

Constitutive Sp1 Activity Is Essential for Differential Constitutive Expression of Vascular Endothelial Growth Factor in Human Pancreatic Adenocarcinoma¹

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ABSTRACT

Vascular endothelial growth factor (VEGF) is a key angiogenic molecule that plays an important role in the growth and metastasis of many types of human cancer, including pancreatic adenocarcinoma. In this study, we explored the regulation of VEGF in human pancreatic cancer cells. Over 70% of the human pancreatic cancer cell lines studied *in vitro* secreted constitutively high levels of VEGF. High VEGF-secreting cells also generally expressed an elevated steady-state level of VEGF mRNA. Kinetic analysis revealed that the elevated steady-state level of VEGF mRNA was due to enhanced VEGF gene transcription and increased constitutive VEGF promoter activity. Deletive mutation analyses of the VEGF promoter revealed that the region from -109 to -38 bp was essential for constitutive VEGF promoter activity. Further deletion and point mutation analyses indicated that mutation of individual or all of the putative Sp1 binding sites reduced or eliminated the constitutive VEGF promoter activity and abrogated the differential activity of the promoter in high and low VEGF-expressing cells. Consistent with the constitutive VEGF transcription activation, a high level of constitutive Sp1 expression and activity was detected in pancreatic cancer cell lines and pancreatic cancer tissue specimens overexpressing VEGF. Collectively, our data demonstrated that constitutive Sp1 activation is essential for the differential overexpression of VEGF, which in turn plays an important role in the angiogenesis and progression of human pancreatic cancer.

INTRODUCTION

Pancreatic adenocarcinoma is currently the fifth leading cause of cancer-related death in the United States, and the incidence of this disease appears to be increasing. Despite improvements in early diagnosis, surgical techniques, and chemotherapy, the majority of pancreatic cancer patients die of the physiological effects of metastasis to the lymph nodes, liver, lungs, and/or peritoneum (1). The aggressive nature of this disease is related to several abnormalities in growth factors and their receptors, which affect the downstream signal transduction pathways involved in the control of growth and differentiation. Other contributing molecular changes in pancreatic adenocarcinoma include mutation and inactivation of various oncogenes and tumor suppressor genes (1–3). These perturbations confer a tremendous growth advantage to pancreatic cancer cells.

Like other solid tumors, the growth and metastasis of pancreatic adenocarcinoma is dependent on angiogenesis, the formation of new blood vessels from a preexisting network of capillaries (4). Of the

numerous angiogenic factors discovered thus far, VEGF³ (5), also known as VPF (6), has been identified as a key mediator of tumor angiogenesis (5, 6). Elevated expression of VEGF in human tumor biopsies has been reported in various cancers, including pancreatic adenocarcinoma (7, 8). Additionally, blockade of VEGF signal transduction can inhibit tumor growth and metastasis (9–12) using neutralizing anti-VEGF monoclonal antibodies (13), antisense VEGF mRNA-expressing constructs (14, 15), VEGF-toxin conjugates (16), antagonistic VEGF mutants (17), inhibitory soluble VEGF receptors (18–20), inhibitors of VEGF receptor function (12, 21, 22), interference of the VEGF receptor system by specific antibodies (9, 23), and overexpression of a dominant-negative VPF/VEGF receptor mutant (10).

The mechanism of VEGF expression and its regulation in human pancreatic cancer are mostly unknown. Increasing evidence suggests that for many types of normal and malignant cells, VEGF expression is regulated by a plethora of external factors, *e.g.*, cytokines, growth factors, gonadotropins, and many other extracellular molecules. Major stimulators of VEGF expression include hypoxia and hypoglycemia (24), which occur frequently within the expanding tumor mass and particularly in regions surrounding necrotic areas within diverse types of tumors (24). The major role of hypoxia in the angiogenesis switch has been clearly demonstrated in a mouse model (25). Hypoxia-mediated VEGF induction involves the transactivation of a VEGF promoter by HIF-1 (26, 27), as well as the stabilization of the VEGF mRNA by proteins that bind to sequences located in the 3' untranslated region of VEGF mRNA (28–30). However, in many types of tumors, an elevated level of VEGF production can often be detected in tumor cells located in the extreme periphery of the tumor where there is no apparent hypoxia. This observation is consistent with numerous recent findings indicating that such exogenous factors as hormones, cytokines, and growth factors modulate VEGF expression and then modulate angiogenesis. These factors include epidermal growth factor, insulin-like growth factor I (31), fibroblast growth factor (32), IL-1 β (33), IL-6 (34), IL-8 (35), keratinocyte growth factor (36), platelet-derived growth factor (37), transforming growth factor β (38), and tumor necrosis factor (39). Other cytokines, such as IL-10, IL-4, and IL-13, can inhibit the release of VEGF. Initial analysis of the VEGF promoter region has revealed several potential transcription factor binding sites, such as HIF-1, AP-1, AP-2, Egr-1, Sp1 (40), and many others (41–44), suggesting that they may be involved in VEGF transcription regulation.

Although VEGF can be induced dramatically by external factors, such as hypoxia, many tumors can constitutively express VEGF without any apparent external stimuli. For example, loss or inactivation of tumor suppressor genes and activation of oncogenes are associated with VEGF overexpression (45–53). Given the prominent role of VEGF in tumor angiogenesis, it is critical to understand the

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³ The abbreviations used are: VEGF, vascular endothelial growth factor; VPF, vascular permeability factor; HIF-1, hypoxia-inducible factor 1; EMSA, electrophoretic mobility shift assay; IL, interleukin; AP, activator protein; vHL, von Hippel-Lindau.

molecular basis of constitutive VEGF gene expression. Therefore, in the present study, we defined the *cis*-acting elements and transcription factors involved in constitutive VEGF expression and regulation in human pancreatic adenocarcinoma. We found that human pancreatic adenocarcinoma cells constitutively expressed VEGF and that having differential levels of constitutive Sp1 expression was strongly correlated with the differential levels of constitutive VEGF expression, suggesting that Sp1 plays an important role in tumor angiogenesis and contributes to the aggressive biology of human pancreatic adenocarcinoma.

MATERIALS AND METHODS

Cell Line and Culture Conditions. The following human pancreatic adenocarcinoma cell lines were used in this study: AsPc-1, Bxpc-3, CaPan-1, CaPan-2, HPAFII, HS766T, MiaPaca-2, PANC-1, and SW-1990, which were purchased from the American Type Culture Collection (Manassas, VA); MDA Panc-3, MDA Panc-28, and MDA Panc-48 were gifts from Dr. Paul J. Chiao (The University of Texas M. D. Anderson Cancer Center, Houston, Texas); and COLO357, which was originally established from a pancreatic adenocarcinoma metastasis by Morgan *et al.* (54), and its fast-growing (FG) and liver metastatic variant (L3.3) in nude mice, which were established by Vezeridis *et al.* (55). All cell lines were maintained in plastic flasks as adherent monolayers in MEM supplemented with 15% fetal bovine serum, sodium pyruvate, non-essential amino acids, L-glutamine, and vitamin solution (Flow Laboratories, Rockville, MD).

Analysis of VEGF Gene Expression. The human pancreatic carcinoma cells were incubated at 70–80% confluence. Cellular mRNA was extracted using the FastTrack mRNA isolation kit (Invitrogen Co., San Diego, CA). The mRNA (2 μ g) was then separated electrophoretically on a 1% denaturing formaldehyde agarose gel, transferred to a GeneScreen nylon membrane (DuPont Co., Boston, MA) in 20 \times SSC, and UV cross-linked using a UV-Stratalinker 1800 (Stratagene, La Jolla, CA). The VEGF cDNA probe used was a 0.204-kb *Bam*HI/*Eco*RI cDNA fragment corresponding to human VEGF. The cDNA probes were labeled with [α -³²P]deoxycytidine triphosphate using a random labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Equal loading of mRNA samples was monitored by hybridizing the same membrane filter with a human β -actin cDNA probe (35). To measure VEGF secretion, the VEGF level in culture supernatants was determined using the Quantikine VEGF ELISA kit (R&D Systems, Minneapolis, MN), which is a quantitative immunometric sandwich enzyme immunoassay. A curve of the absorbance versus the concentration of VEGF in the standard wells was plotted. By comparing the absorbance of the samples with the standard curve, we determined VEGF concentration in the unknown samples (35).

