

PACE4 regulates apoptosis in human pancreatic cancer Panc-1 cells via the mitochondrial signaling pathway

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Abstract. Previous studies have demonstrated the overexpression of paired basic amino acid cleaving enzyme 4 (PACE4) mRNA in prostate cancer tissues. This overexpression is correlated with higher circulating protein levels in certain patients, however, the role of PACE4 in apoptosis and the potential molecular mechanisms of pancreatic cancer remain to be elucidated. The aim of the present study was to investigate the effect and potential molecular mechanisms of PACE4 on apoptosis in the Panc-1 pancreatic cancer cell line. Cell proliferation was assessed using a Cell Counting Kit-8 assay. Apoptotic nuclear shrinkage was monitored using Hoechst 33258 staining. Caspase-3/7 activities were measured using a colorimetric caspase-glo 3/7 assay. Alterations in protein expression were monitored using Western blot analysis. The results indicated that PACE4 small interfering (si)RNA inhibited cell proliferation and activated caspase-3/7 activities. In addition, PACE4 siRNA significantly increased apoptosis via the activation of caspase-3 and the downregulation of anti-apoptotic proteins, X-linked inhibitor of apoptosis protein and phosphorylated-Akt. In addition, the results showed deregulation of the B cell lymphoma-2 (Bcl-2)-associated X protein/Bcl-2 ratio which led to the release of cytochrome *c* following PACE4 siRNA transfection. In conclusion, PACE4 siRNA may exert antitumor activity through the mitochondrial pathway and is expected to be a promising therapeutic strategy for the treatment of pancreatic cancer.

Introduction

Pancreatic cancer, one of the most aggressive types of human malignancy, has a poor prognosis with a five-year survival

rate of <5% worldwide (1). It is characterized by rapid disease progression without specific symptoms, thus, early diagnosis and curative treatment are almost impossible (1,2). The standard treatment strategies for pancreatic cancer include surgery, which is restricted to early disease stages, radiation and/or gemcitabine-based chemotherapies. However, pancreatic cancer is among the most intrinsically resistant types of malignancy to radiation and chemotherapy, resulting in an overall survival rate of <7%. Therefore, scientists and oncologists are attempting to identify novel and more efficient anti-pancreatic cancer agents or adjuvants (3-5). A previous report showed that ~90% of patients succumb to the disease within 1 year following diagnosis, and the five-year survival rate is <5% worldwide (6). The incidence of pancreatic cancer has been increasing in previous years, demonstrating the importance of investigating its pathogenesis. Alterations in gene and protein expression levels, and the activation of signaling pathways are associated with the occurrence and progression of pancreatic cancer (7).

Protein convertases (PCs) are a family of enzymes, which are responsible for the activation of numerous protein precursors. At present, nine PCs have been identified, namely, furin, paired basic amino acid cleaving enzyme 4 (PACE4), PC1/3, PC2, PC4, PC5/6, PC7, PCSK9 and SKI-1/S1P (8). PACE4 is considered to be important in the development and progression of cancer. It activates several biologically relevant substrates; and a number have been shown to be significantly involved in tissue homeostasis and cancer growth (9,10). Among these are numerous metalloproteinases, growth factors, growth factor receptors and adhesion molecules, which are directly associated with tumor development (11-14). PACE4 is expressed at low levels in several mammalian tissues and has been demonstrated to be upregulated in certain tumor cell lines, including murine squamous cell carcinoma (15). In addition, mice overexpressing PACE4 have been found to exhibit tumors with increased growth rates (16).

Previously, two independent studies demonstrated the overexpression of PACE4 mRNA in prostate cancer tissues (17,18). This overexpression was correlated with higher circulating protein levels in certain patients (18). However, the possible role of PACE4 in apoptosis and the potential molecular mechanisms of pancreatic cancer remain to be elucidated. In the present study, molecular silencing with small interfering (si)RNA was used to knock down endogenously expressed

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PACE4 in the Panc-1 cell line, following which cell proliferation and the apoptotic response was examined.

