

# Regulation of Vascular Endothelial Growth Factor Receptor-1 Expression by Specificity Proteins 1, 3, and 4 in Pancreatic Cancer Cells

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## Abstract

**Vascular endothelial growth factor receptor-1 (VEGFR1) is expressed in cancer cell lines and tumors and, in pancreatic and colon cancer cells, activation of VEGFR1 is linked to increased tumor migration and invasiveness. Tolfenamic acid, a nonsteroidal anti-inflammatory drug, decreases Sp protein expression in Panc-1 and L3.6pl pancreatic cancer cells, and this was accompanied by decreased VEGFR1 protein and mRNA and decreased luciferase activity on cells transfected with constructs (pVEGFR1) containing VEGFR1 promoter inserts. Comparable results were obtained in pancreatic cancer cells transfected with small inhibitory RNAs for Sp1, Sp3, and Sp4 and all three proteins bound to GC-rich elements in the VEGFR1 promoter. These results show that VEGFR1 is regulated by Sp proteins and that treatment with tolfenamic acid decreases expression of this critical angiogenic factor. Moreover, *in vitro* studies in Panc-1 cells show that activation of VEGFR1 by VEGFB to increase mitogen-activated protein kinase 1/2 phosphorylation and cell migration on collagen-coated plates is also inhibited by tolfenamic acid. Thus, targeted degradation of Sp proteins is highly effective for inhibiting VEGFR1 and associated angiogenic responses in pancreatic cancer.** [Cancer Res 2007;67(7):3286–94]

## Introduction

Angiogenesis is a process involving new blood vessel formation from existing vessels and is essential for tissue regeneration and remodeling during wound healing and for the female reproductive cycle (1–3). Angiogenesis also plays a role in several diseases, including rheumatoid arthritis, proliferative retinopathy, psoriasis, macular degeneration, and cancer, where this pathway is activated during tumor progression, growth, and metastasis (1–6). Vascular permeability factor or vascular endothelial growth factor (VEGF) was initially identified as a key regulator of angiogenesis and is a member of the VEGF/platelet-derived growth factor (PDGF) family of proteins that include VEGF and related splice variants (VEGF<sub>121</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub>, and VEGF<sub>206</sub>) and VEGFB-E placenta growth factors 1 and 2 (1–3). These growth factors activate angiogenesis

through their specific interactions with VEGF receptors (VEGFR), which are transmembrane tyrosine kinases and members of the PDGF receptor gene family.

VEGFR1 (Flk-1), VEGFR2 (Flt-1/KDR), and VEGFR3 (Flt-4) are the three major receptors for VEGF and related angiogenic factors. The former two receptors are primarily involved in angiogenesis in endothelial cells, whereas VEGFR3 promotes hematopoiesis and lymphoangiogenesis (2, 3, 7, 8). VEGFR2 plays a critical role in angiogenesis; homozygous knockout mice were embryonic lethal [gestation day (GD) 8.5–9.0] and this was associated with the failure to develop blood vessels (9). VEGFA binds with high affinity to VEGFR2 and induces formation of a homodimer, which subsequently activates downstream signaling pathways similar to other receptor tyrosine kinases. Deletion of the *VEGFR1* gene in mice is also embryolethal at GD of 8.5; however, the embryonic phenotype is different from that observed in the VEGFR2 knockouts because embryonic endothelial cells are highly overgrown and disorganized (10, 11). There is evidence that VEGFR1 acts, in part, as a decoy receptor that inhibits VEGFR2-mediated angiogenesis (12–14); however, under some conditions, VEGFR1 may also cooperatively activate angiogenesis with VEGFR2 and this response is both ligand and cell context dependent (14).

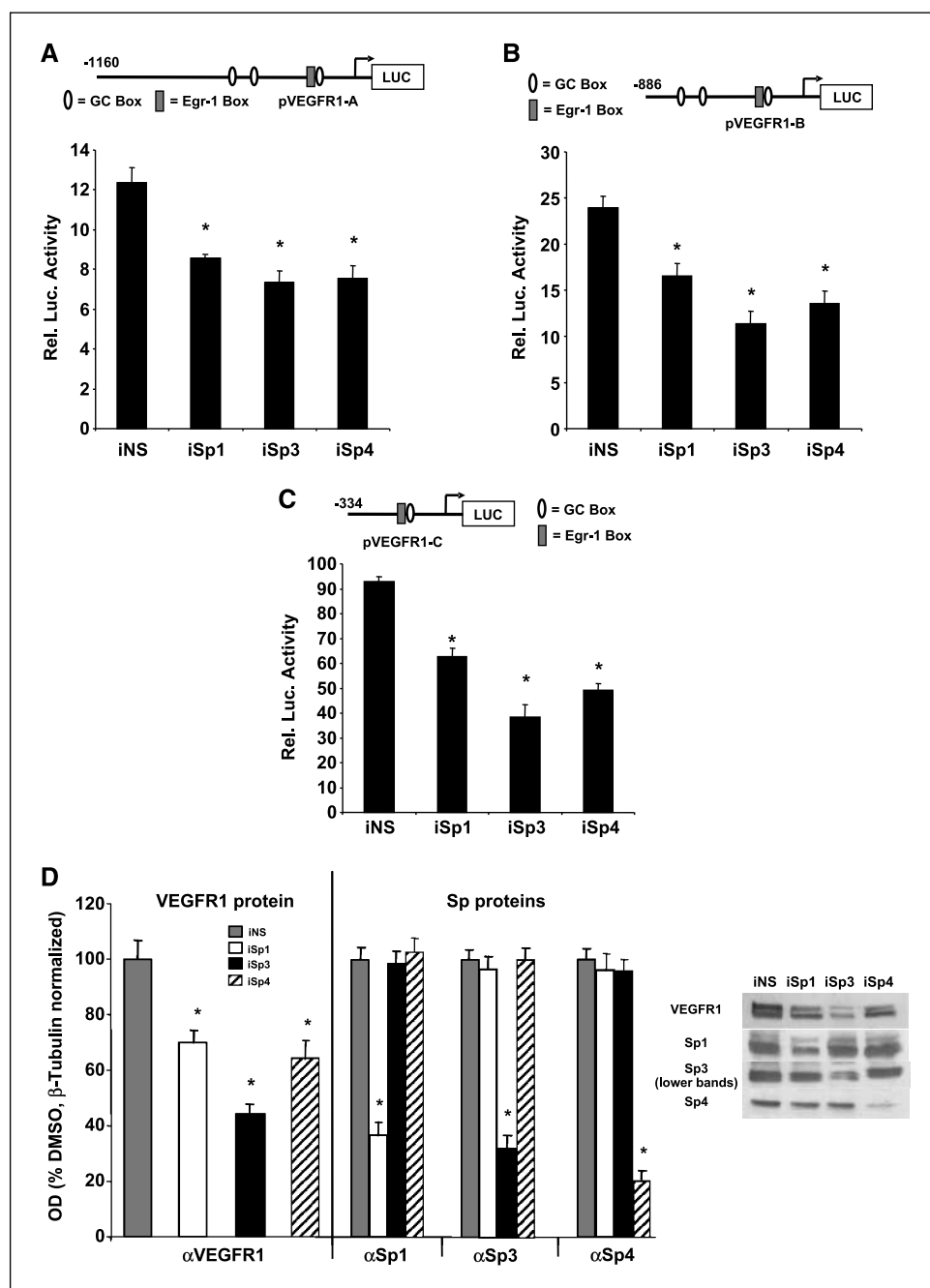
VEGFR1 is also expressed as a soluble truncated (sVEGFR1) form, which does not contain the intracellular receptor tyrosine kinase domains, and sVEGFR1 acts primarily to bind VEGF and inhibit angiogenesis (15–18). VEGFR1 is expressed in a variety of cancer cell lines and tumors (19–25) and, in colon and pancreatic cancer cells, activation of VEGFR1 results in epithelial to mesenchymal transition, which is linked to increased invasion and migration of tumor cells (23–25). For example, VEGFB induced phosphorylation of mitogen-activated protein kinase (MAPK) and enhanced migration in pancreatic cancer cells, and VEGFR1-neutralizing antibody inhibited these responses.

