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Biochemical and Biophysical Research Communications 345 (2006) 292–301
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Regulation of vascular endothelial growth factor receptor-2 expression in pancreatic cancer cells by Sp proteins ☆

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Received 30 March 2006
Available online 2 May 2006

Abstract

Vascular endothelial growth factor receptor-2 (VEGFR2/KDR) is an important mediator of angiogenesis, and VEGFR2 mRNA is expressed in several pancreatic cancer cell lines. Deletion analysis of the VEGFR2 promoter in Panc-1, AsPC-1, and MiaPaCa-2 pancreatic cancer cells shows that the proximal region of the promoter is primarily responsible for VEGFR2 expression, and two GC-rich sites at −58 and −44 are critical elements in all three cell lines. Panc-1, AsPC-1, and MiaPaCa-2 cells also express Sp1, Sp3, and Sp4 proteins which bind to the GC-rich region of the VEGFR2 promoter in electrophoretic mobility shift and chromatin immunoprecipitation assays. RNA interference with small inhibitory RNAs for Sp1, Sp3, and Sp4 decreases VEGFR2 mRNA and reporter gene activity in transfection assays, confirming that VEGFR2 expression in pancreatic cancer cells is regulated by Sp proteins. These results suggest that VEGFR2 cannot only be targeted by receptor tyrosine kinase inhibitors but also by drugs that downregulate Sp proteins or block Sp-dependent transactivation.

Keywords: VEGFR2; Sp; Regulation; Angiogenesis; Pancreatic

Pancreatic cancer ranks fourth among cancer deaths in the United States, and it is estimated that approximately 32,000 new pancreatic cancer cases were diagnosed in 2004 [1]. Since diagnosis of this disease usually occurs at a late stage, the prognosis for patient survival is low and the five-year survival rate is <5% [2]. Successful management of pancreatic cancer requires more sensitive methods for early diagnosis, the development of improved surgical and chemotherapies, and a more comprehensive understanding of the underlying biological basis for pancreatic tumor development and metastasis [3]. A number of genetic determinants and medical conditions have been identified as risk factors for this disease [3,4]. For example, several heritable gene mutations such as Peutz–Jeghers, hereditary pancreatitis, hereditary non-polyposis colorectal cancer syndromes, familial breast cancer, and familial atypical multiple-mole melanoma are associated with increased risks for pancreatic cancer. In addition, medical conditions such as chronic pancreatitis gastrectomy, diabetic mellitus, and certain polymorphisms associated with DNA repair and drug/carcinogen metabolism are also associated with increased risks for pancreatic cancer [3–5]. Epidemiology studies also show that several environmental and lifestyle factors such as cigarette smoking, intakes of red and processed meats and their methods of preparation, and low dietary intakes of fruits and vegetables are correlated with increased incidence of pancreatic cancer [6,7]. Many of

☆ *Abbreviations:* ChIP, chromatin immunoprecipitation; DME, Dulbecco's modified Eagle's; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; PBS, phosphate-buffered saline; siRNA, small inhibitory RNA; Sp1, specificity protein 1; TBP, TATA binding protein; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.04.111

these same factors are associated with increased risks for other cancers; however, identification of specific substances that modulate these risks has not been determined.

Development of pancreatic cancer is also accompanied by several acquired mutations of both oncogenes and tumor suppressor genes [3–7]. *K-ras* oncogene mutations are observed in >85% of pancreatic tumors and are generally detected during the early stages of cancer development. In contrast, the tumor suppressor genes *p*16, *p*53, and *SMAD4* are detected in up to 95%, 98%, and 75% of pancreatic cancer cases, respectively, and are generally observed in later stages of tumor development. Mutations of these and other genes contribute to the high proliferative rates and metastases of pancreatic cancers. Current chemotherapies commonly used for treatment of pancreatic cancer involve cytotoxic drugs such as gemcitabine alone or in combination with radiation or other drugs; however, therapies that inhibit k-ras, receptor tyrosine kinases, and matrix metalloproteinases are promising new approaches for treatment of this disease [3,8,9].

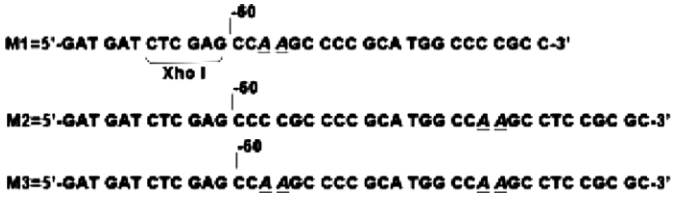
Research in our laboratory has focused on expression and regulation of the important angiogenic factor vascular endothelial growth factor (VEGF) in cancer cell lines [10–14]. Several studies show that VEGF expression is due, in part, to specificity protein 1 (Sp1) expression in pancreatic and other cancer cell lines [12–16]. However, using RNA interference, it has recently been shown that VEGF regulation in pancreatic cancer cell lines is due to Sp1, Sp3, and Sp4 [12]. In this study, using real-time PCR, we have identified vascular endothelial growth factor receptor-2 (VEGFR2) expression in Panc-1, AsPC-1, Panc-28, HPAFII, BxPC-3, and MiaPaCa-2 pancreatic cancer cells, and this has been confirmed by immunofluorescent staining for VEGFR2 protein in Panc-1 cells. Analysis of the VEGFR2 promoter shows that two proximal GC-rich sites at −58 and −44 are important for expression of VEGFR2, and RNA interference studies show that Sp proteins (Sp1, Sp3, and Sp4) are critical transcription factors that mediate expression of VEGFR2 in pancreatic cancer cells.

Materials and methods

Chemicals, plasmids, and gifts. Phosphate-buffered saline (PBS) and 100× antibiotic/antimycotic solution were purchased from Sigma Chemical Company (St. Louis, MO); 5× lysis buffer, luciferase reagent, restriction enzymes (*Xho*I and *Hin*dIII), and ligase were purchased from Promega (Madison, WI). β-Galactosidase reagents were purchased from Tropix (Bedford, MA). *Taq* polymerase and other PCRs were purchased from Perkin-Elmer (Boston, MA). pCDNA3.1-His-LacZ expression plasmid was obtained from Invitrogen (Carlsbad, CA). VEGFR2 promoter luciferase constructs pVEGFR2A, pVEGFR2B, and pVEGFR2C (previously named pKDR-716/+268, pKDR-225/+268, and pKDR-95/+268) were provided by Dr. Arthur Mu-EnLee (deceased) and Dr. Koji Maemura (Cardiovascular Biology Laboratory, Boston, MA). pGL2 basic luciferase reporter vector was purchased from Promega.

Cell lines and tissue culture. The human pancreatic cancer cell lines Panc-1, AsPC-1, MiaPaCa-2, HPAFII, and BxPC-3 were obtained from American Type Culture Collection (ATCC, Manassas, VA), and Panc-28 cells were obtained from Dr. J. Abbruzzese, M.D. Anderson Cancer Center (Houston, TX). Panc-1, MiaPaCa-2, and Panc-28 cells were cultured in Dulbecco's modified Eagle's (DME) medium/F12 (Sigma) supplemented with 5% or 10% fetal bovine serum (FBS) (Summit Biotechnology, Fort Collins, CO; Intergen, Des Plains, IA; JRH Biosciences, Lenexa, KS; or Atlanta Biologicals, Inc., Norcross, GA). Medium was further supplemented with 2.2 g/L sodium bicarbonate and 100× antibiotic/antimycotic solution (Sigma). AsPC-1 and BxPC-3 cells were maintained in RPMI 1640 media (Sigma) supplemented with 10% FBS (Summit Biotechnology; Intergen; JRH Biosciences; or Atlanta Biologicals, Inc.). Medium was further supplemented with 1.5 g/L sodium bicarbonate, 2.38 g/L Hepes, 0.11 g/L sodium pyruvate, and 100× antibiotic/antimycotic solution (Sigma). HPAFII cells were cultured in Eagle's minimal essential medium (Sigma) supplemented with 10% FBS (Summit Biotechnology; Intergen; JRH Biosciences; or Atlanta Biologicals, Inc.). Medium was further supplemented with 1.5 g/L sodium bicarbonate and 100× antibiotic/antimycotic solution (Sigma). Cells were maintained at 37 °C with a humidified CO_2:air (5:95) mixture.

