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Essential contribution of Ets-1 to constitutive Pim-3 expression in human pancreatic cancer cells

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We previously demonstrated that the proto-oncogene Pim-3 with serine/threonine kinase activity was aberrantly expressed in cancer cells but not in the normal cells of the pancreas. In order to elucidate the molecular mechanism underlying aberrant Pim-3 expression in pancreatic cancer cells, we constructed luciferase expression vectors linked to 5′-flanking deletion mutants of the human *Pim-3* gene and transfected human pancreatic cancer cells with the resultant vectors. The region up to –264 bp was essential for constitutive *Pim-3* gene expression, and the mutation in the Ets-1 binding site (between –216 and –211 bp) reduced luciferase activities. Moreover, Ets-1 mRNA and protein were constitutively expressed together with Pim-3 in human pancreatic cancer cell lines. Chromatin immunoprecipitation assay demonstrated constitutive binding of Ets-1 to the 5′-flanking region of human *Pim-3* gene between –249 and –183 bp. Pim-3 promoter activity and its protein expression were induced by transfection with wild type-Ets-1 and were reduced by transfection with dominant negative-Ets-1 or Ets-1 small-interfering RNA (siRNA). Furthermore, dominant negative-Ets-1 and *Ets-1* siRNA reduced the amount of Bad phosphorylated at its Ser[112] and induced apoptosis, when they were transfected into human pancreatic cancer cells. Finally, *Pim-3* cDNA transfection reversed Ets-1 siRNA-induced increase in apoptosis and decrease in Bad phosphorylation at its Ser[112]. These observations would indicate that the transcription factor Ets-1 can induce aberrant Pim-3 expression and subsequently prevent apoptosis in human pancreatic cancer cells. (*Cancer Sci* 2009; 100: 396–404)

Pancreatic cancer is often diagnosed at its advanced stage, when liver metastasis and peritoneal dissemination are already evident. The prognosis is further worsened by resistance to chemotherapy and/or radiotherapy, which are standard treatments for advanced pancreatic cancer. Thus, pancreatic cancer continues to be a highly fatal cancer, with less than 5% overall 5-year survival rates.[1] Thus, a better understanding of the molecular mechanism of pancreatic carcinogenesis and subsequent identification of a novel molecular target are necessary to develop novel and more effective therapeutics.

We previously observed that Pim-3, a member of the proto-oncogene Pim family with serine/threonine kinase activity, was aberrantly expressed in precancerous and cancerous lesions but not normal tissues of endoderm-derived organs including the pancreas, liver, colon, and stomach.[2–5] Other Pim family members, Pim-1 and Pim-2, are presumed to be crucially involved in the carcinogenesis of various organs.[6–9] Deneen and colleagues demonstrated that *Pim-3* gene transcription was enhanced in EWS/ETS-induced malignant transformation of NIH 3T3 cells.[10] These observations suggest that aberrantly expressed Pim-3 could contribute to carcinogenesis of endoderm-derived organs such as the pancreas and liver. This assumption was further supported by our previous observation that Pim-3 can inactivate a potent proapoptotic molecule, Bad, in human pancreas and colon carcinoma cell lines, by phosphorylating its Ser[112] and eventually promoting survival.[2,4]

Kinase activation generally requires a post-translational modification, particularly phosphorylation in its regulatory domain. In contrast, Pim-1 is constitutively active without any further alteration in its conformation, probably due to its lack of any regulatory domains.[11] Pim-3 shows a high sequence identity with Pim-1 even in the kinase domain and also lacks any regulatory domains.[3] Thus, it is probable that Pim-3 can exhibit its kinase activity when its gene product is translated after transcription. Because gene transcription is regulated by transcription factors, which bind to specific *cis*-elements present in the promoter region of the target gene, the identification of the transcription factor(s) regulating *Pim-3* gene transcription can elucidate the molecular mechanism of aberrant *Pim-3* gene expression and its protein expression during the course of carcinogenesis.

In the present study, we tried to characterize the *cis*-elements and the transcription factor(s) required for constitutive *Pim-3* gene expression in human pancreatic cancer cell lines. We revealed that the transcription factor Ets-1 constitutively bound to the promoter region of the human *Pim-3* gene and was colocalized with Pim-3 protein in human pancreatic cancer cells. Transfection of dominant negative Ets-1 and Ets-1 small-interfering RNA (siRNA) decreased Pim-3 protein expression and the amount of phospho-Ser[112]Bad, thereby enhancing apoptosis. Moreover, *Pim-3* cDNA transfection reversed Ets-1 siRNA-induced increase in apoptosis and decrease in Bad phosphorylation at its Ser[112]. These observations suggest that Ets-1 was involved in constitutive expression of Pim-3, a kinase that can counteract the apoptosis of human pancreatic cancer cells.

Materials and Methods

Cell culture and antibodies. The human pancreatic cancer cell lines PCI35, PCI55, PCI66,[12] MiaPaca-2,[13] and PANC-1[14] were maintained in RPMI-1640 medium, while L3.6pl[15] was maintained in minimum essential medium (MEM) medium. All media were supplemented with 10% fetal bovine serum (FBS) and the cells were cultured in 5% CO_2 at 37°C. The following antibodies were used: mouse anti-Bad and rabbit anti-Ets-1 (C-20) antibodies, rabbit antiphospho-Ser[112]Bad antibodies, rabbit anti-β-actin antibodies, Alexa Fluor 488 donkey antirabbit IgG and Zenon Alexa Fluor 555 rabbit IgG labeling kits (Molecular Probes Inc.), and Immunopure peroxidase-conjugated goat antimouse and goat antirabbit antibodies (Pierce Biotechnology, Rockford, IL, USA). Rabbit anti-Pim-3 antibodies were prepared as described previously.[4]

Plasmids. A series of Pim-3 promoter fragments were amplified by polymerase chain reaction (PCR) using human

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genomic DNA as a template and subcloned into pGL4 *firefly* luciferase reporter gene vector. The structure and fidelity of the resulting constructs were confirmed by restriction mapping and sequencing. Plasmids were purified using the Plasmid DNA Purification NucleoBond PC2000 (Macherey-Nagel). At least two independent plasmid preparations were used for each construct in the following reporter assays. Wild-type Ets-1 (WT-Ets-1) and dominant-negative Ets-1 (DN-Ets-1) expression vectors were a kind gift from H. Sato (Kanazawa University). DN-Ets-1 lacks a transcription activation domain, which corresponds to amino acid residues 306–441.[16] Human *Pim-3* full-length cDNA was subcloned into pcDNA4.

Dual Luciferase reporter assay. For reporter gene assays, 1×10^5 PCI55 and MiaPaca-2 cells were cultured in a 24-well plate for 16–18 h. Then, the cells were transfected with the pGL4 vectors containing deleted or mutated Pim-3 promoter fragment (100 ng) by using Lipofectamine LTX and Plus (Invitrogen) according to the manufacturer's instructions. Transfection efficiency was normalized by cotransfecting 5 ng of the SV40 promoter *Renilla* luciferase reporter gene (pRL-SV40). In some experiments, WT-Ets-1 or DN-Ets-1 expression vector were cotransfected together with luciferase expression vectors. Cells were lyzed in passive lysis buffer 36 h after the transfection. Luciferase assays were conducted with the use of the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase values were normalized to Renilla luciferase activities and were expressed as a fold-increase in luciferase activities compared with pGL4 empty vector control.