To develop agents that will modulate VEGFR1 expression, we have investigated the molecular mechanism regulation of VEGFR1 expression in pancreatic cancer cells. The VEGFR1 promoter contains multiple *cis* elements, including at least three GC-rich sites that bind Sp proteins and a proximal early growth response-1 (Egr-1) binding sequence (26). Based on results of RNA interference assays and the effects of the nonsteroidal anti-inflammatory drug (NSAID) tolfenamic acid, which decreases Sp protein expression (27), we have now shown that VEGFR1 expression is primarily dependent on Sp proteins. Moreover, VEGFB-dependent activation of VEGFR1, which increases MAPK phosphorylation and migration in Panc-1 cells, is also inhibited by tolfenamic acid. Results of this study show that, like VEGF and VEGFR2 (27–38), VEGFR1 expression is Sp protein dependent, indicating that agents, such

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**Figure 1.** VEGFR1 expression in pancreatic cancer cells is Sp protein dependent. Transfection with pVEGFR1A (A), pVEGFR1B (B), and pVEGFR1C (C) and small inhibitory RNAs for Sp1 (iSp1), Sp3 (iSp3), or Sp4 (iSp4). Panc-1 cells were transfected with the pVEGFR1 constructs and iSp1, iSp3, or iSp4, and luciferase (*LUC*) activity was determined as described in Materials and Methods. As a control for the transfection experiment, cells were transfected with a nonspecific small inhibitory RNA (iNS), and luciferase activity for transfection with iNS was set at 100%. All experiments were replicated at least thrice. Columns, mean; bars, SD. \*,  $P < 0.05$ , significantly decreased luciferase activity. D, effects of iSp1, iSp3, and iSp4 on Sp and VEGFR1 proteins. Panc-1 cells were transfected with iNS, iSp1, iSp3, or iSp4, and whole-cell lysates were analyzed by Western blot analysis as described in Materials and Methods. The experiment was replicated (thrice) and relative expression of individual Sp proteins and VEGFR1 was compared with levels in cells treated with iNS (set at 100%). \*,  $P < 0.05$ , significantly decreased protein expression.



as tolfenamic acid, which decreased expression of these transcription factors, are an important class of mechanism-based anti-angiogenic drugs.

## Materials and Methods

**Cell lines, chemicals, biochemical, constructs, and oligonucleotides.** Panc-1 cells were obtained from the American Type Culture Collection (Manassas, VA). L3.6pl cell line was developed at the M. D. Anderson Cancer Center (Houston, TX) and kindly provided by Dr. I.J. Fidler. VEGFR1 promoter luciferase constructs were kindly provided by Dr. Koji Maemura (Department of Cardiovascular Medicine, University of Tokyo, Tokyo, Japan). DMEM/F-12 with and without phenol red, 100 $\times$  antibiotic/antimycotic solution, and lactacystin were purchased from Sigma Chemical Co. (St. Louis, MO). Collagen IV-coated plates were

purchased from Becton Dickinson Labware (Bedford, MA). Diff-Quik staining kit was obtained from Dade Behring (Newark, DE). Fetal bovine serum (FBS) was purchased from InterGen (Purchase, NY). [ $\gamma$ - $^{32}$ P]ATP (300 Ci/mmol) was obtained from Perkin-Elmer Life Sciences. Poly(deoxyinosinic-deoxycytidylic acid) [poly(dI-dC)] and T4 polynucleotide kinase were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Antibodies for Sp1, Sp3, Sp4, histone deacetylase (HDAC),  $\beta$ -tubulin, and VEGFR1 proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphorylated ERK1/2 (pERK1/2) were obtained from Zymed Laboratories, Inc. (San Francisco, CA). Lysis buffer and luciferase reagent were obtained from Promega Corp. (Madison, WI). VEGF promoter constructs have been described previously (27).

**Transfection of pancreatic cancer cells and preparation of nuclear extracts.** Cells were cultured in six-well plates in 2 mL DMEM/F-12