Cloning and oligonucleotides. VEGFR2 promoter-derived oligonucleotides, PCR primers, and primers employed in plasmid construction were synthesized by Genosys/Sigma (The Woodlands, TX) or Integrated DNA Technologies (IDT) (Coralville, IA). VEGFR2 promoter deletion constructs pVEGFR2D, pVEGFR2E, pVEGFR2F, and pVEGFR2G were created by PCR amplification using pVEGFR2A as the template. Forward primers were designed with *Xho*I restriction enzyme sites at the 5′-end. A reverse luciferase primer was used for PCR. PCR products were digested with *Xho*I and *Hin*dIII, and subsequently ligated into the pGL2 basic vector. All constructs are in pGL2 basic luciferase reporter vector and all constructs were sequenced to verify their identity. Mutation constructs pVEGFR2Em1, pVEGFR2Em2, and pVEGFR2Em3 were constructed by PCR amplification using the reverse luciferase primer paired with the forward primer containing the desired mutations. Forward primers are as follows (mutated bases are underlined):



Transient transfection assays. Cells were seeded in 12-well plates at a concentration of 1.5 to 3.0×10^5 cells per well in phenol red-free DME/F12 media supplemented with 2.5% charcoal-stripped FBS. Panc-1, AsPC-1, and MiaPaCa-2 cells were transiently cotransfected with 500 ng of the appropriate VEGFR2 luciferase reporter plasmid and 250 ng pCDNA3.1-His-LacZ. Four to eight hr after transfection, cells were shocked with 25% glycerol in PBS to increase transfection efficiency, washed with PBS, and fresh serum-free DME/F12 medium was replaced. Cells were harvested by scraping the plates in 100–200 μL of 1× lysis buffer (Promega). An aliquot of soluble protein was obtained by one cycle of freezing/thawing the cells, vortexing (30 s), and centrifuging at 12,000*g* (1 min). Cell lysates (30 μL) were assayed for luciferase activity using Luciferase Assay Reagent (Promega) and β-galactosidase activity using Tropix Galacto-Light Plus assay system (Tropix) in a Lumicount micro-well plate reader (Packard Instrument Co., Downers Grove, IL). Relative luciferase activity was normalized to relative β-galactosidase units for each transfection experiment.

Transient transfection of siRNA. Cells were cultured in phenol red-free DME/F12 medium supplemented with 2.5% charcoal stripped FBS in 12-well plates until 50–70% confluent. Cells were washed once with serum free, antibiotic free, phenol red-free DME/F12 media. The amount of siRNA to give a maximal decrease of each target protein was determined experimentally (5–20 nM final concentration in the well). Pancreatic cancer cells were cotransfected with siRNA, 400 ng of the appropriate VEGFR2 luciferase reporter plasmid, and 200 ng pCDNA3.1-His-LacZ using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Cells were harvested ~48 h later. Cell lysates

were assayed for luciferase and β-galactosidase activity as described earlier.

The Lamin A/C duplex (target sequence: 5′-CTG GAC TTC CAG AAG AAC A-3′) and the Luciferase GL2 duplex RNA (target sequence: 5′-CGT ACG CGG AAT ACT TCG A-3′) from Dharmacon (Lafayette, CO) were used for controls in siRNA transfections. The siRNA oligonucleotides for Sp1, Sp3, and Sp4 were also ordered from Dharmacon as follows:

Sp1: 5′-AUC ACU CCA UGG AUG AAA UGA dTdT-3′
Sp3: 5′-GCG GCA GGU GGA GCC UUC ACU dTdT-3′
Sp4: 5′-GCA GUG ACA CAU UAG UGA GCdT dT-3′

Western-blot analysis. Cells were seeded into six-well plates in DME/F12 medium supplemented with 2.5% charcoal-stripped FBS. The next day, cells were transfected with siRNA as described above. Cellular protein was obtained by harvesting cells in a high salt lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/mL aprotinin, 50 mM phenylmethylsulfonyl flouride, and 50 mM sodium orthovanadate) on ice for 45–60 min and centrifugation at 20,000*g* for 10 min at 4 °C. Thirty to sixty micrograms of protein was diluted with Laemmli's loading buffer, boiled, and loaded onto a 7.5% SDS–polyacrylamide gel. Samples were resolved using electrophoresis at 150–180 V for 3–4 h and transferred (transfer buffer: 48 mM Tris–HCl, 29 mM glycine, and 0.025% SDS) to a PVDF membrane (Bio-Rad, Hercules, CA) by electrophoresis at 0.2 Å for ~12–16 h.

Membranes were blocked with excess protein and then probed with polyclonal primary antibodies for Sp1 (PEP2), Sp3 (D20), and Sp4 (V20) from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Sp1 and Sp3 were each diluted 1:1000 and incubated overnight. Sp4 was diluted 1:250 or 1:500 and incubated overnight as well. Membranes were probed with a horseradish peroxidase-conjugated secondary antibody (1:5000) for 3–6 h. Blots were visualized using the chemiluminescent substrate ECL detection system (NEN-DuPont, Boston, MA) and exposure on Kodak X-O Mat autoradiography film (Eastman Kodak Co., Rochester, NY). Band intensity values were obtained by scanning the film on a Sharp JX-330 scanner (Sharp Electronics, Mahwah, NJ) and by densitometry using the Zero-D Scanalytics software package (Scanalytics, Sunnyvale, CA).

Real-time PCR. For experiments involving siRNA, pancreatic cancer cells were transfected as described previously. Total RNA was isolated using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was eluted with 30 μL RNase-free water and stored at −80 °C. RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. PCR was carried out using SYBR Green PCR Master Mix from PE Applied Biosystems (Warrington, UK) on an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The 25-μL final volume contained 0.5 μM of each primer and 2 μL cDNA template. TATA binding protein (TBP) was used as an exogenous control to compare the relative amount of target gene in different samples. The PCR profile was as follows: 1 cycle of 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The comparative C_T method was used for relative quantitation of samples. Primers were purchased from Integrated DNA Technologies (Coralville, IA). The following primers were used:

KDR (F): 5′-CAC CAC TCA AAC GCT GAC ATG TA-3′
KDR (R): 5′-CCA ACT GCC AAT ACC AGT GGA T-3′
TBP (F): 5′-TGC ACA GGA GCC AAG AGT GAA-3′
TBP (R): 5′-CAC ATC ACA GCT CCC CAC CA-3′

Preparation of nuclear extracts. Cells were cultured in medium without phenol red, supplemented with 2.5% charcoal-stripped FBS. The next day, cells were switched to serum-free media without phenol red for 1–3 days. Cells were washed in PBS (2×), scraped in 1 ml of 1× lysis buffer, incubated at 4 °C for 15 min, and centrifuged 1 min at 14,000*g*. Cell pellets were washed in 1 ml lysis buffer (3×). Lysis buffer supplemented with 500 mM KCl was then added to the cell pellet and incubated for 45 min at 4 °C with frequent vortexing. Nuclei were pelleted by centrifugation at 14,000*g* for 1 min at 4 °C, and aliquots of supernatant were stored at −80 °C until needed.