RNA isolation and semiquantitative reverse transcription–PCR (RT-PCR). Total RNAs were extracted from PCI35, PCI55, PCI66, MiaPaca-2, PANC-1, and L3.6pl with RNA-Bee (Tel.Test, Friends Woods, TX, USA) according to the manufacturer's instructions. After treatment with ribonuclease-free deoxyribonuclease (DNase) I, 2 µg of total RNA was reverse-transcribed at 42°C for 1 h in 20-µL reaction mixture containing mouse moloney leukemia virus reverse transcriptase and hexanucleotide random primers. Serially two-fold diluted cDNA products were amplified for GAPDH (glyceraldehydes-3-phosphate dehydrogenase) using the specific set of primers with 25 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min in 25 µL of reaction mixture containing Taq polymerase to evaluate the amount of the transcribed cDNA. Then equal amounts of cDNA products were amplified for the *Pim-3* and *Ets-1* genes using the following sets of primers (Pim-3: forward primer, 5′-AAGCAGTGACC-TCTACCCCTGGTGACC-3′, reverse primer, 5′-CAAATAAATT-AAACAATAAATAGCCCC-3′; Ets-1: forward primer, 5′-AAGT-CGTCACCCCAGACAAC-3′, reverse primer, 5′-TTCCTCTTT-CCCCATCTCCT-3′) with 30 cycles of 94°C for 30 s, 58°C for 30 s, and 68°C for 1 min. The resultant PCR products were fractionated on 1.5% agarose gel and visualized by ethidium bromide staining under ultraviolet light transillumination.

Immunohistochemical analysis. Human pancreatic cancer tissues were obtained from patients upon surgery at Toyama University Hospital for pancreatic cancer. The patients provided consent for the use of tumor tissue for clinical research and the Toyama University Ethical Committee approved the research protocol. Pancreatic tissue, which was apart from the edge of tumor foci and was judged histologically to be free from adenocarcinoma cells, was used as a normal tissue control. Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol (70–100%). After incubation with 0.3% hydrogen peroxide, sections were digested by trypsin as antigen retrieval, followed by blocking with Non-Specific Staining Blocking reagent (DakoCytomation, Glostrup, Denmark). Subsequently, the slides were treated with rabbit anti-Pim-3 IgG (3 µg/mL), followed by the incubation with goat antirabbit IgG at room temperature for 1 h. For Ets-1 immunohistochemical staining, after deparaffinization and rehydration, the sections were heated in a microwave in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval. After blockade of endogenous peroxidase and non-specific protein binding, the slides were treated with rabbit anti-Ets-1 IgG, followed by incubation with goat antirabbit IgG antibodies. As a negative control, rabbit IgG was used instead of specific primary antibodies. Then, the immunoreactivity was visualized by using the Vectastain Elite ABC kit and Vectastain DAB substrate kit (both Vector Laboratories). The slides were counterstained with ChemMate Hematoxylin (Dako Cytomation), mounted, and observed under a microscope (BX-50; Olympus, Tokyo, Japan).

Double-color immunofluorescence analysis. Human pancreatic cancer cell lines were seeded onto a Laboratory-Teck chamber slide (Nalge Nunc International, Naperville, IL, USA) and incubated at 37°C with 5% CO_2. Forty-eight hours later, they were fixed with cold acetone at –20°C for 5 min. Immunostaining was performed by incubating the slides with rabbit polyclonal anti-Pim-3 IgG (3 µg/mL) at 4°C, overnight, followed by incubation with Alexa Fluor 488 donkey antirabbit antibodies (1:100) for 1 h at room temperature in the dark. After washing with tris-buffed saline–0.1% Tween 20 (TBS-T), the sections were further incubated with Zenon Alexa Fluor 555–labeled rabbit polyclonal anti-Ets-1 (2 µg/mL) for 1 h at room temperature in the dark. Finally, the sections were washed with TBS-T and cell images were captured on a Carl Zeiss LSM510 confocal microscope (Carl Zeiss Microimage, Thornwood, NY, USA).

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed using the ChIP assay kit (Upstate, Lake Placid, NY, USA) according to the manufacturer's instructions. Briefly, PCI55 cells in a subcofluent state were cross-linked with 1% formaldehyde for 10 min at 37°C, harvested in sodium dodecylsulfate (SDS) lysis buffer, and the chromatin was sheared to fragments of 500 bp by sonication. Then, anti-Ets-1 polyclonal IgG or control rabbit IgG were added to each aliquot of precleared chromatin and incubated at 4°C overnight. Then, Protein A agarose was added to the mixtures, which were incubated for additional 2 h at 4°C. After reversing the cross-links, DNA was recovered by phenol–chloroform extraction and subsequent ethanol precipitation. The resultant DNA underwent PCR amplification using the forward primer (5′-CCAAGCG-CAGGTCGCCTCCCGC-3′) and the reverse primer (5′-GAAGGTCACCCGCCGGCCCCGAA-3′) to amplify a 67 bp-long fragment corresponding to the region spanning from –249 bp to –183 bp of the human *Pim-3* promoter harboring the Ets-1 binding site. The PCR conditions consisted of an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 68°C for 1 min, with a final extension at 68°C for 5 min. The resultant PCR products were fractionated on 12% polyacrylamide gel and visualized by ethidium bromide staining under ultraviolet light transillumination.

Transient transfection and western blot analysis. Subconfluent MiaPaca-2 cells in a 10-cm dish were transfected with either 15 µg of WT-Ets-1 or 15 µg of DN-Ets-1 expression vector by using Lipofectamine LTX and Plus, according to the manufacturer's instructions. As a negative control, the cells were transfected with 15 µg of pCEP4 empty vector. In some experiments, MiaPaca-2 cells in a 10-cm dish were transfected with the 100 nM (final concentration) of Ets-1 siRNA (Ambion, siRNA ID: 115623) together with or without 10 µg of *Pim-3* cDNA expression vector by using Lipofectamine 2000, according to the manufacturer's instructions. As a negative control, the cells were treated with the same amount of Lipofectamin 2000. Whole cell lysates were prepared 48 h later by using CelLytic-M mammalian Cell Lysis/Extraction Reagent (Sigma Chemicals), with a complete protease inhibitor cocktail (Roche Diagnostics). After centrifugation for 15 min, aliquots (100 µg) of the obtained supernatants were subjected to immunoblotting analysis to

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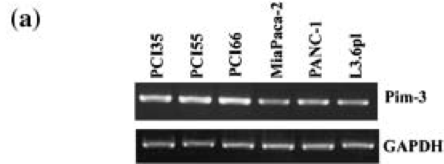

(b)

