

Interleukin-32 α inactivates JAK2/STAT3 signaling and reverses interleukin-6-induced epithelial–mesenchymal transition, invasion, and metastasis in pancreatic cancer cells

Jingfeng Chen^{1,2,*}
 Silu Wang^{1,*}
 Jiadong Su¹
 Guanyu Chu¹
 Heyi You¹
 Zongjing Chen¹
 Hongwei Sun¹
 Bicheng Chen^{1,3}
 Mengtao Zhou¹

¹Department of Surgery, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou,

²Department of Surgery, The Sixth Affiliated Hospital of Wenzhou Medical University, Lishui, ³Zhejiang Provincial Top Key Discipline in Surgery, Wenzhou Key Laboratory of Surgery, Department of Surgery, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, People's Republic of China

*These authors contributed equally to this work

Abstract: Interleukin (IL)-32 is a newly discovered cytokine that has multifaceted roles in inflammatory bowel disease, cancer, and autoimmune diseases and participates in cell apoptosis, cancer cell growth inhibition, accentuation of inflammation, and angiogenesis. Here, we investigated the potential effects of IL-32 α on epithelial–mesenchymal transition, metastasis, and invasion, and the JAK2/STAT3 signaling pathway in pancreatic cancer cells. The human pancreatic cancer cell lines PANC-1 and SW1990 were used. Epithelial–mesenchymal transition-related markers, including E-cadherin, N-cadherin, Vimentin, Snail, and Zeb1, as well as extracellular matrix metalloproteinases (MMPs), including MMP2, MMP7, and MMP9, were detected by immunofluorescence, Western blotting, and real-time polymerase chain reaction. The activation of JAK2/STAT3 signaling proteins was detected by Western blotting. Wound healing assays, real-time polymerase chain reaction, and Western blotting were performed to assess cell migration and invasion. The effects of IL-32 α on the IL-6-induced activation of JAK2/STAT3 were also evaluated. In vitro, we found that IL-32 α inhibits the expressions of the related markers N-cadherin, Vimentin, Snail, and Zeb1, as well as JAK2/STAT3 proteins, in a dose-dependent manner in pancreatic cancer cell lines. Furthermore, E-cadherin expression was increased significantly after IL-32 α treatment. IL-32 α downregulated the expression of MMPs, including MMP2, MMP7, and MMP9, and decreased wound healing in pancreatic cancer cells. These consistent changes were also found in IL-6-induced pancreatic cancer cells following IL-32 α treatment. This study showed that reversion of epithelial–mesenchymal transition, inhibition of invasiveness and metastasis, and activation of the JAK2/STAT3 signaling pathway could be achieved through the application of exogenous IL-32 α .

Keywords: EMT, MMPs, IL-32 α , JAK2, STAT3

Introduction

Pancreatic cancer, a common malignant neoplasm of the digestive system, is an extremely malignant tumor that is characterized by locally advanced or metastatic disease at diagnosis. Its 5-year survival rate is <5%.¹ Only ~10% to 20% of people undergo resection, yet the majority of them (~80%) still achieve <2 years' median survival after surgery.² At present, researchers are devoting great attention to identifying biologically targeted therapies and chemotherapeutic drugs to develop a comprehensive treatment for pancreatic tumors.

Interleukin (IL)-32 was previously reported as natural killer transcript 4, which has been described.^{3,4} This cytokine is primarily elevated in activated T lymphocytes,

Correspondence: Mengtao Zhou; Bicheng Chen
 Department of Surgery, The First Affiliated Hospital of Wenzhou Medical University, 2 Fuxue Lane, Wenzhou 325000, Zhejiang Province, People's Republic of China
 Tel +86 577 5557 9220
 Fax +86 577 8806 9555
 Email zmt0417@hotmail.com; bisonch@163.com

natural killer cells, and epithelial cells. The gene encoding IL-32 is located on human chromosome 16p13.3 and comprises a 705 bp coding sequence that is organized into eight exons. There are six major splice variants (IL-32 α , IL-32 β , IL-32 γ , IL-32 δ , IL-32 ϵ , and IL-32 ζ), and a particular subtype has been reported. IL-32 α is the most common transcript.⁵⁻⁹ This protein has various roles in inflammation, cancer, and autoimmune diseases and participates in cell apoptosis, cancer cell growth inhibition, accentuation of inflammation, and angiogenesis. Several studies have indicated that IL-32 is a typical pro-inflammatory cytokine that enhances the secretion of IL-1 β , tumor necrosis factor- α , IL-6, and IL-8 through the p38 mitogen-activated protein kinase, nuclear factor- κ B, and JNK signal transduction pathways.^{10,11} However, the role of IL-32 α in pancreatic cancer invasion and metastasis has not yet been elucidated.

Metastasis of pancreatic tumor cells is associated with epithelial–mesenchymal transition (EMT), and epithelial cells obtain new mesenchymal features through EMT. EMT is a key biological process in cancer progression by which incipient tumor cells lose their apical–basal polarity, dissolve cell–cell junctions, gain invasive and migratory properties, and increase in drug resistance, resulting in escape from the preinvasive neoplasm, invasion to the edge of the normal tissue, and migration to distant areas.^{12,13} Previous studies have shown that EMT is an essential process in invasion and metastasis in various human epithelial carcinomas, including pancreatic cancer. The loss of E-cadherin expression and the overexpression of many mesenchymal markers, such as Vimentin, N-cadherin, Snail, Slug, Twist, Zeb1, and Zeb2, are generally regarded as markers of the EMT process.^{14,15} It is well known that tumor invasion and metastasis occur through an intricate and multistep process. Dissolving the extracellular matrix is also a key sign of invasion and metastasis in pancreatic cancer. Extracellular matrix metalloproteinases (MMPs) can degrade the cell basal lamina and extracellular matrix, and these enzymes maintain a balance with tissue inhibitors of metalloproteinases, which play vital roles in the invasion and metastasis of malignant tumors.^{16,17}

Signal transducer and activator of transcription 3 (STAT3) plays an important role in EMT. STAT3, a potential therapeutic target in pancreatic cancer, results from the phosphorylation of a conserved tyrosine residue. Two STAT3 monomers form a homodimer (p-STAT3) through reciprocal phosphotyrosine–SH2 tyrosine interaction. The dimer translocates into the nucleus and binds to response elements, thus regulating the transcription of target genes and modulating fundamental cellular processes, such as apoptosis, invasion, and metastasis.^{18,19} Several extracellular signals, including

some cytokines and growth factors, can trigger the JAK/STAT3 signaling pathway and induce a cascade of biological processes.^{20,21} IL-6 secretion increases remarkably in the pancreatic tumor microenvironment, and this increase is significantly related to the invasion and metastasis of pancreatic cancer.²² Moreover, JAK/STAT3 signaling is constitutively activated by IL-6 and frequently observed in various human cancers, including pancreatic cancer.²³⁻²⁵

In this context, we attempted to assess the function of IL-32 α in pancreatic cancer. Our results show that IL-32 α can inactivate novel JAK2/STAT3 signaling, with the additional effect of reversing IL-6-induced EMT, invasion, and metastasis in pancreatic cancer cells.