Electrophoretic mobility shift assay (EMSA). VEGFR2 oligonucleotide (−64 5′-CCG GCC CCG CCC CGC ATG GCC CCG CCT CCG-3′-35) was synthesized and annealed, and 5 pmol aliquots were ^{32}P-labeled at the 5′-end using T4 polynucleotide kinase (Invitrogen) and [γ^{32}P]ATP (NEN-Dupont, Boston, MA). A 30-μL EMSA mixture contained ~100 mM KCl, 3 μg of crude nuclear protein, 1 μg poly(dI–dC) (Roche Molecular Biochemicals, Basel, Switzerland), with or without unlabeled competitor oligonucleotide, and ~10 fmol of radiolabeled probe. After incubation for 20 min on ice, antibodies against Sp1, Sp3, or Sp4 proteins were added and incubated another 20 min on ice. Protein–DNA complexes were resolved by 5% polyacrylamide gel electrophoresis as previously described [10,12]. Specific DNA–protein and antibody-supershifted complexes were observed as retarded bands in the gel, and were visualized by exposure to a phosphor-storage screen, followed by scanning on a STORM 860 (Molecular Dynamics, Sunnyvale, CA).

Chromatin immunoprecipitation (ChIP) assay. MiaPaCa-2, Panc-1, and AsPC-1 cells (1×10^7 each) were fixed with 1.5% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. Cells were scraped, pelleted, and hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to desired chromatin length (~500 bp). The chromatin was precleared by addition of protein A-conjugated beads (Pierce Biotechnology, Rockford, IL). The precleared chromatin supernatants were immunoprecipitated with antibodies specific to IgG, TFIIB, Sp1, Sp3, Sp4, and estrogen receptor α (ERα) (Santa Cruz Biotechnology) at 4 °C overnight. The protein–antibody complexes were collected by addition of protein A-conjugated beads for 1 h, and the beads were extensively washed. The protein–DNA cross-links were eluted and reversed. DNA was purified by Qiaquick Spin Columns (Qiagen) and followed by PCR amplification. The VEGF primers were: 5′-GGT CGA GCT TCC CCT TCA-3′ (forward) and 5′-GAT CCT CCC CGC TAC CAG-3′ (reverse), which amplified a 202-bp region of human VEGF promoter containing GC-rich/Sp1 binding sites. The VEGFR2/KDR primers were: 5′-GTC CAG TTG TGT GGG GAA AT-3′ (forward) and 5′-GAG CTG GAG CCG AAA CTC TA-3′ (reverse), which amplified a 169-bp region of human VEGFR2/KDR promoter containing GC-rich/Sp1 binding sites. The positive control primers were: 5′-TAC TAG CGG TTT TAC GGG CG-3′ (forward) and 5′-TCG AAC AGG AGG AGC AGA GAG CGA-3′ (reverse), which amplified a 167-bp region of human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. The negative control primers were: 5′-ATG GTT GCC ACT GGG GAT CT-3′ (forward) and 5′-TGC CAA AGC CTA GGG GAA GA-3′ (reverse), which amplified a 174-bp region of genomic DNA between the GAPDH gene and the CNAP1 gene. PCR products were resolved on a 2% agarose gel in the presence of 1:10,000 SYBR gold (Molecular Probes-Invitrogen, Carlsbad, CA).

Immunofluorescence. Rabbit polyclonal antibody for VEGFR2/KDR was purchased from Santa Cruz Biotechnology. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) or Santa Cruz Biotechnology. Panc-1 cells were seeded in Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) at 100,000 cells/well in phenol red-free DME/F12 medium supplemented with 5% charcoal-stripped FBS. Cells were then transfected with iRNAs as described previously and, after 48 h, were fixed with cold methanol at −20 °C for 5 min. After washing with PBS, cells were blocked with 4% goat serum at 4 °C overnight and incubated with the primary rabbit polyclonal antibody against VEGFR2 (1:25) at 37 °C for 1 h. After washing with PBS/0.1% Tween 3 × 10 min, the samples were incubated with FITC-conjugated goat anti-rabbit IgG (1:500) at room temperature for 1 h. After PBS/Tween rinsing, glass coverslips were mounted over the samples with mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), and cells were examined with a fluorescence microscope.

Statistical analysis. Results of transient transfection studies are presented as means (±) standard error (SE) for at least three replicates for each treatment group. All other experiments were carried out at least two times to confirm a consistent pattern of responses. Significant statistical differences between treatment groups were determined by analysis using SuperANOVA and Scheffe's test or Fisher's Protected LSD ($p < 0.05$).

Results

VEGFR2 expression in human pancreatic cancer cells

Several studies have reported expression of VEGF in pancreatic tumors and cancer cells and have identified a role for this protein and other angiogenic factors in tumor growth and metastasis [14,15,17]. A recent study did not detect expression of VEGFR2 in pancreatic cancer cells using reverse transcriptase-PCR [18]. Real-time PCR was used in this study to analyze expression of VEGFR2 mRNA in several pancreatic cancer cell lines including Panc-1, AsPC-1, Panc-28, HPAFII, BxPC-3, and MiaPaCa-2. VEGFR2 mRNA was detected in all cell lines tested, and the relative expression levels between cell lines were determined by comparison with TBP (Table 1). Relatively high VEGFR2 expression levels were observed in Panc-1 and AsPC-1 cells, lower levels of VEGFR2 mRNA were detected in Panc-28 and HPAFII cells, and among these six cell lines, the lowest levels were observed in BxPC-3 and MiaPaCa-2 cells. Subsequent transfection studies have used Panc-1, AsPC-1, and MiaPaCa-2 cells as models since they express both high and low VEGFR2 mRNA levels and are readily transfectable.

Analysis of VEGFR2 gene promoter constructs in Panc-1, AsPC-1, and MiaPaCa-2 pancreatic cancer cells

The proximal region of the VEGFR2 promoter contains multiple *cis*-elements [19,20], and the relative contributions of these motifs to expression of VEGFR2 were investigated in transient transfection studies. Panc-1 human pancreatic cancer cells were transiently transfected with pVEGFR2A which contained the −716 to +268 promoter insert and also a series of 5′ deletion constructs including pVEGFR2B, pVEGFR2C, pVEGFR2D, and pVEGFR2E (Fig. 1). Luciferase activity was comparable, even after deletion of the −716 to −78 region of the VEGFR2 promoter; however, activity was significantly decreased by approximately 35% in Panc-1 cells after deletion of the promoter region between −77 and −61. This suggests that overlapping GC-rich/AP-2 motifs may contribute to the basal activity of VEGFR2. Upon further deletion of the two GC-rich sites between −60 and −37, basal activity was decreased by >80%. Mutation analysis of the proximal GC-rich motifs showed that basal activity was also decreased in cells transfected with constructs containing single mutations of each of these sites (pVEGFR2Em1 and pVEGFR2Em2), and a further decrease was observed in cells transfected with the double mutant (pVEGFR2Em3). Thus, results of deletion/mutation analysis of the VEGFR2 promoter in Panc-1 cells showed that basal activity was primarily due to two proximal GC-rich motifs between −60 and −37.

The pattern of activity of the VEGFR2 deletion and mutated constructs was also investigated in two additional pancreatic cancer cell lines which are also known to express Sp proteins that bind GC-rich motifs [12]. The results obtained in AsPC-1 cells showed that there was a significant 45% decrease in activity after deletion of the −95 to −78 region of the promoter, and further deletion of the overlapping GC-rich/AP-2 motifs (−77 to −61) did not significantly result in further decreased luciferase activity (data not shown; see Supplemental materials 1 and 2). Thus, in contrast to Panc-1 cells, the AP-2/NFκB sites (−95 to −78) contributed to basal activity of the VEGFR2 promoter constructs in AsPC-1 cells; however, analysis of the proximal −60 to −37 region of the promoter showed that both proximal GC-rich sites were the major *cis*-elements required for basal activity in both AsPC-1 and Panc-1 (Fig. 1) cells. The role of the proximal GC-rich motifs in modulating basal activity of VEGFR2 constructs in MiaPaCa-2 cells which express lower levels of the VEGFR2 mRNA transcript (Table 1) was also investigated. The results of transfection studies with the constructs shown in Fig. 1 indicate that both the −58 and −44 GC-rich motifs were required for maximal activity (data not shown). These results (Fig. 1; Supplemental materials 1 and 2) indicate that the proximal GC-rich sites at −58 and −44 were critical *cis*-elements for constitutive expression of VEGFR2 in pancreatic cancer cells.