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–2130 GTGCAAGGGTCCTCGCTGCACACACAAGCACGTACAACAGTAAATCGACAACATACACCAGTGTCTGGAC
–2060 AGACGTGGTTACAAAGATGTCACACTTTAGGGAAAGGGCTGCCTCTGGCCCTGCCCTGCCCTCCACTCCA
                               STAT3
–1990 GGGCCAGGCTGCTGGACCCCCACGCAGGAGCCCTCCGGAGGCTTTCACCGAGGCCTGTGTGGCAGGAATC
–1920 CCATCTGGAGGCCTGGAGGAATCTCCAGCACACCTGGAGTGAGCGGGAGGAGGGCCGTGGAGGACAGGCA
–1850 CACCGTGCTTTCTGAAGGAGCAAGTCCTCCTGGATCTTAGTTAAGGGACACTTGAGGGTTTCAGAGCAAG
                                          STAT3                 STAT3
–1780 ACCCGGGGCCTAGGGAGAGCCAGGGTGGGGTGCCTGCCCGCCAGAGCCTGGTCTTCTACGGCCAGGATGA
–1710 CTCAGGGAAGACTGGCCTGGAGCCTGACATGTCAGACTCAAGGAAGACTAGCCTGACATGTCAGGCTGAG
–1640 GACCCTCGCCCCTCCAGACTGACATGAACTCAACTGTAGTGGTCACCCCCTAGACCTACCGTGCTCTCCA
–1570 GCATCTCTGCCCACCTCTGCTGCGTGTCCAGCACAGAAGAGGGCAGCCCCACTGGACCCTCAGGTAGGTC
–1500 CCCGGGAGGACCCACATGGGCAGCAGCCCCTCCTCCTTCGCAGGTGCTGTGTGGATACTGCTGGAGGGGA
–1430 AGGCTTGGGGTGTCATCCTACCAGGGATCTGATCTCGGGGGAGCCCTGTGCTGGGTGCGTGGTGGGTGGG
–1360 GGAAGGCTAACCCACCAGCCCCAACCCTGCCCTCGTACTGCGGGAGAGAACTGGACAGAGCTCTGGGAAG
–1290 CAGCGGCTGGAGGGGGAGGGGCCTGGGGACAAACAATGTCGCGTTGGCTTTGATCCTCAGGACCCAAGGG
–1220 ACAGGAAGAGATGAGCTAGGCAAGAGAGGAGCCCCCAGGAGCACAGGCATGGGCCCAGGCAGCGGGCGAC
–1150 GAAGCGAGCCCCAGAGGGCGCCCAGATGGCAGGTGGGCCTGGGCCAGTCCCGAGAGCCACAGCCTCAGAA
–1080 GAGCTGAACAGGGGAGTGGTGTGTGAGTGGTCAGCGCAGGGACAGCGATGGTGACTGTATAGAGATGAGA
–1010 GGTCAGACTTAGGGGAGGCTGGAAGCTCCGAGGCAAGTGTGTGTGCGCGCTGACGGTCACACCAGACCGC
 –940 CCGGGGATGTCTGCCACAATATTTAGACCCCTGAGGGCCACTGAGAACGCCTGGCAGTGCCAAGAGGACC
 –870 AGCCCCTGGGCCCTCTGGCCCCCAACTCTCAACCCACTAGCCCTGGGGCTGGCGGGTGGCCACTCACGTC
 –800 CTGGAACCCTGAGCTGCTCTCAGGCCGGTGACTTTGGACCAATGTTGGCAATCTGTGGGTTTGCACCAAA
                                        STAT3                  STAT3
 –730 CCACACAGGTGAGTCAGACTTCTGTACAGAGAGCAGGGAAGGGGCACGACAGGGTGAAGTCCAGACCAGG
 –660 GCAATGTGTACCCCGGCTGTTCAGCCTTTGAGTGCGGCTGTCGCAGCAATCAAGGTGGGCTCCCTGGTGG
 –590 AGGTGGACAGCAGCTGCTGGAGAGCCCGGAGGGCAGCCAGGACCGTGTCAGACGGCAGGAGTCGGGCGGG
 –520 GCCACTCCAGGCACCGGCCAGCAGGGCAGAGGTACCAGGGAGGGCCCTCGGGCTGCGGGCGTGAGGTCAG
 –450 AGCCCAGGGTGAGGAGGAAGCATCTCCAGCCAGGTGCGCTCTCGGCCCCCGCCGGGGCTGCGAGGCTGGG
 –380 GGCGCTGCCGCTGCGAGGGCTTCCAGAGGAGCCGGCGCTACCTCCCCGCGCGCCCGGCCACGTGCGCGAC
 –310 GGGATGCGCAGACAGCCGGGGACGCAGACTGGCGGCGTGTGTCACCTCGAGGCCGCGGGACAAGCGCAGG
                                                         Sp1
 –240 TCGCCTCCCGCACTCCCACACCCCCAGGAACCCTGCTCGGGGCCGGCGGGTGACCTTCCTAGGCACTGCT
                              Ets-1                      NF-kB
 –170 GGGGGCGGAGCCAGGAAACGGCTCGCCCCGCCATTGGCTGGGGCGCCGGATTTCTTTAAAAACTCCGGCT
         Sp1      Ets-1               NF-1          Putative TATA Box
 –100 TCCATTGGCTCGTGCAGTCCCTCCTCGTTCTGCCCGAGGCGCCACGGCCGCCACCCGCGCGGGCACTGCG
  –30 CCTGCGCGGAGTGAGAGCGTGAGCGCGGAGAGCGGACCGACGCGACACGCCGTGCGCCTCCGCGGCTGCG
                                +1 Exon1
   41 CTACGAAAACGAGTCCCGGAGCGGCCCCGCGCCCGCCGCACCCGGCCCTCGCCCGCCCGAAGACAGGCGC
  111 CAAGCTGCCCCGCCGTCTCCCCAGCTAGCGCCCGGCCGCCGCCGCCTCGCGGGCCCCGGGCGGAAGGGGG
  181 CGGGGTCCCGATTCGCCCCGCCCCCGCGGAGGGATACGCGGCGCCGCGGCCCAAAACCCCCGGGCGAGGC
  251 GGCCGGGGCGGGTGAGGCGCTCCGCCTGCTGCGCGTCTACGCGGTCCCCGCGGGCCTTCCGGGCCCACTG
  321 CGCCGCGCGGACCGCCTCGGGCTCGGACGGCCGGTGTCCCCGGCGCGCCGCTCGCCCGGATCGGCCGCGG
  391 CTTCGGCGCCTGGGGCTCGGGGCTCCGGGGAGGCCGTCGCCCGCGATG
                                                   +436
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Fig. 1. Pim-3 mRNA expression in human pancreatic cancer cell lines. (a) Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of Pim-3 mRNA in human pancreatic cancer cell lines. Total RNAs were extracted from PCI35, PCI55, PCI66, MiaPaca-2, PANC-1, and L3.6pl cells to determine the expression of endogenous Pim-3 at mRNA by RT-PCR. Amplification of GAPDH (glyceraldehydes-3-phosphate dehydrogenase) was used to confirm that an equal amount of total RNA was used for RT-PCR. Representative results from three independent experiments are shown here. (b) The nucleotide sequence of the 5′-flanking region of the human *Pim-3* gene. Putative transcription factor binding sites are underlined. The boundary of the first exon, putative TATA box, and the translation initiation site are indicated in bold and with underlining.

detect Bad, phosphoSer^{112}Bad, Pim-3, Ets-1, or β-actin in a similar way to that previously described.[2]

Cell apoptosis analysis. After transfection with either empty vector, DN-Ets-1, WT-Ets-1 expression vectors, or Ets-1 siRNA together with or without *Pim-3* cDNA expression vector, the cells were harvested and the phosphatidyl serine exposure level was determined by staining the cells with the human Annexin V-FITC Kit (Bender MedSystem), according to the manufacturer's instructions. At least 20 000 stained cells were analyzed on a FACS Caliber system (Becton Dickinson, Bedford, MA, USA) for each determination.