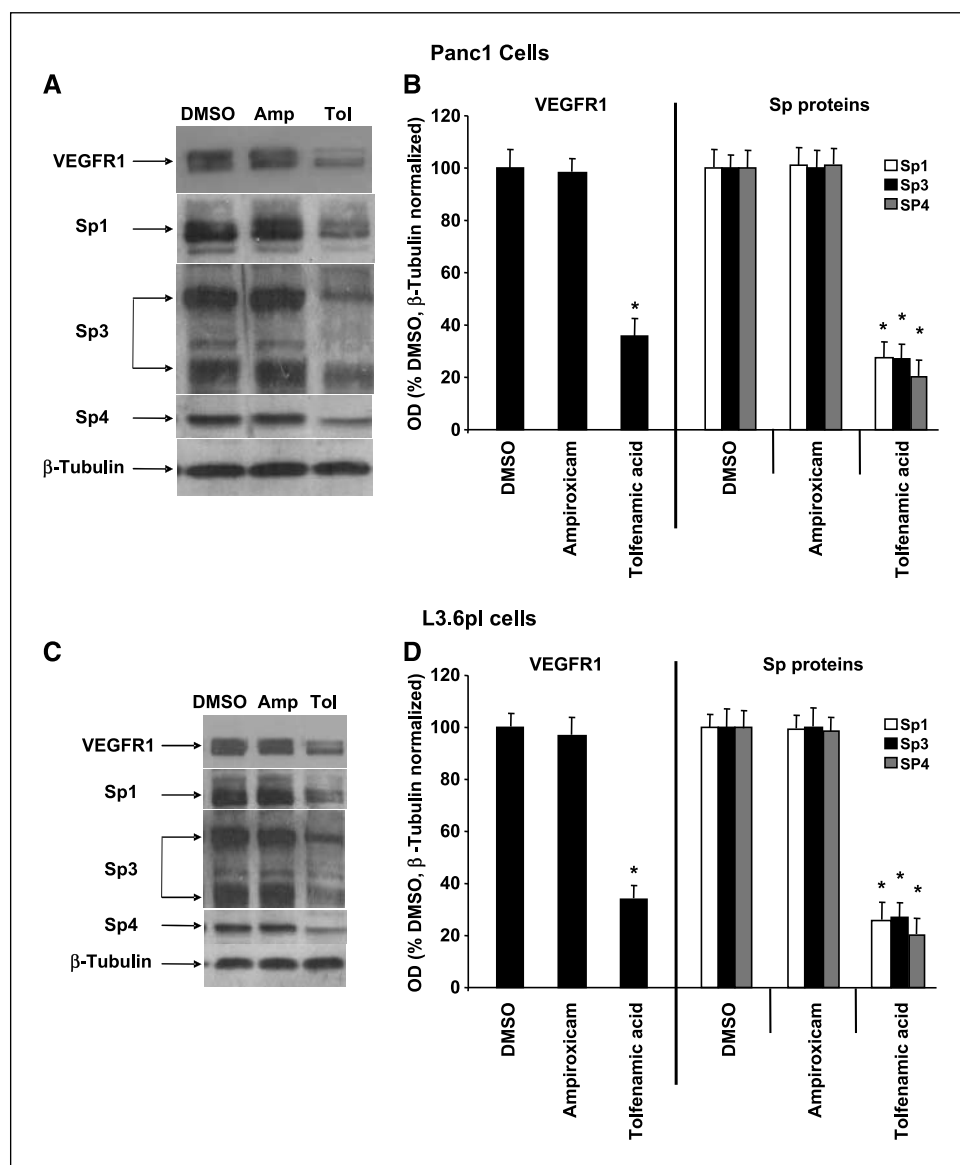
supplemented with 5% FBS. After 16 to 20 h when cells were 50% to 60% confluent, reporter gene constructs were transfected using LipofectAMINE reagent (Invitrogen, Carlsbad, CA). The effects of tolfenamic acid and amproxicam on transactivation was investigated in Panc-1 and L3.6pl cells cotransfected with (500 ng) different VEGFR1 constructs. Cells were treated with DMSO (control) or with the indicated concentration of NSAIDs for 48 h, and then luciferase activity of lysates (relative to  $\beta$ -galactosidase activity) was determined. For electrophoretic mobility shift assay (EMSA) assay, nuclear extracts from Panc-1 and L3.6pl cells were isolated as described previously (30, 36), and aliquots were stored at  $-80^{\circ}\text{C}$  until used (27).

**Western Immunoblot.** Cells were washed once with PBS and collected by scraping in 200  $\mu\text{L}$  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, 5  $\mu\text{L}/\text{mL}$  Protease Inhibitor Cocktail (Sigma Chemical)]. Cell lysates were incubated on ice for 1 h with intermittent vortexing followed by centrifugation at  $40,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . Equal amounts of protein (60  $\mu\text{g}$ ) from each treatment group were diluted with loading buffer, boiled, and loaded onto 10% and 12.5% SDS-polyacrylamide gel. Samples were electrophoresed and proteins were detected by incubation with polyclonal primary antibodies Sp1 (PEP2), Sp3 (D-20), Sp4 (V-20), HDAC (H-51), VEGFR1 (C-17), ERK1/2, pERK1/2(Thr<sup>202</sup>/Tyr<sup>204</sup>), and  $\beta$ -tubulin (H-235) followed by blotting with appropriate horseradish

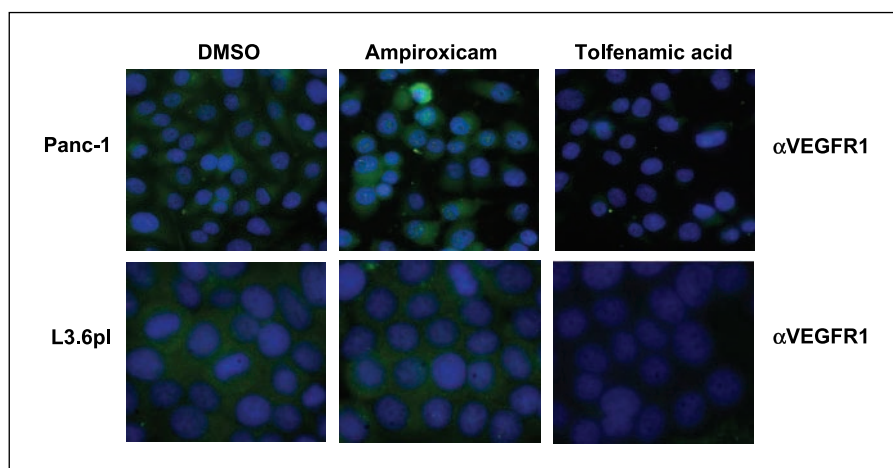
peroxidase-conjugated secondary antibody as described previously (27). After autoradiography, band intensities were determined by a scanning laser densitometer (Sharp Electronics Corp., Mahwah, NJ) using Zero-D Scanalytics software (Scanalytics Corp., Billerica, MA).

**EMSA.** VEGFR1 oligonucleotides [5'-CTCGTCGGCCCCGCCCTCT-3' (sense) and 5'-AGAGGGGGGGGGCCGACGAG-3' (antisense)] were synthesized and annealed, and 5-pmol aliquots were 5'-end labeled using T4 kinase and [ $\gamma$ -<sup>32</sup>P]ATP. A 30- $\mu\text{L}$  EMSA reaction mixture contained  $\sim 100$  mmol/L KCl, 3  $\mu\text{g}$  crude nuclear protein, 1  $\mu\text{g}$  poly(dI-dC), with or without unlabeled competitor oligonucleotide, and 10 fmol radiolabeled probe. After incubation for 20 min on ice, antibodies against Sp1, Sp3, or Sp4 proteins were added and incubated for another 20 min on ice. Protein-DNA complexes were resolved by 5% PAGE as described previously (27). Specific DNA-protein and antibody-supershifted complexes were observed as retarded bands in the gel.