Table 1
Relative VEGFR2 expression in pancreatic cancer cells[a]

Cell line	VEGFR2 mRNA
Panc-1	426
AsPC-1	419
Panc-28	5.2
HPAFII	4.9
BxPC-3	2.8
MiaPaCa-2	1

[a] VEGFR2 mRNA levels in pancreatic cancer cells were determined by real-time PCR as described in the Materials and methods. VEGFR2 mRNA levels in each cell line were normalized to TBP mRNA.

Role of Sp proteins in regulating VEGFR2 expression in pancreatic cancer cells

Results in Fig. 2A summarize the Western-blot analysis of whole cell lysates from MiaPaCa-2, Panc-1, and AsPC-1 cells, and show that Sp1, Sp3, and Sp4 were expressed in all three cell lines. The role of Sp proteins in mediating regulation of VEGFR2 expression in pancreatic cancer cells was investigated by RNA interference in Panc-1, AsPC-1, and MiaPaCa-2 cells using small inhibitory RNAs (siRNAs) for Sp1 (iSp1), Sp3 (iSp3), and Sp4 (iSp4). Initial studies on the effectiveness of these siRNAs were carried out in Panc-1 cells transfected with different amounts of iSp1 (Fig. 2B). The results showed that 20 nM iSp1

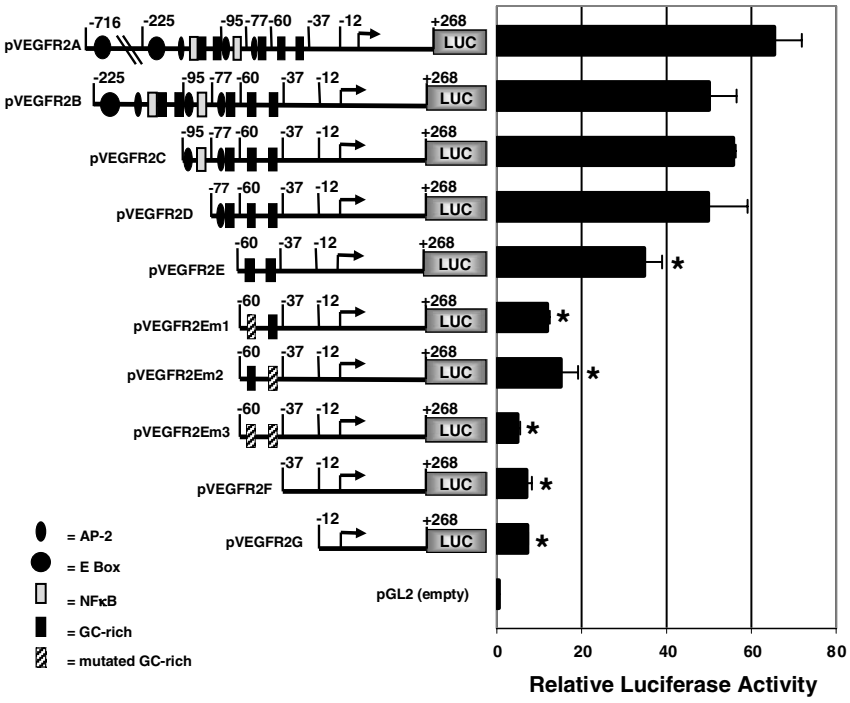


Fig. 1. Deletion and mutation analysis of the VEGFR2 gene promoter in Panc-1 cells. Panc-1 cells were transiently transfected with 500 ng of each pVEGFR2 reporter construct (or pGL2 empty vector), and luciferase activity was determined as described in the Materials and methods. Significantly ($p < 0.05$) decreased activity compared to that observed for pVEGFR2A is indicated by an asterisk. Results are presented as means ± SE for at least three determinations for each treatment group. Results obtained using these same constructs in AsPC-1 and MiaPaCa-2 cells are included in the Supplemental material.

decreased Sp1 protein expression by 45–58% based on Western-blot analysis of whole cell lysates. Since transfection efficiencies vary from 60% to 95% in this cell line, the results represent a relatively high percentage of Sp1 protein knockdown in the transfected cells. In this study, expression of Sp3 and Sp4 proteins was unaffected by iSp1 (data not shown), and this has previously been observed with this same siRNA oligonucleotide in Panc-1 cells [12]. Using a comparable approach, 20 nM iSp3, 20 nM iSp4, and iLamin (control) were also transfected into Panc-1 cells and protein levels relative to those in iLamin transfected cells were determined by Western-blot analysis (Fig. 2C). Both iSp3 and iSp4 were highly effective in decreasing expression of Sp3 and Sp4 proteins, respectively. Protein expression was decreased using 5–20 nM of the siRNAs, and higher levels of siRNAs appeared to be less effective (data not shown). A similar approach was used for MiaPaCa-2 (Fig. 2D) and AsPC-1 cells (Fig. 2E), and the results show the iSp1, iSp3, and iSp4 specifically knockdown Sp1, Sp3, and Sp4 proteins, respectively, as determined by Western-blot analysis of whole cell lysates.

The relative contributions of Sp1, Sp3, and Sp4 proteins in regulating VEGFR2 expression were investigated in pancreatic cancer cells cotransfected with the pVEGFR2A or pVEGFR2E constructs and iSp1, iSp3, or iSp4. Panc-1 cells were cotransfected with pVEGFR2A (Fig. 3A) and pVEGFR2E (Fig. 3B) and iLamin (non-specific control), iGL2 (positive control), iSp1, iSp3, and iSp4. The results show that all three siRNAs for Sp proteins decreased luciferase activity in Panc-1 cells transfected with either construct. Transfection with iGL2 decreased luciferase activity by >90–95% and served as a control showing the effectiveness of RNA interference in the transfected cells. The effects of iSp1, iSp3, and iSp4 were also investigated in AsPC-1 and MiaPaCa-2 cells transfected with pVEGFR2A and pVEGFR2E (Fig. 3C–F). The results showed that all three siRNAs decreased activity in AsPC-1 (Fig. 3C and D) and MiaPaCa-2 (Fig. 3E and F) cells transfected with pVEGFR2A or pVEGFR2E, and confirm a role for Sp1, Sp3, and Sp4 proteins in regulating VEGFR2 expression in pancreatic cancer cells. The results are similar to those observed for Sp-dependent regulation of VEGF in pancreatic cancer cells [12], and suggest an important role for Sp1, Sp3, and Sp4 proteins in mediating expression of two critical angiogenic factors in pancreatic cancer cells.

The effects of Sp proteins on VEGFR2 mRNA expression were also determined in Panc-1 and AsPC-1 cells transfected with a combination of siRNAs for Sp1, Sp3, and Sp4 (Fig. 4A and B). These cells were used in this study because of their relatively high expression of VEGFR2 mRNA (Table 1). Real-time PCR analysis of mRNA from both cell lines show that knockdown of Sp1, Sp3, and Sp4 resulted in a significant decrease in VEGFR2 mRNA expression in both cell lines. These results complement the VEGFR2 promoter studies and confirm that VEGFR2 expression in pancreatic cancer cells is regulated by Sp1,