Statistical analysis. Data were analyzed statistically using one-way ANOVA followed by the Tukey–Kramer test. $P < 0.05$ was considered statistically significant.

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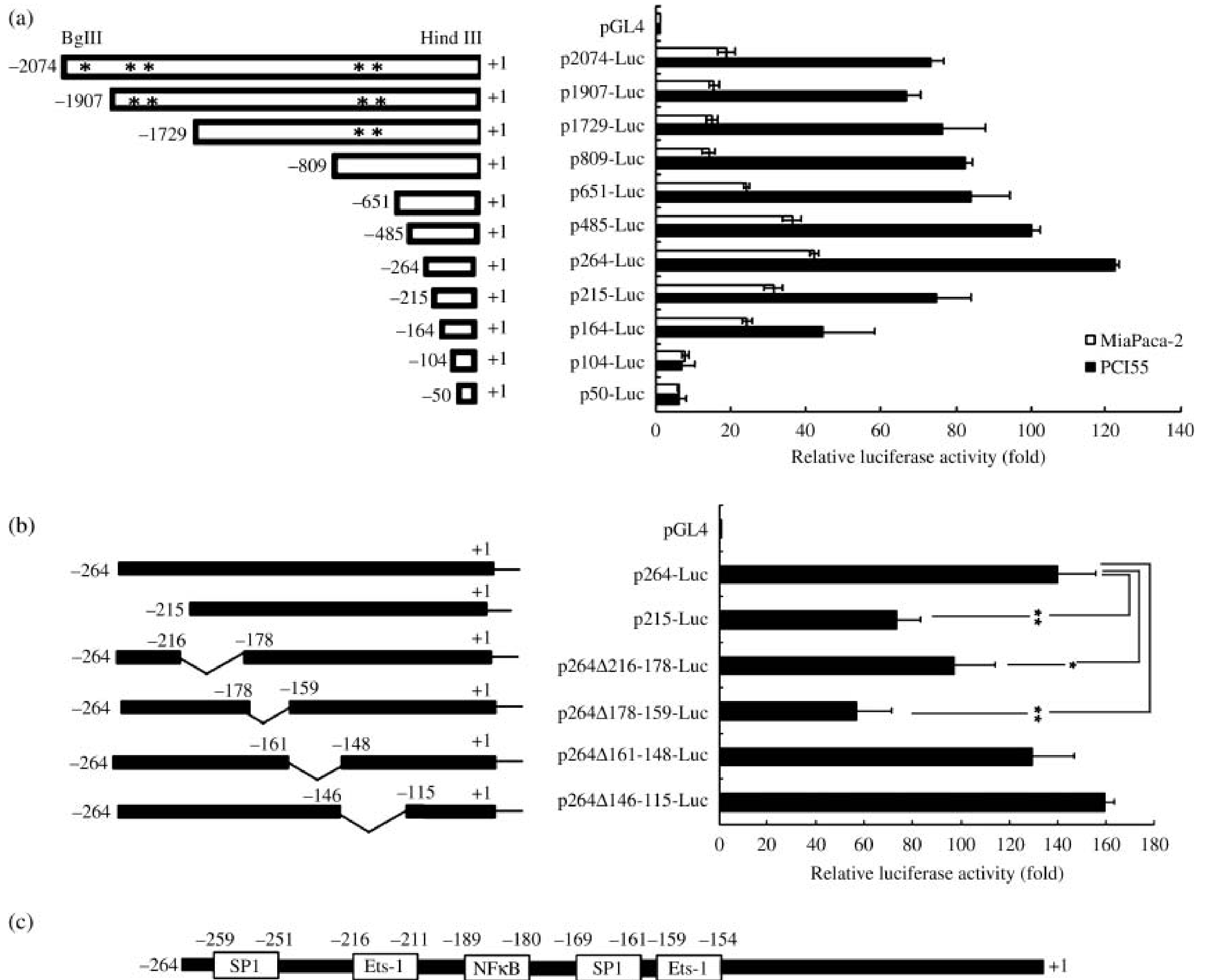

Fig. 2. Determination of promoter activities of the 5′-flanking region of the human *Pim-3* gene. (a, left) Schematic structures of luciferase expression vectors linked with various deleted 5′-flanking regions of the human *Pim-3* gene. (right) Luciferase activities were determined when the indicated luciferase vectors were transfected into PCI55 (closed boxes) or MiaPaca-2 cells (open boxes), as described in 'Materials and Methods'. Relative activities were calculated on the assumption that the luciferase activity was 1.0 when promoter/enhancer-less (pGL4) luciferase vector was transfected. Mean and 1SD were calculated on three independent experiments and are shown here. (b, left) Schematic structures of luciferase expression vectors linked with internally deleted 5′-flanking region of the *Pim-3* gene. Right. Luciferase activities were determined when the indicated luciferase vectors were transfected into PCI55 cells. Relative activities were calculated on the assumption that the luciferase activity was 1.0 when the empty pGL4 luciferase vector was transfected. Mean and 1SD were calculated for three independent experiments and are shown here. $*P < 0.05$; $**P < 0.01$ *versus* p264-Luc. (c) Potential binding sites for known transcription factors, in the 5′-flanking region between –260 and 150 bp.