Nuclear Run-On Assays for VEGF. The pancreatic cancer cells were plated into 15-cm plates at 80% confluence, and nuclei were isolated 24 h after cell seeding. *In vitro* transcription was performed as described previously (35). Nuclear RNA labeled with [α -³²P]UTP (Amersham Corp., Arlington Heights, IL) was then hybridized with VEGF and β -actin cDNA immobilized on a filter. The filter was washed and then exposed to X-ray film. Quantitative results were obtained using densitometric analysis of autoradiograms using the ImageQuant sample measurement software, standardized to β -actin, and expressed as fold change (35).

Determination of mRNA Stability. To evaluate VEGF mRNA stability in different pancreatic cancer cells, we measured the half-life of VEGF mRNA in the cells 24 h after incubation at 80% confluence. Actinomycin D (5 μ g/ml) was added to the culture to block further gene transcription. mRNA was isolated 0, 1, 2, 4, 8, and 12 h after the addition of actinomycin D. The amount of VEGF and β -actin mRNA at each time point was quantified after Northern blot analysis using densitometry; the amount of VEGF mRNA was corrected for loading differences using the amount of β -actin mRNA. Also, the half-life of VEGF mRNA was calculated by drawing the best-fit linear curve on a plot of VEGF versus time; the time at the point of half-maximal VEGF was taken to be the half-life.

Construction of Reporter Plasmids and Mutagenesis. A 2.324-kb fragment containing 5' VEGF sequences from -2274 to +50 relative to the transcription initiation site was subcloned into the *Kpn*I and *Nhe*I sites of pGL3-basic (Promega, Madison, WI), which contains firefly luciferase coding

sequences as well as SV40 intron and polyadenylation signals but lacks eukaryotic promoter or enhancer elements. The final full-length reporter plasmid was designated pGL3-V2274. Its deletion and site-directed mutation reporters were generated as follows. All constructs were verified by sequencing the inserts and flanking regions of the plasmids.

To generate progressive 5'-unidirectional deletion mutants, a series of forward primers was used in combination with the same reverse primer in a PCR with pGL3-V2274 as a template. The reverse primer with the *Nhe*I site (*underlined*) was 5'-CAGAGCGCTGGTCTAGCCC-3', whereas the forward primers, each of which had the *Kpn*I site (*underlined*), were 5'-GCTGGGTAC-CACCATGGAGG-3' (pGL3-V2274), 5'-GCTGGGTACACGAATGATGG-3' (pGL3-V1181), 5'-GCTGGGTACTCCCCCTTTGG-3' (pGL3-V1012), 5'-GCTGGGTACCAAGATGAGGGTCC-3' (pGL3-V789), 5'-GCTGGGTACCG-TGTGCCCTCC-3' (pGL3-V411), 5'-GCTGGGTACCGCGGTGTCTCTGG-3' (pGL3-V267), and 5'-GCTGGGTACCCCCCTTTTTTTTAAAAAG-3' (pGL3-V38). The resulting PCR products were gel-purified, digested with *Kpn*I and *Nhe*I, and subcloned into the *Kpn*I/*Nhe*I sites of the pGL3-basic vector. These products were designated as pGL3-V1181, pGL3-V1012, pGL3-V789, pGL3-V411, pGL3-V267, pGL3-V109, pGL3-V88, pGL3-V61, and pGL3-V38.

To generate site-directed mutants of Sp1, AP-2, and Egr-1 elements at -109 to +50, respectively, the QuickChange mutagenesis kit (Stratagene) was used according to the manufacturer's instructions. The primers (mutations are shown in *bold* and *underlined* throughout) for mutation of the AP-2 elements were 5'-CGGGGCGGGCTAGGGCGGGTCCC-3' (sense) and 5'-GGGACCCCCGCCCTAGGCCCGCCCCG-3' (antisense), and the primers for mutation of the Egr-1 elements were 5'-GGGGCGGGCTAGGGCGGGGTCCC-3' (sense) and 5'-CATGGCTCCGCCCTAGCCGGGACCCCCGCCCTAGGCCCGCCCCG-3' (antisense). In addition, the primers for mutation of the Sp1.1 elements were 5'-GGTCCCGCGGTTCCG-GAGCCATGC-3' (sense) and 5'-GCATGGCTCCGAACCGCCGGGACC-3' (antisense), the primers for mutation of the Sp1.2 elements were 5'-GGGCGGGC-CGGGTTCCGGGTCCC-3' (sense) and 5'-GCCGGGACCCCGAACCCG-GCCCC-3' (antisense), and the primers for mutation of the Sp1.3 elements were 5'-CCGCCCCCGGTTCCGGGCGGGG-3' (sense) and 5'-CCCCCG-GCCGAACCGGGGGCGG-3' (antisense). The primers for mutation of the Sp1.4 elements were 5'-CGCCTGTCCCCGAACCCCGGGGCGGG-3' (sense) and 5'-CCGCCCCCGGTTCCGGGACAGGCG-3' (antisense). The primers for mutation of all four Sp1 elements were 5'-GCTCGCTGTCCCCGAAC-CCC-3' (sense) and 5'-GCATGGCTCCGAACCGCCGGGACCCCGAACCCCGCC-CAACCGGGTTCCGGGACAGGCGAGC-3' (antisense). The resulting reporter plasmids were designated as pGL3-V/AP-2m, pGL3-V/Egr-1m, pGL3-V/Sp1.1m, pGL3-V/Sp1.2m, pGL3-V/Sp1.3m, pGL3-V/Sp1.4m, and pGL3-V/Sp1.ALLm, respectively.

Analysis of VEGF Promoter Activities. VEGF promoter plasmids containing firefly luciferase reporters were cotransfected into tumor cells in triplicate with an internal control pBA-RL using a calcium-phosphate method (Mammalian Transfection Kit; Stratagene). The pBA-RL contained a full-length renilla luciferase gene under the control of a human β -actin promoter. In some experiments, the reporters were cotransfected with pCMV4-Sp1, pCMV4-Sp3, or pCMV4 (obtained from Dr. Jonathan M. Horowitz, the North Carolina State University, North Carolina; reviewed in Ref. 56). Six h after cotransfection, the plates were washed three times with PBS and refed with fresh MEM. The activity of both firefly and renilla luciferase was determined 48 h later using the Dual Luciferase Assay kit (Promega). Specific VEGF promoter activity was calculated as described previously (35).

EMSA. EMSA was performed using nuclear extracts prepared from different pancreatic cancer cells plated into the 15-cm plates at 80% confluence. The following double-stranded oligonucleotides were used: a wild-type Sp1 consensus sequence (5'-ATTCGATCGGGGCGGGGCGAGC-3') and its mutant Sp1 consensus sequence (5'-ATTCGATCGGTTCCGGGCGAGC-3') and Sp1 binding sequences corresponding to Sp1 binding sites in the VEGF promoter region (as described in "Results"). The oligonucleotides were annealed and 5' end-labeled with [³²P]ATP (Amersham Corp.) plus T4 polynucleotide kinase using standard procedures (35). A binding reaction was carried out by preincubating 5 μ g of nuclear extract protein in 20 mM HEPES (pH 7.9), 50 mM NaCl, 5% glycerol, 0.1 mM DTT, and 1 μ g of poly(dI-dC) at room temperature for 15 min, which was followed by the addition of the double-stranded ³²P-labeled oligonucleotide and a second incubation at room temper-

ature for 15 min. For competition assays, a 50-fold molar excess of unlabeled oligonucleotides (or as indicated) was added to the binding reaction. For supershift reactions, extracts were preincubated with anti-Sp1 or anti-Sp3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 45 min on ice. Samples were then loaded on a 5% polyacrylamide gel, and electrophoresis was performed at room temperature for 3 h at 120 V. The gel was then dried for 2 h at 80°C and exposed to Kodak film (Eastman Kodak Co., Rochester, NY) at -70°C.

Western Blot Analysis. Whole-cell lysates were prepared from human pancreatic cancer cell lines. A standard Western blot was performed using a polyclonal rabbit antihuman and antimouse Sp1 (Santa Cruz Biotechnology) and a second antibody [antirabbit Ig, horseradish peroxidase-linked F(ab')₂ fragment from a donkey; Amersham Corp.]. Equal protein sample loading was monitored by hybridizing the same membrane filter with an anti- β -actin antibody (35). The probe proteins were detected using the Amersham enhanced chemiluminescence system according to the manufacturer's instructions.

Immunohistochemistry. Human tumor and normal tissue sections (5- μ m thick) of formalin-fixed, paraffin-embedded specimens were processed for immunostaining using a 1:500 dilution of rabbit anti-VEGF and anti-Sp1 antibodies and appropriate peroxidase-conjugated antirabbit IgG second antibody as described previously (35). The slides were then examined under a bright-field microscope. A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm and nucleus. Negative controls were done using nonspecific IgG.