Materials and methods

Reagents and antibodies. The following polyclonal antibodies were purchased from Proteintech Group, Inc. (Wuhan, China): Rabbit anti-human cleaved caspase-3 (19677-1-AP; 1:3,000), rabbit B cell lymphoma (Bcl)-2 (12789-1-AP; 1:3,000), rabbit Bcl-2-associated X protein (Bax; 50599-2-Ig; 1:3,000), rabbit cytochrome *c* oxidase subunit IV and X-linked inhibitor of apoptosis protein (XIAP; 10037-1-Ig; 1:2,000) antibodies, and the mouse anti-GAPDH (MM-0163-P; 1:1,000) antibody. Polyclonal rabbit anti-human PACE4 (AB151562; 1:3,000) and cytochrome *c* (ab154476; 1:3,000) antibodies were purchased from Abcam (Cambridge, UK). Polyclonal rabbit anti-human phosphorylated-Akt (p-Akt; 13038; 1:2,000) and Akt (4685; 1:3,000) antibodies were purchased from Cell Signaling Technologies, Inc. (Danvers, MA, USA). Anti-rabbit (15134-1-AP) and anti-mouse (30000-0-AP) IgG-horseradish peroxidase antibodies were purchased from Proteintech Group, Inc. A Cell Counting Kit-8 (CCK-8) and Hoechst 33258 were purchased from Beyotime Institute of Biotechnology (Haimen, China). Other reagents were of analytical grade.

Cell culture and small interfering RNA transfection. Human pancreatic cancer Panc-1 cells were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences, Beijing, China) containing 10% fetal bovine serum (FBS; GE Healthcare Life Sciences), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 100 μ g/ml streptomycin (Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO₂. The cultures were replaced every 2-3 days and the cultures were divided into two at 80% confluence.

The Panc-1 cells were transfected with 100 nM of PACE4 siRNA (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. sc-45482; Genbank ID for PACE4, NM_174936) or control siRNA (scrambled siRNA, a universal negative control; Sangon Biotech Co., Ltd., Shanghai, China) with GeneSilencer siRNA transfection reagent (Genlantis, San Diego, CA, USA) at 37°C, according to the manufacturer's protocol. At 48 h post-transfection, the efficiency of siRNA-mediated PACE4 knockdown was determined using Western blot analysis.

Cell proliferation assay. The Panc-1 cells were plated in each well of a 96-well plate at a density of 5x10³ cells/well in the culture medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C. Following 24 h of incubation, the cells were transfected with PACE4 siRNA or control siRNA, as described above, for 12, 24, 36 and 48 h, followed by the addition of 10 μ l CCK-8 solution at 37°C. The cells were then incubated for 3 h at 37°C. Absorbance was measured at 450 nm using a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Inc.).

Morphological analysis following Hoechst 33258 staining. The Panc-1 cells were seeded in 24-well plates (6x10⁴ cells per well) overnight, and transfected with PACE4 siRNA or

control siRNA for 48 h. The cells were then fixed and stained with Hoechst 33258 (Invitrogen; Thermo Fisher Scientific, Inc.). The apoptotic cells were visualized under a fluorescence microscope (M165 FC; Leica Microsystems GmbH, Wetzlar, Germany).

Detection of caspase-3/7 protein activity. The activities of caspase-3/7 were measured using a colorimetric method, according to the manufacturer's protocol, using a Caspase-glo 3/7 Assay kit (G8093; Promega Corporation, Madison, WI, USA). Briefly, 2x10⁴ Panc-1 cells were seeded in 96-well plates and, after 24 h, the cells were transfected with PACE4 siRNA or control siRNA for another 48 h. Subsequently, the lysates (lysed using Sigma-Aldrich lysis buffer) of the Panc-1 cells were mixed with equilibrated caspase-glo 3/7 reagents for 1 h at room temperature. Luminescence was measured using a GloMax 96 luminometer (Promega Corporation).