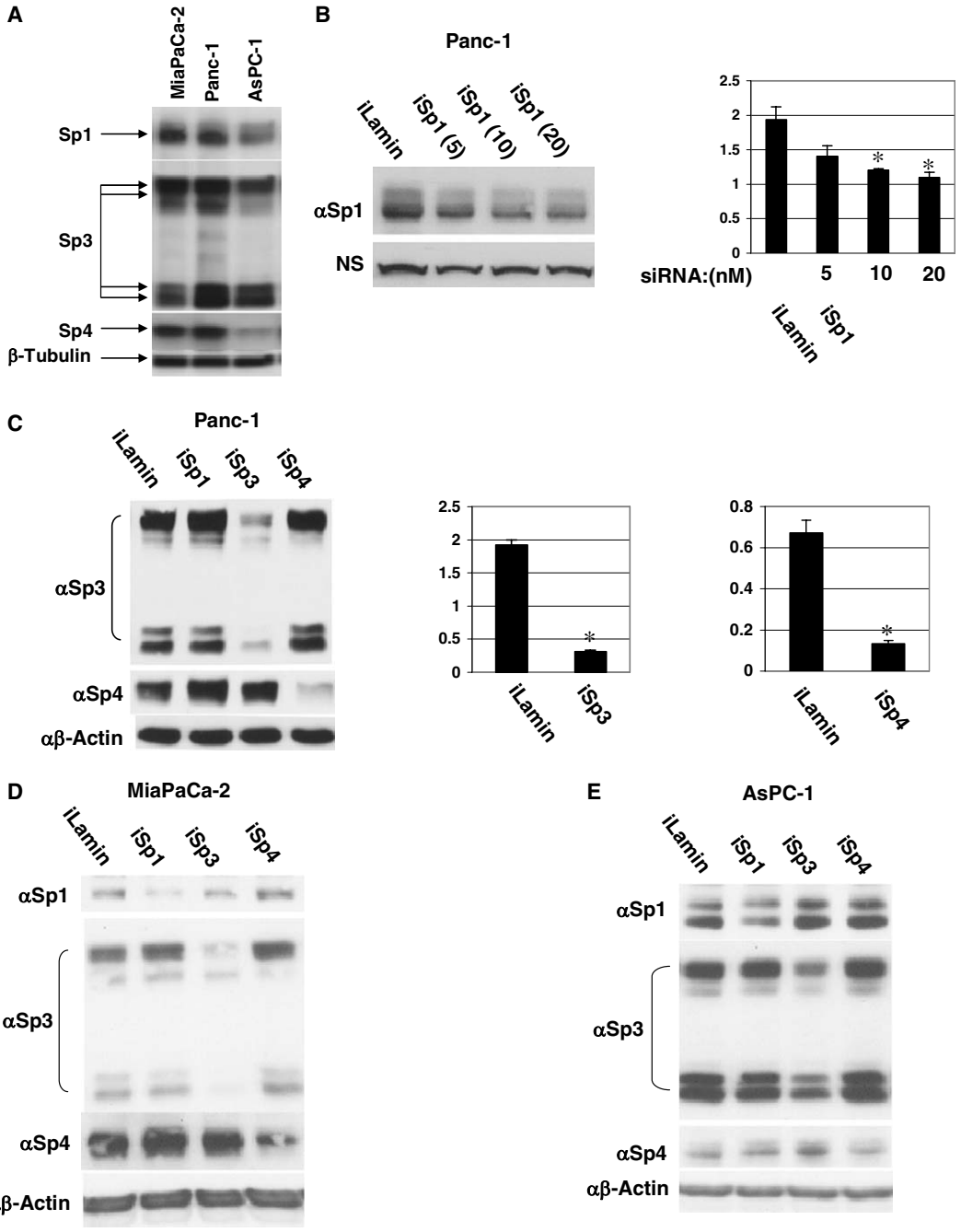


Fig. 2. Sp protein expression and Sp protein knockdown in pancreatic cancer cells by RNA interference. (A) Sp protein expression. Whole cell lysates from Panc-1, AsPC-1, and MiaPaCa-2 cells were analyzed for Sp1, Sp3, and Sp4 by Western-blot analysis as described in the Materials and methods. (B) Effects of iSp1 in Panc-1 cells. Different amounts of iSp1 were transfected in Panc-1 cells, and protein levels were determined by Western-blot analysis as described in the Materials and methods. (C) Effects of iSp3 and iSp4 in Panc-1 cells. Panc-1 cells were transfected with 20 nM iSp3 or iSp4, and protein levels were determined by Western-blot analysis as described in the Materials and methods. Knockdown of Sp proteins in MiaPaCa-2 (20 nM) (D) and AsPC-1 cells (5 nM) (E) by RNA interference. Cells were transfected with iLamin, iSp1, iSp3, or iSp4, and whole cell lysates were analyzed by Western-blot as described in the Materials and methods. Protein expression was quantitated relative to levels in cells treated with iLamin (control), and results are expressed as means ± SE for at least three determinations for each treatment group. A significant ($p < 0.05$) decrease in protein expression level is indicated by an asterisk.

Sp3, and Sp4. The role of Sp proteins in VEGFR2 expression was also investigated in Panc-1 cells by immunofluorescent staining (Fig. 4C). Cytoplasmic green staining for VEGFR2 was observed in cells transfected with iScr (non-specific) (panel a), and intensity of this staining was decreased after transfection of iSp1 (panel b) or Sp4 (panel c). Nuclei are stained blue with DAPI. These data confirm expression of VEGFR2 protein in this cell line and the role of Sp proteins in mediating VEGFR2 expression.

The direct binding of Sp proteins to the proximal region of the VEGFR2 promoter was initially investigated in