**VEGFB activation of MAPK and cell migration.** Panc-1 cells were pretreated with DMSO, 50  $\mu\text{mol}/\text{L}$  amproxicam, or 50  $\mu\text{mol}/\text{L}$  tolfenamic acid for 48 h and then treated with 50 ng/mL VEGFB for 5 and 10 min. MAPK phosphorylation in the various treatment groups was then determined by Western immunoblot analysis as described above. For migration assays, Panc-1 cells were seeded in triplicates in six-well collagen IV-coated plates and then treated with the selected NSAIDs for 24 h before



**Figure 2.** Tolfenamic acid decreases Sp and VEGFR1 proteins in pancreatic cancer cells. Effects of tolfenamic acid in Panc-1 (A and B) and L3.6pl (C and D) cells. Cells were treated with DMSO, 50  $\mu\text{mol}/\text{L}$  tolfenamic acid (Tol), or 50  $\mu\text{mol}/\text{L}$  amproxicam (Amp) for 48 h, and whole-cell lysates were analyzed by Western blot analysis. The experiment was replicated (thrice), and the Sp and VEGFR1 protein levels were set at 100%. \*,  $P < 0.05$ , significantly decreased expression of Sp1, Sp3, Sp4, and VEGFR2.



**Figure 3.** Immunohistochemical analysis of VEGFR1 in pancreatic cancer cells treated with tolafenamic acid. Panc-1 and L3.6pl cells were treated with DMSO, 50  $\mu\text{mol/L}$  ampiroxicam, or 50  $\mu\text{mol/L}$  tolafenamic acid for 48 h, and cells were immunostained by VEGFR1 antibodies as described in Materials and Methods.

the scratch was made. A scratch through the central axis of the plate was gently made using a sterile pipette tip. Cells were 70% confluent when the scratch was made. Cells were then washed and treated with the DMSO control, selected NSAIDs alone, or NSAIDs and VEGFB. Migration of the cells into the scratch was observed at nine preselected points (three points per well) at 0, 8, and 16 h. Results of this study were obtained at a 16 h time point and one plate was stained using Diff-Quik.

**Immunocytochemistry.** Panc-1 cells were seeded in LabTek chamber slides (Nalge Nunc International, Naperville, IL) at 100,000 cells per well in DMEM/F-12 supplemented with 5% FBS. Cells were then treated with the selected NSAIDs, and after 48 h, the medium chamber was detached and the remaining glass slides were washed in Dulbecco's PBS. The immunostaining for VEGFR1 was determined essentially as described previously (27). Briefly, the glass slides were fixed with cold ( $-20^{\circ}\text{C}$ ) methanol for 10 min, and then slides were washed in 0.3% PBS/Tween 20 for 5 min (twice) before blocking with 5% goat serum in antibody dilution buffer [stock solution: 100 mL PBS/Tween 20, 1 g bovine serum albumin, 45 mL glycerol (pH 8.0)] for 1 h at  $20^{\circ}\text{C}$ . After removal of the blocking solution, VEGFR1 rabbit polyclonal antibody was added in antibody dilution buffer (1:200) and incubated for 12 h at  $4^{\circ}\text{C}$ . Slides were washed for 10 min with 0.3% Tween 20 in 0.02 mol/L PBS (thrice) and incubated with FITC-conjugated goat anti-rabbit antibodies (1:1,000 dilution) for 2 h at  $20^{\circ}\text{C}$ . Slides were then washed for 10 min in 0.3% PBS-Tween 20 (four times). Slides were mounted in ProLonged antifading medium with 4',6-diamidino-2-phenylindole for nuclear counterstaining (Molecular Probes, Inc., Eugene, OR), and coverslips were sealed using Nailslicks nail polish (Noxell Corp., Hunt Valley, MD). Fluorescence imaging was done using Carl Zeiss Axiophoto 2 (Carl Zeiss, Inc., Thornwood, NY) and Adobe Photoshop 5.5 was used to capture the images. Previous studies showed that tolafenamic acid decreased Sp protein expression in an orthotopic model for pancreatic cancer, and tumor tissue from these animals was stained for VEGFR1 essentially as described (27).

**Semiquantitative reverse transcription-PCR analysis.** Panc-1 cells were treated with DMSO (control) or with the indicated concentration of NSAIDs for 24 h before total RNA collection. RNA concentration was measured by UV 260:280 nm absorption ratio, and 200 ng/ $\mu\text{L}$  RNA was used in each reaction for reverse transcription-PCR (RT-PCR). RNA was reverse transcribed at  $42^{\circ}\text{C}$  for 25 min using oligo d(T) primer (Promega) and subsequently PCR amplified of reverse transcription product using 2 mmol/L  $\text{MgCl}_2$ , 1  $\mu\text{mol/L}$  of each gene-specific primer, 1 mmol/L deoxynucleotide triphosphates, and 2.5 units AmpliTaq DNA polymerase (Promega). The gene products were amplified using 22 to 25 cycles ( $95^{\circ}\text{C}$  for 30 s,  $56^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 30 s). The sequence of the oligonucleotide primers used in this study was as follows: VEGFR1 5'-TGGGACAGTAGAAAGGCTT-3' (forward) and 5'-GGTCCACTCCTTACACGACAA-3' (reverse) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5'-AATCCCATCACCATCTTCCA-3' (forward) and 5'-GTCATCATTTGGCAGGT-3' (reverse).

Following amplification in a PCR express thermal cycler (Hybaid US, Franklin, MA), 20  $\mu\text{L}$  of each sample were loaded on a 2% agarose gel containing ethidium bromide. Electrophoresis was done at 80 V in  $1\times$  TAE buffer for 1 h, and the gel was photographed by UV transillumination using Polaroid film (Waltham, MA). VEGFR1, GAPDH, Sp1, and Sp4 band intensity values were obtained by scanning the Polaroid on a Sharp JX-330 scanner; background signal was subtracted; and densitometric analysis was done on the inverted image using Zero-D software. Results are expressed as VEGFR1 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 of cell culture studies are expressed as mean  $\pm$  SD for at least three separate (replicate) experiments for each treatment.

## Results

The VEGFR1 gene promoter contains multiple GC-rich sites that bind Sp proteins (26), and the role of these transcription factors in mediating VEGFR1 expression was initially investigated in Panc-1 cells by RNA interference using small inhibitor RNAs for Sp1 (iSp1), Sp3 (iSp3), and Sp4 (iSp4). In cells transfected with a construct (pVEGFR1A) containing a  $-1,160$  to  $+304$  VEGFR1 promoter insert linked to luciferase cotransfection with iSp1, iSp3, and iSp4 significantly decreased luciferase activity (42–33%; Fig. 1A). Similar results were observed in Panc-1 cells cotransfected with iSp1, iSp3, and iSp4 and deletion constructs pVEGFR1B and pVEGFR1C, which contain the  $-886$  to  $+304$  and  $-334$  to  $+304$  VEGFR1 promoter inserts, respectively (Fig. 1B and C). Previous studies have shown that Sp1, Sp3, and Sp4 are expressed in Panc-1 cells (30), and the results suggest that VEGFR1 expression is Sp dependent. Further confirmation that Sp proteins regulated VEGFR1 expression was determined in parallel studies, which show that transfection with the small inhibitory RNAs iSp1, iSp3, and iSp4 decreased Sp1, Sp3, and Sp4 protein levels, respectively, compared with cells transfected with a nonspecific small inhibitory RNA (iNS; Fig. 1D). Moreover, decreased Sp protein expression was paralleled by decreased levels of VEGFR1 protein (Fig. 1D), and these results were observed in three replicate experiments showing that Sp1, Sp3, and Sp4 proteins were significantly decreased only by their corresponding small inhibitory RNAs, whereas VEGFR1 was significantly decreased by iSp1, iSp3, and iSp4 (Fig. 1D).