Preparation of mitochondria and cytosol. A mitochondria/cytosol kit (C3601; Beyotime Institute of Biotechnology, Beijing, China) was used to isolate the mitochondria and cytosol, according to the manufacturer's protocol. Following transfection, as described above, the cells (5x10⁷ cells) were collected by centrifugation at 600 x g for 5 min at 4°C, washed twice with ice-cold phosphate-buffered saline and then resuspended in 500 μ l isolation buffer (Tiangen Biotech Co., Ltd., Beijing, China) containing protease inhibitors for 10 min on ice. The cells were then mechanically homogenized using a Dounce grinder (ATX810; Tiangen Biotech Co., Ltd.). The unbroken cells, debris and nuclei were discarded by centrifugation at 800 x g for 10 min at 4°C. The supernatants were centrifuged at 12,000 x g for 15 min at 4°C. The supernatant of the cytosol was collected and the pellet fraction mitochondria was dissolved in 50 μ l lysis buffer (Sigma-Aldrich).

Western blot analysis. The Panc-1 cells were transfected, as described above, and were then lysed using radioimmunoprecipitation assay buffer (Sigma-Aldrich). A Bicinchoninic Acid Protein Assay kit (NEP045-2; Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) was used to measure the protein concentrations. The total protein (0.2 μ g) was loaded onto 12% SDS-PAGE gels (Tiangen Biotech Co., Ltd.) and transferred onto polyvinylidene fluoride membranes (Sigma-Aldrich). The membranes were probed overnight at 4°C with the indicated primary antibodies in Tris-buffered saline with Tween-20 (Tiangen Biotech Co., Ltd.), containing 1% bovine serum albumin (w/v; Gibco; Thermo Fisher Scientific, Inc.). The blots were then incubated for 1 h with anti-rabbit or mouse secondary antibodies at 37°C. The immune complexes were detected using an ECL Detection kit (32132; Thermo Fisher Scientific, Inc.) and quantified using a scanning densitometer (SD4; Tobias Associates, Inc., Ivyland, PA, USA) with molecular analysis software (Quantity One 1-D Analysis software; version 4.6.9; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Quantification of band density was additionally performed using Quantity One 1-D Analysis software with normalization to the GAPDH signal. The expression levels of the proteins of interest in the

various treatment groups were expressed relative to those under non-treated conditions.

Statistical analysis. All data are presented as the mean \pm standard deviation, and were analyzed using Student's *t*-test and one-way analysis of variance to determine the levels of significance. $P < 0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS/Windows 11.0 software (SPSS Inc., Chicago, IL, USA).

Results

***PACE4* reduces *Panc-1* cell proliferation.** *Panc-1* cell proliferation was examined using a CCK-8 assay following transfection of the cells with *PACE4* siRNA or control siRNA. As shown in Fig. 1, *PACE4* siRNA inhibited cell proliferation, with a significant reduction observed 48 h post-transfection, compared with that in the control siRNA-treated group ($P < 0.05$). Thus, these data indicated that *PACE4* may affect cellular proliferation in *Panc-1* cells.

***PACE4* siRNA induces the apoptosis of *Panc-1* cells.** In the present study, the *Panc-1* cells were transfected with *PACE4* siRNA. The results of the Western blot analysis indicated that the protein expression of *PACE4* was inhibited in the *PACE4* siRNA-transfected group, compared with the control siRNA-transfected group (Fig. 2A and B). In order to evaluate whether the proliferation inhibition induced by *PACE4* siRNA in the *Panc-1* cells was associated with apoptosis, the present study examined the morphological changes in the cells using Hoechst 33258 staining. The *Panc-1* cells were transfected with *PACE4* siRNA for 48 h, and the apoptotic morphological changes were observed and compared with the appearances in the control group. In the control siRNA group, the nuclei of the *Panc-1* cells were round and homogeneously stained (Fig. 2C). However, the *PACE4* siRNA-transfected cells exhibited evident apoptotic characteristics, including cell shrinkage and membrane integrity loss or deformation, nuclear fragmentation and chromatin compaction of a late apoptotic appearance. Together, these data indicated that *PACE4* siRNA induced apoptosis in the *Panc-1* cells.

***PACE4* siRNA induces apoptosis via a caspase-dependent pathway.** Caspase-3 is a critical executioner of apoptosis, and its activation is essential for DNA fragmentation and a number of the typical biochemical and morphological changes observed in cells undergoing apoptosis. Therefore, to evaluate whether *PACE4* siRNA-induced apoptosis is involved in the activation of caspase-3/7, the present study investigated the activities of caspase-3/7 through measuring the bioluminescence intensities. The activities of caspase-3/7 were significantly activated following *PACE4* siRNA transfection (Fig. 3A).