Statistics. Each experiment was performed independently at least twice with similar results; one representative experiment was presented. The significance of the data was determined using Student's *t* test (two-tailed). *P*s < 0.05 were deemed significant.

RESULTS

VEGF Expression in Human Pancreatic Cancer Cell Lines. In the first set of experiments, we determined the VEGF expression in human pancreatic cancer cell lines. A variety of established human pancreatic adenocarcinoma cell lines were cultured *in vitro*. VEGF expression was detected by analyzing VEGF protein secreted from the tumor cells into the culture supernatant using ELISA. We found that most of the cell lines constitutively secreted VEGF with a range of 32–450 pg/10⁶ cells every 24 h (Fig. 1A). Also, 11 of the 15 cell lines constitutively secreted a high level of VEGF protein (more than 100 pg/10⁶ cells every 24 h).

Next, we measured the steady-state level of VEGF mRNA in pancreatic cancer cells. Cellular mRNA was extracted from the cells, and Northern blot analysis was performed. As shown in Fig. 1B, all of the cell lines expressed a detectable level of VEGF mRNA. Specifically, AsPc-1, Bxpc-3, FG, PANC-1, and SW-1990 cells expressed a relatively high level of VEGF mRNA, whereas CaPan-2, HPAFII, HS766T, MDA Panc-3, and MDA Panc-48 cells expressed a relatively low level of VEGF mRNA. Therefore, the level of VEGF protein secretion (Fig. 1A) was correlated with the steady-state level of VEGF mRNA (Fig. 1B).

Regulation of the Steady-State Level of VEGF mRNA. The steady-state level of VEGF mRNA expression has been shown to be dynamically regulated by many factors at both the transcriptional and posttranscriptional level (18–53) and determined using nuclear run-on (transcription) and transcript degradation rate (mRNA stability) assays of the VEGF gene. In the present study, we first measured the VEGF mRNA half-life in both CaPan-2 cells expressing a low level of VEGF and PANC-1 cells expressing a high level of VEGF. Actinomycin D (5 μ g/ml) was added to the culture to block further gene transcription. The total RNA was isolated 0, 1, 2, 4, 8, and 12 h after the addition of actinomycin D. The amount of VEGF mRNA and β -actin mRNA at each time point was quantified after Northern blot analysis (Fig. 2A). The VEGF mRNA half-life was calculated by drawing the best-fit linear curve on a plot of VEGF *versus* time (Fig.

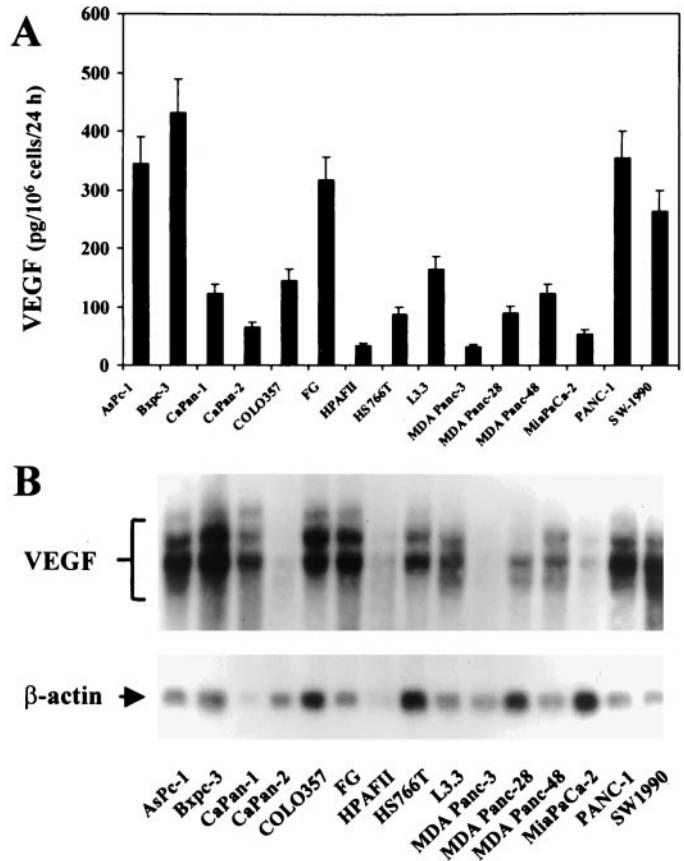


Fig. 1. Constitutive VEGF expression in human pancreatic adenocarcinoma cells. Cells were cultured in 100-mm dishes to 80% confluence. After 24 h, the medium was changed (10 ml/dish), and the cells were incubated for 24 h. A, culture supernatants were collected, and the amount of VEGF protein was determined using ELISA and expressed as pg/10⁶ cells every 24 h. B, cellular mRNA was extracted, Northern blot analysis was performed, and β -actin expression was probed to monitor the equal mRNA sample loading.

2B). We found that the VEGF mRNA half-life in PANC-1 and SW-1990 cells was slightly shorter than that in CaPan-2 and HS766T cells (Fig. 2B). Therefore, stability did not appear to be the major factor for the different expression levels of VEGF mRNA.

To determine whether increased transcription contributed to the differential VEGF expression, we measured the VEGF transcription rate in CaPan-2 and PANC-1 cells using a standard nuclear run-on assay. The cell nuclei were harvested 24 h after the medium was changed. Next, the VEGF transcription rate was determined and normalized with the transcription rate of the housekeeping gene β -actin. We found that the rate of VEGF transcription in PANC-1 cells was significantly higher than that in CaPan-2 cells (Fig. 2C). This experiment was repeated three times using two more cell lines (HS766T and SW-1990), and the mean transcription rate was calculated. The transcription rate of the VEGF gene in PANC-1 and SW-1990 cells was 5-fold higher than that in CaPan-2 and HS766T cells (Fig. 2D). These data demonstrated that transcription activation was the major contributing factor for constitutive VEGF expression in the human pancreatic cancer cells and that the stability of VEGF mRNA was not a significant factor for the differential VEGF expression in the cells. We concluded that the constitutive VEGF expression was due to elevated transcription of the VEGF gene but not prolonged VEGF mRNA stability.

Constitutive VEGF Promoter Activity. To characterize the DNA sequences involved in constitutive transcriptional activation of the VEGF gene, we used luciferase reporter plasmids in which VEGF 5'-flanking sequences were fused to the firefly luciferase coding