Results

Characterization of 5′-flanking region of the human *Pim-3* gene. We previously reported that Pim-3 protein was constitutively expressed in human pancreatic cancer cell lines and cancer cells in human pancreatic cancer tissues with its predominant localization in cytoplasm.[2] RT-PCR analysis further demonstrated constitutive *Pim-3* mRNA expression in all human pancreatic cancer cell lines that we examined (Fig. 1a), whereas *Pim-3* mRNA was not detected in normal murine pancreas (data not shown). Constitutive *Pim-3* gene expression in human pancreatic cancer cell lines prompted us to delineate its molecular mechanisms in human pancreatic cancer cells. We first cloned the human *Pim-3* gene up to –2074 bp from the start of the first exon, based on the data deposited on GenBank and confirmed its nucleotide sequence. The human *Pim-3* gene contains canonical TATA box and contains putative binding sites for several known transcription factors such as Stat3, Sp1, Ets-1, NF-κB, and NF-1 (Fig. 1b). To determine the promoter activities of 5′-flanking region of the human *Pim-3* gene, we subcloned various deleted 5′-flanking regions of the human *Pim-3* gene into a firefly luciferase reported vector (Fig. 2a, left panel) and transiently transfected the resultant vectors into PCI55 and Miapaca-2, two human pancreatic cancer cell lines that constitutively express *Pim-3* mRNA at different levels (Fig. 1a). Luciferase activities were retained with the deletion

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(a) **Wild type**

−267

ACCTCGAGGC CGCGGGACAA GCGCAGGTCG
Sp1

CCTCCCGCAC TCCCACACCC CCAGGAACCC
Ets-1

TGCTCGGGGC CGGCGGGTGA CCTTCCTAGG
NF-κB

CACTGCTGGG GGCGGAGCCA
Sp1 −158

Mutations

p264MutSp1BS1-Luc:	CGCGGGA	→	CGCGTTA
p264MutEBS-Luc:	CAGGAA	→	CATTAA
p264MutNFBS-Luc:	ACCTTCCT	→	ACCTCACT
p264MutSp1BS2-Luc:	GGGGCGG	→	GCAGGCGG

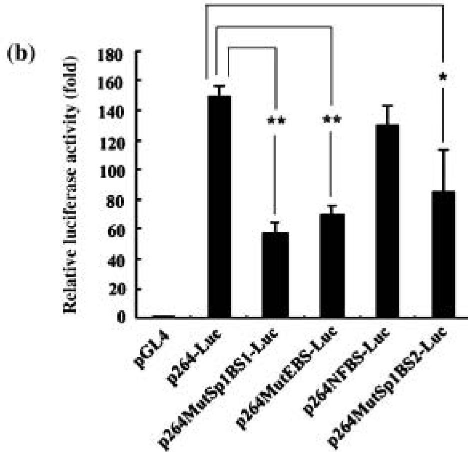

Fig. 3. Essential roles of the Ets-1 binding site in *Pim-3* expression. (a, left) Potential binding sites for the known transcription factors, which are present in the human *Pim-3* gene between –267 and –158 bp region. (right) The mutation, which was introduced into each transcription factor binding site in p264-Luc, is indicated here. (b) Luciferase activities were determined when the indicated luciferase vectors were transfected into PCI55 cells. Relative activities were calculated on the assumption that the luciferase activity was 1.0 when empty pGL4 luciferase vector was transfected. Mean and 1SD were calculated for three independent experiments and are shown here. $*P < 0.05$; $**P < 0.01$ *versus* p264-Luc.

below –264 bp from the start of the first exon (Fig. 2a, right panel). By contrast, the deletion below –104 bp almost completely abrogated enhancer activities (Fig. 2a, right panel). We further examined the activities of p264-Luc with various internal deletions between –264 and –115 bp (Fig. 2b, left panel). The reporter activities were not significantly decreased with p264Δ161-148-Luc or p264Δ146-115-Luc. On the contrary, luciferase activities were reduced significantly with p215-Luc, p264Δ216-178-Luc, and p264Δ178-159-Luc when they were transfected into PCI55 cells, although the activities were still higher than the basal activity (Fig. 2b, right panel). Similar results were obtained when Miapaca-2 was used (data not shown). These observations would indicate that the region between –264 and –159 bp was minimally required for constitutive *Pim-3* gene expression in human pancreatic cancer cells. This region contains two Sp1-, one Ets-1-, and one NF-κB-binding sites (Fig. 2c). We introduced a mutation into each potential binding site (Fig. 3a, right panel) in p264-Luc and transfected the resultant vectors into PCI55 cells. Luciferase activities were reduced when the cells were transfected with p264-Luc with the mutation in the Sp1 or Ets-1 binding site, whereas the mutation in the NF-κB binding site had little effect on luciferase activities (Fig. 3b). These observations would indicate that the Sp1 and Ets-1 binding sites were operational for *Pim-3* gene expression in human pancreatic cancer cells. Sp1 is ubiquitously expressed in normal organs including the pancreas,[17] while Ets-1 expression was selectively enhanced in human pancreatic cancer, compared with normal pancreatic tissue.[18] Moreover, Deneen and colleagues demonstrated that *Pim-3* gene transcription was enhanced in EWS/ETS-mediated malignant transformation of mouse NIH3T3 cells.[10] These observations prompted us to examine the involvement of Ets-1 but not Sp1 in *Pim-3* gene expression.

Coexpression of Ets-1 and Pim-3 proteins in human pancreatic cancer cells and tissues. In order to define the roles of Ets-1, we first examined Ets-1 expression in human pancreatic cancer cell lines and tissues. *Ets-1* mRNA was constitutively expressed in all human pancreatic cancer cells that we examined (Fig. 4a), and Ets-1 protein was detected in PCI35, PCI55, and MiaPaca-2 cells, together with Pim-3 protein (Fig. 4b). Moreover, a double-color immunofluorescence analysis demonstrated that PCI55 cells constitutively expressed Ets-1 protein in the nuclei and Pim-3 protein in the cytoplasm (Fig. 4c). Similar results were obtained for PCI35 and MiaPaca2 cells (data not shown). Ets-1 protein was detected faintly in normal pancreatic tissue (Fig. 4d), consistent with the previous report.[18] In contrast, Ets-1 protein was abundantly detected in the nuclei of cancer cells in all human pancreatic cancer tissues that we examined. Moreover, Ets-1 and Pim-3 protein were mainly localized in the nuclei and cytoplasm of the same cancer cells, as revealed by immunohistochemical analysis on serial sections of human pancreatic cancer tissues that we examined (Fig. 4e). These observations would indicate that Ets-1 protein was constitutively expressed in human pancreatic cancer cells, particularly in its nuclei, and could transactivate the *Pim-3* gene.

Essential contribution of Ets-1 to Pim-3 expression in human pancreatic cancer cells. In order to prove the functionality of the Ets-1 binding site, we conducted ChIP assays. In PCI55 cells (Fig. 5a) and Miapaca-2 cells (data not shown), Ets-1 bound to the 5′-flanking region of the human *Pim-3* gene between –249 and –183 bp, the region that contains Ets-1 but not the Sp1-binding site. The use of control rabbit IgG failed to give rise to any bands, indicating the specificity of the ChIP assay. Moreover, cotransfection with WT-Ets-1 further enhanced luciferase activities of p264-Luc whereas that with DN-Ets-1 reduced the luciferase activities in PCI55 cells, compared with the cells transfected with p264-Luc alone (Fig. 5b). These observations would indicate that Ets-1 could interact with the Ets-1 binding site of *Pim-3* gene and confer the enhancer activity.