To further assess the role of *PACE4* in *Panc-1* cell apoptosis, the present study evaluated the expression levels of apoptosis-associated proteins. These included pro-apoptotic cleaved caspase-3 (c-caspase-3), anti-apoptotic XIAP and p-Akt. The results of the Western blot analysis and subsequent statistical analysis indicated that *PACE4* siRNA increased the levels of c-caspase-3 by ~ 2.2 -fold ($P < 0.05$). By contrast, the

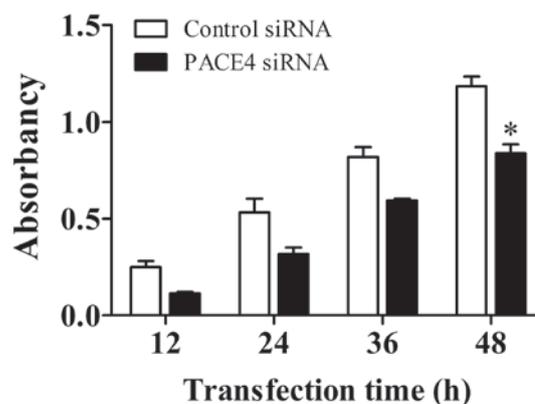


Figure 1. Proliferation of *Panc-1* cells is inhibited by *PACE4* siRNA. *Panc-1* cells were transfected with *PACE4* siRNA or control siRNA for the indicated durations. Cell proliferation was examined using a Cell Counting Kit-8 assay. The OD value in each well was read at the wavelengths of 450 on a microtiter plate reader. Data are presented as the mean \pm standard deviation of OD450 at 12, 24, 36 and 48 h of siRNA transfection. Each concentration was assessed in triplicate. * $P < 0.05$; *PACE4* siRNA vs. control siRNA. *PACE4*, paired basic amino acid cleaving enzyme 4; siRNA, small interfering RNA; OD, optical density.

levels of XIAP and p-Akt were decreased by $\sim 53\%$ ($P < 0.05$) and $\sim 40\%$ ($P < 0.05$), respectively, following *PACE4* siRNA transfection (Fig. 3B and C).

***PACE4* siRNA induces apoptosis via the mitochondrial signaling pathway.** In order to further understand the molecular mechanisms by which *PACE4* siRNA exerts pro-apoptotic effects, the present study examined the protein expression of mediators in the mitochondrial signaling pathway. Initially, the present study determined whether *PACE4* siRNA stimulated the release of cytochrome *c* into the cytosolic fraction in *Panc-1* cells. As expected, cytochrome *c* was redistributed following *PACE4* siRNA transfection. The level of cytochrome *c* in the mitochondria was significantly decreased by 64% (Fig. 4A and B; $P < 0.05$). Correspondingly, the levels of cytochrome *c* in the cytosol were increased by 1.83-fold (Fig. 4C and D; $P < 0.05$).

As the Bcl-2 family of proteins are critical in regulating the release of cytochrome *c*, the present study subsequently investigated the possible involvement of Bax and Bcl-2 in the process of *PACE4* siRNA-mediated *Panc-1* cell apoptosis. As shown in Fig. 4E and F, the level of Bax was significantly increased and the level of Bcl-2 was markedly decreased in the *PACE4* siRNA-transfected cells, compared with the control cells. Statistical analysis showed that *PACE4* siRNA increased the ratio of Bax/Bcl-2 by ~ 5.5 -fold ($P < 0.05$).