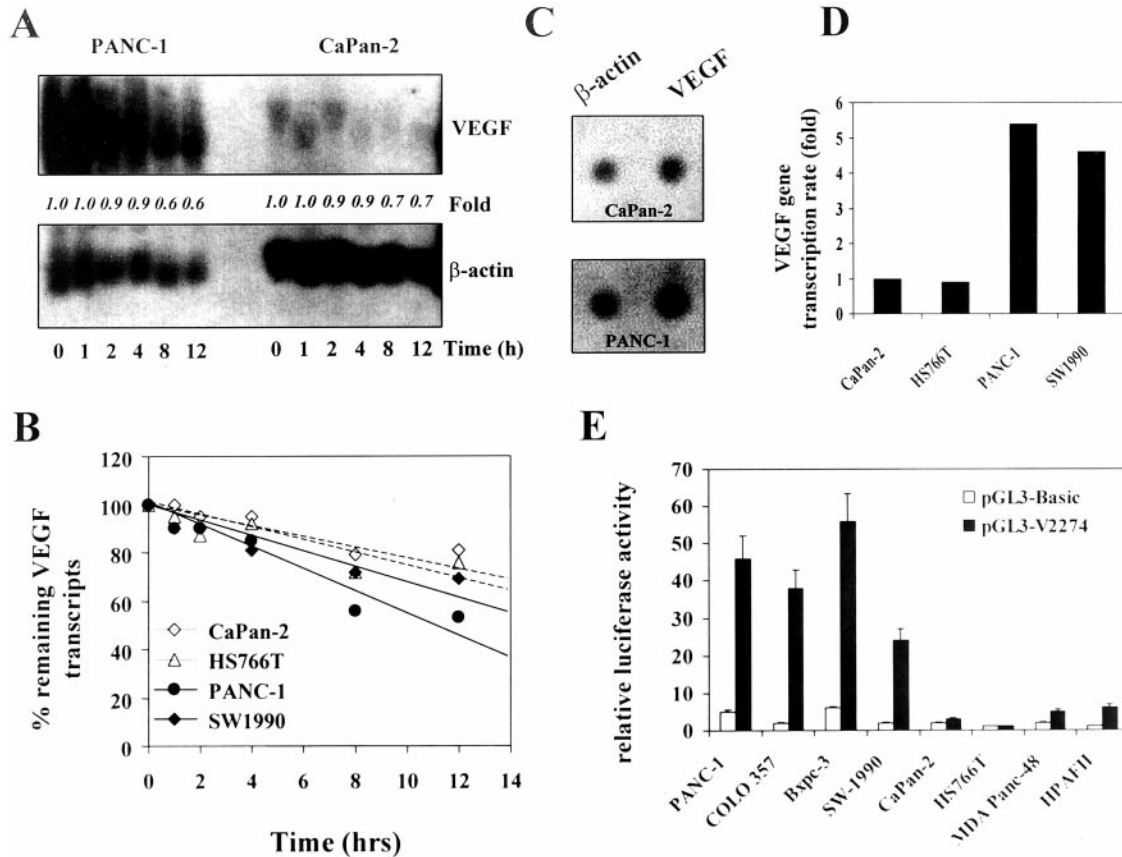


Fig. 2. Regulation of the steady-state level of *VEGF* gene transcript. Pancreatic adenocarcinoma cells were seeded at 90% confluence. Actinomycin D (5 μ g/ml) was added to the culture 24 h later to block further gene transcription. The mRNA was isolated 0, 1, 2, 4, and 8 h after the addition of actinomycin D. A, the amount of *VEGF* and β -actin mRNA at each time point was quantified after Northern blot analysis using densitometry. B, the half-life of *VEGF* mRNA was calculated. For the nuclear run-on assay, the cells were seeded at 90% confluence and incubated for 24 h. Nucleus isolation and *in vitro* transcription were performed. Nuclear RNA labeled with [α - 32 P]UTP was then hybridized with *VEGF* and β -actin cDNA immobilized on a filter. C, the filter was washed and then exposed to X-ray film. D, quantitative results were obtained by densitometric analysis, standardized to β -actin, and expressed as fold change. For full-length *VEGF* promoter activity, the cells with high or low *VEGF* expression were cotransfected with pGL3-V2274 and pBA-RL. E, the cells were incubated for 48 h, and firefly and renilla luciferase activity was measured for the calculation of specific *VEGF* promoter activity.

sequences in the pGL3-basic vector. The full-length sequence (defined as pGL3-2274) spanned -2274 to $+50$ relative to the transcription initiation site. Additionally, a luciferase reporter without any *VEGF* promoter sequences (defined as pGL3-basic) was used as a control (Fig. 2E). The plasmids were cotransfected into the cells with pBA-RL, which was used as an internal control to monitor transfection efficiency (35). The transfected cells, which were 40–50% confluent, were incubated for 48 h; the activity of both luciferases was then measured using the Dual Luciferase Assay. As shown in Fig. 2E, a high level of *VEGF* promoter activity was detected in PANC-1, COLO357, Bxpc-3, and SW-1990 cells, whereas a low level of *VEGF* promoter activity was detected in CaPan-2, HS766T, MDA Panc-48, and HPAFII cells.

Characterization of the Minimal Responsive Region. To determine the essential responsive region or regions of a high level of *VEGF* promoter activity, progressive deletion mutants from the pGL3-V2274 were generated as described in “Materials and Methods” and designated pGL3-V1181, pGL3-V1012, pGL3-V789, pGL3-V411, pGL3-V267, pGL3-V109, pGL3-V88, pGL3-V61, and pGL3-V38. This series of mutant reporters was cotransfected into the PANC-1 cells with pBA-RL, and the relative luciferase activity was determined as described above (Fig. 2E). There was no significant loss of *VEGF* promoter activity with deletions from -2274 to -267 , whereas further deletion of -267 to -38 totally eliminated the constitutive *VEGF* promoter activity (Fig. 3A). These results suggested that the sequence from -267 bp to -38 bp was required for constitutive *VEGF* gene expression.

To further define the responsive region or regions, small-scale deletion mutants from pGL3-V267 were generated and designated pGL3-V109, pGL3-V88, pGL3-V61, and pGL3-V38 (Fig. 3B). Deletion of the region from -267 to -109 reduced promoter activity by approximately 25%, whereas deletion up to -61 totally abrogated the promoter activity (Fig. 3B), which suggested that the regions around -109 to -61 contain the minimal essential elements to confer constitutive *VEGF* promoter activity.

Characterization of *cis*-responsive Elements. It has been reported that there are five Sp1 binding sites within the region from -267 to -38 : one is located between -267 and -109 (Sp1.5); whereas the remaining four are clustered between -109 and -38 (Sp1.4, Sp1.3, Sp1.2, and Sp1.1). In addition, there are several other putative response elements in this region (one AP-2 and two Egr-1 binding sites) that overlap with the Sp1 cluster as shown in Fig. 3B.

To determine whether these *cis*-elements were essential for the constitutive *VEGF* expression, point-mutated pGL3-V109 luciferase reporters were generated (Fig. 3C) and transfected into both PANC-1 and CaPan-2 cells. As compared with the control pGL3-V109 (wild-type) reporter, point mutations of AP-2 or Egr-1 had no significant effects on luciferase activity, suggesting that these putative sites did not contribute significantly to the constitutive *VEGF* promoter activity. Mutations in individual Sp1 sites variably reduced the luciferase activity, and mutations in all four Sp1 sites eliminated the luciferase activity and the difference in promoter activity in PANC-1 and CaPan-2 cells (Fig. 3C). We further compared the pGL3-V109 (Sp1-mediated) and pGL3-V/Sp1.ALLm (non-Sp-mediated) promoter ac-