Ets-1-mediated Pim-3 expression and subsequent apoptosis in human pancreatic cancer cells. In order to examine whether Ets-1 could affect endogenous Pim-3 protein expression in human pancreatic cancer cells, we transfected a human pancreatic

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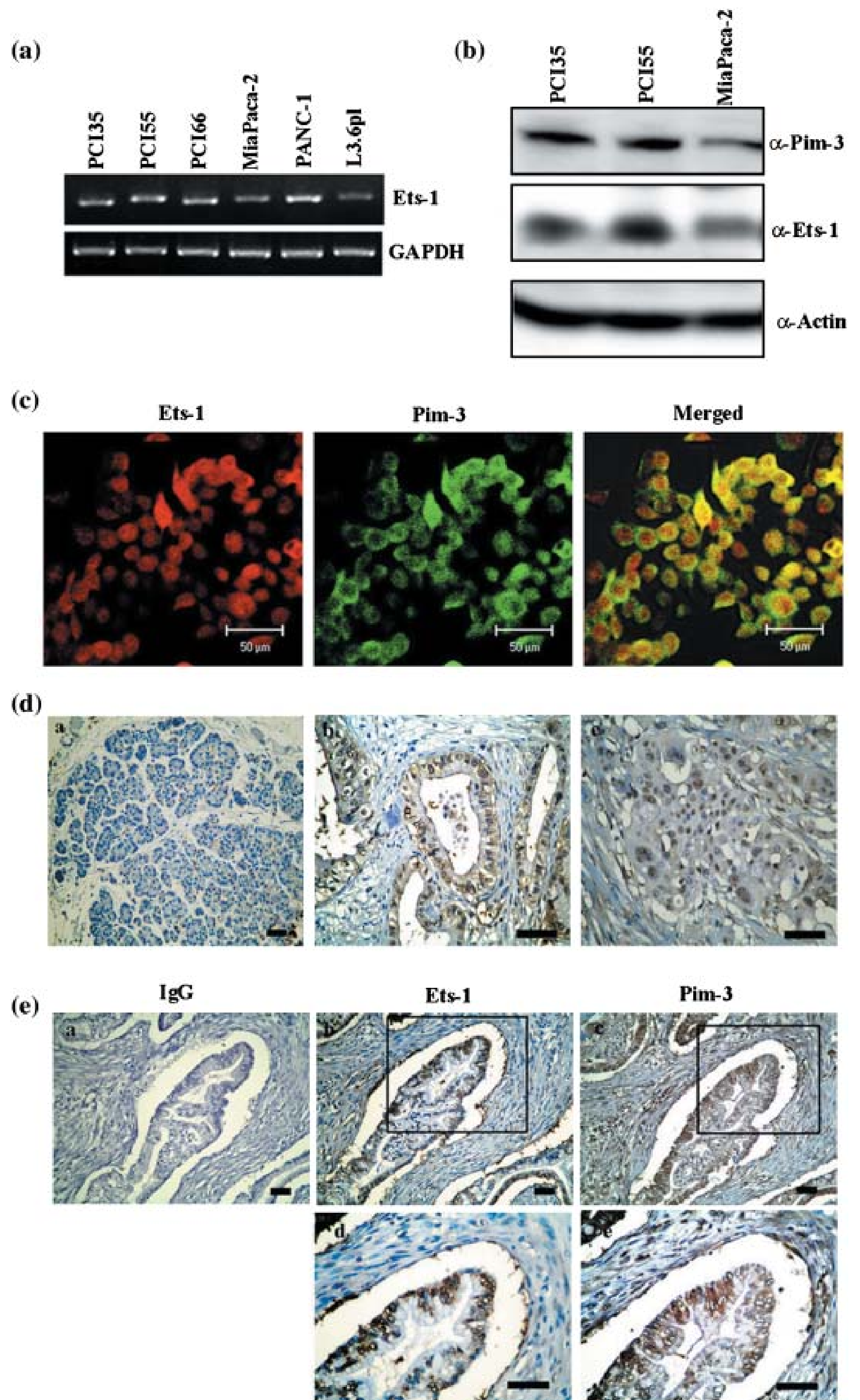

Fig. 4. Co-expression of Ets-1 and Pim-3 in human pancreatic cancer cell lines and tissues. (a) Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of Ets-1 mRNA in human pancreatic cancer cell lines. Total RNAs were extracted from PCI35, PCI55, PCI66, MiaPaca-2, PANC-1, and L3.6pl cells to determine the expression of endogenous Ets-1 at mRNA by RT-PCR. Amplification of GAPDH (glyceraldehydes-3-phosphate dehydrogenase) was used to confirm that an equal amount of total RNA was used for RT-PCR. Representative results from three independent experiments are shown here. (b) Constitutive expression of Ets-1 and Pim-3 protein in human pancreatic cancer cell lines. The proteins were extracted from PCI35, PCI55, and MiaPaca-2 cells to determine the expression of endogenous Ets-1 and Pim-3 protein by immunoblotinng. Immunoblotting with anti-β-actin antibodies was used to confirm that an equal amount of protein was loaded onto each lane. Representative results from three independent experiments are shown here. (c) A double-color immunofluorescence analysis was performed on a human pancreatic cancer cell line, PCI55, using the combination of anti-Ets-1 antibodies (red, middle panel) and anti-Pim-3 (green, left panel) as described in 'Materials and Methods'. The fluorescent images were digitally merged (right panels). Representative results from three independent experiments are shown here. Original magnification, ×400. Scale bars, 50 μm (d) Immunohistochemical analysis was performed on human pancreatic cancer tissues (b, c) and normal pancreas tissues (a), using the anti-Ets-1 antibodies (a, b, and c) as described in 'Materials and Methods'. Representative results from 10 samples are shown here. Original magnification, ×200 (a); ×400 (b, c). Scale bars, 50 μm. (e) Immunohistochemical analysis on serially sectioned human pancreatic cancer tissues, using the anti-Ets-1 antibodies (b, d) and anti-Pim-3 antibodies (c, e) or control rabbit IgG (a) as described in 'Materials and Methods'. Serial sections from the same patient with pancreatic ductal adenocarcinoma were used (a, b, c, d, e). (d, e) represent box areas in (b, c) observed under higher magnification. Representative results from six human pancreatic cancer tissues are shown here. Original magnification, ×200 (a, b, c); ×400 (d, e). Scale bars, 50 μm.

cancer cell line, MiaPaca-2, with WT-Ets-1, DN-Ets-1, or Ets-1 siRNA. WT-Ets-1 increased Pim-3 protein and the amount of phospho-Ser^{112}Bad (Fig. 6a). In contrast, DN-Ets-1 reduced Pim-3 protein and the amount of phospho-Ser^{112}Bad in MiaPaca-2 cells, with little effect on the total amount of Bad protein. Likewise, transfection with Ets-1 siRNA markedly diminished Pim-3 protein expression and the amount of phospho-Ser^{112}Bad but not the total amount of Bad (Fig. 6b). Moreover, Ets-1 siRNA significantly enhanced the proportions of both early (Annexin V-positive, PI-negative) and late (Annexin V-positive, PI-positive) apoptotic cells, as DN-Ets-1 did (Fig. 6c). Cotransfection with Pim-3 reversed the decrease in the amount of phosphor-Ser^{112}Bad and the increase in apoptosis induced by Ets-1 siRNA (Fig. 6b,c). Taken together, constitutively expressed Ets-1 in human pancreatic cancer cells can induce gene expression and eventually protein expression of Pim-3, a serine/threonine kinase that can regulate cell apoptosis by inactivating the antiapoptosis molecule, Bad, through phosphorylation of its Ser^{112}.

