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# Role of Sp Proteins in Regulation of Vascular Endothelial Growth Factor Expression and Proliferation of Pancreatic Cancer Cells

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## ABSTRACT

**Sp proteins play an important role in angiogenesis and growth of cancer cells, and specificity protein 1 (Sp1) has been linked to vascular endothelial growth factor (VEGF) expression in pancreatic cancer cells. RNA interference was used to investigate the role of Sp family proteins on regulation of VEGF expression and proliferation of Panc-1 pancreatic cancer cells. Using a series of constructs containing VEGF promoter inserts, it was initially shown that Sp1 and Sp3 were required for transactivation, and this was primarily dependent on proximal GC-rich motifs. We also showed that Sp4 was expressed in Panc-1 cells, and RNA interference assays suggested that Sp4 cooperatively interacted with Sp1 and Sp3 to activate VEGF promoter constructs in these cells. However, the relative contributions of Sp proteins to VEGF expression were variable among different pancreatic cancer cell lines. Small inhibitory RNAs for Sp3, but not Sp1 or Sp4, inhibited phosphorylation of retinoblastoma protein, blocked $G_0/G_1 \rightarrow$ S-phase progression, and up-regulated p27 protein/promoter activity of Panc-1 cells; similar results were observed in other pancreatic cancer cells, suggesting that Sp3-dependent growth of pancreatic cancer cells is caused by inhibition of p27 expression.**

## INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a major cause of cancer-related deaths in developed countries, and it is estimated that in 2003, >30,000 new cases will be diagnosed in the United States (1). PDAC is a highly aggressive disease that invariably evades early diagnosis (2–5). The mean survival time for patients with metastatic disease is only 3 to 6 months, and the 1-year survival time for all of the pancreatic cancer cases is ~20 to 30% (1). Several factors are associated with increased risk for pancreatic cancer, including chronic pancreatitis, previous gastric surgery, smoking, diabetes, exposure to certain classes of organic solvents, and radiation (6–18).

Heritable germline mutations in several genes also are associated with increased risks for pancreatic cancer (4, 5, 11, 19–22). For example, Peutz-Jeghers, hereditary pancreatitis, familial atypical multiple melanoma (FAMM), familial breast cancer 2, and hereditary nonpolyposis colorectal cancer syndromes, which are linked to specific heritable gene mutations, markedly increase the risk for pancreatic cancer. However, the gene(s) involved has not been identified. In addition to heritable mutations, several acquired gene mutations have been identified in sporadic pancreatic tumors, and these mutations typically lead to dysregulated growth and deficiencies in DNA repair (22–27). For example, the K-*ras* oncogene is primarily mutated in codon 12 in >90% of pancreatic tumors, and the mutation results in a constitutively active form of *ras*, which can lead to increased cell proliferation. Mutations in the cyclin-dependent kinase inhibitor *p16*, the tumor suppressor gene *p53*, and *SMAD4*, a downstream target of transforming growth factor $\beta$, also exhibit high mutation frequencies in pancreatic tumors (19, 20).

Specificity protein 1 (Sp1) is expressed in pancreatic tumors and in pancreatic cells in culture, and there is evidence suggesting that Sp1 plays an important role in regulation of vascular endothelial growth factor (VEGF) expression in Panc-1 and other pancreatic cancer cells (28). Sp family proteins play a complex role in regulation of cancer cell growth and expression of genes required not only for growth but also for apoptosis and angiogenesis (29–31). Panc-1 cells express high levels of Sp1, and this correlated with high expression of VEGF protein and mRNA levels and has been linked to interactions of Sp1 with GC-rich motifs (−109 to −61) in the proximal region of the *VEGF* gene promoter (28). Studies in other cell lines also have shown the importance of this region of the VEGF promoter, which interacts not only with Sp1 protein but also with early growth response 1 and activator protein 2 (AP-2) proteins (32–37). In this study, we used RNA interference to investigate the role of Sp proteins in VEGF expression and cell cycle progression in Panc-1 pancreatic cancer cells. Our results show for the first time that Sp4 is expressed in pancreatic cancer cells and, along with Sp1 and Sp3, plays an important role in regulating expression of VEGF in Panc-1 cells. However, RNA interference studies in other pancreatic cancer cell lines show variable contributions of Sp proteins to VEGF expression. In contrast, Sp3 was identified as a key regulator of $G_0/G_1 \rightarrow$ S-phase progression and retinoblastoma (Rb) protein phosphorylation in Panc-1 cells, and this was linked to Sp3-dependent suppression of the cyclin-dependent kinase inhibitor *p27*. Similar results were observed in other pancreatic cancer cell lines. These studies show that individual Sp proteins and their cooperative interactions play a pivotal role in regulating growth and VEGF expression in pancreatic cancer cells.

## MATERIALS AND METHODS

**Cell Lines, Chemicals, Biochemical, Constructs, and Oligonucleotides.** Panc-1, Panc-28, AsPC-1, MIA-PaCa-2, HPAFII, HepG2, 22RV1, and MCF7 cells were obtained from the American Type Culture Collection (Manassas, VA). DME/F12 with and without phenol red, 100× antibiotic/antimycotic solution, and propidium iodide (PI) were purchased from Sigma (St. Louis, MO). Fetal bovine serum was purchased from Intergen (Purchase, NY). [$\gamma$-$^{32}$P]ATP (300 Ci/mmol) was obtained from Perkin-Elmer Life Sciences (Wellesley, MA). Poly(dI·dC) and T4 polynucleotide kinase were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Antibodies for Sp1, Sp3, Sp4, Rb, p27, cyclin D1, and cyclin E proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Lysis buffer, luciferase reagent, and RNase were obtained from Promega Corp. (Madison, WI). Consensus GC-rich oligonucleotides and VEGF promoter constructs have been described previously (36, 38, 39). $p27^{Kip1}$ promoter luciferase constructs p27 PF (−3568/−12), p27 no. 2 (−549/−12), and p27 *Sac*II (−311/−12) were provided by Dr. Toshiyuki Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan). Small inhibitory RNA (siRNA) duplexes were prepared by Dharmacon Research (Lafayette, CO) and targeted coding regions of the Sp1 (1811 to 1833), Sp3 (1681 to 1701), Sp4 (1181 to 1201), lamin A/C (608 to 626), and luciferase (GL2; 153 to 171). Previous studies in this laboratory have reported oligonucleotide sequences for Sp1, GL2, and lamin A/C siRNA (36, 38, 39), and the inhibitory RNA (iRNA) duplex for Sp3 and Sp4 is provided here: Sp3,

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5′-GCGGCAGGUGGAGCCUUCACUTT and TCGCCGUCCACCUCGGAAGUGA-5′; and Sp4, 5′-GCAGUGACACAUUAGUGAGCTT and TTCGUCACUGUGUAAUCACUCG-5′.

**Transfection of Pancreatic Cells and Preparation of Nuclear Extracts.** Cells were cultured in six-well plates in 2 mL of DME/F12 medium supplemented with 5% fetal bovine serum. After 16 to 20 hours when cells were 50 to 60% confluent, iRNA duplexes and/or reporter gene constructs were transfected using Oligofectamine reagent (Invitrogen, Carlsbad, CA). The effects of iSp1, iSp3, and iSp4 on transactivation were investigated in Panc-1 cells cotransfected with (500 ng) different VEGF and $p27^{Kip}$ constructs. Briefly, iRNA duplex was transfected in each well to give a final concentration of 50 nmol/L. Cells were harvested after 48 to 56 hours, and luciferase activity of lysates (relative to $\beta$-galactosidase activity) was determined. Relatively high transfection efficiencies were observed for Panc-1, Panc-28, MIA-PaCa-2, and AsPC-1 cells, whereas inconsistent results were obtained for HPAF-II cells because of cell clumping. For electrophoretic mobility shift assay (EMSA) assay, nuclear extracts from Panc-1 cells were isolated as described previously, and aliquots were stored at −80°C until used (36, 38, 39).