Materials and methods

Reagents

Recombinant human IL-32 α was purchased from Sino Biological Inc. (Beijing, People's Republic of China), and recombinant IL-6 was obtained from Pepro Tech (Rocky Hill, NJ, USA). Dimethyl sulfoxide and fetal calf serum were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute medium-1640 (RPMI-1640), trypsin-ethylenediaminetetraacetic acid penicillin, streptomycin, and TRIzol were obtained from Gibco-BRL (Thermo Fisher Scientific, Waltham, MA, USA). Antibodies against phospho-JAK2, JAK2, phospho-STAT3 (Tyr705), STAT3, E-cadherin, N-cadherin, Vimentin, Snail, Zeb1, MMP2, MMP9, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Cell Signaling Technology Inc. (Boston, MA, USA). Horseradish peroxidase-conjugated goat antirabbit/mouse secondary antibodies were purchased from Bio-World Co (Nanjing, People's Republic of China).

Cell lines and culture conditions

The human pancreatic cancer cell lines PANC-1 and SW1990 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, People's Republic of China). The PANC-1 cell line was cultured with DMEM, and the SW1990 cell line was maintained in RPMI-1640 supplemented with 10% fetal calf serum containing 100 μ /mL penicillin and 100 μ g/mL streptomycin. Cancer cells were cultured in a humidified incubator with 5% CO₂ and at 37°C. No ethical approval was required for this set of experiments because the experiments were performed on commercially available cell lines, and the ethical committee of the First Affiliated Hospital of Wenzhou Medical University deems approval unnecessary for such studies.

Quantification by real-time PCR

Total RNA was isolated from pancreatic cancer cells according to the manufacturer's instructions for the TRIzol Reagent. Subsequently, a spectrophotometer was used to determine the purity and concentration of the RNA. Single-stranded cDNA was then synthesized using a One-Step RT-polymerase chain reaction (PCR) kit (Thermo Fisher Scientific, Waltham, MA, USA). We obtained the amplified cDNAs by PCR using an ABI Prism 7500 real-time system (Applied Biosystems). The RT-PCR thermal cycler protocol consisted of initial denaturation at 95°C for 10 minutes, denaturation at 95°C for 15 seconds, and annealing and extension at 62°C for 60 seconds. The primer sequences are listed in Table 1.

Western blotting analysis

Pancreatic cancer cells were collected and lysed in RIPA Lysis Buffer (Beyotime, Shanghai, People's Republic of China) after treatment. Subsequently, protein concentrations were determined using a protein assay kit (Beyotime). Lysates containing 50 μ g of protein were dissolved in loading buffer with SDS and heated for 5 minutes at 100°C. Next, the sample was separated by 8%–12% SDS-PAGE at 55 V for 30 minutes and 110 V for 90 minutes. Then, the protein was transferred onto PVDF membranes by wet blotting. The PVDF membranes were incubated in Tris-Buffered Saline with Tween 20 (TBS-T) buffer containing 5% defatted milk and TBS-T at room temperature for 1.5 hours, after which the membranes were immersed in solutions containing the primary antibodies, including anti-p-JAK2 and anti-JAK2 (1:1,000 dilution), anti-p-STAT3, and anti-STAT3 (1:2,000 dilution), anti-MMP2 and anti-MMP9 (1:1,000 dilution), anti-E-cadherin, anti-N-cadherin, anti-Vimentin, anti-Snail, and anti-ZEB1 (1:1,000 dilution), and anti-GAPDH (1:1,000 dilution) at 4°C overnight.

After incubation with primary antibodies, the membrane was washed three times with TBS-T for 5 minutes each time. The membranes were probed with a horseradish peroxidase-conjugated rabbit IgG or mouse IgG secondary

antibody (1:5,000 dilution) for 1.5 hours at room temperature. After washing with TBS-T, the signal was detected using Amersham TM ECL TM Prime, and the expression levels of the specific proteins were quantified and captured using Image Quant TM 400.

Immunofluorescence staining

The pancreatic cancer cell lines PANC-1 and SW1990 were maintained on cover glasses in an incubator for 24 hours according to a previously described method. Then, the cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 minutes at 37°C. Cell membranes were perforated using PBS containing 0.3% Triton X-100 for 10 minutes. After the cells were saturated with PBS containing 10% goat serum (Beyotime) for 1.5 hours, they were incubated with anti-N-cadherin (1:200 dilution) and anti-Vimentin (1:200 dilution) in PBS overnight. After that, the cells were washed three times with PBS at room temperature, followed by incubation with secondary antibody conjugated with AlexaFluor 488 to detect Vimentin and N-cadherin. The samples were washed with PBS three times, and the cells nuclei were stained with 4',6-diamidino-2-phenylindole (Beyotime). Finally, the glass slides were photographed using an automated upright microscope system (Leica, DM4000B Leica Microsystems, Wetzlar, Germany).

Wound healing assay

To assess cell motility, confluent cells were seeded in 6 cm culture dishes, and after the cells were grown to 80%–90% confluence, a wound ~500 μ m was created as a linear scratch using a sterile p200 pipette tip. The cells were washed twice with PBS and incubated for 24 hours in serum-free DMEM (for PANC-1) or serum-free RPMI-1640 (for SW1990) containing 25 μ g/mL IL-32 α and/or 100 ng/mL IL-6. The images of cell migration were captured microscopically at 0 and 24 hours after wounding. The wound area was captured using Image Pro Plus. The migration rate = (wound area/wound height after 0 hour – wound area/wound height after 24 hours)/24.

Table 1 The primers used in real-time PCR

Gene	Sense (5'-3')	Antisense (5'-3')
E-cadherin	TCACGCTGTGTCATCCAACGG	TAGGTGTTACATCATCGTCCGC
N-cadherin	CATCATCATCCTGCTTATCCTTGT	GGTCTTCTTCTCCTCCACCTTCTT
Vimentin	AATCCAAGTTTGCTGACCTCTCTGA	ACTGCACCTGTCTCCGGTACTC
Snail	CTTCTCCTTACTTCAGTCTCTCC	TGAGGTATTCCTTGTTCAGTATT
Zeb1	CGCTTCTCACACTCTGGGTCTTATT	CCTTCTCCCTTGTAAACTCTCT
MMP2	GGGGGAAGATGCTGCTGTT	AGCGGTCCTGGCAGAAATAG
MMP9	CAGTCCACCCTTGTGCTCTTCC	CATCTCTGCCACCCGAGTGTAAC
MMP7	AGTGAGCTACAGTGGGAACAGGC	CATTATTTCTATGACGCGGGAGT
GAPDH	GGGTGTGAACCATGAGAAGTATG	GATGGCATGGACTGTGGTCAT

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP, matrix metalloproteinase; PCR, polymerase chain reaction.