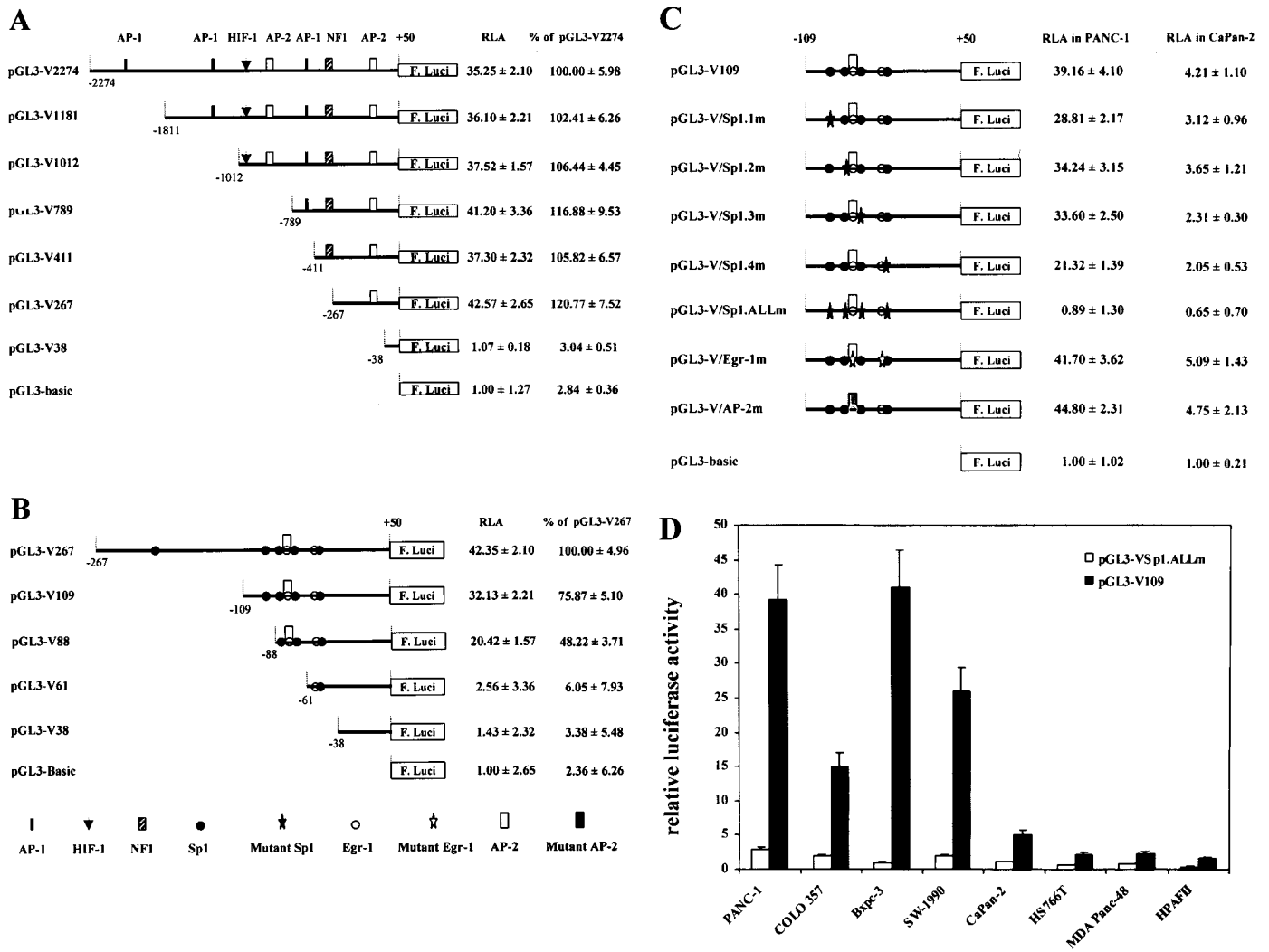


Fig. 3. Contribution of Sp1 elements to the constitutive activity of *VEGF* promoter. *A*, large-scale deletion mutation analysis. pGL3-V2274 and its progressive deletion mutants were transfected with pBA-RL into PANC-1 cells. *B*, small-scale deletion mutation analysis. pGL3-V267 and its small-scale deletion mutants generated by PCR were transfected with pBA-RL into PANC-1 cells. *C*, point mutation analysis. pGL3-V109 and its point mutants generated using PCR were transfected with pBA-RL into both PANC-1 and CaPan-2 cells. *D*, cell line comparison. pGL3-V109 and pGL3-V/Sp1.ALLm were transfected with pBA-RL into both high and low *VEGF*-expressing cells. The cells were incubated for 48 h, and firefly and renilla luciferase activity was measured for the calculation of specific *VEGF* promoter activity.

tivity in cell lines with low or high *VEGF* expression. As shown in Fig. 3D, a high level of pGL3-V109 activity was observed in PANC-1, COLO357, Bxpc-3, and SW-1990 cells expressing a high level of *VEGF*, whereas a low level of pGL3-V109 activity was observed in CaPan-2, HS766T, MDA Panc-48, and HPAFII cells expressing a high level of *VEGF*. There was no significant pGL3-V/Sp1.ALLm promoter activity in either high or low *VEGF*-expressing cells (Fig. 3D). These results indicated that Sp1 factor binding elements were mainly responsible for the constitutive *VEGF* promoter in human pancreatic cancer cells. Additionally, a maximal level of constitutive *VEGF* promoter activity appeared to require the presence and cooperation of all four Sp1 binding sites, suggesting that the proximal Sp1 cluster in the *VEGF* promoter is responsible for the high constitutive transcription activity of *VEGF* in human pancreatic cancer cells.

Elevated Sp1 Expression in Human Pancreatic Cancer Cells.

To further determine the *trans*-acting factors involved in the constitutive expression of *VEGF*, EMSA was performed. The five pairs of probes used contained the putative Sp1 binding sites (Sp1.1, Sp1.2, Sp1.3, Sp1.4, and Sp1.5) and their corresponding mutants; their spanning positions in the *VEGF* promoter are outlined in Fig. 4A. Incu-

bation of nuclear extracts from PANC-1 cells with these wild-type Sp1.1, Sp1.2, and Sp1.4 probes resulted in the formation of three major shifted DNA-protein complexes (indicated as Sp1 and Sp3 in Fig. 4A) but not with their corresponding mutants (Fig. 4B). The shifted complexes were competed with cold Sp1 consensus oligonucleotides but not with cold mutant Sp1 consensus oligonucleotides. In supershift assays, we clearly showed that there were two Sp3-containing complexes and one Sp1-containing complex (Fig. 4C). In contrast, incubation of nuclear extracts from PANC-1 cells with wild-type Sp1.3 and Sp1.5 probes failed to form the three shifted DNA-protein complexes described above (Fig. 4B). These data indicated for the first time that Sp1.1, Sp1.2, and Sp1.4 binding sites were functionally active, whereas Sp1.3 and Sp1.5 binding sites were not.

To determine whether Sp1 binding activity was differentially expressed in the human pancreatic cancer cells, EMSA was performed using the Sp1.1 probe (functionally active) or Sp1.3 and Sp1.5 probes (functionally inactive). Incubation of nuclear extracts from all of the human pancreatic cancer cell lines with the Sp1.1 probe resulted in the formation of three major shifted DNA-protein complexes (indicated as Sp1 and Sp3 in Fig. 4C) but at significantly different levels. A high level of binding activity was detected in PANC-1, Bxpc-3, COLO357,

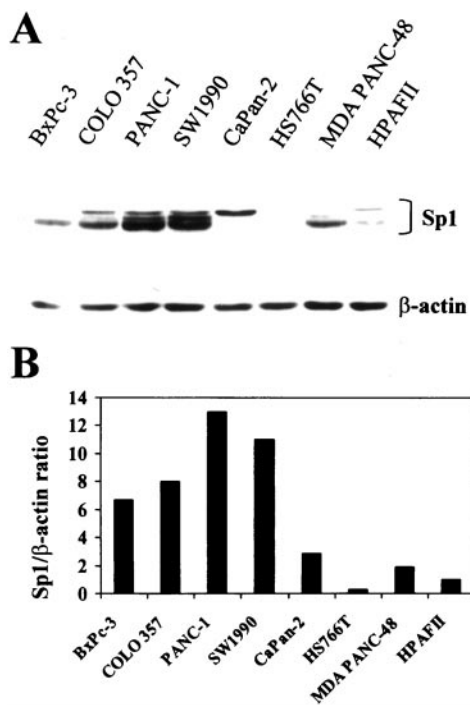


Fig. 5. Sp1 protein expression. Human pancreatic cancer cells were plated in 100-mm dishes at 80% confluence. Twenty-four h after seeding, cells were lysed for cytosolic protein extraction. Cytosolic protein was separated on a 10% PAGE and blotted onto a membrane. *A*, specific anti-Sp1 and β -actin antibodies were used. *B*, the expression level of Sp1 was expressed as the ratio of Sp1: β -actin.

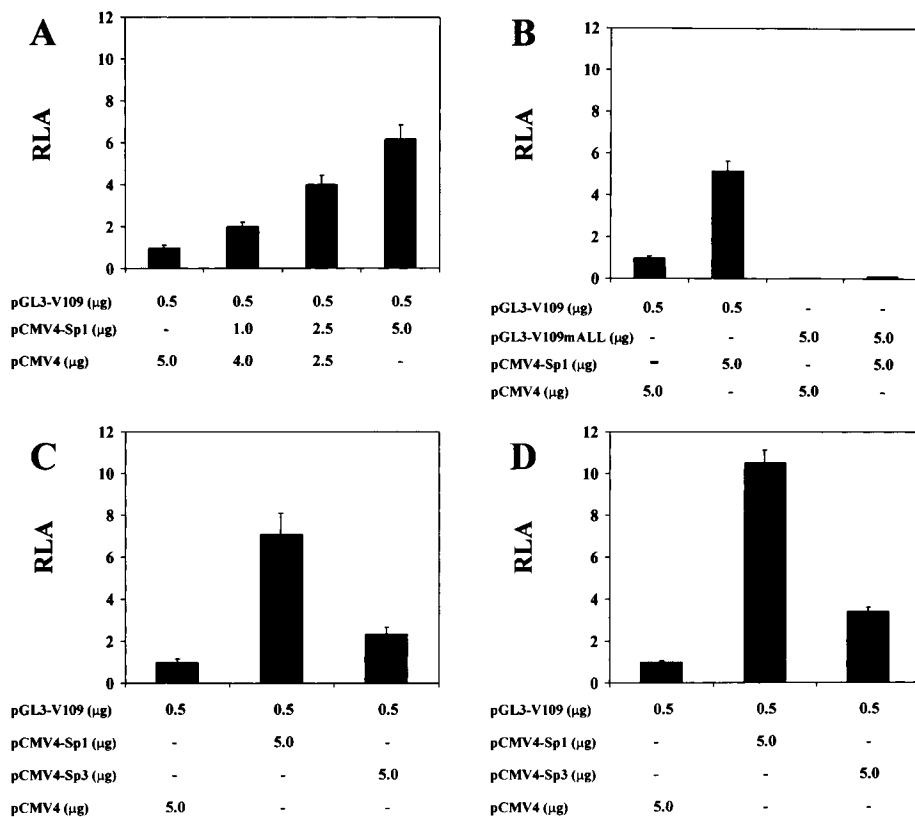
Western blot analysis. As shown in Fig. 5, *A* and *B*, a high level of Sp1 protein was detected in PANC-1, COLO357, SW-1990, and Bxpc-3 cells constitutively expressing a high level of VEGF, whereas a low level of Sp1 proteins was detected in CaPan-2, HS766T, MDA

Panc-3, and HPAFII cells constitutively expressing a low level of VEGF. The expression level of Sp3 was proportional to that of Sp1, and no significant ratio difference was observed in the cells expressing different levels of VEGF (data not shown), suggesting that different VEGF expression levels may not be due to an Sp1 and Sp3 expression imbalance.