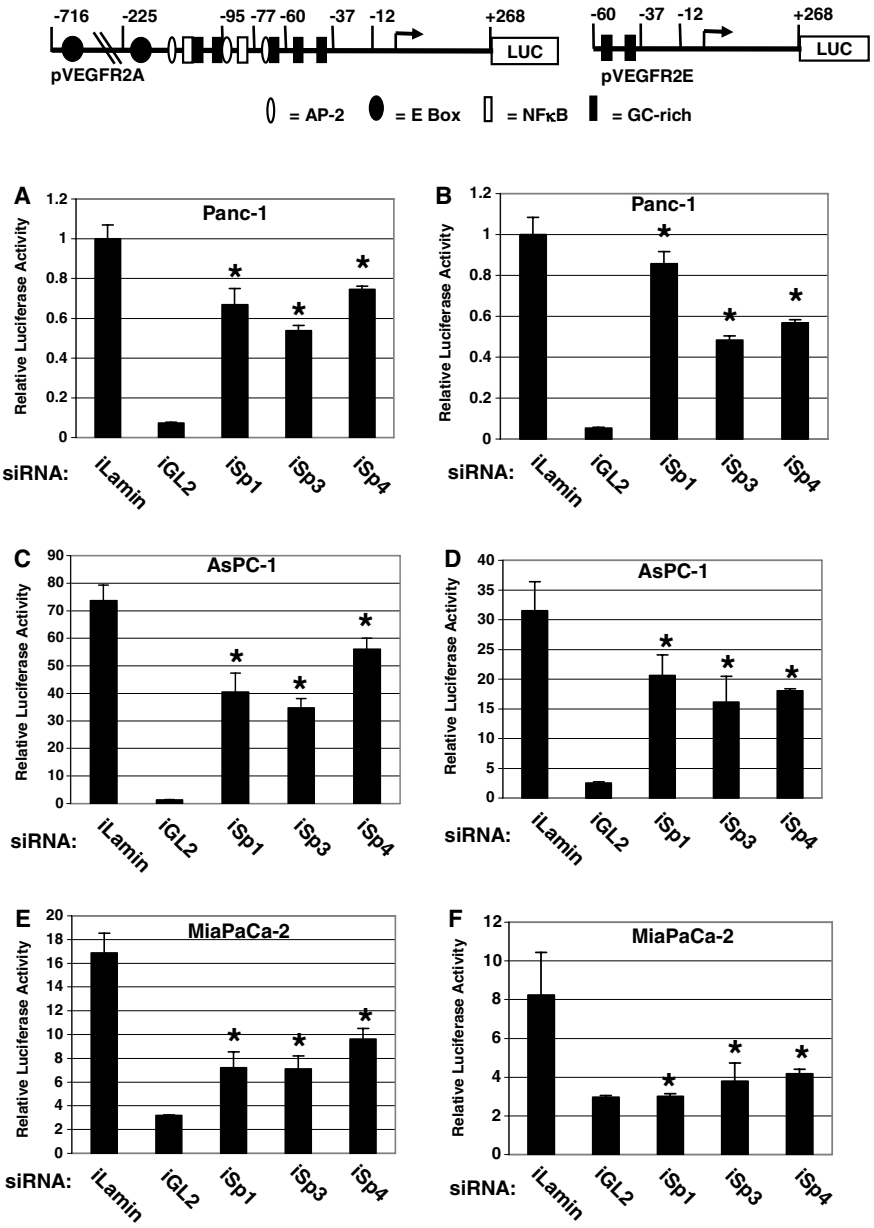


Fig. 3. Effects of Sp proteins on regulation of VEGFR2. pVEGFR2A or pVEGFR2E constructs were transfected in Panc-1 (A,B), AsPC-1 (C,D), or MiaPaCa-2 (E,F) cells, cotransfected with iLamin, iGL2, iSp1, iSp3, or iSp4, and luciferase activity was determined as described in the Materials and methods. Results are expressed as means ± SE for three replicate determinations for each treatment group, and significant ($p < 0.05$) inhibition of luciferase activity is indicated by an asterisk. iLamin serves as a non-specific control plasmid, and iGL2 is a positive control siRNA that targets the luciferase mRNA as described [38].

EMSA using an oligonucleotide (VEGFR2 ^{32}P) derived from the −64 to −35 region of the VEGFR2 promoter (Fig. 5A). Extracts from Panc-1, MiaPaCa-2, and AsPC-1 cells (lanes 2–4) gave a pattern of protein-DNA complexes in which the least mobile band contained Sp1, Sp3, and Sp4 as previously reported [12], and the more mobile band contained Sp3 protein. These assignments were confirmed in supershift experiments with antibodies for Sp1 (lane 5), Sp3 (lane 6), and Sp4 (lane 7). Non-specific IgG (lane 8) did not affect the pattern of retarded bands and, in the absence of cell extracts [lane 1, free probe (FP)], only the radiolabeled oligonucleotide probe was observed. Interactions of Sp proteins with the GC-rich region of the VEGFR2 promoter were further investigated in a ChIP assay using primers that targeted the proximal region of the VEGFR2 promoter (Fig. 5B and C). The results show that Sp1, Sp3, and Sp4 bound to the VEGFR2 promoter in MiaPaCa-2, AsPC-1, and Panc-1 cells, and we also show that these Sp proteins bound to the corresponding GC-rich region of the VEGF promoter (Fig. 5B and C). As a control for the ChIP assay, we show that TFIIB bound to the proximal region of the GAPDH promoter but not exon-1 of CNAP1 as previously described [19]. The ChIP assay confirmed that Sp1, Sp3, and Sp4 bound to the

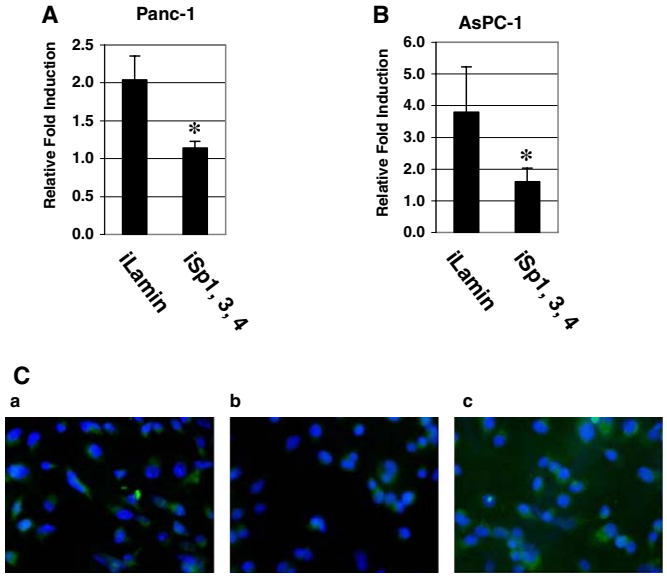


Fig. 4. Decreased VEGFR2 expression by RNA interference. Downregulation of VEGFR2 mRNA by siRNA for Sp-proteins in Panc-1 (A) and AsPC-1 (B) human pancreatic cancer cells. Panc-1 cells were transfected with siRNA for Sp1 (10 nM), Sp3 (20 nM), and Sp4 (20 nM) or iLamin (50 nM) (control), and AsPC-1 cells were transfected with siRNA for Sp1 (5 nM), Sp3 (5 nM), and Sp4 (5 nM) or iLamin (15 nM) (control). After 48 h, RNA was isolated using the RNeasy Protect Mini Kit (Qiagen), and samples were analyzed by Real-Time PCR as described in the Materials and methods (A,B). Results are presented as means ± SE for at least three determinations for each treatment group. Significant ($p < 0.05$) inhibition of VEGFR2 mRNA levels (relative to iLamin) is indicated by an asterisk (*). Immunofluorescence detection of VEGFR2 in Panc-1 cells transfected with siRNA for Sp proteins (C). Panc-1 cells were transiently transfected with iSc (a), iSp1 (b), and iSp4 (c), and stained for VEGFR2 (green) and nucleus with 4′,6-diamidino-2-phenylindole-stained (blue) as described in the Materials and methods. Photographs were taken at the magnification of 400×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

VEGFR2 promoter, and this is consistent with the role of these transcription factors in mediating the expression of VEGFR2 in pancreatic cancer cell lines.

Discussion

Angiogenesis is a key process in both physiologic and carcinogenic pathways where angiogenic factors play a critical role in tumor cell growth and metastasis [20]. VEGF proteins and related placental growth factors regulate angiogenesis through interactions with the transmembrane receptors VEGFR1, VEGFR2, VEGFR3, neurophilins, and a soluble form of VEGFR1 which does not express the transmembrane or tyrosine kinase domains [21,22].