Previous studies in this laboratory showed that the NSAID tolafenamic acid induced proteasome-dependent degradation of

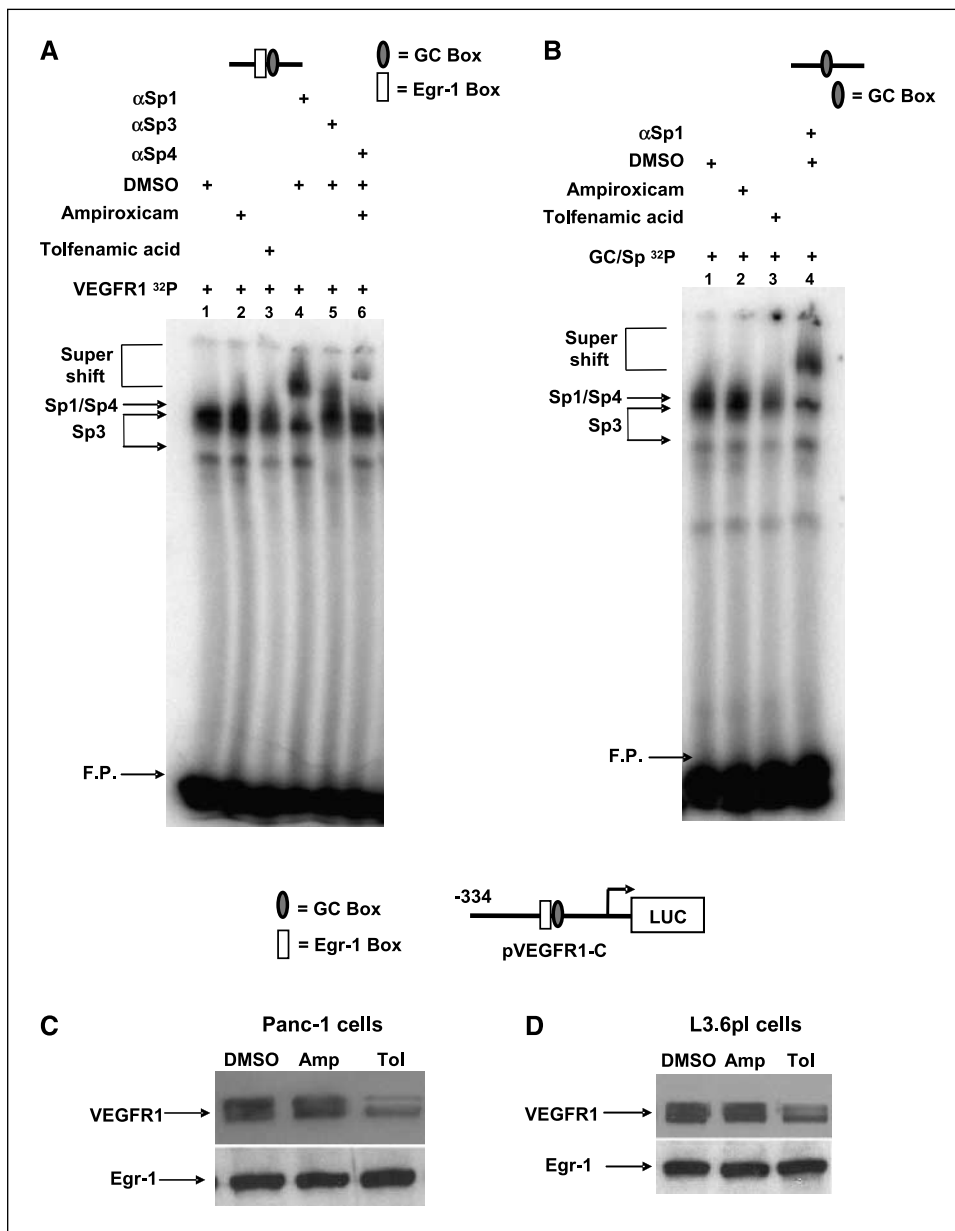


Sp1, Sp3, and Sp4 in pancreatic cancer cells, whereas ampiroxicam did not affect expression of these transcription factors (27). Treatment of Panc-1 cells with solvent (DMSO), 50  $\mu\text{mol/L}$  ampiroxicam, or 50  $\mu\text{mol/L}$  tolfenamic acid resulted in decreased expression of Sp1, Sp3, Sp4, and VEGFR1 proteins as determined by Western blot analysis (Fig. 2A). Replicate (3) experiments showed that only tolfenamic acid significantly decreased Sp proteins and VEGFR1 in Panc-1 cells (Fig. 2B), whereas ampiroxicam did not affect expression of these proteins. A parallel experiment was also carried out in the highly metastatic L3.6pl pancreatic cancer cell line, which also expresses Sp1, Sp3, and Sp4 (27). The results show that 50  $\mu\text{mol/L}$  tolfenamic acid but not DMSO or 50  $\mu\text{mol/L}$  ampiroxicam significantly decreased levels of Sp1, Sp3, Sp4, and VEGFR1 proteins in L3.6pl cells (Fig. 2C and D).