**Western Immunoblot Analysis.** Cells were washed once with PBS and collected by scraping in 200 $\mu$L of lysis buffer [50 mmol/L HEPES, 0.5 mol/L sodium chloride, 1.5 mmol/L magnesium chloride, 1 mmol/L EGTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 $\mu$L/mL of protease inhibitor mixture (Sigma)]. Brain tissue was obtained from B6C3F1C mice, washed with cold PBS, and homogenized in 1× lysis buffer (Promega). The lysates from cells and brain tissues were incubated on ice for 1 hour with intermittent vortexing, followed by centrifugation at 40,000 × *g* for 10 minutes at 4°C. Equal amounts of protein from each treatment group were diluted with loading buffer, boiled, and loaded onto 10 and 12.5% SDS-polyacrylamide gel. TNT quick-coupled transcription/translation system (Promega) was used according to the manufacturer's protocol to provide *in vitro* translated Sp4 protein using Sp4 expression plasmid. One microgram of pSp4 expression plasmid was added to an aliquot of the TNT Quick Master Mix and incubated in a 50-$\mu$L reaction volume for 60 minutes at 30°C; 25 $\mu$L of the reaction were used to load the gel and served as a standard marker for this protein in Western blot analysis. Samples were electrophoresed, and proteins were detected by incubation with polyclonal primary antibodies Sp1 (PEP2), Sp3 (D-20), Sp4 (V-20), lamin A/C (N-18), cyclin D1 (M-20), cyclin E (C-19), Rb (C-15), and p27 (C-19), followed by blotting with appropriate horseradish peroxidase-conjugated secondary antibody as described previously (36, 38, 39). After autoradiography, band intensities were determined by a scanning laser densitometer (Sharp Electronics Corporation, Mahwah, NJ) using Zero-D Scanalytics software (Scanalytics Corporation, Fairfax, VA).

**Fluorescence-Activated Cell Sorting Analysis.** Cells were transfected with iRNAs for Sp1, Sp3, Sp4, or GL2, and after 48 to 56 hours, cells then were trypsinized, and ~$2 \times 10^6$ cells were centrifuged and resuspended after removal of trypsin in 1 mL of staining solution containing 50 $\mu$g/mL PI, 4 mmol/L sodium citrate, 30 units/mL RNase, and 0.1% Triton X-100 (pH 7.8). Cells were incubated at 37°C for 10 minutes, and then before fluorescence-activated cell sorting (FACS) analysis, sodium chloride was added to give a final concentration of 0.15 mol/L. Cells were analyzed on an FACS Calibur flow cytometer (BD PharMingen, San Diego, CA) using CellQuest (BD PharMingen) acquisition software. PI fluorescence was collected through a 585/42-nm bandpass filter, and list mode data were acquired on a minimum of 12,000 single cells defined by a dot plot of PI width *versus* PI area. Data analysis was performed in ModFit LT (Verity Software House, Topsham, ME) using PI width *versus* PI area to exclude cell aggregates. FlowJo (Treestar, Inc., Palo Alto, CA) was used to generate plots shown in Fig. 4.

**Electrophoretic Mobility Shift Assay.** Consensus Sp1 and GT-box oligonucleotides were synthesized and annealed, and 5-pmol aliquots were 5′-end–labeled using T4 kinase and [$\gamma$-$^{32}$P]ATP. A 30-$\mu$L EMSA reaction mixture contained ~100 mmol/L KCl, 3 $\mu$g of crude nuclear protein, 1 $\mu$g poly(dI·dC), with or without unlabeled competitor oligonucleotide, and 10 fmol of radiolabeled probe. After incubation for 20 minutes on ice, antibodies against Sp1, Sp3, and/or Sp4 proteins were added and incubated another 20 minutes on ice. Protein-DNA complexes were resolved by 5% polyacrylamide gel electrophoresis as described previously (36, 38, 39). Specific DNA-protein and antibody-supershifted complexes were observed as retarded bands in the gel. GC- and GT-box sequences and the p27 GC-rich oligonucleotide sequence used in gel shift analysis are 5′-AGCTTATTCGATCGGGGCGGGGCGAGCG-3′ (GC-box oligonucleotide); 5′-AGCTTCCGTTGGGGTGTGGCTTCACG-3′ (GT-box oligonucleotide); and GC-1 GC-2, 5′-AGCCTCGGCG**GGGCGG**CT**CCCGCC**GCCGCAA-3′ (−555/−525 p27 promoter).

**Immunocytochemistry.** HepG2 and Panc-1 cells were seeded in Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) at 100,000 cells/well in DME/F12 medium supplemented with 5% fetal bovine serum. Cells then were transfected with iRNAs, and after 48 hours, the media chamber was detached, and the remaining glass slides were washed in Dulbecco's PBS. The immunostaining for Sp4, p27, and pRb was determined essentially as described previously for Sp1 (38). Briefly, the slides were fixed with cold methanol and washed with 0.3% PBS/Tween before blocking with 5% rabbit or goat serum in antibody dilution buffer [stock solution: 100 mL of PBS/Tween, 1 g of BSA, and 45 mL of glycerol (pH 8.0)] for 1 hour at 20°C. After removal of the blocking solution, rabbit Sp4-V20, p27-C19, or goat pRb-Ser249/Thr252 polyclonal antibodies were added in antibody dilution buffer (1:200) and incubated for 12 hours at 4°C. Slides were washed for 10 minutes with 0.3% Tween in 0.02 mol/L PBS (3×) and incubated with FITC antirabbit or antigoat secondary antibodies (1:1000 dilution) for 2 hours at 20°C. Slides then were washed for 10 minutes in 0.3% PBS-Tween (4×). Slides were mounted in ProLonged antifading medium (Molecular Probes, Inc., Eugene, OR). For VEGF staining, rabbit polyclonal (A-20) and FITC antirabbit antibodies were used, and mounting media with 4′,6-diamidino-2-phenylindole were used for nuclear counterstaining. Fluorescence imaging was performed using Zeiss Axiophoto 2 (Carl Zeiss, Inc., Oberkochen, Germany), and Adobe Photoshop 5.5 (San Jose, CA) was used to capture the images.

**Semiquantitative Reverse Transcription-PCR Analysis.** Panc-1 cells were treated with iLMN, iSp1, iSp3, and iSp4 for 48 to 52 hours before total RNA collection. Total RNA was obtained with RNAzol B (Tel-Test, Friendswood, TX) according to the manufacturer's protocol. RNA concentration was measured by UV 260:280 nm absorption ratio, and 200 ng/$\mu$L RNA were used in each reaction for reverse transcription-PCR. RNA was reverse transcribed at 42°C for 25 minutes using oligo d(T) primer (Promega) and subsequently PCR amplified of reverse transcription product using 2 mmol/L $MgCl_2$, 1 $\mu$mol/L of each gene-specific primer, 1 mmol/L dNTPs, and 2.5 units AmpliTaq DNA polymerase (Promega). The gene products were amplified using 22 to 25 cycles (95°C, 30 s; 56°C, 30 s; 72°C, 30 s). The sequence of the oligonucleotide primers used in this study was as follows: p27 forward, 5′-CCA CGAAGAGTTAACCCGGG-3′; p27 reverse, 5′-GTGTGCTCCACAGAACCGGC-3′; GAPDH forward, 5′-AATCCCATCACCATCTTCCA-3′; and GAPDH reverse, 5′-GTCATCATATTTGGCAGGTT-3′.

Following amplification in a PCR express thermal cycler (Hybaid US, Franklin, MA), 20 $\mu$L of each sample were loaded on a 2% agarose gel containing ethidium bromide. Electrophoresis was performed at 80 V in 1×TAE buffer for 1 hour, and the gel was photographed by UV transillumination using Polaroid film (Waltham, MA). GAPDH and p27 band intensity values were obtained by scanning the Polaroid on a Sharp JX-330 scanner (Sharp Electronics, Mahwah, NJ); background signal was subtracted; and densitometric analysis was performed on the inverted image using Zero-D software (Scanalytics). Results were expressed as p27 band intensity values normalized to GAPDH values and then by averaging three separate determinations for each treatment group.

**Statistical Analysis.** Statistical significance was determined by ANOVA and Scheffe's test, and the levels of probability are noted. The results are expressed as mean ± SD for at least three separate (replicate) experiments for each treatment.