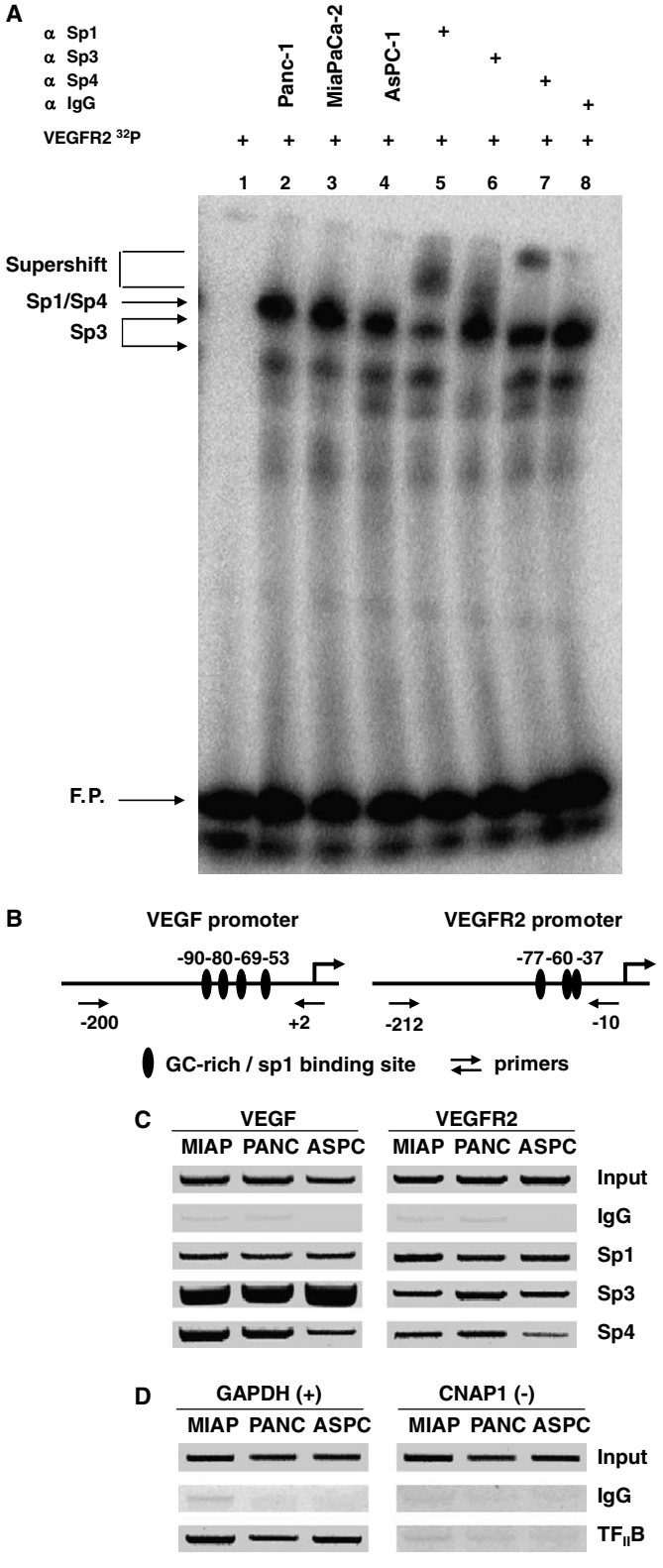


Fig. 5. Sp protein binding to the VEGFR2 promoter. (A) Electrophoretic mobility shift assay. Nuclear extracts from Panc-1, AsPC-1, and MiaPaCa-2 cells were incubated with radiolabeled VEGFR2 ^{32}P alone or in the presence of unlabeled oligonucleotides and/or antibodies, and DNA–protein complexes were separated by EMSA as described in the Materials and methods. Arrows indicate various retarded and supershifted complexes. (B) Summary of primers (→ ←) and targeted regions of the VEGF and VEGFR2 promoters used in ChIP assays. (C) Analysis of protein interactions with the VEGF and VEGFR2 promoter by ChIP. MiaPaCa-2, Panc-1, and AsPC-1 cells were harvested and analyzed in a ChIP assay as described in the Materials and methods. (D) Binding of TFIIB to the GAPDH promoter. The ChIP assay was also used to examine binding of TFIIB to the GAPDH promoter (positive control) and to exon 1 of CNAP1 (negative control) as described in the Materials and methods.

VEGF is overexpressed in multiple tumors and cancer cells and, for some cancers, VEGF is a negative prognostic factor [23,24]. VEGFR1 and VEGFR2 are also expressed in other tumors and cancer cell lines; however, their prognostic significance and function have not been extensively investigated. VEGFR2 was expressed in over 64% of a set of breast tumors and expression was highly correlated with proliferation indices [25]. Also, in another mammary tumor study, there was a correlation between VEGF and VEGFR2 expression [26]. VEGFR2 expression increased while VEGFR1 decreased during prostate tumor progression [27].

The molecular mechanism of VEGFR2 expression has primarily been investigated in endothelial cells using various constructs containing VEGFR2 promoter inserts. The VEGFR2 promoter is highly complex and contains multiple *cis*-elements including GATA, E-box, GC-rich, NFκB, and AP-2 motifs [28–30]. However, deletion analysis and DNA footprinting studies in endothelial cells indicate that interactions of Sp1 protein with the proximal GC-rich (−110 to −25) VEGFR2 promoter are important for basal and sheer-stress-induction of transactivation in cells transfected with pVEGFR2 constructs [22,28–30]. These results are similar to those observed for VEGF expression in breast, colon, and pancreatic cancer cells where proximal GC-rich sites in the VEGF promoter are required for basal and hormone-induced transactivation [10–12,15]. Real-time PCR showed that VEGFR2 mRNA was expressed in a series of pancreatic cancer cell lines (Table 1), and we also detected VEGFR2 by immunostaining (Fig. 4C). In addition, we investigated the molecular biology of VEGFR2 regulation in three cell lines that are readily transfected and express high (Panc-1, AsPC-1) and low (MiaPaCa-2) VEGFR2 mRNA levels (Table 1). In cells transfected with a series of deletion constructs (Fig. 1 and Supplemental Materials 1 and 2), basal luciferase activity was primarily dependent on two GC-rich sites at −58 and −44 in Panc-1 and MiaPaCa-2 cells, and in AsPC-1 cells there was also a significant contribution from the −95 to −78 region of the promoter which also contained AP-2/NFκB sites. These results illustrate that the proximal GC-rich sites at −58 and −44 in the VEGFR2 promoter are important for transactivation, and this parallels results obtained for regulation of VEGF in Panc-1 cells where proximal GC-rich sites are also critical for expression [12].

Several studies show that Sp1 is overexpressed in tumors, and this transcription factor regulates expression of *VEGF* and other genes associated with cancer cell proliferation [14,16,17]. Our results show that Sp1 is expressed in Panc-1, AsPC-1, and MiaPaCa-2 cells; however, these cells also contain Sp3 and Sp4 proteins (Fig. 2A). Sp1 and Sp3 are often coexpressed in cancer cell lines and cooperatively activate some GC-rich promoters, although Sp3 also inhibits other Sp1-dependent genes. For example, Sp3 attenuates Sp1-mediated activation of VEGFR2 in endothelial cells [29]. Electrophoretic mobility shift and ChIP assays (Fig. 5) show that Sp1, Sp3, and Sp4 are expressed in these pancreatic cancer cell lines and bind to proximal GC-rich motifs in the VEGFR2 promoter. RNA interference studies with inhibitory RNAs for Sp1, Sp3, and Sp4 demonstrate that all three proteins not only regulate transactivation in cells transfected with pVEGFR2 constructs (Fig. 3) but are also important for VEGFR2 mRNA (Fig. 4A and B) and protein (Fig. 4C) expression. These results demonstrate that, like VEGF [12], VEGFR2 expression is regulated by multiple Sp transcription factors in pancreatic cancer cell lines.

Chemotherapies targeting the tyrosine kinase domains of VEGFR2 are currently being developed for inhibiting tumor angiogenesis and metastasis [31,32]. Results of this study also suggest that drugs such as mithramycin that target GC-rich promoters or cyclooxygenase inhibitors that induce Sp protein degradation will also exhibit antiangiogenic activity in pancreatic and other cancer cells through their effects on VEGF/VEGFR2 expression [13,33,34]. Current studies in this laboratory are investigating chemotherapies that specifically target Sp transcription factors alone or in combination with other agents such as gemcitabine or tyrosine kinase inhibitors as novel drug combinations for treatment of pancreatic cancer and for inhibition of angiogenesis through downregulation of Sp-dependent genes such as VEGFR2.

Acknowledgments

This research was supported by the National Institutes of Health (CA104116 and ES09106) and the M.D. Anderson Pancreatic Cancer Spore (P20-CA10193).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.04.111.

References

[1] American Cancer Society, Cancer Facts and Figures—2004, American Cancer Society, Atlanta, GA, 2004.
[2] A. Jemal, L.X. Clegg, E. Ward, L.A. Ries, X. Wu, P.M. Jamison, P.A. Wingo, H.L. Howe, R.N. Anderson, B.K. Edwards, Annual report to the nation on the status of cancer, 1975–2001, with a special feature regarding survival, Cancer 101 (2004) 3–27.
[3] D. Li, K. Xie, R. Wolff, J.L. Abbruzzese, Pancreatic cancer, Lancet 363 (2004) 1049–1057.
[4] R.H. Hruban, G.M. Petersen, P.K. Ha, S.E. Kern, Genetics of pancreatic cancer. From genes to families, Surg. Oncol. Clin. N. Am. 7 (1998) 1–23.
[5] P. Ghadirian, H.T. Lynch, D. Krewski, Epidemiology of pancreatic cancer: an overview, Cancer Detect. Prev. 27 (2003) 87–93.
[6] D. Li, Molecular epidemiology of pancreatic cancer, Cancer J. 7 (2001) 259–265.
[7] E.B. Gold, S.B. Goldin, Epidemiology of and risk factors for pancreatic cancer, Surg. Oncol. Clin. N. Am. 7 (1998) 67–91.
[8] S. McKenna, M. Eatock, The medical management of pancreatic cancer: a review, Oncologist 8 (2003) 149–160.
[9] D.G. Haller, Chemotherapy for advanced pancreatic cancer, Int. J. Radiat. Oncol. Biol. Phys. 56 (2003) 16–23.