Discussion

We previously observed that the protein expression of the proto-oncogene Pim-3 with serine/threonine kinase activity was selectively enhanced in precancerous and cancerous lesions of

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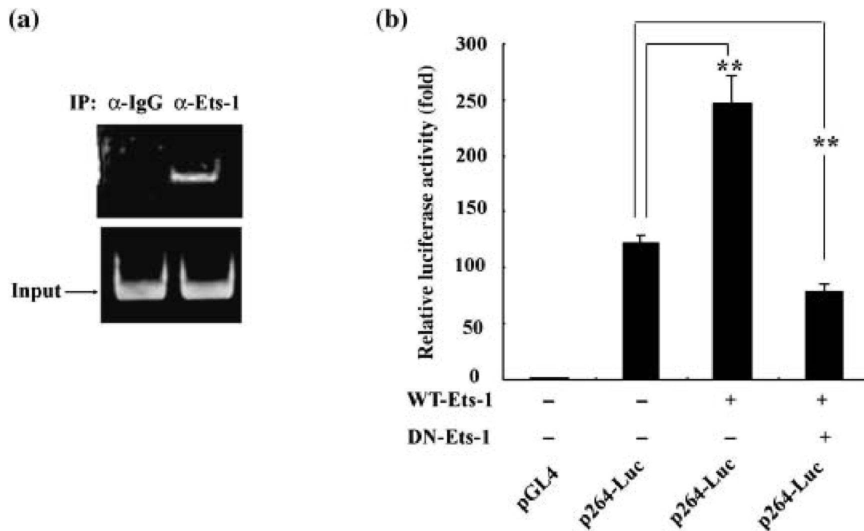

Fig. 5. Regulation of Pim-3 promoter activities by Ets-1. (a) Determination of Ets-1 bound to the enhancer region of the Pim-3 gene by ChIP assay. ChIP assay was conducted on nuclear extracts obtained from PCI55 cells, with the use of anti-Ets-1 (3 µg) or normal rabbit IgG as a negative control, as described in Materials and Methods. Input indicates the results when total nuclear lysates were used without any immunoprecipitation. Representative results from three independent experiments are shown here. (b) The effects of the co-transfection with WT-Ets-1 or DN-Ets-1 expression vector on the activities of luciferase expression vectors. PCI55 cells were transfected with the p264-Luc luciferase expression vectors, together with WT-Ets-1 or DN-Ets-1 expression vectors. Then, luciferase activities were determined as described in Materials and Methods. Relative activities were calculated on the assumption that the luciferase activity was 1.0 when the pGL4 luciferase vector was transfected. Mean and 1 SD were calculated on three independent experiments and are shown here. ***P* < 0.01 vs. transfection with p264-Luc alone.

endoderm-derived organs including the liver, pancreas, colon, and stomach.[2–5] We further demonstrated that Pim-3 can inactivate Bad by phosphorylating its Ser^{112}, maintain the expression of $Bcl\text{-}X_L$, and eventually prevent apoptosis of human pancreatic and colon cancer cells.[2,4] Protein expression is generally controlled at various steps and the first one is transcriptional control by a transcription factor(s). Hence, the identification of the transcription factor(s) regulating *Pim-3* gene transcription can elucidate the molecular mechanism of aberrant *Pim-3* gene expression and its subsequent protein expression in these cancer tissues. Moreover, the clarification of the molecular mechanism of Pim-3 protein expression can clarify one aspect of Pim-3 activation mechanism, because its protein expression can lead directly to its activation due to its lack of a regulatory domain like other Pim kinase family members.

STAT3 activities are enhanced in a wide variety of human tumors including pancreatic cancer.[19–22] Moreover, STAT3 regulates a number of pathways important for oncogenesis including cell cycle progression, apoptosis, and tumor angiogenesis.[23] Of interest is that other Pim kinase members, Pim-1 and Pim-2, are targets for the gp130-mediated Stat3 signal in hematopoietic cells.[24,25] Furthermore, the leukemia inhibitory factor/gp130/Stat3 pathway enhanced the expression of Pim-3 and Pim-1, which were indispensable for the self-renewal of mouse embryonic stem cells.[26] These observations raised the possibility that STAT3 can up-regulate *Pim-3* gene expression. Likewise, the human *Pim-3* gene contains several potential binding sites for Stat3 in its 5′-flanking region. However, luciferase activities were still retained even when Stat3 binding sites were completely eliminated by a deletion below –264 bp. Moreover, luciferase activity of p2074-Luc was not reduced in PCI55 cells, when the dominant negative form of Stat3 expression vector was cotransfected (our unpublished data). Thus, Stat3 may contribute little to constitutive *Pim-3* gene expression in human pancreatic cancer cell lines.

A CD40-mediated signal induced *Pim-1* gene expression in an NF-κB-dependent manner.[27] A potential binding site for NF-κB is present between –189 and –177 bp of the human *Pim-3* gene. Constitutive NF-κB activation in pancreatic cancer cells[28] prompted us to investigate the role of NF-κB in *Pim-3* gene expression. However, the mutation in this NF-κB binding site failed to reduce luciferase activities; therefore, it is a remote possibility that NF-κB contributed to constitutive *Pim-3* gene expression in human pancreatic cancer cells.

Deneen and colleagues demonstrated that *Pim-3* gene transcription was enhanced in the EWS/ETS-mediated malignant transformation of mouse NIH3T3 cells.[10] EWS/ETS fusion proteins retain an intact Ets DNA-binding domain and can bind to a *cis*-acting site in the target genes through this domain.[29] Thus, it is plausible that *Pim-3* gene transcription can be regulated by other Ets family members as well as EWS/ETS fusion proteins. A major Ets family member, Ets-1, binds to specific DNA sequences containing GGAA/T with additional flanking nucleotides.[30] Moreover, its expression is enhanced in human pancreatic carcinoma, compared with normal pancreatic tissue.[18,31,32] Consistent with the previous reports, we also observed that Ets-1 protein was constitutively and abundantly expressed in the nuclei of cancer cells in the human pancreatic cancer cell lines and tissues that we examined. Moreover, the deletion of the Ets-1 binding site between –216 and –211 bp reduced markedly promoter activities as revealed by luciferase assay. Furthermore, ChIP assay demonstrated that Ets-1 bound to the 5′-flanking region of the human *Pim-3* gene between –249 and –183 bp, the region that contain Ets-1 binding site. These observations revealed the indispensable role of Ets-1 in constitutive *Pim-3* gene expression. This assumption was further supported by the observations that Pim-3 promoter activity was reduced by transfection with DN-Ets-1. Finally, transfection with DN-Ets-1 and *Ets-1* siRNA decreased endogenous Pim-3 protein expression and subsequently induced cell apoptosis together with reducing the amount of phosphor-Ser^{112}Bad. The cotransfection of Pim-3 cDNA reverted the Ets-1 siRNA-mediated decrease in the amount of phospho-Ser^{112}Bad and the increase in apoptosis. Thus, constitutive Ets-1 expression may counteract apoptosis in pancreatic cancer cells by inducing the expression of Pim-3, a kinase that can inactivate an antiapoptotic molecule, Bad, through phosphorylating its Ser^{112}.