Discussion

PACE4 has already been highlighted for its potential role in several types of neoplasia, including oral tongue carcinoma (19), hepatocellular carcinoma (20), glioma (21), skin cancer (16,22) and prostate cancer (17). Whereas previous studies have predominantly examined *PACE4* overexpression, the present study focused on gene silencing as a predictive approach to define potential therapeutic benefits. In the present study, the effect of *PACE4* siRNA on apoptosis in a cellular

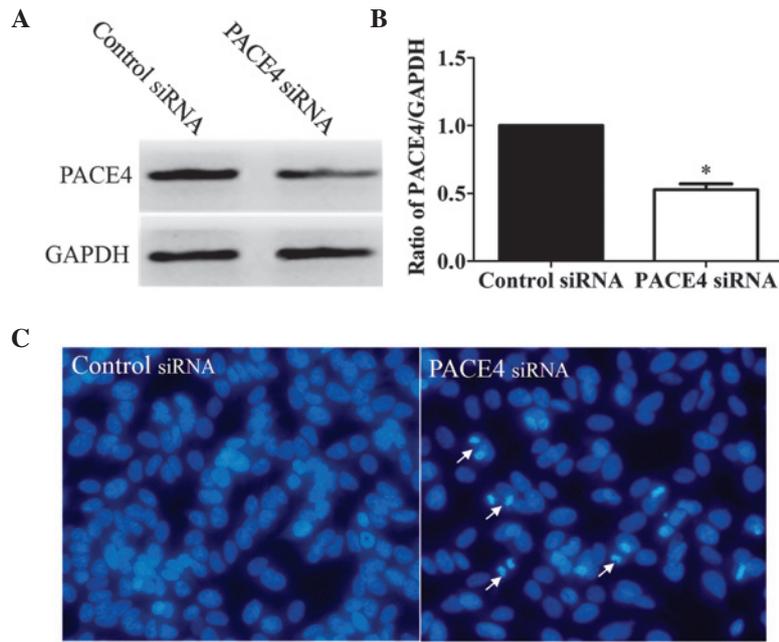


Figure 2. PACE4 siRNA significantly increases apoptosis of Panc-1 cells. Panc-1 cells were transfected with PACE4 siRNA or control siRNA for 48 h. (A and B) Expression of PACE4 in the Panc-1 cells following siRNA transfection. GAPDH was included as a loading control (* $P < 0.05$, vs. control). The data are presented as the mean \pm standard deviation (n=3). (C) At 48 h post-transfection, the cells were incubated with Hoechst 33258 staining buffer. Healthy cells appeared round with intact nuclei, whereas apoptotic cells exhibited nuclear karyopyknosis or fragmentation, as indicated by the white arrows. Magnification, x100. PACE4, paired basic amino acid cleaving enzyme 4; siRNA, small interfering RNA.