## RESULTS

**Regulation of VEGF Expression by Sp1 and Sp3.** Previous studies showed that for several pancreatic cancer cell lines, there was a correlation between levels of VEGF protein (secretion)/mRNA, and Sp1 protein expression (28). Cells that expressed high levels of Sp1 also exhibited high transactivation in pancreatic cancer cells transfected with constructs containing VEGF promoter inserts, and this activity was associated with proximal GC-rich sites that bound Sp1 and Sp3 proteins (28). RNA interference assays have shown that Sp1 and Sp3 proteins cooperatively enhanced basal and hormone-induced VEGF expression in ZR-75 breast cancer cells, and transactivation

also depended on proximal GC-rich sites (36). Therefore, we further investigated the role of Sp1 and Sp3 proteins in mediating transcriptional activation of VEGF constructs in Panc-1 cells, which highly express both proteins (28). Sp1 and Sp3 expression was decreased in Panc-1 cells transfected with siRNAs for Sp1 (iSp1) or Sp3 (iSp3) as reported previously in other cancer cell lines (36, 38, 39). The results show that transfected siRNA for Sp1 decreased Sp1 protein >50% in whole cell lysates, and similar results were obtained using iSp3 (Fig. 1*A*). The effects of both iRNAs were highly specific, and the decreased Sp1 or Sp3 protein levels in whole cell lysates were caused by the high transfection efficiencies obtained in Panc-1 cells using Oligofectamine. Gel mobility shift assays (Fig. 1*B*) were carried out using an oligonucleotide that contains consensus GC-rich motifs that also are present in the proximal region of the VEGF promoter (−131 to −47). The specifically bound retarded band formed with whole cell lysates from untreated (CTL) cells (*Lane 2*) or cells transfected with siRNA for lamin (iLMN; *Lane 3*) was decreased in intensity after transfection with siRNA for Sp1 (*Lane 4*) or Sp3 (*Lane 5*). These results were consistent with decreased expression of both proteins in Panc-1 cells (Fig. 1*A*). Experiments with Sp1 and Sp3 antibodies gave supershifted bands (*Lanes 6* through *8*), and 100-fold excess of unlabeled oligonucleotide decreased intensities of the retarded bands (*Lane 9*). These results were consistent with the reported expression of Sp1 and Sp3 in Panc-1 cells (28); however, in this experiment, treatment with iSp1 plus iSp3 did not completely deplete the retarded band, and a residual complex remained (*Lane 8*). This was observed in several experiments.

The role of Sp1 and Sp3 in regulation of VEGF was investigated using a series of constructs containing different inserts from the *VEGF* gene promoter. The results (Fig. 1*C* and *D*) show that in Panc-1 cells transfected with pVEGF1, pVEGF2, pVEGF3, or pVEGF4 and cotransfected with iSp1 or iSp3, there was a decrease in luciferase activity that depended on the promoter insert. iSp1 was more effective than iSp3 in decreasing luciferase activity in cells transfected with pVEGF1, which contains the −2018 to +54 VEGF promoter insert; however, iSp3 was a more effective inhibitor in cells transfected with pVEGF2, pVEGF3, and pVEGF4, which contain −133 to +54, −67 to +54, and −66 to −47 VEGF promoter inserts, respectively. Sp

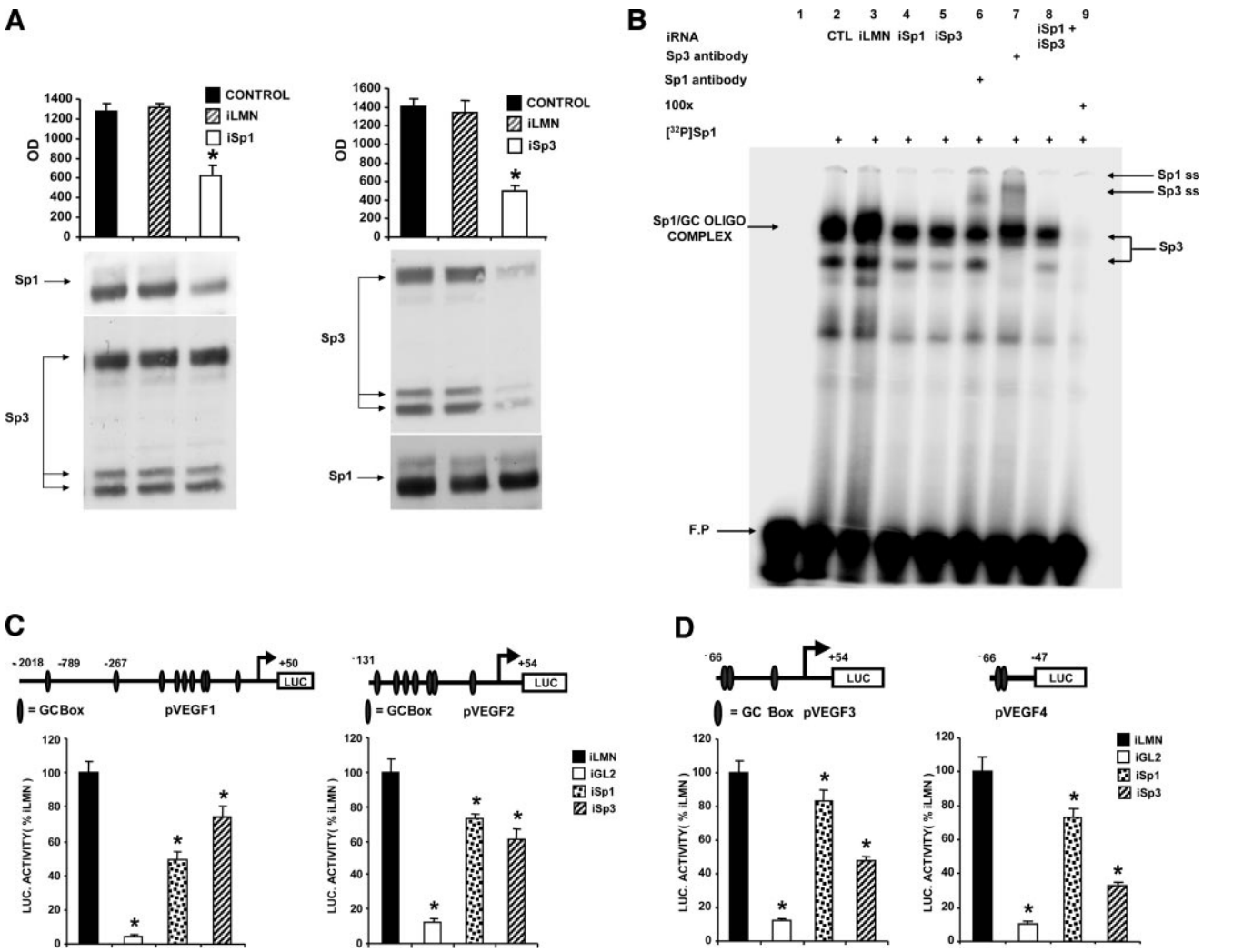


Fig. 1. Activation of VEGF by Sp1 and Sp3 in Panc-1 cells. *A.* siRNAs for Sp1 and Sp3 down-regulate their corresponding proteins in Panc-1 cells. Cells were transfected with siRNAs, and whole cell lysates were analyzed by Western blot analysis as described in Materials and Methods. Protein band intensities were determined by scanning laser densitometry. Results are expressed as mean ± SD for three separate experiments, and iSp1 and iSp3 significantly ($*P < 0.05$) down-regulated their corresponding proteins. *B,* EMSA analysis of nuclear extracts from Panc-1 cells transfected with iLMN, iSp1, or iSp3. Nuclear extracts from Panc-1 cells were incubated with a GC-rich oligonucleotide ($^{32}$P-Sp1) and various oligonucleotides/antibodies and were analyzed by gel mobility shift assays as described in Materials and Methods. Specifically bound complexes and supershifted complexes are indicated (arrows). *C* and *D,* transfection with iSp1, iSp3, or iLMN and various VEGF promoter constructs. Panc-1 cells were transfected with VEGF promoter constructs and iLMN, iGL2, iSp1, or iSp3, and luciferase activity (normalized by β-Gal) was determined as described in Materials and Methods. Results are expressed as mean ± SD for three separate experiments for each treatment group, and significant ($*P < 0.05$) decreases in activity (compared with control) are indicated.