Transactivation of the VEGF Promoter by Sp1 and Sp3. To assess the effect of Sp1 and Sp3 on VEGF transcription, the VEGF promoter-luciferase construct, pGL3-V109, was cotransfected into PANC-1 cells with increasing concentrations of an expression vector encoding for Sp1 (pCMV4-Sp1) or with the control vector lacking Sp1 (pCMV4). Using the pBA-RL vector as a control to normalize for transfection efficiency, we showed that the luciferase activity driven by the VEGF promoter was activated by Sp1 in PANC-1 cells in a dose-dependent manner (Fig. 6A). Additionally, a 6.3-fold stimulation was observed in cells cotransfected with 5 μ g of the plasmid expressing the Sp1 protein, which was not detected in transfections with the control vector.

To provide direct evidence that the Sp1 sites within the 109-bp proximal Sp1 promoter actually contribute to its activity, we performed site-directed mutagenesis of all four Sp1 sites located upstream of the VEGF transcription initiation site (Fig. 3C). Disruption of the upstream Sp1 sites totally eliminated reporter activity in the PANC-1 cells and promoter transactivation in the cells cotransfected with 5 μ g of expression vector for Sp1 (Fig. 6B). Furthermore, when the pGL3-V109 construct was cotransfected with pCMV4-Sp1 into PANC-1 cells, which expressed a high level of VEGF (Fig. 6C), and CaPan-2 cells, which expressed a low level of VEGF (Fig. 6D), the luciferase activity was increased in these cells. A more pronounced increase in VEGF promoter activity was seen in the CaPan-2 cells, which may have reflected the existence of different levels of endogenous Sp1. Similarly, cotransfection of pCMV4-Sp3 led to lower transactivation of VEGF promoter in both PANC-1 and CaPan-2 cells,

Fig. 6. Activation of VEGF promoter by transient transfection of Sp1. *A*, dose-dependent activation by Sp1. pGL3-V109 and pCMV4-Sp1 in different ratios were transfected with pBA-RL into PANC-1 cells. *B*, disruption by mutation of Sp1 binding sites. pGL3-V109 and its Sp1 mutant pGL3-V109mALL (or pGL3-V/Sp1mALL) were transfected with pBA-RL into PANC-1 cells. *C*, activation by Sp1 and Sp3 in PANC-1 cells. pGL3-V109 and pCMV4-Sp1 or pCMV4-Sp3 were transfected with pBA-RL into PANC-1 cells. *D*, activation by Sp1 and Sp3 in CaPan-2 cells. pGL3-V109 and pCMV4-Sp1 or pCMV4-Sp3 were transfected with pBA-RL into CaPan-2 cells. The cells were incubated for 48 h, and firefly and renilla luciferase activity was measured for the calculation of specific VEGF promoter activity.



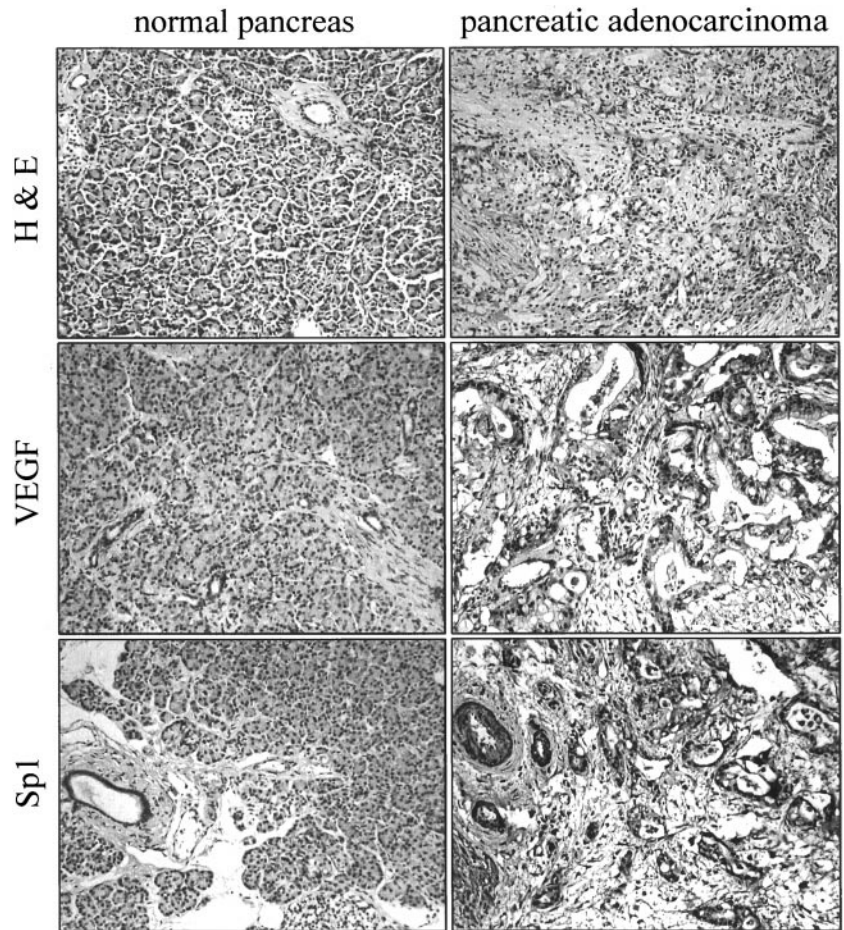


Fig. 7. Expression of VEGF and Sp1 in pancreatic cancer tissue. Normal human pancreatic tissue and pancreatic adenocarcinoma tissue specimens were collected and processed for H&E staining and immunostaining of VEGF (1:200) and Sp1 (1:200) expression. As a negative control, staining of section was performed without the first antibody or second antibody or with preimmune rabbit serum replacing the first or second antibody (data not shown).

suggesting that Sp3 is a weak activator compared with Sp1. These experiments indicated that the presence of functional Sp1 elements within the *VEGF* promoter regulates VEGF expression in human pancreatic cancer cells.

Expression of VEGF and Sp1 in Pancreatic Adenocarcinoma Cells. To determine the biological relevance of Sp1-mediated constitutive expression of VEGF, VEGF and Sp1 expression was determined in human pancreatic adenocarcinoma cells. Ten human pancreatic adenocarcinoma specimens and 10 normal human pancreatic tissue specimens were used for immunostaining with specific anti-VEGF and anti-Sp1 antibodies. As compared with the normal pancreatic tissue specimens, all of the human pancreatic cancer specimens overexpressed the Sp1 protein, which was correlated with elevated expression of VEGF (Fig. 7). To further confirm this finding, VEGF expression was determined by Northern blot analysis, and Sp1 expression was determined by both Western blot analysis and EMSA. Consistent with the results of immunostaining (Fig. 7), tumor tissues expressed a higher level of VEGF than normal tissue (Fig. 8A). The tumor tissue also expressed a higher level of Sp1 protein (Fig. 8B) and binding activity (Fig. 8C) than normal tissues. These data demonstrated clearly that constitutive expression of the transcription factor Sp1 contributes to VEGF overexpression and hence to angiogenesis and progression of human pancreatic adenocarcinoma.

DISCUSSION

In the present study, we demonstrated that the majority of the human pancreatic cancer cell lines studied constitutively expressed VEGF and that human pancreatic cancer tissues commonly overex-

pressed VEGF. The constitutive VEGF expression appeared to result from constitutive VEGF promoter activity, which correlated directly with the expression and activity of the transcription factor Sp1. Elevated Sp1 expression was confirmed in human pancreatic cancer cells. These findings suggest that elevated Sp1 activity is essential for constitutive VEGF overexpression. Because VEGF is a key angiogenic molecule, it is expected that overexpression of Sp1 and subsequent overexpression of VEGF play an important role in pancreatic cancer angiogenesis and progression.