Transwell invasion assay

The invasive abilities of PANC-1 and SW1990 were investigated using a particular invasion chamber (BD Biosciences, San Jose, CA, USA), and an 8 μm porosity polyethylene terephthalate membrane with basement membrane matrix was inserted (Corning Incorporated, Corning, NY, USA). Culture medium containing 10% fetal bovine serum (FBS) was placed in the lower compartment as a chemical inducer. After treatment with IL-32 α (25 $\mu\text{g}/\text{mL}$) and/or IL-6 (100 ng/mL), 10^5 pancreatic cancer cells (PANC-1 or SW1990) that had been cultivated in 0.2 mL medium RPMI-1640 (for SW1990) or DMEM (for PANC-1) with 0.2% bovine serum albumin were placed in the upper compartment and incubated at 37°C for 24 hours. Then, the cells on the upper surface of the filter were removed by scraping. Subsequently, the filters were washed with PBS three times and fixed for 20 minutes using 4% paraformaldehyde. Afterward, the cells in the lower compartment were stained with crystal violet. Finally, the cells that invaded across the basement membrane to the lower compartment of the filter were counted under an automated upright microscope system (Leica, DM4000B).

Statistical analysis

All experiments were repeated three times, and data are expressed as the mean \pm standard deviation. Student's *t*-test was used to assess significance by checking the statistical correlation of data between groups. Differences with $P < 0.05$ were considered to be statistically significant. All analyses were performed using SPSS 19.0 software (IBM Corporation, Armonk, NY, USA).

Results

Inhibition of EMT by IL-32 α in a dose-dependent manner in pancreatic cancer cell lines

In our experiment, we used serum-free medium to avoid the growth factors that are contained in serum. We treated PANC-1 and SW1990 with 0, 10, 25, and 50 $\mu\text{g}/\text{mL}$

concentrations of IL-32 α , respectively. Then, the EMT of pancreatic cancer cells was assessed after treatment with IL-32 α for 24 hours.

First, we investigated EMT-related genes in pancreatic cancer cell lines. The mRNA levels of E-cadherin gene (CDH1), N-cadherin gene (CDH2), Vimentin, Snail, and Zeb1 were quantified by real-time PCR as shown in Figure 1A and B, and the results suggested that IL-32 α reduced the mRNA expression of N-cadherin gene (CDH2), Vimentin, Snail, and Zeb1 and increased the mRNA expression of E-cadherin gene (CDH1) in a dose-dependent manner. However, the expression of Snail mRNA was increased when treated with IL-32 α in SW1990 cell lines.

We also detected several protein levels by Western blotting, including the epithelial-like marker E-cadherin and the mesenchymal-like markers N-cadherin, Vimentin, Snail, and Zeb1. As shown in Figure 1C–F, N-cadherin, Vimentin, Snail, and Zeb1 expression levels are reduced, while the protein levels of E-cadherin are increased after treatment with IL-32 α in a dose-dependent manner.

To verify that IL-32 α suppressed the process of EMT, the cellular protein levels of N-cadherin and Vimentin were examined by immunofluorescence, and the results showed that these two proteins, which can inhibit EMT, were increased after IL-32 α treatment in a dose-dependent manner (Figure 1G and H). N-cadherin and Vimentin are well-defined mesenchymal-like markers for EMT. The progression of EMT is always associated with expression levels of N-cadherin and Vimentin. Taken together, our findings revealed that IL-32 α was able to reverse the EMT process in the PANC-1 and SW1990 cell lines.

Effect of IL-32 α on the expression of genes associated with invasion and metastasis in pancreatic cancer cell lines

Cadherin switching (from E-cadherin to N-cadherin) and increased expression of MMPs are related to the acquisition

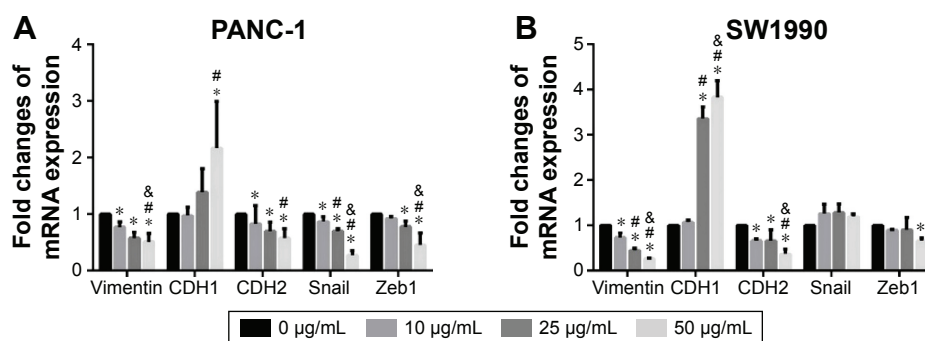


Figure 1 (Continued)