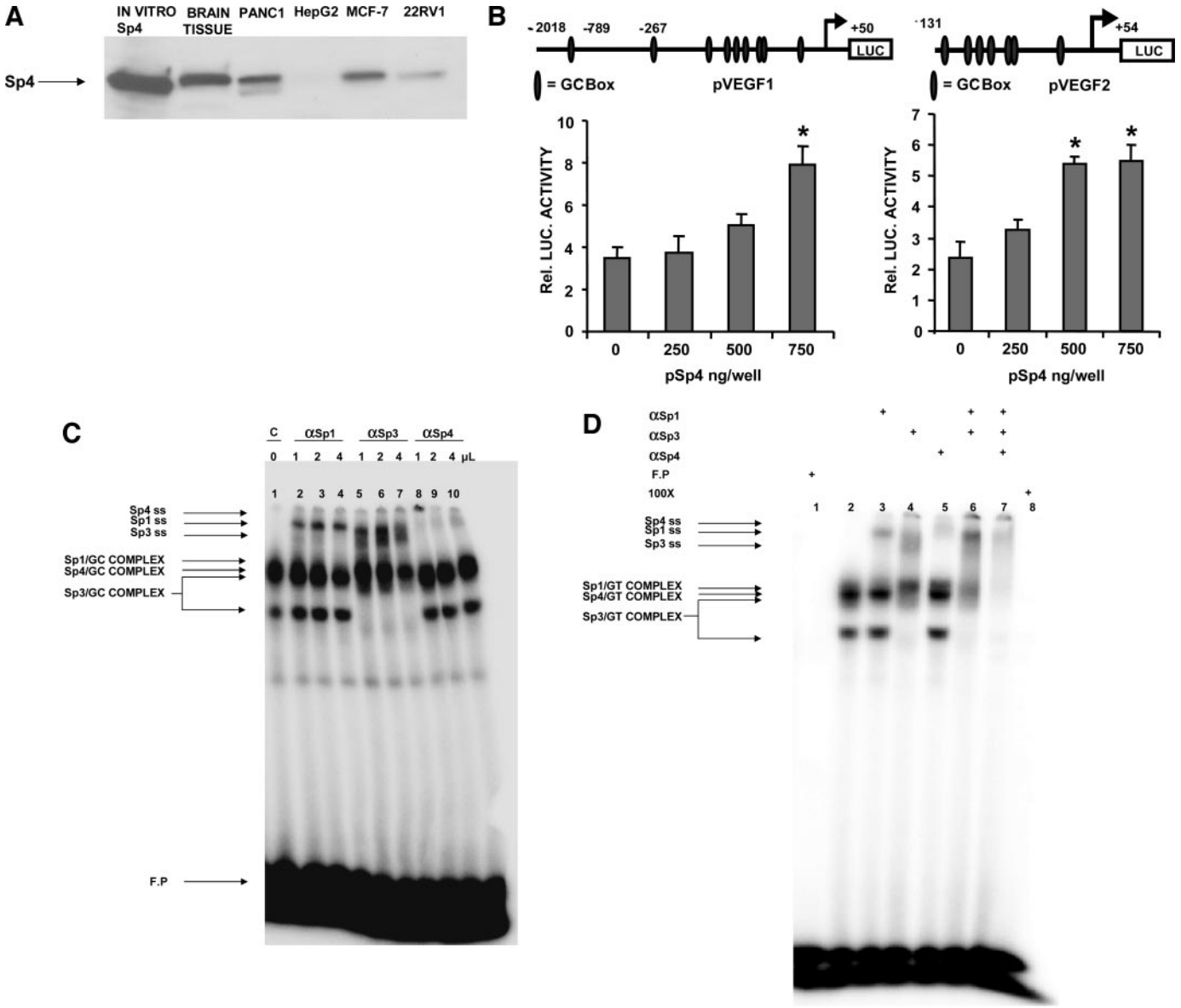


Fig. 2. Sp4 expression in Panc-1 cells and regulation of VEGF. *A*, Western blot analyses. Whole cell and mouse brain lysates were analyzed for Sp4 and Sp1 by Western immunoblot analysis as described in Materials and Methods. *In vitro*-translated Sp4 protein was used as a reference protein. Sp1 protein also was observed in all of the cells and tissue (data not shown). *B*, induction of VEGF promoter constructs by Sp4. Panc-1 cells were transfected with VEGF promoter constructs and increasing amounts of Sp4 expression plasmid, and luciferase activity was determined as described in Materials and Methods. Empty vector was used to ensure the same amount of DNA was transfected. Results are expressed as mean ± SD for three replicate determinations for each treatment group, and significant ($*P < 0.05$) induction is indicated. *C* and *D*, EMSA assays. Gel mobility shifts were determined using $^{32}$P-labeled GC-rich (*C*) and GT-rich (*D*) oligonucleotides using nuclear extracts from Panc-1 cells and antibodies for Sp1, Sp3, and Sp4. The retarded bands bound to the GC/GT-rich oligonucleotides, and the antibody supershifted bands are indicated.

proteins form homo- and hetero-oligomeric complexes bound to GC-rich motifs (40–43), and the data suggest that Sp1 and Sp3 cooperatively activate VEGF promoter constructs. Moreover, the results of RNA interference indicate that Sp1 and Sp3 may differentially modulate VEGF transactivation from distal and proximal GC-rich sites, respectively. Previous gel mobility shift assays using different GC-rich oligonucleotides from the −224 to +1 region of the VEGF promoter showed that Panc-1 cell extracts gave similar Sp1/Sp3 retarded bands (28) with similar Sp1/Sp3 binding affinities. Thus, differences in transactivation observed after RNA interference (Fig. 1*C* and *D*) might be because of alterations in Sp1/Sp3 bound to these sites *in vivo* or differential interactions with coregulatory proteins.

**Sp4-Dependent Regulation of VEGF.** Gel mobility shift assays (Fig. 1*B*) show that although Sp1 and Sp3 are bound to the consensus GC-rich oligonucleotide, RNA interference experiments with Sp1 and Sp3 show that some residual complex remained after treatment with both siRNAs. Sp4 protein also binds GC/GT-rich oligonucleotides and is primarily expressed in the developing brain in the mouse with lower but detectable levels in many other tissues (28, 44). Results in Fig. 2*A* show that immunoreactive Sp4 protein observed after *in vitro* translation of Sp4 protein also can be detected in Panc-1 cells and brain tissue, MCF-7 breast, and 22Rv1 prostate human cancer cells. In contrast, Sp4 was not detected in the human HepG cancer cell line. Sp4 has not been detected previously in pancreatic cancer cell lines; therefore, we investigated the effects of Sp4 expression on induction of luciferase activity in Panc-1 cells transfected with VEGF constructs. Sp4 clearly activates luciferase activity in Panc-1 cells transfected with pVEGF1 and pVEGF2 (Fig. 2*B*), and we also have observed comparable activation of the same VEGF constructs by Sp1 and Sp3 (data not shown) as reported previously (28).

Expression of Sp4 in Panc-1 cells was further investigated in gel mobility shift assays using nuclear extracts from Panc-1 cells and a $^{32}$P-labeled GC-rich (Fig. 2*C*) and GT-rich oligonucleotide (Fig. 2*D*, *Lanes 1* and *2*, labeled oligonucleotide alone). The retarded bands formed with the GC-rich oligonucleotide (Fig. 2*C*) gave supershifted complexes after incubation with Sp1, Sp3, or Sp4 antibodies; however, only a weak retarded band was observed using the Sp4 antibody. Therefore, we carried out an additional assay using a GT-rich oligonucleotide that also binds Sp proteins (Fig. 2*D*). The retarded band complex (*Lane 2*) gave supershifted bands after coincubation with Sp1, Sp3, and Sp4 antibodies (*Lanes 3* through *5*, respectively). Incubation with Sp1 plus Sp3 antibodies did not completely supershift the major retarded band complex (*Lane 6*); however, coincubation with Sp1, Sp3, plus Sp4 antibodies immunodepleted/supershifted the specifically bound bands (*Lane 7*). Expression of Sp4 protein also was confirmed by immunostaining with Sp4 antibodies (Fig. 3*A*). Sp4 staining was observed in Panc-1 (Fig. 2*D*, *d*) but not in HepG2 cells (Fig. 3*A*, *b*). These results show that Panc-1 cells express Sp4 protein, and the antibody supershift experiment (Fig. 2*D*) suggests that Sp1, Sp3, and Sp4 constitute the major Sp family proteins expressed in this cell line. Transfection of siRNA for Sp4 (iSp4) specifically decreased immunoreactive Sp4 but not Sp1 or Sp3 proteins in Panc-1 cells (Fig. 3*B*). The effects of iSp4 on VEGF promoter constructs are summarized in Fig. 3*C*. siRNA for luciferase (iGL2) significantly decreased luciferase activity by ≥80% in Panc-1 cells transfected with pVEGF1, pVEGF2, or pVEGF3, and iSp4 oligonucleotide decreased luciferase activity by ≥50% in cells transfected with the same constructs. These data, coupled with the RNA interference studies summarized in Fig. 1*C* and *D*, show that Sp4 cooperatively interacts with Sp1/Sp3 to regulate transactivation in Panc-1 cells transfected with VEGF constructs. Fig. 3*D* illustrates the effects of siRNA for Sp proteins on VEGF expression in Panc-1 cells. Immunostaining for VEGF gave broadly diffuse cytoplasmic staining in cells transfected with iScr (nonspecific; Fig. 3*D*, *b*). In cells transfected with iSp1, iSp3, iSp4, and iSp1 plus iSp4 (Fig. 3*D*, *c–f*, respectively), there was decreased staining for VEGF, and iSp1 and iSp4 were more effective than iSp3. The immunostaining results for VEGF complement the effects of the same siRNAs on VEGF promoter activities (Figs. 1*C* and *D* and 3*C*)