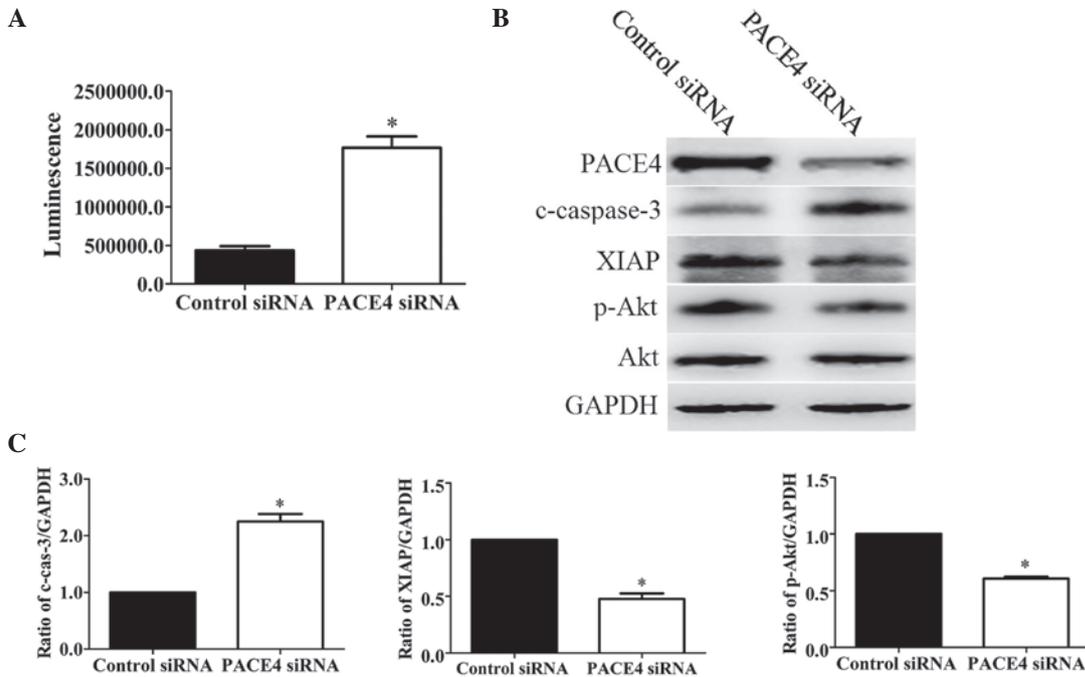


Figure 3. Western blot analysis of apoptotic-associated proteins following PACE4 siRNA transfection. (A) Relative luminescence expression levels of caspase-3/7 in Panc-1 cells transfected with PACE4 siRNA or control siRNA for 48 h. The data are presented as the mean \pm standard deviation of three independent experiments. * $P < 0.05$, compared with the control group. (B) Representative Western blot images. (C) Statistical analyses of the protein expression of c-cas, XIAP and p-Akt. Expression levels in three experiments were quantified by measuring the intensity of the apoptotic-associated proteins relative to GAPDH (loading control). The data are presented as the mean \pm standard deviation (n=3). * $P < 0.05$, vs. control. PACE4, paired basic amino acid cleaving enzyme 4; siRNA, small interfering RNA; c-cas-3, cleaved caspase 3; XIAP, X-linked inhibitor of apoptosis protein; p-Akt, phosphorylated Akt.

model of pancreatic cancer was investigated. The results indicated that PACE4 siRNA inhibited the proliferation of Panc-1 cells. Based on the results of Hoechst 33258 staining,

measurement of caspase-3/7 activities and Western blot analysis, it was concludes that PACE4 siRNA induced apoptosis in the Panc-1 cells. Thus, the present study provided the first