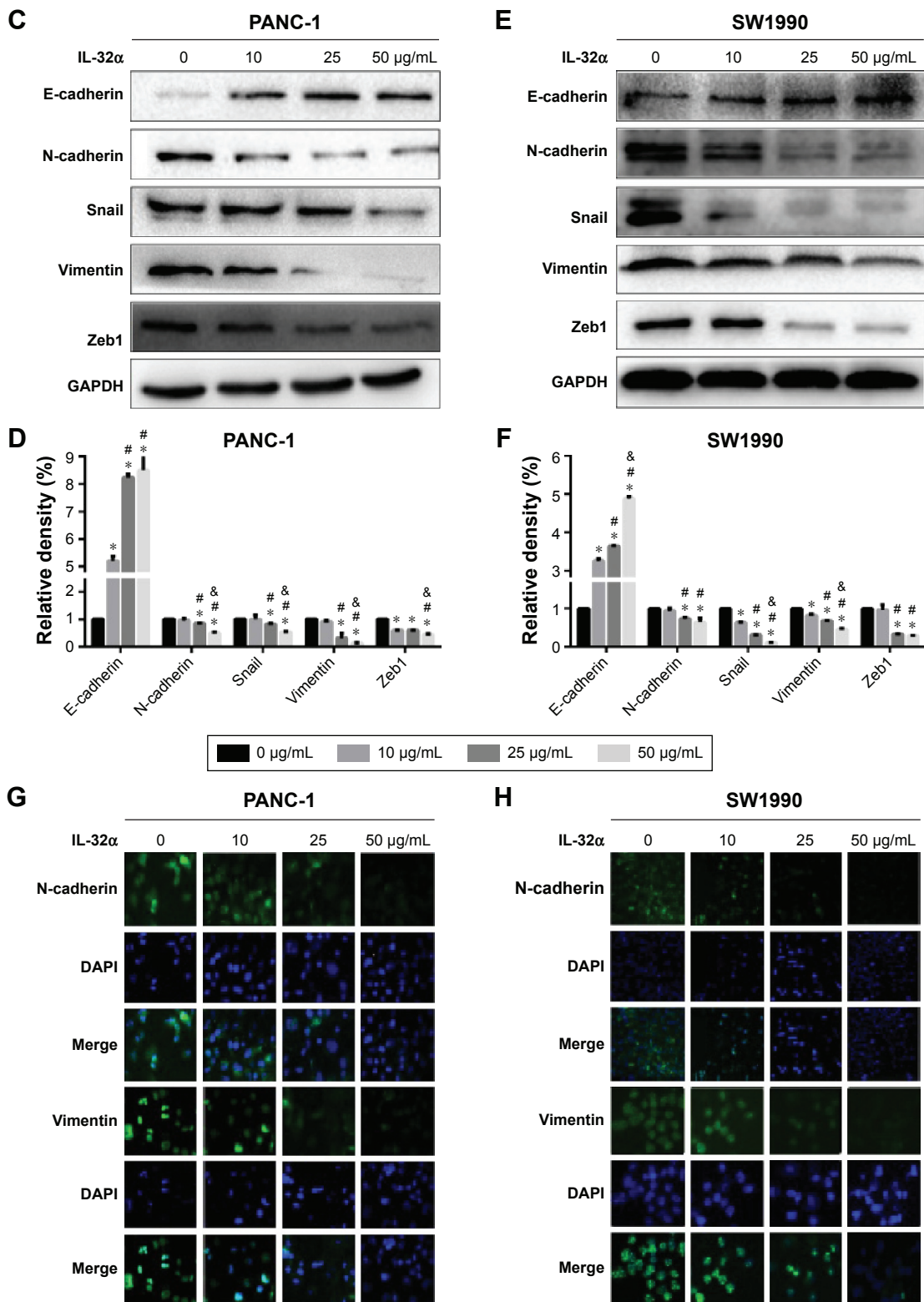


Figure 1 Dose-dependent inhibition of EMT in SW1990 and PANC-1 cell lines by IL-32 α . **Notes:** (A, B) Fold change in mRNA expression of Vimentin, CDH1 (E-cadherin), CDH2 (N-cadherin), snail, and Zeb1 in PANC-1 and SW1990 cells; GAPDH was used as an endogenous control. (C–F) The expression of E-cadherin, N-cadherin, Snail, Zeb1, and Vimentin was measured by Western blotting using IL-32 α in PANC-1 and SW1990. GAPDH was used as a loading control. The relative densities of each group are presented in graphs (D) and (F). (G, H) Expression of N-cadherin and Vimentin detected by immunofluorescence. Minimal staining (membranous expression of N-cadherin and Vimentin) was observed in PANC-1 and SW1990 cells after incubation with IL-32 α for 24 hours. PANC-1 and SW1990 cell lines were treated with IL-32 α at concentrations of 0, 10, 25, and 50 μ g/mL for 24 hours. Data are represented as the mean \pm SEM, n=3, * P <0.05 vs 0 μ M group; # P <0.05 vs 10 μ M group; & P <0.05 vs 25 μ M group. Magnification details are as follows; PANC-1-N-cadherin 400 \times ; PANC-1-Vimentin 400 \times ; SW1990 N-cadherin 200 \times ; SW1990 Vimentin 400 \times . Comparisons among multiple groups were made with a one-way analysis of variance followed by Least Significant Difference or Dunnett's t -test. **Abbreviations:** DAPI, 4',6-diamidino-2-phenylindole; EMT, epithelial–mesenchymal transition; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; mRNA, messenger RNA; SEM, standard error of mean.

of invasiveness and metastatic potential in cancer cells. To investigate the effects of IL-32 α on pancreatic cancer cell invasion and metastasis, we analyzed the mRNA expression of MMP2, MMP7, MMP9, CDH1, and CDH2 by real-time PCR (Figures 1A and B and 2A and B). Decreased mRNA levels of MMP2, MMP7, and CDH2 in combination with elevated mRNA levels of CDH1 and MMP9 after IL-32 α treatment suggested that IL-32 α can reverse EMT in pancreatic cancer cell lines. IL-32 α also suppressed the protein

levels of MMP2 and MMP9 in a dose-dependent manner (Figure 2C–F). Furthermore, IL-32 α upregulated the protein expression of E-cadherin, whereas the N-cadherin protein level was decreased (Figure 1C–F). It is well known that E-cadherin can increase the ability to form cell–cell junctions and, thus, adhesion. As shown earlier, our results indicated that IL-32 α partially inhibited metastasis and invasion by affecting proteolytic activation and adhesive activity.