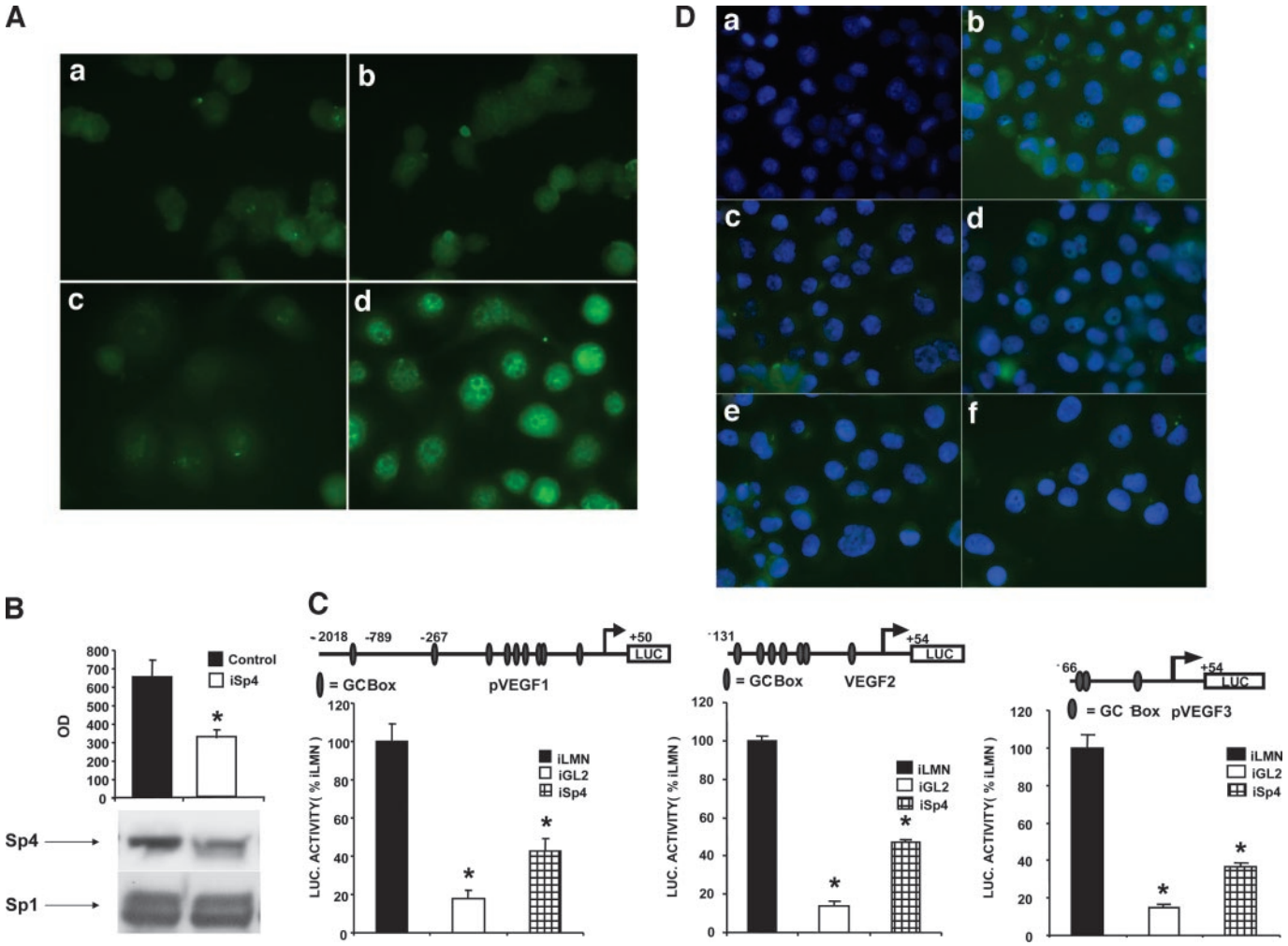


Fig. 3. Sp4 expression in Panc-1 cells and effects of iSp4 on VEGF. *A*, immunostaining with Sp4 antibodies. Immunofluorescence (*green*) was determined in HepG2 (*a*, *b*) and Panc-1 (*c*, *d*) cells stained with Sp4 primary antibody (*b*, *d*) or only with the secondary antibody (*a*, *c*). Increased Sp4 staining was observed only in Panc-1 cells. *B*, effects of iSp4 on Sp4 protein levels in Panc-1 cells. siRNA for Sp4 was transfected in Panc-1 cells, and Sp proteins were detected by Western blot analysis of whole cell lysates as described in Materials and Methods. Sp4 but not Sp1 protein was significantly ($*P < 0.05$) decreased, and Sp3 protein levels also were unchanged (data not shown). Results are expressed as mean ± SD for three replicate experiments. *C*, effect of iSp4 on VEGF expression. Panc-1 cells were transfected with VEGF promoter constructs and iLMN, iGL2, or iSp4, and luciferase activity was determined as described in Materials and Methods. Results of transfection experiments are expressed as mean ± SD for three replicate determinations for each treatment group, and significantly ($*P < 0.05$) decreased reporter gene activity after cotransfection with iSp4 is indicated. *D*, immunostaining for cytoplasmic VEGF in Panc-1 cells. 4′,6-Diamidino-2-phenylindole–stained (*blue*) Panc-1 (*a–f*) cells were transfected with iSc (*b*), iSp1 (*c*), iSp3 (*d*), iSp4 (*e*), and iSp1 + iSp4 (*f*) and stained with VEGF antibody (*b–f*) as described in Materials and Methods. Diffuse cytoplasmic staining (*green*) was decreased by the siRNAs for Sp1 proteins compared with cells transfected with iSc. Although transfection efficiencies are high for Panc-1 cells, there were some regions of the panels that retained high VEGF staining, and this was associated with cells not transfected with siRNAs.

and confirm that Sp4 significantly contributes to VEGF expression in Panc-1 cells.

**Sp3 as a Key Regulator of Panc-1 Cell Cycle Progression.** Sp1 and other Sp proteins also regulate expression of multiple genes associated with cancer cell proliferation (29–31). On the basis of the identification of Sp4 in Panc-1 cells and the effects of Sp4 on VEGF expression, we used RNA interference assays to determine the contribution of Sp1, Sp3, and Sp4 on Rb protein phosphorylation and cell cycle progression in Panc-1 cells. In cells transfected with iSp1 or iSp4, there were minimal changes in Rb phosphorylation compared with results in control cells (untreated) or cells transfected with iLMN (Fig. 4*A*). FACS analysis of cells transfected with iSp4 also showed that the percentage distribution of Panc-1 cells in $G_0/G_1$, S, or $G_2$-M phases of the cell cycle was comparable with those observed in control cells or cells transfected with nonspecific siRNAs (*e.g.*, iGL2; data not shown). In contrast, cells transfected with iSp1 decreased the percentage of cells in S phase (32.97 to 27.23%) and increased the percentage in $G_0/G_1$ from 42.85 to 53.11% (Fig. 4*B*). We previously reported a similar response to iSp1 in MCF-7 breast cancer cells (38), and this was consistent with Sp1-dependent regulation of several genes required for cell growth and DNA synthesis (29–31). The most dramatic effects were observed in Panc-1 cells transfected with iSp3. Rb phosphorylation was markedly decreased (Fig. 4*A*), and FACS analysis (Fig. 4*B*) indicated that transfection with iSp3 inhibited $G_0/G_1 \rightarrow$ S-phase progression in Panc-1 cells. The percentage of cells in $G_0/G_1$ (62.96%) compared with control cells (42.86%) and the percentage of cells in S phase (15.58%) and $G_2$-M (21.45%) compared with control cells (32.97% and 24.18%, respectively) were markedly altered after transfection with iSp3.

iSp3-dependent inhibition of $G_0/G_1 \rightarrow$ S-phase progression is consistent with decreased Rb phosphorylation and suggests that Sp3 regulates gene(s) that enhance proliferation of Panc-1 cells. Therefore, we further investigated expression of proteins associated with $G_0/G_1 \rightarrow$ S-phase progression of the cell cycle and the effects of iSp3 on protein expression patterns (Fig. 5*A*). Low levels of cdk2, cdk4, and p21 proteins were observed in Panc-1 cells (data not shown), and the results in Fig. 5*A* show the effects of iSp3 on cyclin E, cyclin D1, and p27 protein levels in whole cell lysates from Panc-1 cells. The loss of Sp3 did not affect cyclin D1 or cyclin E but resulted in a more than fourfold increase in p27 protein levels, suggesting that Sp3 enhances growth of Panc-1 cells through suppression of the cyclin-dependent kinase inhibitor p27. The role of Sp3 on *p27* expression was further investigated using constructs containing *p27* promoter inserts linked to a luciferase reporter gene. In a parallel experiment, transfection of Panc-1 cells with iSp3 also enhanced p27 mRNA expression, whereas iSp1 and iSp4 did not significantly affect p27 mRNA levels (Fig. 5*B*). The results (Fig. 5*C*) clearly show that iSp3 but not iSp1 increases transactivation in Panc-1 cells transfected with the p27 constructs that contain distal and proximal GC-rich sites and confirm the critical role of Sp3 in modulating growth of Panc-1 cells through inhibition of *p27* expression. Gel mobility shift assay using a GC-rich oligonucleotide from the *p27* gene promoter (−555 to −525; Fig. 5*D*) confirmed that Sp3, Sp1, and Sp4 bound this region of the gene promoter, and the pattern of retarded and antibody supershifted bands was similar to that observed using a consensus GC-rich oligonucleotide (Fig. 2*C*). Confirmation that iSp3 results in up-regulation of p27 protein is given in Fig. 5*E*, where knockdown of Sp3 protein results in up-regulation of p27 protein (*f*) and down-regulation of pRb (*e*) compared with cells transfected with the nonspecific control siRNA for luciferase (*c* and *d*). The data show that Sp3 enhances $G_0/G_1 \rightarrow$ S-phase progression through inhibition of p27 expression in Panc-1 cells.

