EG-1 does indeed bind to c-Src (Fig. 6).

Discussion

We report here that the novel gene *EG-1* can stimulate cellular proliferation. Overexpression of EG-1 in transfected cells resulted in a marked increase of *in vitro* proliferation. On the other hand, siRNA cotransfection resulted in inhibition of proliferation. *In vivo*, mouse xenograft models showed that EG-1 transfectants had a growth advantage as evidenced by the formation of larger s.c. tumors. Because cellular proliferation is an important component of the malignant phenotype, the current observations are consistent with our previous reports on the expression profile of this novel gene. We have shown in our first publication that the expression of EG-1 is significantly elevated in cancerous compared with benign epithelial cells, as seen in Northern blot analyses (16). Subsequent immunohistochemical studies of several human pathologic specimens confirmed our hypothesis that EG-1 is associated with the malignant phenotype of the common epithelial-derived cancers of the breast, colon, and prostate (17).

Our present observations are unique in part because *EG-1* is a novel gene with completely unknown function, which was discovered in our laboratory 2 years ago. The fact that this gene is highly conserved in mammals suggests that it may serve an important and fundamental role in cellular biology. The EG-1 protein product is unique because we could not find any homology of its tryptic peptides with any known proteins in the database. Thus, the major finding is that a unique and novel gene is a positive signal for cellular proliferation. The extent of proliferation correlates with the levels of EG-1 peptide, as seen in Western blot analyses *in vitro* and immunohistochemistry *in vivo*. As further evidence for the stimulatory effect of EG-1, we showed that the knockdown of EG-1 expression by specifically designed siRNAs resulted in inhibition of cellular proliferation.

To investigate possible mechanisms for the stimulatory activity of EG-1, we analyzed the well-known MAPK family kinase signaling pathway which has been shown to be crucial in promoting cellular proliferation (20). We used the stable clones that overexpress EG-1, and compared their MAPK activity with that expressed by stable clones carrying only empty vectors. By Western blot analysis, we found that EG-1 overexpression was correlated with activation of the following MAP kinases: ERK-1 and -2, JNK, and p38. Further work is needed to study the network involved in this interaction, and to determine whether the MAPK pathway really plays a role in EG-1–associated cellular proliferation.

A key to the understanding of cellular signal transduction pathways is to determine whether certain proteins of interest interact with one another. Protein-protein interactions are often mediated by noncatalytic and conserved domains (21). To further clarify the function of EG-1, we analyzed the sequence of this highly conserved gene. EG-1 has two tyrosines at positions 68 and 163. The presence of an NH_2-terminal polyproline region suggests that EG-1 may interact with Src homology 3 domains. This led us to screen more than 100 proteins with Src homology 3 domains by domain array assays, in which we repeatedly observed an association between EG-1 and c-Src (data not shown). Immunoprecipitation with anti-Src antibody, followed by immunoblotting with anti–EG-1 antibody, showed an interaction between these two molecules. C-Src is a member of the Src family of cytoplasmic tyrosine kinases that regulate cell growth, differentiation, cell shape, migration, and survival (22). C-Src has been reported to be overexpressed and to play a role in human carcinomas of the breast, colon, and others (23). Src family tyrosine kinases are often activated by receptor tyrosine kinases, such as epidermal growth factor receptor or platelet-derived growth factor receptor (24). Further work is needed to elucidate the effects of the observed association between Src and EG-1.

The above observations collectively support the hypothesis that the novel gene *EG-1* is a positive stimulator of cellular proliferation. Because cellular proliferation is an important component of the malignant phenotype, our present findings suggest that EG-1 may be an important target in the design of novel cancer therapies. In addition, we have now shown that EG-1 overexpression is potentially associated with the well-known MAP kinase family signaling pathway, which could underline the mechanism of the function of EG-1. Finally, we have observed that EG-1 forms protein-protein complexes with the Src tyrosine kinase, which is critical in the regulation of cell growth, differentiation, migration, and survival. The cross-talk between MAPK family and c-Src has been reported by several groups (25, 26). When c-Src is catalytically active, it is presumed to phosphorylate epidermal growth factor

receptor (EGFR) monomers on tyrosine residues leading to EGFR transactivation. Tyrosine-phosphorylated EGFR forms complex with adapter proteins, including growth factor receptor binding protein 2-Sos, which activate Ras and the ERK pathway (27).

We think that these results will form the basis for further studies of this interesting gene, and the possible translation of the discovery of this molecule into potential use in cancer diagnosis and/or treatment.

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