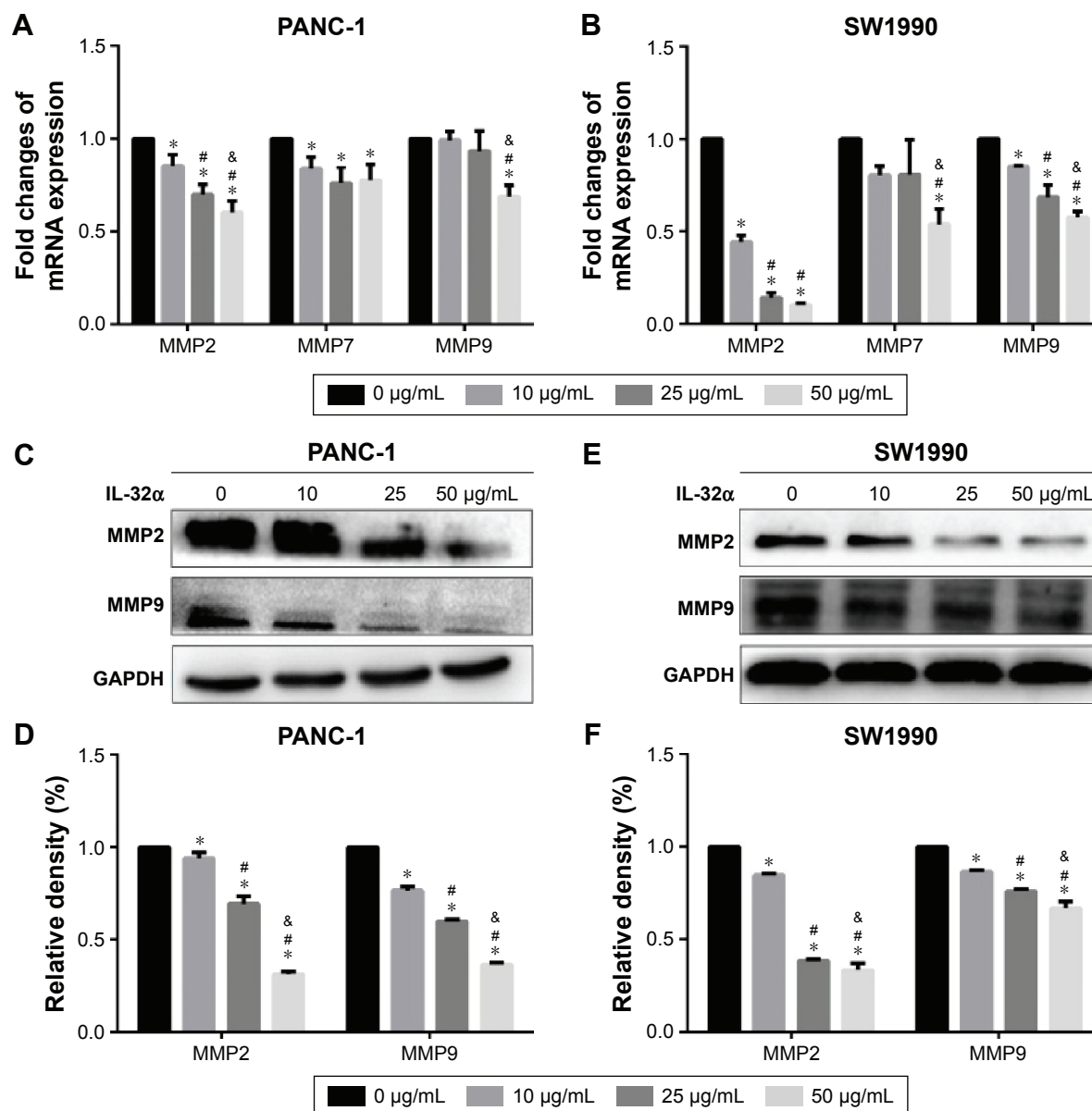


Figure 2 Effect of IL-32 α on the expression of genes associated with invasion and metastasis in pancreatic cancer cell lines.

Notes: (A, B) Fold change in mRNA expression of MMP2, MMP7, and MMP9 in PANC-1 and SW1990 cells. GAPDH was used as an endogenous control. (C–F) The expression of MMP2 and MMP9 was measured by Western blotting after treatment with IL-32 α in PANC-1 and SW1990. GAPDH was used as a loading control. The relative densities of each group are presented in graphs (D) and (F). PANC-1 and SW1990 cell lines were treated with IL-32 α at concentrations of 0, 10, 25, and 50 $\mu\text{g/mL}$ for 24 hours. Data are represented as the mean \pm SEM, $n=3$, * $P<0.05$ vs 0 μM group; # $P<0.05$ vs 10 μM group; & $P<0.05$ vs 25 μM group. Comparisons among multiple groups were made with a one-way analysis of variance followed by Least Significant Difference or Dunnett's t -test.

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; MMPs, metalloproteinases; mRNA, messenger RNA; SD, standard deviation; SEM, standard error of mean.

Suppression of activation of JAK2/STAT3 signaling proteins following exogenous IL-32 α treatment in pancreatic cancer cell lines PANC-1 and SW1990

The JAK/STAT3 pathway is known to be involved in the EMT process and MMP expression in pancreatic cancer. Our Western blotting results showed that exogenous IL-32 α inhibited JAK2, p-JAK2, and p-STAT3 in a dose-dependent manner but had little effect on STAT3 expression (Figure 3A–D).

Exogenous IL-32 α inhibits IL-6-induced EMT of pancreatic cancer cell lines PANC-1 and SW1990

Many studies have shown that IL-6 can induce EMT in various cancers. Our results also revealed that human recombinant IL-6 (100 ng/mL) induced EMT in PANC-1 and SW1990

(Figure 4A–F). We observed changes in cell morphology; in particular, a transition to a spindle-shaped morphology was detectable in the PANC-1 and SW1990 cell lines after treatment with IL-6 for 24 hours (Figure 4G and H). Our studies indicated that EMT characteristics and JAK2/STAT3 activity were both inhibited by IL-32 α (Figures 1 and 3). Growing evidence has suggested that stimulation of the JAK/STAT3 signaling pathway might enhance the process of EMT in cancer cells.^{21,25} IL-6 has been used to activate the JAK2/STAT3 signaling pathway in vitro.²⁴ Hence, we attempted to suppress the JAK2/STAT3 signaling by treatment with IL-32 α and activate it by treatment with IL-6 in pancreatic cancer cells. Then, we observed the effects on EMT-related molecular markers. We found that IL-32 α stimulation decreased levels of p-JAK2, JAK2, and p-STAT3 in cells induced with IL-6, although total STAT3 levels did not change in cells treated