[10] M. Stoner, M. Wormke, B. Saville, I. Samudio, C. Qin, M. Abdelrahim, S. Safe, Estrogen regulation of vascular endothelial growth factor gene expression in ZR-75 breast cancer cells through interaction of estrogen receptor α and Sp proteins, Oncogene 23 (2004) 1052–1063.
[11] M. Stoner, F. Wang, M. Wormke, T. Nguyen, I. Samudio, C. Vyhlidal, D. Marme, G. Finkenzeller, S. Safe, Inhibition of vascular endothelial growth factor expression in HEC1A endometrial cancer cells through interactions of estrogen receptor α and Sp3 proteins, J. Biol. Chem. 275 (2000) 22769–22779.
[12] M. Abdelrahim, R. Smith III, R. Burghardt, S. Safe, Role of Sp proteins in regulation of vascular endothelial growth factor expression and proliferation of pancreatic cancer cells, Cancer Res. 64 (2004) 6740–6749.
[13] M. Abdelrahim, S. Safe, Cyclooxygenase-2 inhibitors decrease vascular endothelial growth factor expression in colon cancer cells by enhanced degradation of Sp1 and Sp4 proteins, Mol. Pharmacol. 68 (2005) 317–329.
[14] S. Safe, M. Abdelrahim, Sp transcription factor family and its role in cancer, Eur. J. Cancer 41 (2005) 2438–2448.
[15] Q. Shi, X. Le, J.L. Abbruzzese, Z. Peng, C.N. Qian, H. Tang, Q. Xiong, B. Wang, X.C. Li, K. Xie, Constitutive Sp1 activity is essential for differential constitutive expression of vascular endothelial growth factor in human pancreatic adenocarcinoma, Cancer Res. 61 (2001) 4143–4154.
[16] M. Ryuto, M. Ono, H. Izumi, S. Yoshida, H.A. Weich, K. Kohno, M. Kuwano, Induction of vascular endothelial growth factor by tumor necrosis factor alpha in human glioma cells. Possible roles of SP-1, J. Biol. Chem. 271 (1996) 28220–28228.
[17] J.C. Yao, L. Wang, D. Wei, W. Gong, M. Hassan, T.T. Wu, P. Mansfield, J. Ajani, K. Xie, Association between expression of transcription factor Sp1 and increased vascular endothelial growth factor expression, advanced stage, and poor survival in patients with resected gastric cancer, Clin. Cancer Res. 10 (2004) 4109–4117.
[18] J.S. Wey, F. Fan, M.J. Gray, T.W. Bauer, M.F. McCarty, R. Somcio, W. Liu, D.B. Evans, Y. Wu, D.J. Hicklin, L.M. Ellis, Vascular endothelial growth factor receptor-1 promotes migration and invasion in pancreatic carcinoma cell lines, Cancer 104 (2005) 427–438.
[19] M. Abdelrahim, S. Liu, S. Safe, Induction of endoplasmic reticulum-induced stress genes in Panc-1 pancreatic cancer cells is dependent on Sp proteins, J. Biol. Chem. 280 (2005) 16508–16513.
[20] D. Hanahan, J. Folkman, Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis, Cell 86 (1996) 353–364.
[21] M. Shibuya, Structure and function of VEGF/VEGF-receptor system involved in angiogenesis, Cell Struct. Funct. 26 (2001) 25–35.
[22] N. Ferrara, H.P. Gerber, J. LeCouter, The biology of VEGF and its receptors, Nat. Med. 9 (2003) 669–676.
[23] G. Gasparini, M. Toi, M. Gion, P. Verderio, R. Dittadi, M. Hanatani, I. Matsubara, O. Vinante, E. Bonoldi, P. Boracchi, C. Gatti, H. Suzuki, T. Tominaga, Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma, J. Natl. Cancer Inst. 89 (1997) 139–147.
[24] M. Toi, K. Inada, H. Suzuki, T. Tominaga, Tumor angiogenesis in breast cancer: its importance as a prognostic indicator and the association with vascular endothelial growth factor expression, Breast Cancer Res. Treat. 36 (1995) 193–204.
[25] L. Nakopoulou, K. Stefanaki, E. Panayotopoulou, I. Giannopoulou, P. Athanassiadou, H. Gakiopoulou-Givalou, A. Louvrou, Expression of the vascular endothelial growth factor receptor-2/Flk-1 in breast carcinomas: correlation with proliferation, Hum. Pathol. 33 (2002) 863–870.
[26] L. Ryden, B. Linderholm, N.H. Nielsen, S. Emdin, P.E. Jonsson, G. Landberg, Tumor specific VEGF-A and VEGFR2/KDR protein are co-expressed in breast cancer, Breast Cancer Res. Treat. 82 (2003) 147–154.
[27] W.J. Huss, C.F. Hanrahan, R.J. Barrios, J.W. Simons, N.M. Greenberg, Angiogenesis and prostate cancer: identification of a molecular progression switch, Cancer Res. 61 (2001) 2736–2743.
[28] C. Patterson, M.A. Perrella, C.M. Hsieh, M. Yoshizumi, M.E. Lee, E. Haber, Cloning and functional analysis of the promoter for KDR/flk-1, a receptor for vascular endothelial growth factor, J. Biol. Chem. 270 (1995) 23111–23118.
[29] Y. Hata, E. Duh, K. Zhang, G.S. Robinson, L.P. Aiello, Transcription factors Sp1 and Sp3 alter vascular endothelial growth factor receptor expression through a novel recognition sequence, J. Biol. Chem. 273 (1998) 19294–19303.
[30] C. Patterson, Y. Wu, M.E. Lee, J.D. DeVault, M.S. Runge, E. Haber, Nuclear protein interactions with the human *KDR/flk-1* promoter *in vivo*. Regulation of Sp1 binding is associated with cell type-specific expression, J. Biol. Chem. 272 (1997) 8410–8416.
[31] J.S. Beebe, J.P. Jani, E. Knauth, P. Goodwin, C. Higdon, A.M. Rossi, E. Emerson, M. Finkelstein, E. Floyd, S. Harriman, J. Atherton, S. Hillerman, C. Soderstrom, K. Kou, T. Gant, M.C. Noe, B. Foster, F. Rastinejad, M.A. Marx, T. Schaeffer, P.M. Whalen, W.G. Roberts, Pharmacological characterization of CP-547,632, a novel vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for cancer therapy, Cancer Res. 63 (2003) 7301–7309.
[32] A.D. Laird, J.G. Christensen, G. Li, J. Carver, K. Smith, X. Xin, K.G. Moss, S.G. Louie, D.B. Mendel, J.M. Cherrington, SU6668 inhibits Flk-1/KDR and PDGFRβ in vivo, resulting in rapid apoptosis of tumor vasculature and tumor regression in mice, FASEB J. 16 (2002) 681–690.
[33] G.H. Zhu, M. Lenzi, E.L. Schwartz, The Sp1 transcription factor contributes to the tumor necrosis factor-induced expression of the angiogenic factor thymidine phosphorylase in human colon carcinoma cells, Oncogene 21 (2002) 8477–8485.
[34] D. Wei, L. Wang, Y. He, H.Q. Xiong, J.L. Abbruzzese, K. Xie, Celecoxib inhibits vascular endothelial growth factor expression in and reduces angiogenesis and metastasis of human pancreatic cancer via suppression of Sp1 transcription factor activity, Cancer Res. 64 (2004) 2030–2038.