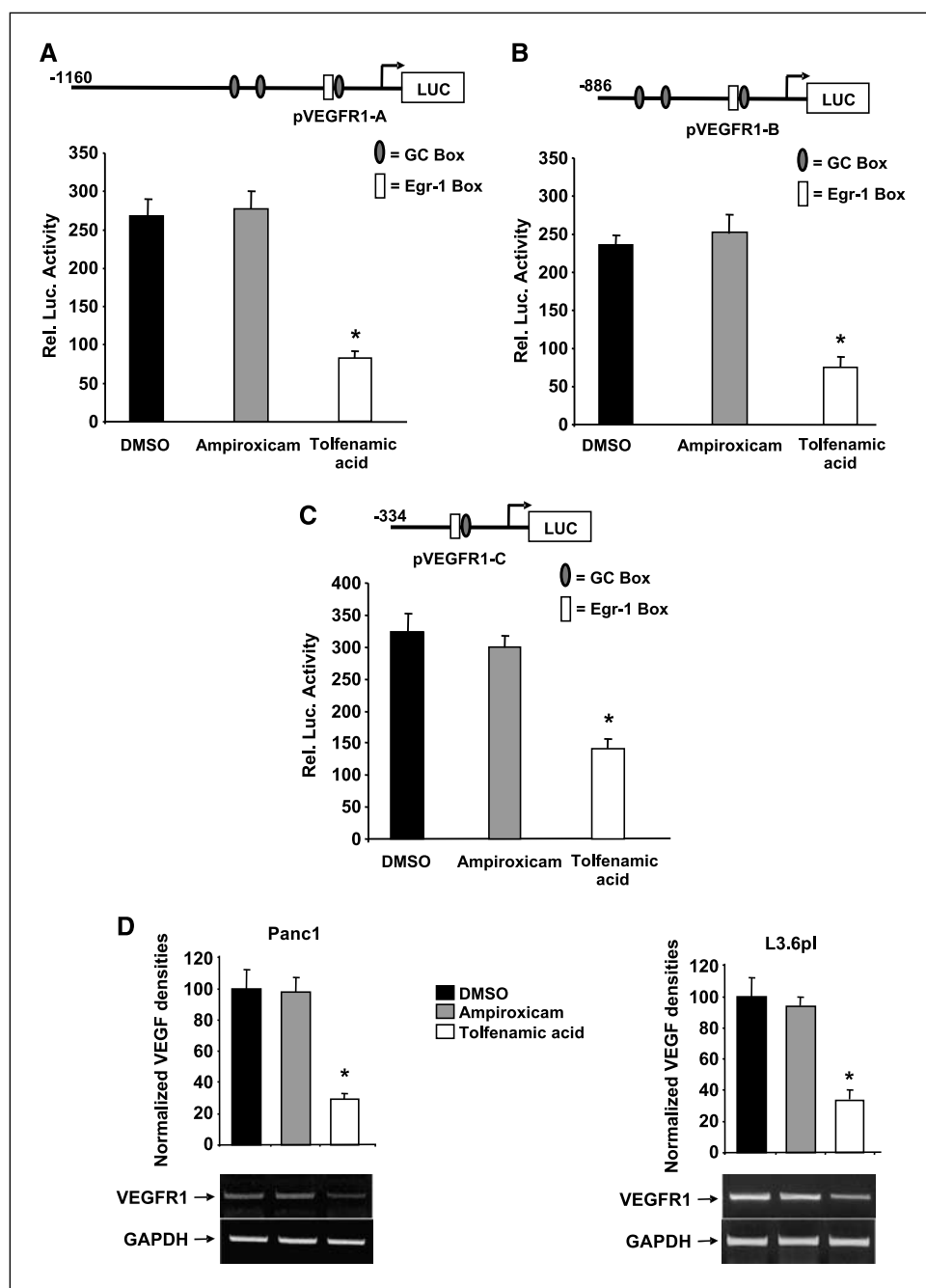
Confirmation that tolfenamic acid decreased VEGFR1 expression in Panc-1 and L3.6pl cells was determined by immunohistochemical analysis (Fig. 3). In Panc-1 cells, VEGFR1 immunostaining was

observed in cells treated with DMSO or ampiroxicam, whereas tolfenamic acid decreased VEGFR1 staining. VEGFR1 expression was lower in L3.6pl cells; however, the pattern of treatment-related effects were comparable in both Panc-1 and L3.6pl cells.

The proximal region of the VEGFR1 promoter contains GC-rich and Egr-1 sites (26), and the effects of tolfenamic acid on VEGFR1 expression through degradation of Sp proteins cannot exclude a role for Egr-1 in this response. Figure 4A and B compare the gel mobility shift and antibody supershift patterns of nuclear extracts from Panc-1 cells bound to the proximal region of the VEGFR1 promoter containing both GC-rich and Egr-1 sites (VEGFR1<sup>32P</sup>) or GC-rich sites alone (GC/Sp<sup>32P</sup>). Extracts from cells treated with DMSO, ampiroxicam, and tolfenamic acid gave similar Sp1, Sp3, and Sp4-DNA retarded bands using both radiolabeled oligonucleotides (Fig. 4A and B, lanes 1–3); however, the retarded band intensity was markedly decreased using extracts from cells treated with tolfenamic acid. Antibodies against Sp1 (Fig. 4A and B,



**Figure 4.** Interactions of Sp proteins with VEGFR1 promoter sequences. Panc-1 cells were treated with DMSO, 50  $\mu\text{mol/L}$  ampiroxicam, or 50  $\mu\text{mol/L}$  tolfenamic acid for 48 h, and nuclear extracts were incubated with VEGFR1<sup>32P</sup> (A) or GC/Sp<sup>32P</sup> (B) oligonucleotides in the presence or absence of Sp1, Sp3, or Sp4 antibodies and analyzed in a gel mobility shift assay as described in Materials and Methods. Arrows, specific Sp protein bands and antibody-supershifted complexes. Effects of tolfenamic acid on VEGFR1 and Egr-1 protein expression in Panc-1 (C) and L3.6pl (D) cells. Cells were treated with DMSO, 50  $\mu\text{mol/L}$  ampiroxicam, or 50  $\mu\text{mol/L}$  tolfenamic acid for 48 h, and whole-cell lysates were examined by Western blot analysis as described in Materials and Methods.



**Figure 5.** Effects of tolfenamic acid on VEGFR1 promoter and mRNA expression. Transfection with pVEGFR1A (A), pVEGFR1B (B), and pVEGFR1C (C). Panc-1 cells were transfected with pVEGFR1 constructs and treated with DMSO, 50  $\mu\text{mol/L}$  ampiroxicam, or 50  $\mu\text{mol/L}$  tolfenamic acid, and luciferase activity was determined as described in Materials and Methods. Columns, mean of replicate experiments for each treatment group; bars, SD (3). \*,  $P < 0.05$ , significantly decreased activity. D, decreased VEGFR1 mRNA in Panc-1 and L3.6pl cells. Panc-1 or L3.6pl cells were treated with DMSO, 50  $\mu\text{mol/L}$  ampiroxicam, or 50  $\mu\text{mol/L}$  tolfenamic acid for 24 h, and relative mRNA expression was determined by semiquantitative RT-PCR as described in Materials and Methods. Results are shown for a single experiment and similar data were obtained in a replicate experiment.

lane 4) supershifted the large Sp1-DNA retarded band and both Sp3 and Sp4 antibodies also induced formation of supershifted complexes (Fig. 4A, lanes 5 and 6). These results show that the presence or absence of the Egr-1 site did not affect Sp protein interactions with the VEGFR1 promoter. Moreover, Western blot analysis of whole-cell lysates from Panc-1 and L3.6pl cells after treatment with DMSO, ampiroxicam, or tolfenamic acid showed that only the latter compound decreased VEGFR1 protein levels, whereas Egr-1 protein was unchanged in all treatment groups (Fig. 4C and D).