**Role of Sp Proteins in Regulation of p27 and VEGF in Other Pancreatic Cancer Cell Lines.** Western blot analysis of whole cell lysates from several pancreatic cancer cell lines showed that the three proteins were expressed in Panc-1, Panc-28, AsPC-1, MIA-PaCa-2, and HPAFII cell lines (Fig. 6*A*). However, relatively low levels of Sp4 were expressed in AsPC-1 and HPAFII cells. Results obtained for Panc-1 cells show that Sp proteins play a role in p27 and VEGF expression, and this was further investigated in other pancreatic cancer cells transfected with p27 no. 2 and pVEGF2 (Fig. 6*A*). Using p27

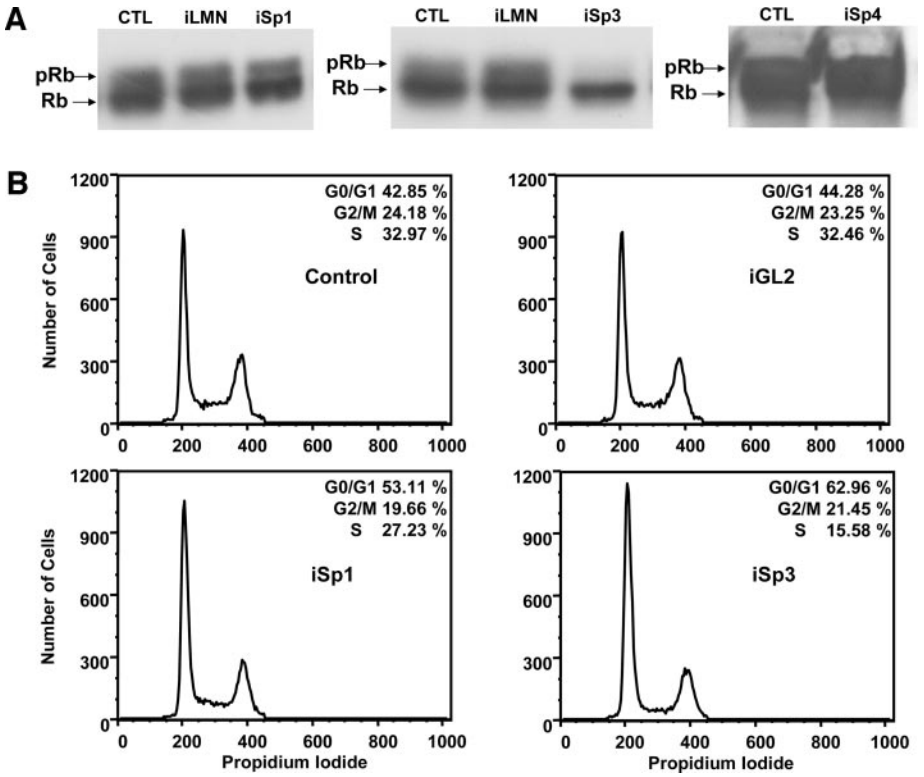


Fig. 4. Role of Sp proteins in Panc-1 cell proliferation. *A*, Rb phosphorylation. Immunoblot analysis of Rb and phospho-Rb (pRb) was determined in whole cell lysates of Panc-1 cells transfected with iSp1, iSp3, or iSp4 as described in Materials and Methods. *B*, FACS analysis. Cells were transfected with iSp1, iSp3, or iSp4, and their subsequent distribution in different phases of the cell cycle was determined by FACS analysis as described in Materials and Methods. Data obtained for Western blot and FACS analyses gave comparable results in duplicate analyses. iSp4 did not affect distribution of the cells in $G_0/G_1$, S, or $G_2$-M phases (data not shown).