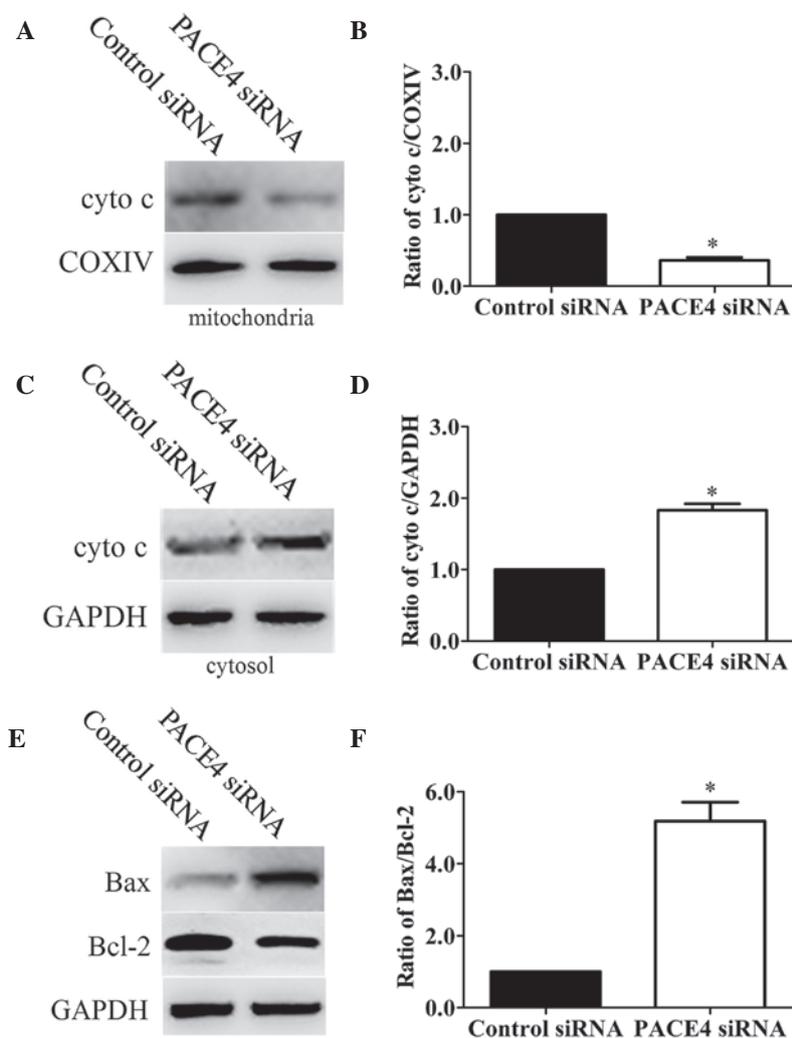


Figure 4. Regulation of mediators in the mitochondrial pathway in apoptotic Panc-1 cells by PACE4 siRNA. Panc-1 cells were transfected with PACE4 siRNA or control siRNA for 48 h, and the levels of (A and B) cyto c in the mitochondria (COXIV as loading control) and (C and D) cytosol (GAPDH as a loading control) were measured using Western blot analysis. (E and F) PACE4 siRNA inhibited the expression of Bcl-2 and increased the expression of Bax, as detected by Western blot analysis. Intensities of the proteins of interest were measured for quantitative analysis. GAPDH was included as a loading control. The data are presented as the mean \pm standard deviation (n=3). *P<0.05, vs. control. PACE4, paired basic amino acid cleaving enzyme 4; siRNA, small interfering RNA; COXIV, cytochrome c oxidase subunit IV; cyto c, cytochrome c; Bcl-2, B cell lymphoma-2; Bax, Bcl-2-associated X protein.

evidence, to the best of our knowledge, that PACE4 has an anti-apoptotic effect in pancreatic cancer cells.

As a primary executioner caspase in the majority of pathways of the caspase protein family, the activation of caspase-3 often results in the irreversible commitment of a cell to apoptosis. Therefore, the activation of caspase-3 is considered a reliable marker for cells undergoing apoptosis (23). The present study found that the activity of caspase-3/7 was significantly activated following PACE4 siRNA transfection (Fig. 3A). An effective strategy for destroying cancer cells is to induce cell apoptosis. XIAP, a member of the inhibitor of apoptosis protein family, contributes to the apoptosis resistance of cancer cells (24,25). Akt is a promoter of cell proliferation and survival, and has been found to be overexpressed in tumor formation (26). Thus, the present study investigated whether these apoptosis-associated proteins were involved in PACE4 siRNA-induced apoptosis. The results of this investigation confirmed the role of PACE4 in the apoptosis of Panc-1 cells, based on the following lines of evidence: PACE4 siRNA

increased the apoptosis of cells by regulating the expression levels of the apoptosis-associated factors c-caspase-3, XIAP and p-Akt (Fig. 3B and C). The inactivation of XIAP and p-Akt by PACE4 siRNA may prevent the development and progression of cancer.

The Bcl-2 family of proteins are important in the apoptosis of cancer cell apoptosis (27,28). The Bcl-2 family can primarily regulate mitochondrial membrane permeabilization (29). The Bax/Bcl-2 ratio is usually regarded as a criterion for apoptosis (30). The results from the present study demonstrated that the level of cytochrome c in the mitochondria was significantly decreased (Fig. 4A and B), whereas that in cytosol was increased (Fig. 4C and D). In addition, PACE4 siRNA increased the levels of Bax and decreased the level of Bcl-2, leading to changes in the ratio of Bax/Bcl-2 (Fig. 4E and F). These results indicated that PACE4 siRNA had an effect on mitochondrial membrane stability. This was evidenced by the increased Bax/Bcl-2 ratio and the release of cytochrome c into the cytoplasm. Taken together, these data demonstrated

that PACE4 siRNA may exert its anti-tumor activity through the mitochondrial signaling pathway (intrinsic pathway) in pancreatic cancer cells.

In conclusion, the results of the present study suggested PACE4 siRNA possesses anti-proliferation and apoptosis-inducing properties in human pancreatic cancer Panc-1 cells. The PACE4 siRNA-induced apoptosis of Panc-1 cells may be mediated through the mitochondria pathway. These results support the potential of PACE4 to be developed as a promising agent for the treatment of pancreatic cancer.

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