Because Sp proteins regulate expression of VEGFR1, we further investigated the effects of tolfenamic acid on luciferase activity in cells transfected with pVEGFR1A, pVEGFR1B, and pVEGFR1C and

on VEGFR1 mRNA levels. Tolfenamic acid but not DMSO or ampiroxicam decreased luciferase activity in Panc-1 cells transfected with pVEGFR1A, pVEGFR1B, and pVEGFR1C (Fig. 5A-C), and tolfenamic acid also decreased VEGFR1 mRNA levels in Panc-1 and L3.6pl cells (Fig. 5D).

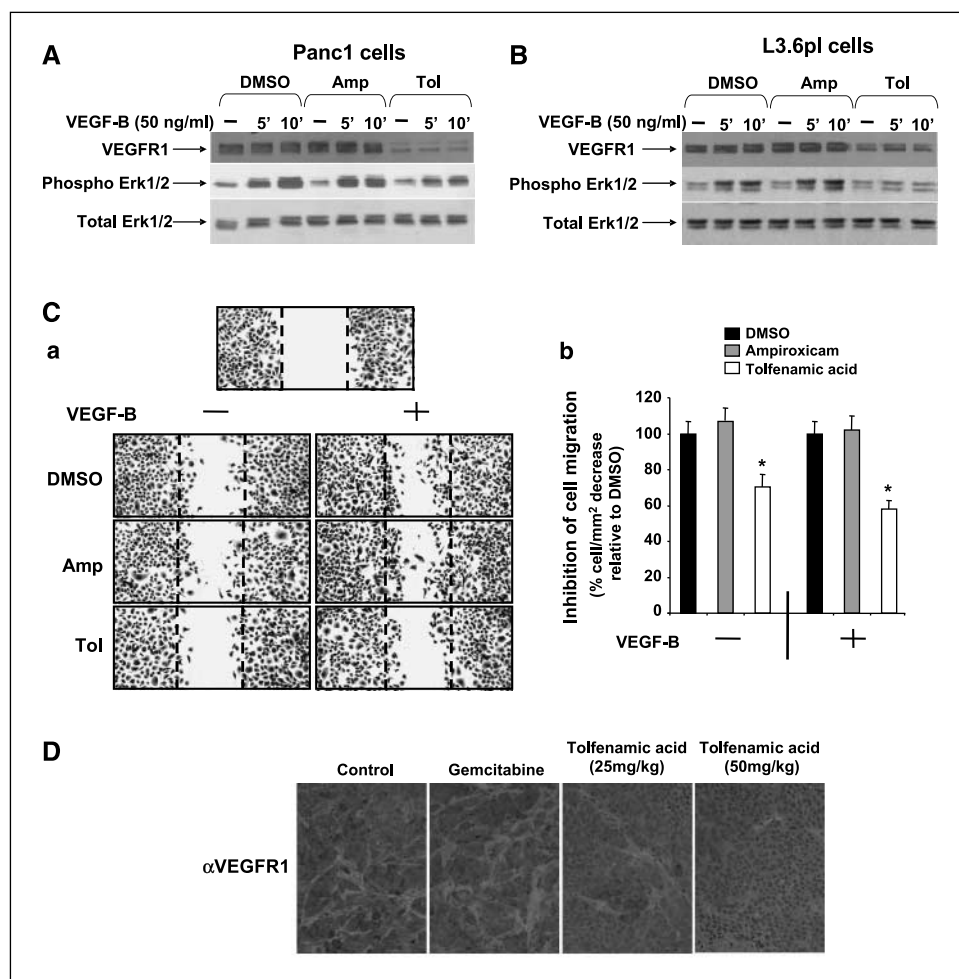
Previous studies have reported that activation of VEGFR1 by VEGFB in pancreatic cancer cells results in enhanced phosphorylation of MAPK and increased cell migration and invasion (23). We therefore investigated the role of Sp proteins in mediating these responses by determining the effects of tolfenamic acid on activation of MAPK by VEGFB. Figure 6A and B shows that, after treatment of Panc-1 and L3.6pl cells, respectively, with VEGFB for 5 or 10 min, there was increased phosphorylation of MAPK1/2 in

cells pretreated with DMSO or 50  $\mu\text{mol/L}$  ampiroxicam for 36 h, and VEGFR1 and total MAPK1/2 levels were unchanged. In contrast, pretreatment with 50  $\mu\text{mol/L}$  tolfenamic acid decreased VEGFR1 expression and this was paralleled by decreased phosphorylated MAPK1/2, whereas total MAPK protein levels were not affected. Thus, inhibition of VEGFB/VEGFR1 signaling by tolfenamic acid was related to decreased VEGFR1 through degradation of Sp proteins and this inhibitory response was similar to that observed in a previous study using neutralizing VEGFR1 antibodies (23). The importance of Sp proteins in VEGFR1-dependent Panc-1 cell migration was determined using a cell migration assay on collagen IV-coated plates. The results show that VEGFB induces Panc-1 cell migration in all treatment groups (Fig. 6C); however, in cells treated with DMSO (set at 100%), 50  $\mu\text{mol/L}$  ampiroxicam, or 50  $\mu\text{mol/L}$  tolfenamic acid, cell migration, which was observed in the absence of VEGFB, was only significantly inhibited by tolfenamic acid (Fig. 6C). VEGFB enhanced cell migration in this assay and, in cells cotreated with VEGFB plus tolfenamic acid, the latter compound significantly inhibited VEGFB-induced cell migration. Previous studies using an orthotopic model for pancreatic cancer (using L3.6pl cells) showed that tolfenamic acid (50 mg/kg) but not gemcitabine decreased Sp proteins, tumor growth, and angiogenesis (27), and tumor tissue from these animals was also stained for VEGFR1 (Fig. 6D). The results show decreased VEGFR1 expression only in tumors from tolfenamic acid-treated mice. These results show that Sp proteins

regulate VEGFR1-mediated responses, including cell migration in pancreatic cancer cells, indicating that agents, such as tolfenamic acid that target Sp proteins (for degradation), are an important new class of mechanism-based antiangiogenic compounds that decrease Sp-dependent expression of VEGF, VEGFR2, and VEGFR1.

## Discussion

VEGF and related angiogenic growth factors and their receptors play a critical role in tumorigenesis and contribute significantly to cancer cell progression and metastasis (1, 4–6). Not surprisingly, VEGF/VEGFR signaling pathways have been extensively targeted for cancer chemotherapy. Antiangiogenic compounds initially discovered in the Folkman laboratory have multiple mechanisms of action (4, 6); however, several alternative approaches have also been reported and these include antibodies that block VEGF and/or VEGFR (39–43) and tyrosine kinase inhibitors that block VEGFR kinase signaling (44, 45). Other approaches include development of arginine-rich peptides that block VEGF action (46) or by blocking downstream factors, such as Src family kinases that mediate some of the VEGFR1-dependent responses in colon cancer cells (47). A construct expressing domains of both VEGFR1 and VEGFR2 that tightly binds VEGF through the extracellular domain of VEGFR1 (VEGF-Trap) has also been used to inhibit tumor growth and metastasis in animal models (48).