Several lines of evidence suggest that enhanced Sp1 activation in K562 cells resulted in aberrant *Pim-1* gene expression,[33] and Kruppel-like factor 5 (KLF5), an Sp1-like zinc-finger protein, can regulate *Pim-1* gene expression in colon cancer cell lines.[34] Moreover, Sp1 and Pim-3 are aberrantly activated and expressed in pancreatic cancer tissues and cells.[2,35–37] Furthermore, we also observed that mutation in the Sp1 binding site significantly affected luciferase activities, suggesting that Sp1 may regulate *Pim-3* gene transcription. Of interest, Ets transcription factors regulate their target gene expression in concert with various transcription factors including Sp1/Sp3, AP-1, and serum

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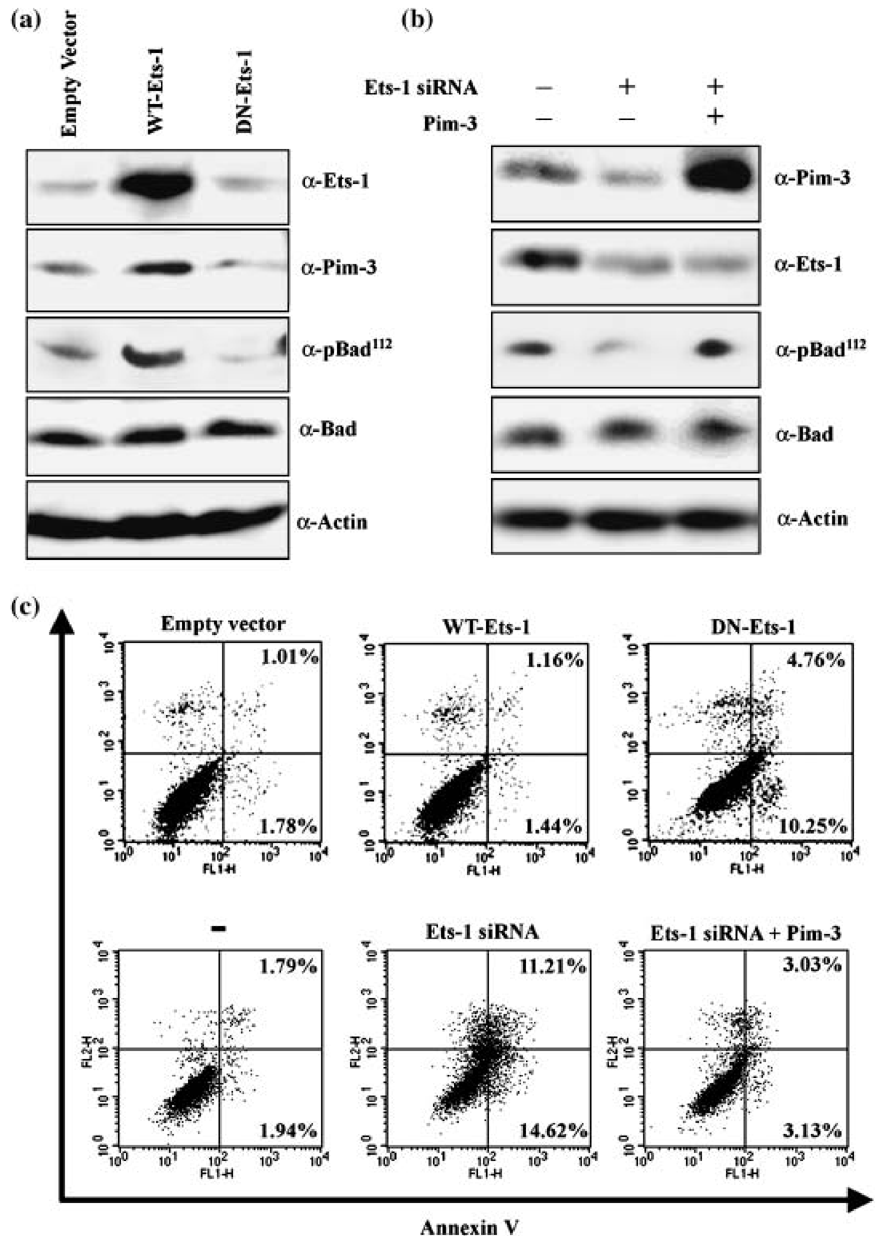

Fig. 6. The effects of Ets-1 siRNA, WT-Ets-1, or DN-Ets-1 on endogenous Pim-3 protein expression and cell apoptosis in human pancreatic cancer cell lines. (a) The effects of DN-Ets-1 or WT-Ets-1 expression vector transfection on endogenous Pim-3 and Bad protein expression. Cell lysates were obtained from Miapaca-2 cells, which were transfected with DN-Ets-1, WT-Ets-1, or an equal amount of empty vector and were subjected to immunoblotting with indicated antibodies as described in Materials and Methods. Representative results from three independent experiments are shown here. (b) Effects of Ets-1 siRNA transfection with or without Pim-3 expression vector on Pim-3 and Bad proteins. Cell lysates were obtained from MiaPaca-2 cells, which were transfected with Ets-1 siRNA with or without Pim-3 expression vector and were subjected to immunoblotting as described in Materials and Methods. Representative results from three independent experiments are shown here. (c) The effects of transfection of WT-Ets-1, DN-Ets-1, Ets-1 siRNA, or Ets-1 siRNA with Pim-3 expression vector on human pancreatic cancer cell apoptosis. MiaPaca-2 cells were transfected with WT-Ets-1, DN-Ets-1, Ets-1 siRNA, or Ets-1 siRNA with Pim-3 expression vector. Cells were harvested 48 hrs after transfection and subjected to combined staining with propidium iodide and Annexin V as described in Materials and Methods. The number in each quadrant indicates the proportion of the cells present in the quadrant. Representative results from three independent experiments are shown here.

response factor (SRF).[30] The 5′-flanking region of the human *Pim-3* gene between –264 and +1 bp contains binding sites for Sp1 but not AP-1 and SRF. Cooperative interactions between Sp1 and Ets-1 have a critical role in regulating the transcription of various genes, such as *Fas* ligand and the α-*11* integrin chain.[38,39] Thus, it is tempting to speculate that Sp1 may also act cooperatively with Ets-1 to induce constitutive *Pim-3* gene expression in human pancreatic cancer cells.

In the present study, we examined the molecular mechanism underlying constitutive Pim-3 expression in human pancreatic cancer cells and revealed the crucial roles of Ets-1. These observations suggest that Ets-1 could be a candidate target molecule for the treatment of pancreatic cancer.

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