Although VEGF expression can be induced dramatically by external factors, such as hypoxia, and modulated by many growth factors and cytokines, many types of cancer can constitutively express VEGF. As shown in the present study, the majority of human pancreatic cancer cells constitutively express VEGF without any apparent external stimuli. As a first approach, we aimed to identify the mechanisms and kinetics implicated by the differential levels of constitutive VEGF mRNA expression observed in various human pancreatic cancer cells. Initial experiments indicated that VEGF mRNA stability varied among the cancer cell lines but could not explain the variable steady-state level of VEGF mRNA. However, the steady-state level of VEGF mRNA was consistent with the *VEGF* gene transcription rate as determined using a nuclear run-on assay. These results suggested that constitutive VEGF expression depends mainly on the constitutively activated VEGF gene transcription process. This conclusion was further supported by our results indicating that the *VEGF* promoter was constitutively active in these cells and correlated with VEGF expression.

Whereas little is currently known about the transcriptional regula-

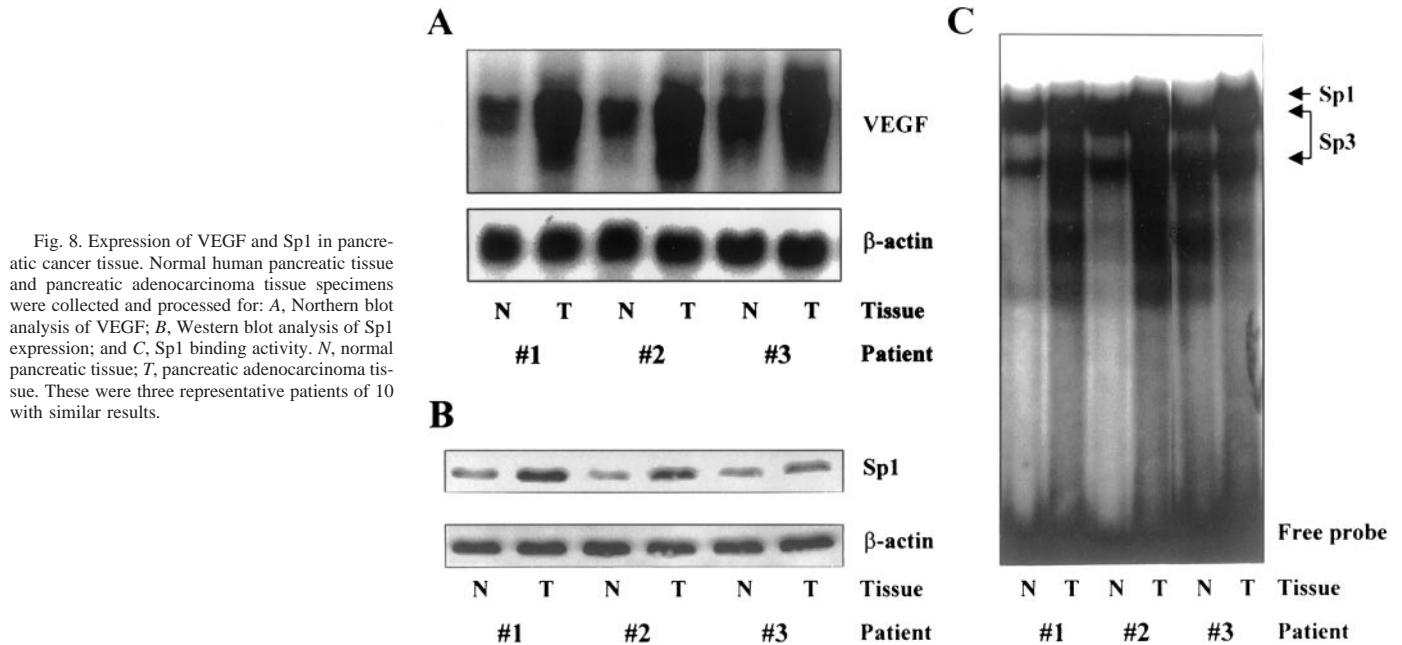


Fig. 8. Expression of VEGF and Sp1 in pancreatic cancer tissue. Normal human pancreatic tissue and pancreatic adenocarcinoma tissue specimens were collected and processed for: A, Northern blot analysis of VEGF; B, Western blot analysis of Sp1 expression; and C, Sp1 binding activity. N, normal pancreatic tissue; T, pancreatic adenocarcinoma tissue. These were three representative patients of 10 with similar results.

tion leading to constitutive VEGF expression, disruption or deregulation of normal signal transduction pathways may be an important contributing factor. This hypothesis is supported by several recent studies showing that the loss or inactivation of the wild-type vHL and/or *p53* tumor suppressor gene is associated with increased angiogenesis in developing tumors (45–53). Also, the *v-src* oncogene increases VEGF expression by inducing HIF-1 expression (57–59), which is not only independent of hypoxia but also actually short circuits the normal HIF-1-dependent hypoxia-sensing mechanism that regulates VEGF expression. Given the prominent role of VEGF in tumor angiogenesis, it is critical to understand the molecular basis of constitutive VEGF gene expression. In the present study, we further defined the *cis*-acting elements and transcription factors involved in constitutive VEGF expression in human pancreatic cancer cells.

Functional analysis of the human VEGF promoter was initially performed using the full-length VEGF promoter reporter in eight human pancreatic cancer cell lines. A high level of VEGF promoter activity was evident in the cells constitutively expressing a high level of VEGF, whereas a low level of VEGF promoter activity was detected in the cells constitutively expressing a low level of VEGF. Therefore, there was a direct correlation between VEGF promoter activity and VEGF expression. In addition, progressive 5'-deletion constructs of the human VEGF promoter were analyzed in PANC-1 and CaPan-2 cells, which had high and low VEGF expression, respectively. Deletion up to -267 had no effect on constitutive VEGF promoter activity compared with pGL3-V2274, which exhibited maximal VEGF promoter activity, whereas further deletion from -267 to -37 reduced significantly or totally eliminated constitutive promoter activity; the difference in VEGF promoter activity between PANC-1 and CaPan-2 cells was consequently reduced or eliminated as well. These results indicate that the region from -37 to -267 is functionally essential for differential constitutive VEGF promoter activity in human pancreatic cancer cells.

Several putative response elements in the region from -267 to -2274 have been identified. These include three AP-1 binding sites, one HIF-1 binding site, two AP-2 binding sites, and one NF1 binding site. VEGF gene regulation by nitric oxide and hypoxia appears to be potentiated by the AP-1 element of the gene in C6 glioma cells (60, 61). Also, a unique site (TGAGTGA) located at position -621 of the

5'-flanking region of the VEGF gene is functional and responsible for the inhibitory effect of retinoids in cultured keratinocytes (62). Furthermore, lipopolysaccharide-induced VEGF production in human pulp cells is mediated in part through AP-1 activation (63). On the other hand, mutant H- and K-*ras* oncogenes, as well as *v-src* and *v-raf*, induce VEGF expression in transformed fibroblasts and epithelial cells in part through *ras*-dependent signal transduction pathways and AP-1 activation, which can presumably bind to relevant sites in the VEGF promoter regions (64). However, hypoxia-mediated VEGF induction in osteoblast-like cells (65) and smooth muscle cells (66, 67) is likely not mediated by AP-1. In addition, VEGF induction by carbon monoxide and anoxia in rat cardiomyocytes does not involve AP-1 binding sites (68). Furthermore, hypoxia-induced VEGF expression is independent of AP-1-mediated transcription (69).

The role of AP-1 in VEGF expression and regulation is not certain at the present time. This might be due to the use of different cell lines or tissues. Our full-length VEGF promoter construct contains three motifs homologous to the AP-1 binding site [5'-TGANT(C/A)NN-3'] at positions -621 , -1527 , and -2265 . Transactivation analysis using different promoter constructs revealed that these distal AP-1 elements do not contribute significantly to constitutive VEGF promoter activity in PANC-1 and CaPan-2 cells because deletion of them (pGL3-V1181, pGL3-V1012, and pGL3-V411; Fig. 3) had no effect compared with pGL3-V2274. Also, *K-ras* and *p53* mutations are very common in pancreatic cancer (3). However, it remains unclear whether these mutations directly influence AP-1 activity and VEGF overexpression in human pancreatic cancer cells.