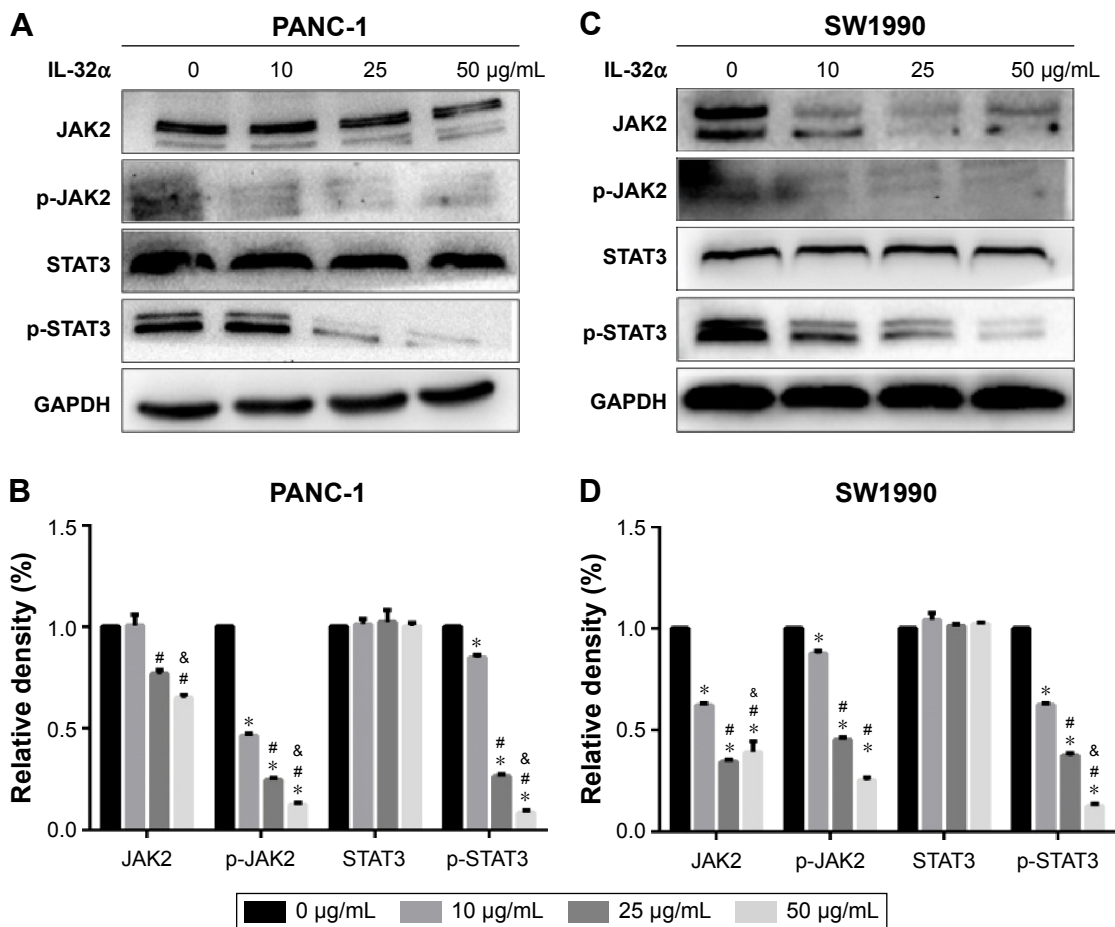


Figure 3 Dose-dependent inactivation of JAK2/STAT3 signaling in SW1990 and PANC-1 cells by IL-32 α .

Notes: (A–D) The expression of the signaling proteins JAK2, p-JAK2, STAT3, and p-STAT3 measured by Western blotting after treatment with various concentrations of IL-32 α for 24 hours in PANC-1 and SW1990 cells. The relative densities of each group are presented in graphs (B) and (D). PANC-1 and SW1990 cell lines were treated with IL-32 α at concentrations of 0, 10, 25, and 50 $\mu\text{g/mL}$ for 24 hours. Data are represented as the mean \pm SEM, $n=3$, * $P<0.05$ vs 0 μM group; # $P<0.05$ vs 10 μM group; & $P<0.05$ vs 25 μM group.

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; mRNA, messenger RNA; SEM, standard error of mean.

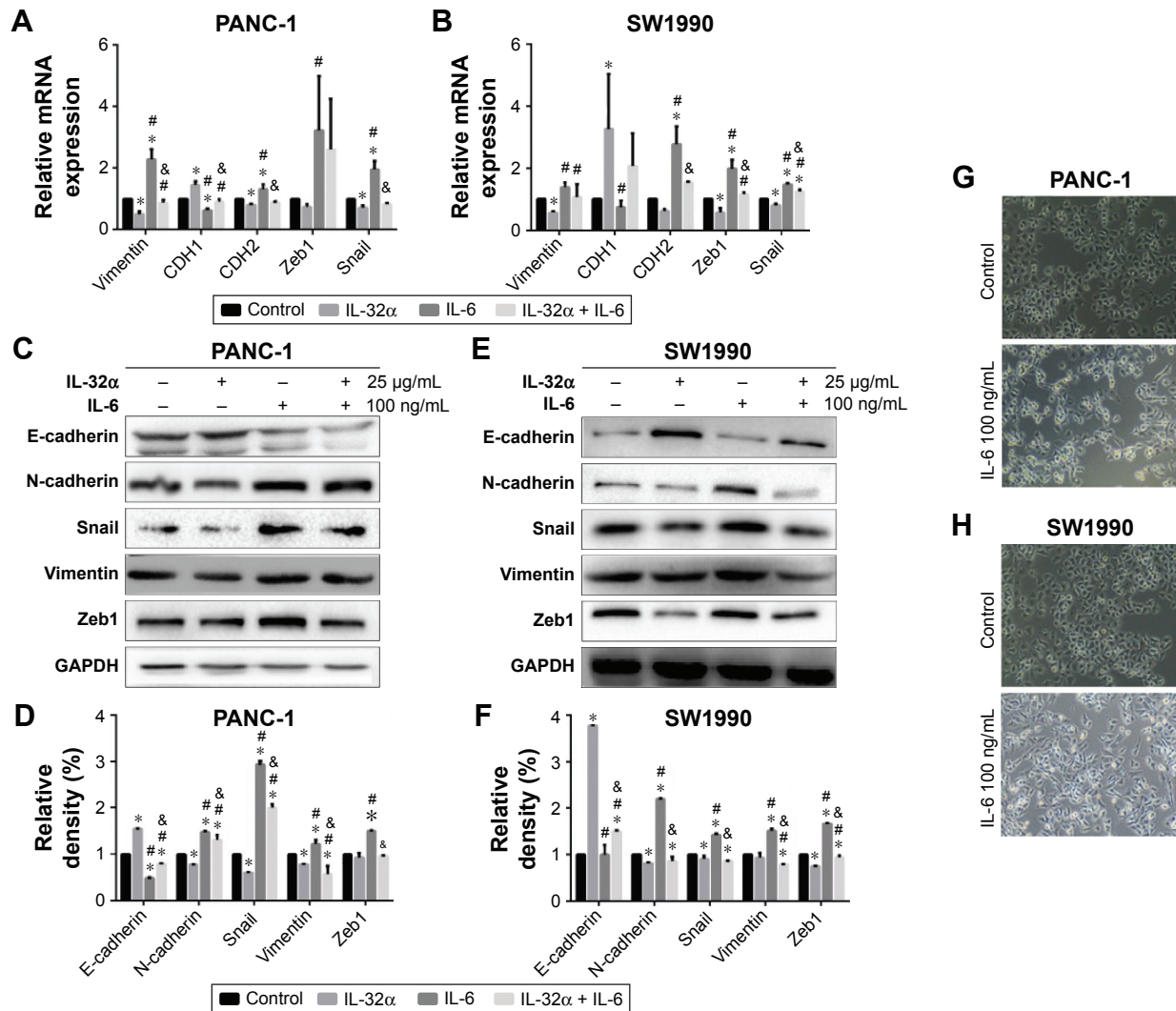


Figure 4 Inhibition of IL-6-induced EMT in PANC-1 and SW1990 cell lines following treatment with IL-32 α .

Notes: (A, B) Fold change in mRNA expression of Vimentin, CDH1, CDH2, Zeb1, and Snail in PANC-1 and SW1990 cells. GAPDH was used as an endogenous control. (C–F) Protein expression levels of E-cadherin, N-cadherin, Vimentin, Snail, and Zeb1 were examined by Western blotting for 24 hours in PANC-1 and SW1990 cells. GAPDH was used as a loading control. The relative densities of each group are presented in graphs (D) and (F). (G, H) Morphological changes after treatment with IL-6 (100 ng/mL) in PANC-1 and SW1990 cells. Cell morphology was observed under an inverted phase-contrast microscope. Original magnification: $\times 100$. PANC-1 and SW1990 cell lines were treated with IL-6 at 100 ng/mL in FBS-free media, and then IL-32 α was added at a concentration of 25 μ g/mL for 24 hours. Data are represented as the mean \pm SEM, $n=3$, $*P<0.05$ vs 0 μ M group; $\#P<0.05$ vs 10 μ M group; $\&P<0.05$ vs 25 μ M group. 200 \times magnification.