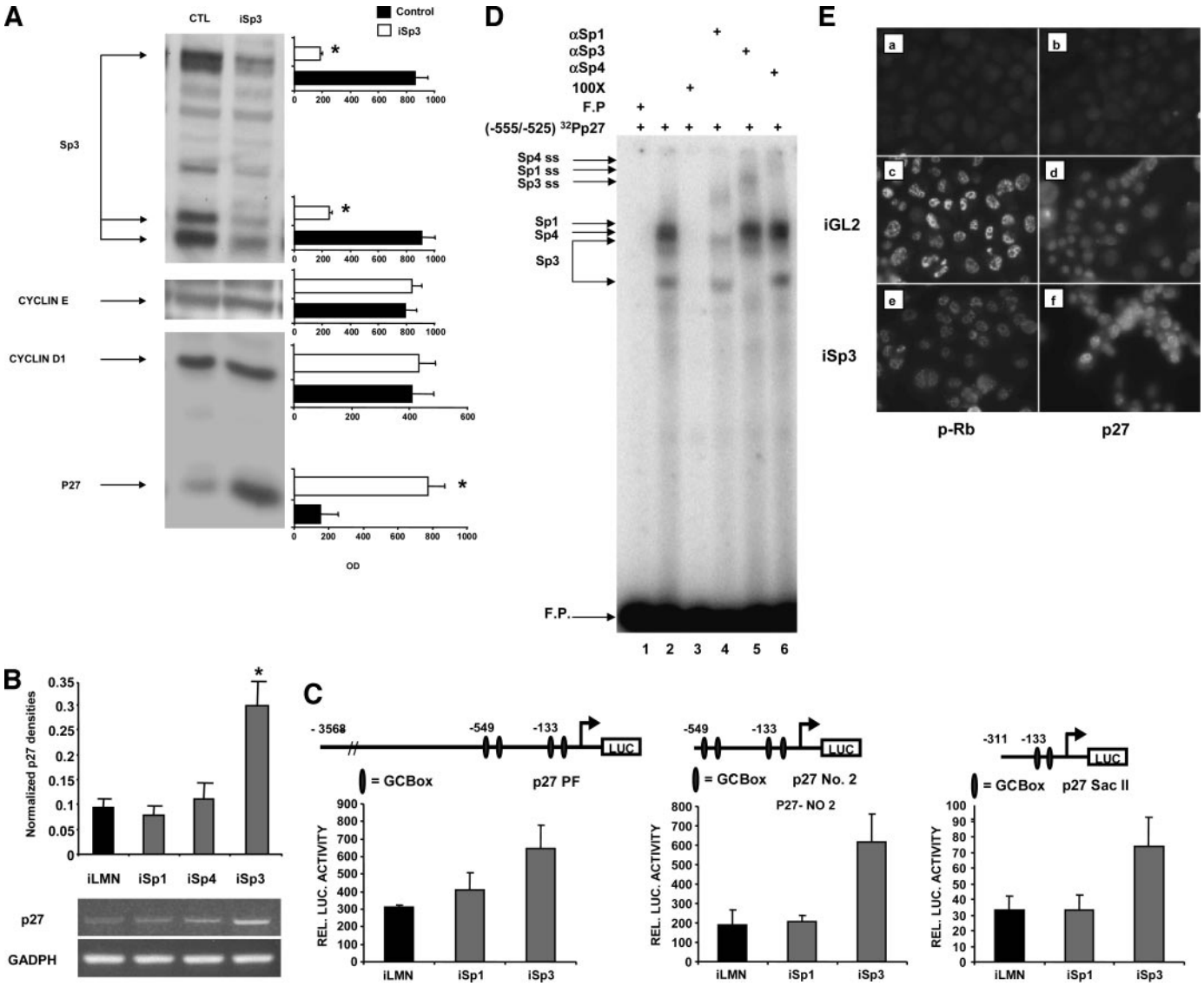


Fig. 5. Sp3-dependent regulation of *p27* in Panc-1 cells. *A*, Western blot analysis. Cells were transfected with iSp3 or control (iLMN); whole cell lysates were obtained; and proteins were analyzed by Western blot analysis as described in Materials and Methods. Levels of other proteins associated with $G_0/G_1 \rightarrow$ S-phase progression (cdk2, cdk4, and p21) were unchanged (data not shown). Results are expressed as mean ± SD for three replicate determinations for each treatment group, and significantly ($*P < 0.05$) increased levels of p27 and decreased levels of Sp3 proteins are indicated. *B*, increased p27 mRNA levels. Cells were transfected with iLMN, iSp1, iSp4, or iSp3, and p27 mRNA levels were determined by reverse transcription-PCR as described in Materials and Methods. Significant ($P < 0.05$) induction of p27 was observed only in cells transfected with iSp3 (compared with iLMN). *C*, p27 promoter activity. Panc-1 cells were transfected with p27 promoter constructs and iLMN, iSp1, or iSp3, and luciferase activity was determined as described in Materials and Methods. Results are expressed as mean ± SD for three replicate determinations for each treatment group, and significantly ($*P < 0.05$) increased activity is indicated. *D*, binding to (−555/−525) p27$^{32}$P. Nuclear extracts from Panc-1 cells were incubated with the GC-rich oligonucleotide from the *p27* gene promoter and analyzed by gel mobility shift assay as described in Materials and Methods. Retarded and supershifted bands are indicated with arrows. *E*, immunostaining of p27 and phospho-Rb. Panc-1 cells were transfected with iGL2 (*c* and *d*) or iSp3 (*e* and *f*) and immunostained with phospho-Rb (*c* and *e*) or p27 (*d* and *f*) antibodies. Panels *a* and *b* represent cells stained only with the secondary antibody.

no. 2 as a probe, the effects of iSp3, iSp1, and iSp4 on luciferase activity in AsPC-1, Panc-28, and MIA-PaCa-2 cells showed that only iSp3 resulted in increased activation of this construct. These results were consistent with those described for Panc-1 cells (Fig. 5*C*), suggesting that Sp3-dependent suppression of p27 might play an important role in proliferation of pancreatic cancer cells as indicated in Fig. 4. HPAFII cells were difficult to transfect because of cell clumping, and consistent results could not be obtained for this cell line. The role of Sp1, Sp3, and Sp4 in regulating VEGF also was investigated in pancreatic cancer cells transfected with pVEGF2 and iSp1, iSp3, or iSp4. iSp1 consistently decreased transactivation in all of the cell lines, and small, but significant, inhibition also was observed for iSp3 in AsPC-1 and MIA-PaCa-2 cells. The effects of iSp4 were variable, and the pattern of inhibition observed in Panc-1 cells (Figs. 1 and 3) was observed only in MIA-PaCa-2 cells, suggesting that the role of Sp4 in regulation of VEGF in pancreatic cancer cells is variable and depends on the cell line.

Further confirmation of the specificity of Sp3 as an inhibitor of p27 expression also was investigated in Panc-1, AsPC-1, Panc-28, and MIA-PaCa-2 cells transfected with iSp3 (Fig. 6*C*), iSp1, or iSp4 (Fig. 6*D*). The results show that iSp3 coordinately decreased Sp3 and increased p27 protein expression in pancreatic cancer cells, whereas knockdown of Sp1 or Sp4 proteins did not affect p27 protein levels. The results clearly show that Sp3 plays an important role in cell cycle progression of pancreatic cancer cells by suppressing p27.

## DISCUSSION

Pancreatic cancer is a complex and devastating disease that is usually detected in advanced stages or after metastases. Not surpris-

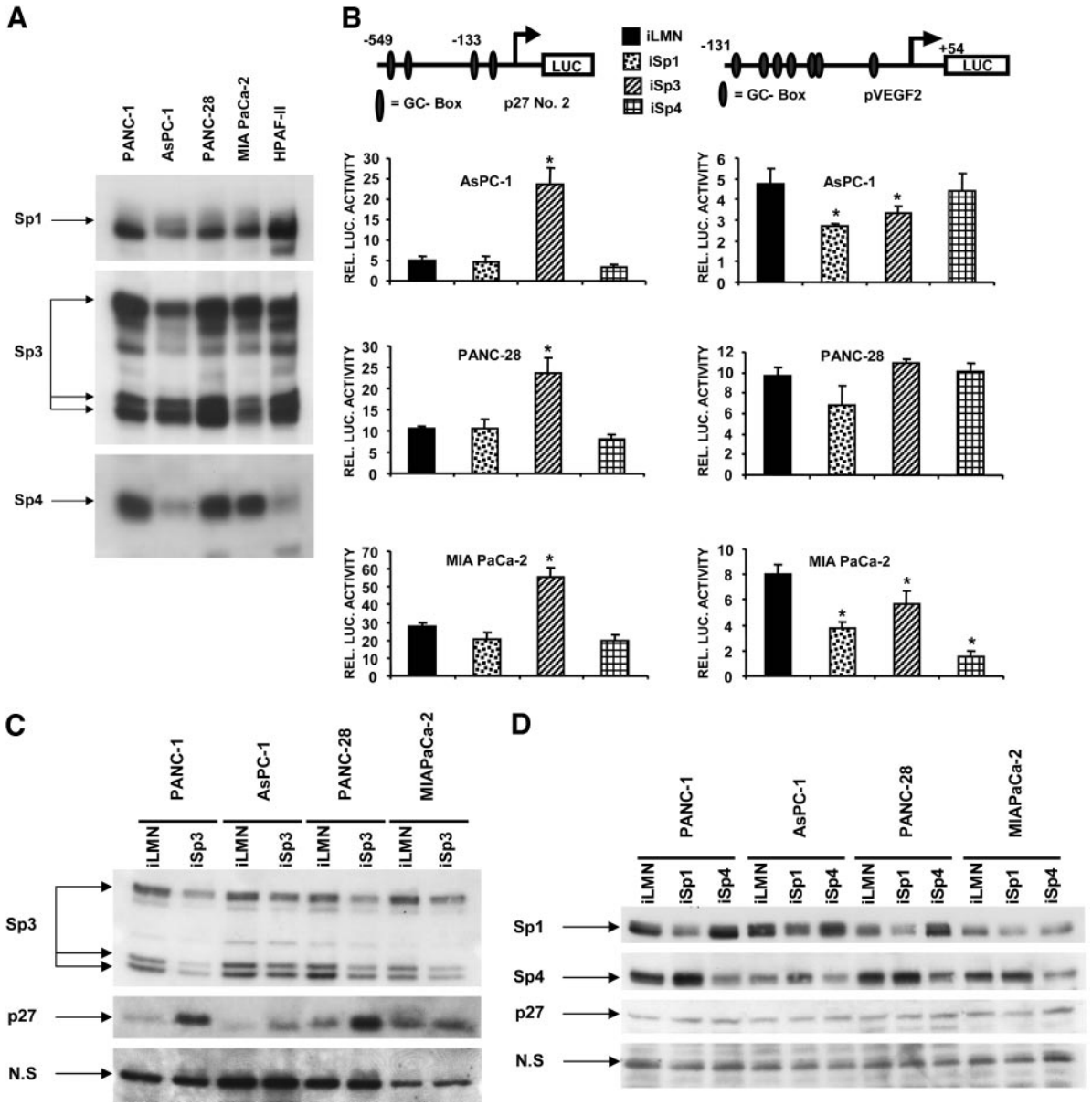


Fig. 6. Regulation of VEGF and p27 by Sp proteins in AsPC-1, Panc-28, MIA-PaCa-2, and HPAFII pancreatic cancer cell lines. *A*, Western blot analysis. Whole cell lysates from Panc-1, AsPC-1, Panc-28, MIA-PaCa-2, and HPAFII cells were obtained and analyzed for Sp1, Sp3, and Sp4 proteins by Western blot analysis as described in Materials and Methods. *B*, effects of Sp proteins on transactivation in cells transfected with p27 no. 2 and pVEGF2. Cells were transfected with p27 no. 2 or pVEGF2 and iLMN, iSp1, iSp3, or iSp4, and luciferase activity was determined as described in Materials and Methods. Luciferase activity significantly ($P < 0.05$) different from that observed in cells transfected with iLMN is indicated (*), and results are expressed as mean ± SD for three separate determinations for each treatment group. p27 expression in pancreatic cancer cells transfected with iSp3 (*C*) or iSp1 and iSp4 (*D*). The pancreatic cancer cell lines were transfected with iLMN (control) or siRNAs for Sp proteins, and whole cell lysates were analyzed by Western blot analysis for Sp3 and p27 proteins (*C*) or Sp1/Sp4 and p27 proteins (*D*) as described in Materials and Methods. A nonspecific band expressed in all of the cell lines was used as a loading control.

ingly, current chemotherapies for this disease have limited efficacy, and the 1- and 5-year survival times are ~21% and 5%, respectively (1, 2). Development of new strategies for detection and management of this disease will depend on several factors, which include a more comprehensive understanding of critical genes and pathways that control pancreatic tumor growth. PDAC cell lines have been developed for *in vitro* studies, and Panc-1 cells were derived from a primary tumor (40) and exhibit K-*ras*, *p53*, and *p16* mutations, which are typically observed in PDAC.