HIF-1 is a heterodimeric basic helix-loop-helix protein that activates transcription of the human erythropoietin gene in hypoxic cells. HIF-1 also activates VEGF transcription by binding VEGF 5'-flanking sequences in hypoxic Hep3B cells (27, 67). The oncogenic *Ha-ras* enhances the induction of VEGF by hypoxia via HIF-1 (70). *c-src* expression is neither required nor critical for expression of HIF-1 or transcriptional activation of the VEGF gene (71), whereas cells expressing the *v-src* oncogene have increased expression of HIF-1 and VEGF under both hypoxic and nonhypoxic conditions (59). Additionally, the loss of PTEN during malignant progression contributes to tumor expansion through the deregulation of Akt activity and HIF-1-regulated gene expression (72). Therefore, the HIF-1 element is

crucial in hypoxia-dependent and -independent induction of VEGF. At least one HRE has been clearly identified and shown to be responsive functionally to hypoxia and oncogenic transformation (67). In the present study, both deletion and site-directed (data not shown) mutation of this HRE apparently did not affect the constitutive VEGF promoter activity, suggesting that this HRE was not functionally active and likely did not contribute significantly to the constitutive VEGF promoter activity observed in human pancreatic cancer cells.

The transcription factor AP-2 is a 52-kDa protein that binds as a dimer to the palindromic sequence 5'-GCCNNGGC-3', although some AP-2 binding sites deviate from this consensus sequence. It has been suggested that AP-2 plays a role in the regulation of VEGF expression in hamster cells by hypoxia and p42/p44 mitogen-activated protein kinase (73) and in bovine ovarian granulosa cells by growth factors (74). Also, AP-2 protein appears to be functionally important in transforming growth factor α -induced VPF/VEGF gene expression in keratinocytes (75). In the present study, we showed that neither distal AP-2 (pGL3-V789 *versus* pGL3-V1012) nor distal NF1 (pGL3-V267 *versus* pGL3-V411) elements contributed to the constitutive VEGF promoter activity.

Our findings described above indicated that these distal putative elements are not constitutively active in human pancreatic cancer cells. In contrast, they may play an essential role in dynamic regulation of VEGF by hypoxia, which mainly involves transcription factor HIF-1 α . In a mouse model, it has been clearly demonstrated that hypoxia becomes a primary mediator of the angiogenic switch in advanced cancers (25). We therefore focused our current study of constitutive VEGF expression on the proximal region of the VEGF promoter. Small-scale deletion of the pGL3-V267 VEGF promoter revealed that the proximal 71-bp region (-38 to -109) is essential for constitutive VEGF promoter activity in pancreatic cancer cells (Fig. 3). Within this region, there are four Sp1 elements (Sp1.1, Sp1.2, Sp1.3, and Sp1.4), two Egr-1 elements (Egr-1.1 and Egr-1.2), and one AP-2 element. To define the contribution of these elements to VEGF promoter activity, site-directed mutagenesis was performed. We found that mutation of the AP-2 element did not affect the constitutive VEGF promoter activity. Therefore, both distal and proximal AP-2 binding sites were not of functional significance in constitutive VEGF promoter activity. Mutation of the two Egr-1 elements did not affect the constitutive VEGF promoter activity either. These results were consistent with previous reports showing that enhanced VEGF gene expression in platelet-derived growth factor-BB-induced NIH3T3 cells is mediated by Sp1 and/or Sp3 transcription factors bound to the promoter region of the VEGF gene (37). In our site-directed mutagenesis study, mutation of the four individual Sp1 elements resulted in a variable (12.36–45.56%) reduction in VEGF promoter activity compared with that of the wild-type pGL3-V109 construct (Fig. 4), and mutation of all four Sp1 elements totally eradicated the constitutive VEGF promoter activity and its difference in PANC-1 and CaPan-2 cells. These results clearly demonstrated that the proximal Sp1 elements are essential for the differential constitutive promoter activity of the VEGF gene in human pancreatic cancer cells. Additionally, we determined the Sp1 binding activity in various pancreatic cancer cells. Only three of five putative Sp1 binding sites were functionally active. Also, a high level of Sp1 binding activity was observed in the cells constitutively expressing high levels of VEGF, whereas low levels of Sp1 binding activity were detected in the cells constitutively expressing a low level of VEGF. Therefore, the Sp1 binding activity was correlated directly with VEGF promoter activity, mRNA expression, and VEGF protein secretion.

Sp1 is a well-characterized, sequence-specific, DNA-binding protein that is important in the transcription of many cellular and viral genes that contain GC boxes in their promoter (41). Additional tran-

scription factors (Sp2, Sp3, and Sp4) have been cloned that are similar in their structural and transcriptional properties to Sp1, thus forming a Sp1 multigene family (76). In the present study, we analyzed the factor or factors binding to the functional Sp1.1 element using EMSA and supershift analysis. Three expressed constitutively DNA-protein complexes were detected using labeled Sp1.1 oligonucleotides. Analysis using antibodies against Sp1 and Sp3 revealed that complex 1 contained Sp1, complex 2 contained both Sp1 and Sp3, and complex 3 contained only Sp3. However, whereas Sp1 stimulates transcriptional activity, the function of Sp3 is less clear. Sp3 has been shown to act as a dual-function regulator whose activity depends on the context of DNA binding sites in a promoter. Sp3 also functions as a repressor when it is bound to a promoter through multiple DNA binding sites and as an activator when it is targeted to a promoter through a single DNA binding site (77–80). In addition, Sp3 has the ability to repress Sp1-mediated transcriptional activation of the human alcohol dehydrogenase 5 gene, possibly by competing with Sp1 for binding (80). With regard to the VEGF gene, we have determined that Sp1 potently activates the VEGF promoter in both PANC-1 and CaPan-2 cells, as well as Sp-deficient SL2 cells (data not shown), whereas Sp3 functions as a weak transcriptional activator.

Finally, we compared Sp1 activity in different pancreatic cancer cells. We found that the level of Sp1 activity directly correlated with the VEGF promoter activity, the steady-state level of mRNA, and VEGF secretion. Furthermore, Sp1 activity was directly correlated with Sp1 expression as determined using Western blot analysis, which showed a high level of Sp1 protein in the cells constitutively expressing a high level of VEGF, whereas a low level of Sp1 protein was seen in the cells constitutively expressing a low level of VEGF. Data from pancreatic cancer tissues were consistent with these biochemical findings. As compared with normal pancreatic tissue specimens, most of the human pancreatic cancer specimens overexpressed Sp1 protein as determined using immunostaining, Western blot, and EMSA and which correlated directly with VEGF overexpression.

The underlying mechanism resulting in different levels of Sp1 activity is currently unknown. This difference could be due to several mechanisms, such as the Sp1:Sp3 ratio (81), methylation status of Sp1 binding sites (82), and possible inhibitory proteins that bind to Sp1/Sp3 (56). It may also involve many other cellular factors, including the functional status of oncogenes and suppressor genes. Sp1/Sp3 has been shown to interact with several oncogenes and suppressor genes, *e.g.*, retinoblastoma protein (56, 83) and the vHL tumor suppressor gene (50). Also, tumor cells harboring the inactivated vHL gene are associated with increased VEGF expression and angiogenesis (47–49). vHL gene-mediated repression of VEGF has convincingly been shown to be mediated by transcriptional and posttranscriptional mechanisms (50–52). At the transcriptional level, vHL forms a complex with the Sp1 transcription factor and inhibits Sp1-mediated VEGF expression (50). Whether the status of vHL, p53, and Ras is involved in this constitutive VEGF expression in human pancreatic cancer cells is unknown. Our present study was the first to demonstrate that differential Sp1 expression and activity directly regulate the constitutive levels of VEGF expression in human pancreatic cancer cells, thus providing a novel mechanism for constitutive VEGF regulation. Interestingly, the VEGF level can be further induced by hypoxia via the HIF-1 α pathway in pancreatic cancer cells expressing high levels of VEGF (data not shown). We are currently investigating the possible interaction between Sp1 and HIF-1 α , which has been shown to be a major mediator of VEGF regulation by hypoxia and oncogenic transformation (26–30).

In summary, we have demonstrated the importance of elements within the proximal region of the human VEGF promoter for differential constitutive VEGF expression in human pancreatic cancer. This

analysis also indicated that the major contributing factor was the differential expression of Sp1 in human pancreatic cancer cells and that Sp1 was aberrantly overexpressed in pancreatic adenocarcinoma specimens compared with normal pancreatic tissue specimens. Therefore, abnormal activation of Sp1 may play an important role in tumor angiogenesis, growth, and metastasis. A further understanding of the molecular basis of Sp1, as well as of VEGF regulation, will have functional implications in suppressing pancreatic cancer progression and will ultimately lead to the design of new therapeutic modalities. On the basis of these results, we propose that increased Sp1 DNA-binding activity may augment the angiogenic and metastatic capacity of tumor cells through overexpression of Sp1-responsive genes, including the key angiogenic factor VEGF.

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