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; mRNA, messenger RNA; SEM, standard error of mean; EMT, epithelial-mesenchymal transition.

with IL-6 and/or IL-32 α (Figure 5A–D). Moreover, our Western blotting analysis demonstrated that IL-32 α exerted significant effects on the expression of important EMT markers, including E-cadherin, N-cadherin, Vimentin, Snail, and Zeb1, in PANC-1 and SW1990 cells induced with IL-6 for 24 hours (Figure 4C–F).

Using real-time PCR, we also determined the mRNA levels of E-cadherin, N-cadherin, Vimentin, Snail, and Zeb1 in IL-6-induced PANC-1 and SW1990 cells after treatment with IL-32 α for 24 hours (Figure 4A and B). The real-time PCR results were in line with the Western blotting results, which indicated that the mesenchymal markers N-cadherin,

Snail, Vimentin, and Zeb1 presented the same variation on this trend as p-STAT3, whereas E-cadherin changed oppositely. Taken together, these data showed that exogenous IL-32 α inhibited IL-6-induced EMT as well as inactivating the JAK2/STAT3 signaling pathway in the pancreatic cancer cell lines PANC-1 and SW1990.

Exogenous IL-32 α inhibits IL-6-induced migration and invasion of pancreatic cancer cell lines PANC-1 and SW1990

Our results also demonstrated that IL-6 can enhance the expression of genes that facilitate metastasis and invasion,

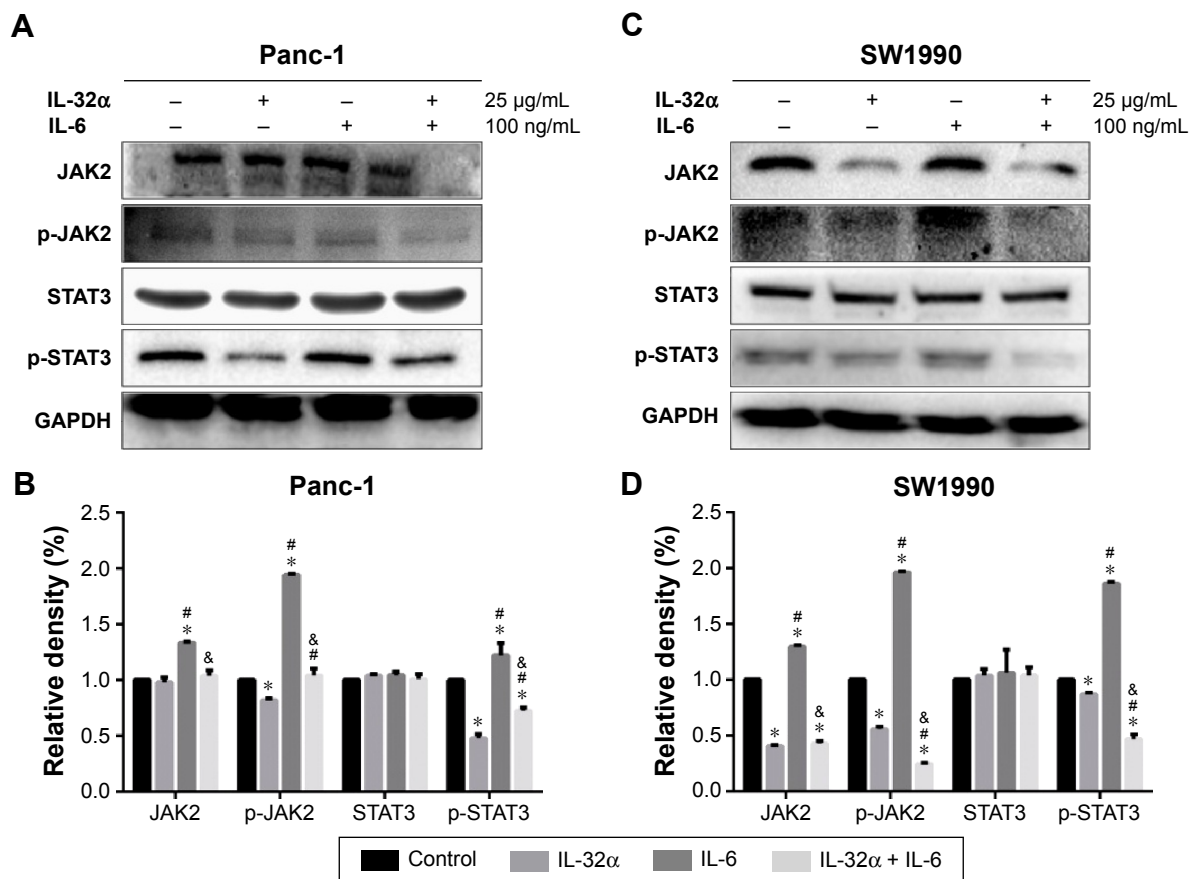


Figure 5 Inactivation of IL-6-induced JAK2/STAT3 signaling pathway in PANC-1 and SW1990 cell lines following treatment with IL-32 α .

Notes: (A–D) Protein expression levels of JAK2, p-JAK2, STAT3, and p-STAT3 were examined by Western blotting for 24 hours in PANC-1 and SW1990 cells. GAPDH was used as a loading control. The relative densities of each group are presented in graphs (B) and (D). PANC-1 and SW1990 cell lines were treated with IL-6 at 100 ng/mL in FBS-free media and treated with IL-32 α at a concentration of 25 μ g/mL for 24 hours. Data are represented as the mean \pm SEM, $n=3$, * $P<0.05$ vs 0 μ M group; # $P<0.05$ vs 10 μ M group; & $P<0.05$ vs 25 μ M group.

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; SEM, standard error of mean.

as determined by Western blotting and real time-PCR (Figure 6A–F). Additionally, to evaluate the effects on the metastasis and invasiveness of pancreatic cancer cells treated with IL-32 α and IL-6, we performed wound assays and invasion assays in vitro. Our experimental results indicated that IL-6 not only enhanced the migration rate but also promoted the invasiveness of the pancreatic cancer cell lines PANC-1 and SW1990 ($P<0.05$) (Figure 7A–D). Subsequently, we investigated whether IL-32 α could weaken the IL-6-induced invasiveness and metastasis in PANC-1 and SW1990 cells induced with IL-6. After treatment with IL-32 α , the mRNA levels of MMP2, MMP7, and MMP9 and the protein expression of MMP2 and MMP9 were significantly decreased in IL-6-induced PANC-1 and SW1990 (Figure 6A–F). According to the migration rate determined for each image and the number of invasive cells found on every filter, IL-32 α markedly reduced the metastasis and invasiveness of PANC-1 and SW1990 cells ($P<0.05$)

compared with IL-6-treated cells (Figure 7A–D). Taken together, our study results indicated that IL-32 α was able to weaken IL-6-induced migration and invasion in the pancreatic cancer cell lines PANC-1 and SW1990.