Previous studies reported that Panc-1 and other pancreatic cancer cell lines expressed high levels of Sp1 and Sp3 proteins, and Sp1 protein levels correlated with expression of VEGF protein and VEGF promoter activity (28). The results are consistent with other studies showing that basal expression of *VEGF* is caused, in part, by interactions of Sp1 with proximal GC-rich sites in the *VEGF* gene promoter (32, 36, 37). Studies in this laboratory have shown that the −131 to −47 region of the promoter is important for basal and estrogen-inducible expression of VEGF/VEGF promoter constructs through estrogen receptor (ER)/Sp protein interactions. Moreover, in ZR-75 breast cancer cells, hormone-induced transactivation of VEGF was caused by ER$\alpha$/Sp1 and ER$\alpha$/Sp3 as determined in RNA interference assays (36). Our first objective was to use RNA interference in Panc-1 cells to investigate the role of Sp1 and Sp3 in regulating VEGF promoter constructs as described previously for Panc-1 and other pancreatic cancer cell lines (28). The results show that iSp1 and iSp3 efficiently decrease Sp1 and Sp3 proteins in Panc-1 cells (Fig. 1*A*), and in transient transfection studies (Fig. 1*C* and *D*), it was apparent that Sp1 and Sp3 were involved in basal expression of

VEGF. Moreover, using a series of deletion constructs (Fig. 1*C* and *D*), it was evident that Sp1-mediated transactivation was most effective in cells transfected with pVEGF1, which contains the −2018 to +50 VEGF promoter insert. In contrast, Sp3-dependent transactivation was higher in cells transfected with constructs containing proximal (−131 to −47) VEGF promoter inserts. Previous studies show that Sp1 primarily binds as an oligomer to GC boxes (41–43), and the results of RNA interference suggest that Sp1/Sp3 heteroligomers of different composition may form on the VEGF promoter.

Results of gel mobility shift assays using a GC-rich oligonucleotide containing motifs present in the VEGF promoter showed that Sp1 and Sp3 antibodies did not completely supershift the major retarded band, suggesting that other GC-rich binding proteins are expressed in Panc-1 cells. Expression patterns of Sp family proteins 2 to 4 in tissues and cancer cell lines have not been extensively investigated, and only Sp4 but not Sp2 binds GC-rich motifs (29, 45). There is evidence for expression and transactivation function of Sp4 in the brain and epithelial tissues and cells (29, 44, 46, 47); therefore, we investigated the expression of Sp4 in Panc-1 cells. Western blot analysis confirmed that Sp4 was expressed in brain, Panc-1, and other cancer cell lines (Fig. 2*A*), and Sp4 antibodies induced formation of a weak supershifted band using nuclear extracts from Panc-1 cells incubated with a GC-rich oligonucleotide (Fig. 2*C*). Gel mobility shift assays with a GT oligonucleotide (Fig. 2*D*) gave a more pronounced supershifted band after incubation with Sp4 antibody and confirmed that Sp1, Sp3, and Sp4 proteins were expressed in Panc-1 cells. Sp4 protein expression activated reporter gene activity in Panc-1 cells transfected with pVEGF1 and pVEGF5 (Fig. 2*B*), and RNA interference clearly showed that Sp4 plays an important role in VEGF promoter activity (Fig. 3*C*) and cytoplasmic protein expression (Fig. 3*D*). On the basis of results of siRNA studies, it is evident that Sp4, Sp1, and Sp3 cooperatively regulate basal expression of VEGF in Panc-1 cells. We also investigated Sp protein expression and effects of RNA interference on transactivation in other pancreatic cancer cells transfected with pVEGF2 (Fig. 6). Although iSp1 decreased activity in these cell lines as observed in Panc-1 cells (Fig. 1), the relative contributions of individual Sp proteins on VEGF expression was highly variable. For example, in MIA-PaCa-2 cells transfected with pVEGF2, iSp4 decreased transactivation by >75%, whereas decreased Sp4 expression in Panc-28 or AsPC-1 cells did not affect luciferase activity (Fig. 6*B*). These data suggest that the role of individual Sp proteins in VEGF regulation in pancreatic cancer cells is variable and may depend not only on their relative expression but also on other factors.

Sp proteins also regulate genes required for cancer cell proliferation (29–31), and we used RNA interference assays with iSp1, iSp3, and iSp4 to investigate their role in Panc-1 cell growth. Transfection with iSp4 followed by FACS analysis indicated that distribution of the cells in $G_0/G_1$, $G_2$-M, or S phase and Rb phosphorylation (Fig. 4*A* and *B*) was not significantly affected. Decreased Sp1 protein expression did not decrease Rb phosphorylation but partially inhibited $G_0/G_1 \rightarrow$ S-phase progression. This was consistent with several reports showing that Sp1 regulates expression of genes involved in cell growth and cell cycle progression (28, 48). In contrast to results obtained for iSp1 and iSp4, transfection with iSp3 decreased in Rb phosphorylation (Figs. 4*A* and 5*E*) and significantly blocked $G_0/G_1 \rightarrow$ S-phase progression. These results are surprising and imply that Sp3 is an important regulator of Panc-1 cell growth. Sp3 can activate and inhibit genes but is not usually associated with cell growth (29–31). Subsequent analysis of cell cycle proteins in Panc-1 cells transfected with iSp3 showed that knockdown of Sp3 protein resulted in increased p27 mRNA, protein, and *p27* reporter gene expression (Fig. 5*A*–*C*). The regulation of p27 expression has been extensively investigated, and decreased expression of this protein in many cancer cell lines is associated with increased rates of proteasome-dependent degradation (49, 50). Moreover, loss of this tumor suppressor gene is a negative prognostic indicator for many tumors. The p27 promoter is complex and contains multiple *cis*-elements (51, 52), including several GC-rich sites (Fig. 5*C*), which bind Sp3, Sp1, and Sp4 in a gel mobility shift assay (Fig. 5*D*). Previous studies have shown that GC-rich sites in the p27 promoter mediate induction of p27 by vitamin D3 and tamoxifen in human myelomonocytic U937 and H358 lung cancer cells (53, 54), and up-regulation of p27 involves cooperative interactions of Sp1 and other factors. In contrast, our results show that siRNA for Sp1 has minimal effects on p27 expression or luciferase activity in Panc-1 cell-transfected constructs containing p27 promoter inserts. Sp3 is the major regulator of p27 in Panc-1 cells, and Sp3-dependent suppression of p27 is correlated with $G_0/G_1 \rightarrow$ S-phase progression of Panc-1 cells (Fig. 4*B*). The role of Sp3 in suppressing p27 expression also was investigated in other pancreatic cancer cell lines transfected with p27 no. 2 (Fig. 6), and the results showed that increased transactivation was observed only after cotransfection with iSp3 and not iSp1 or iSp4. The activity of Sp3 as a negative transcription factor, which suppresses p27 promoter activity, is consistent with the inhibitory effects of Sp3 on many other gene promoters in cancer cell lines (29–31).

In summary, results of this study illustrate that Sp proteins play an important role in angiogenic and cell proliferation pathways in pancreatic cancer cells by regulating expression of VEGF and p27 in Panc-1 cells. Sp4 has been identified for the first time in pancreatic cancer cells and shown to cooperatively activate VEGF along with Sp1 and Sp3 only in some pancreatic cancer cell lines. Sp3 expression significantly contributes to $G_0/G_1 \rightarrow$ S-phase progression in Panc-1 cells through inhibition of the tumor suppressor gene *p27*. Moreover, using reporter gene and RNA interference assays, it is apparent that Sp3 inhibits p27 expression in AsPC-1, Panc-28, and MIA-PaCa-2 pancreatic cancer cell lines (Fig. 6*C* and *D*). Current studies are extending these observations on the role of Sp proteins in regulating tumor growth and angiogenesis in other pancreatic cancer cell lines and identifying specific cellular targets for chemotherapy.

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# Role of Sp Proteins in Regulation of Vascular Endothelial Growth Factor Expression and Proliferation of Pancreatic Cancer Cells

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