**Figure 6.** Tolfenamic acid inhibits activation of VEGFR1 in pancreatic cancer cells. Inhibition of ERK1/2 phosphorylation in Panc-1 (A) and L3.6pl (B) cells. Cells were pretreated with DMSO, 50  $\mu\text{mol/L}$  ampiroxicam, or 50  $\mu\text{mol/L}$  tolfenamic acid for 36 h; VEGFB (50 ng/mL) was added for 5 or 10 min, and whole-cell lysates were obtained and analyzed by Western blot analysis as described in Materials and Methods. C, a, cell migration assay. Panc-1 cells were treated with DMSO, 50  $\mu\text{mol/L}$  ampiroxicam, or 50  $\mu\text{mol/L}$  tolfenamic acid for 24 h. VEGFB (50 ng/mL) was added and the inhibition of cell migration (relative to DMSO-treated cells set at 100%) was determined 16 h after addition of VEGFB as described in Materials and Methods. b, the experiments were replicated (thrice). Columns, mean; bars, SD. \*,  $P < 0.05$ , significantly decreased cell migration. D, immunostaining of pancreatic tumors. Pancreatic tumors from athymic nude mice treated with solvent (control), gemcitabine (50 mg/kg), or tolfenamic acid (25 and 50 mg/kg; ref. 27) were stained with VEGFR1 antibodies as described in Materials and Methods and in a previous report (27).

Previous studies have shown that expression of both VEGF and VEGFR2 in pancreatic and other cancer cell lines was regulated by Sp1, Sp3, and Sp4, and RNA interference with small inhibitory RNAs targeting these proteins decreased VEGF and VEGFR2 expression (30, 38). We also reported recently that, in pancreatic cancer cells and tumors, the NSAID tolfenamic acid induced proteasome-dependent degradation of Sp1, Sp3, and Sp4, and not surprisingly, tolfenamic acid inhibited angiogenesis and decreased liver metastasis in an orthotopic model of pancreatic cancer using the highly metastatic L3.6pl cells (27). Because VEGFR1 also plays a pivotal role in pancreatic tumor migration and invasion (23, 25), we investigated the molecular mechanism of VEGFR1 regulation in pancreatic cancer cells. Takeda et al. (26) identified *VEGFR1* as a gene regulated by the basic helix-loop-helix endothelial PAS domain protein 1, which forms a heterodimer with hypoxia-inducible factor 1B to activate VEGFR1 and other proangiogenic genes. However, the *VEGFR1* gene has three consensus GC-rich motifs that bind Sp proteins, and results from RNA interference studies show that knockdown of Sp1, Sp3, or Sp4 also decreased VEGFR1 protein expression in pancreatic cancer cells (Fig. 1). Moreover, similar results were observed using a series of deletion constructs containing VEGFR1 promoter inserts (Fig. 1).

These results suggest that, like VEGF and VEGFR2 (30, 38), VEGFR1 expression in pancreatic cancer cells is Sp dependent; therefore, compounds, such as tolfenamic acid, which decrease Sp1, Sp3, and Sp4 (Fig. 3), should also decrease VEGFR1. This was confirmed in a series of experiments showing that tolfenamic acid but not the NSAID amproxicam (a negative control; ref. 27) decreases VEGFR1 protein (Figs. 2A and C, 3, and 4) and mRNA (Fig. 5D) as well as luciferase activity in cells transfected with VEGFR1 constructs (Fig. 5A and B). Moreover, decreased VEGFR1 expression (Fig. 6D) was observed in pancreatic tumors from mice treated with tolfenamic acid and this was accompanied by decreased levels of VEGF and Sp protein in these tumors (27). Because VEGFR1 mediates VEGFB-induced migration and invasion of pancreatic and colon cancer cells (23–25), we also investigated the role of Sp proteins in mediating Panc-1 cell migration in a “scratch” test in monolayer cultures grown on collagen IV-coated plates (Fig. 6C). Previous reports show that VEGFA and VEGFB induce migration of colon and pancreatic cancer cells using a Boyden chamber assay (23–25), and results in Fig. 6C confirm that VEGFB also induced migration of Panc-1 cells. Inhibition of VEGFB-induced migration of colon and pancreatic cancer cells was observed in cells treated with the VEGFR1 antibody (18F1; refs. 23–25), and similar inhibitory effects were observed in this study in Panc-1 cells cotreated with

VEGFB plus tolfenamic acid (Fig. 6C). In contrast, tolfenamic acid also inhibited Panc-1 cell migration in solvent-treated (DMSO) cells, whereas the VEGFR1 antibody did not affect migration of untreated/solvent control cells. The differences between tolfenamic acid and VEGFR1 antibodies must be due to the overall decrease in VEGFR1 levels (Fig. 2) in cells treated with tolfenamic acid, whereas the antibodies do not affect VEGFR1 expression. We also examined VEGFB-induced phosphorylation of MAPK in Panc-1 and L3.6pl cells and, like the VEGFR1 antibody (23), tolfenamic acid decreased MAPK phosphorylation in Panc-1 and L3.6pl cells (Fig. 6A and B). This was consistent with the parallel down-regulation of VEGFR1 protein in these cells. Thus, the inhibition of VEGFB-induced migration of pancreatic cancer cells by tolfenamic acid is also accompanied by inhibition of VEGFR1-dependent downstream signaling. Amproxicam, a NSAID that does not induce Sp protein degradation in Panc-1 cells, was also used as a control in the cell migration and MAPK phosphorylation studies, and this compound was inactive in all assays.

In summary, results of this study show that, like VEGF and VEGFR2, VEGFR1 is also regulated by Sp proteins in pancreatic cancer cells. Although extensive studies have not been carried out on Sp protein expression in tumors, there is evidence that Sp1 is overexpressed in cancer cells and tumors (16, 35, 49–52), and this is also consistent with the up-regulation of angiogenic factor expression in many cancers. Selective cyclooxygenase-2 (Cox-2) inhibitors have also shown promise as anticancer agents and these compounds also decrease Sp1 and Sp4 but not Sp3 protein expression in colon and pancreatic cancer cells (27, 31). Because the use of Cox-2 inhibitors has now been limited, selective NSAIDs, such as tolfenamic acid, may be preferable for applications in cancer chemotherapy. Results of this study on VEGFR1 coupled with previous reports showing that Sp proteins also regulate expression of VEGF and VEGFR2 (30, 38) suggest that compounds, such as tolfenamic acid that target Sp transcription factor degradation, represent an important class of antiangiogenic drugs for treatment of pancreatic and other cancers where Sp proteins play a role in cell growth and angiogenesis.

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## Regulation of Vascular Endothelial Growth Factor Receptor-1 Expression by Specificity Proteins 1, 3, and 4 in Pancreatic Cancer Cells

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