Discussion

Previous studies indicated that IL-32 α was involved in many tumor biological processes, including promoting inflammation, angiogenesis, and cell apoptosis. However, its effects on pancreatic cancer metastasis and EMT-relevant signaling pathways had not yet been revealed. In this study, we found that exogenous IL-32 α could deactivate JAK2/STAT3 signaling and suppress EMT and MMP secretion in pancreatic cancer cells in a dose-dependent manner. IL-6 was used to induce EMT and facilitate invasiveness and metastasis in pancreatic tumor cells in our study, and we found that IL-32 α could reverse IL-6-induced EMT, invasiveness, and metastasis in pancreatic cancer cells in vitro.

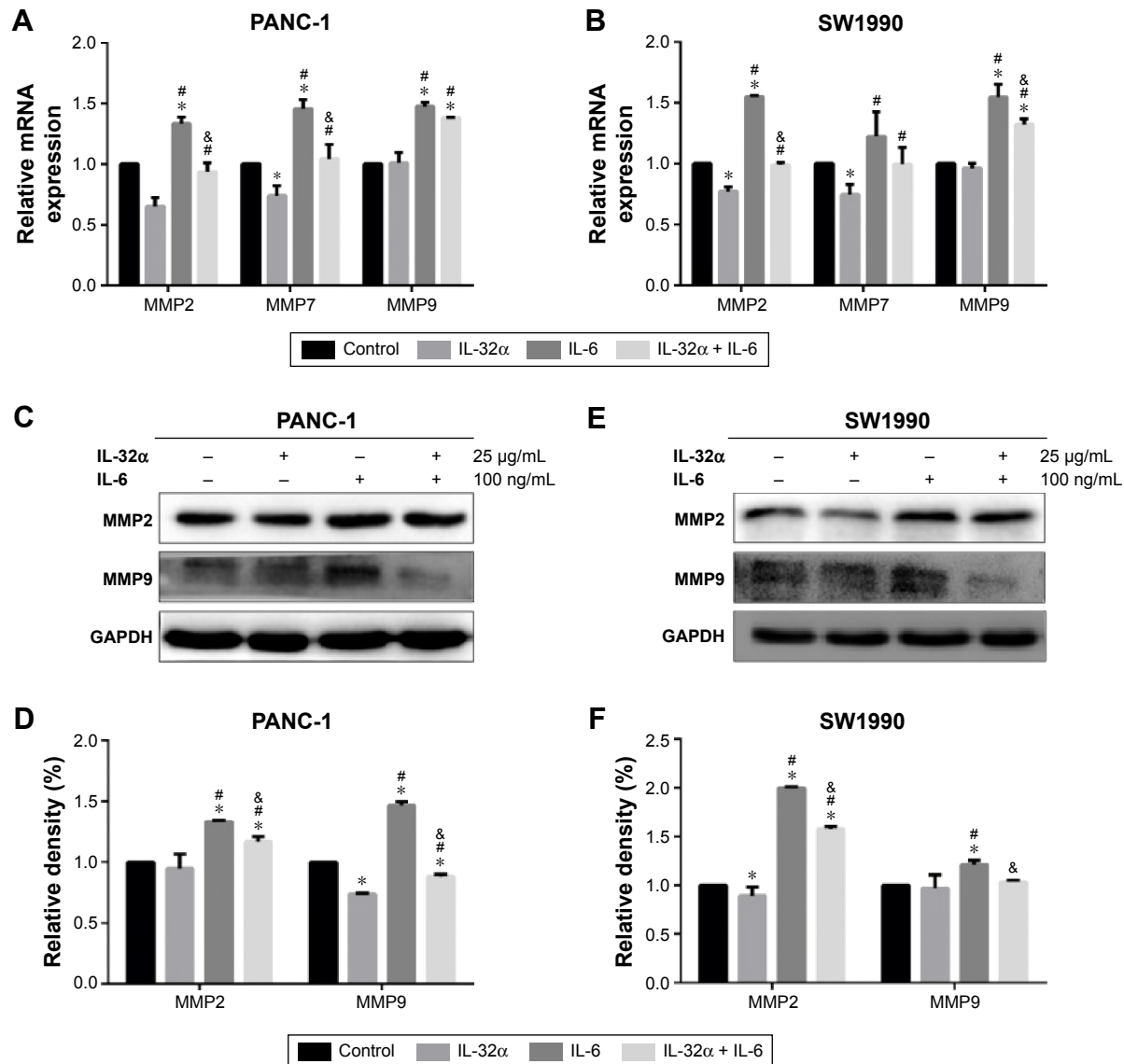


Figure 6 IL-32 α inhibition of IL-6-induced MMP secretion in PANC-1 and SW1990 cell lines.

Notes: (A, B) Fold change in mRNA expression of MMP2, MMP7, and MMP9 in PANC-1 and SW1990 cells. GAPDH was used as an endogenous control. (C–F) Protein expression levels of MMP2 and MMP7 were examined by Western blotting for 24 hours in PANC-1 and SW1990 cells. GAPDH was used as a loading control. The relative densities of each group are presented in graphs (D) and (F). PANC-1 and SW1990 cell lines were treated with IL-6 at 100 ng/mL in FBS-free media, and then IL-32 α was added at a concentration of 25 μ M for 24 hours. Data are represented as the mean \pm SEM, n=3, * P <0.05 vs 0 μ M group; # P <0.05 vs 10 μ M group; & P <0.05 vs 25 μ M group.

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; MMPs, matrix metalloproteinases; mRNA, messenger RNA; SEM, standard error of mean.

Pancreatic carcinoma is one of the most malignant tumor diseases. It is believed that the high invasiveness of pancreatic cancer cells plays a critical role in the disastrous prognoses associated with this disease. EMT is a pivotal biological process in cancer progression that enables the initial tumor cells to obtain invasive and metastatic properties.¹² It has been reported that EMT has close relationships with lymph node metastasis, portal vein invasion, and long-term survival in pancreatic carcinoma.^{26,27} Therefore, EMT is becoming an increasingly important potential clinical target of pancreatic cancer therapy. In our research, IL-32 α was shown to

reverse the EMT phenotype of pancreatic cancer (Figure 1). We believe that this finding indicates that IL-32 α has the potential power to influence the biological properties of pancreatic cancer. Another significant factor was MMPs, which have been regarded as effective regulators of invasion and metastasis in pancreatic cancer.²⁸ MMPs are a family of endopeptidases with proteolytic activity to degrade the basement membrane in the process of EMT. MMP secretion has been verified to increase invasiveness, induce chemoresistance, and promote angiogenesis in pancreatic cancer.^{29–31} In our research, we confirmed that the pancreatic cancer cell lines

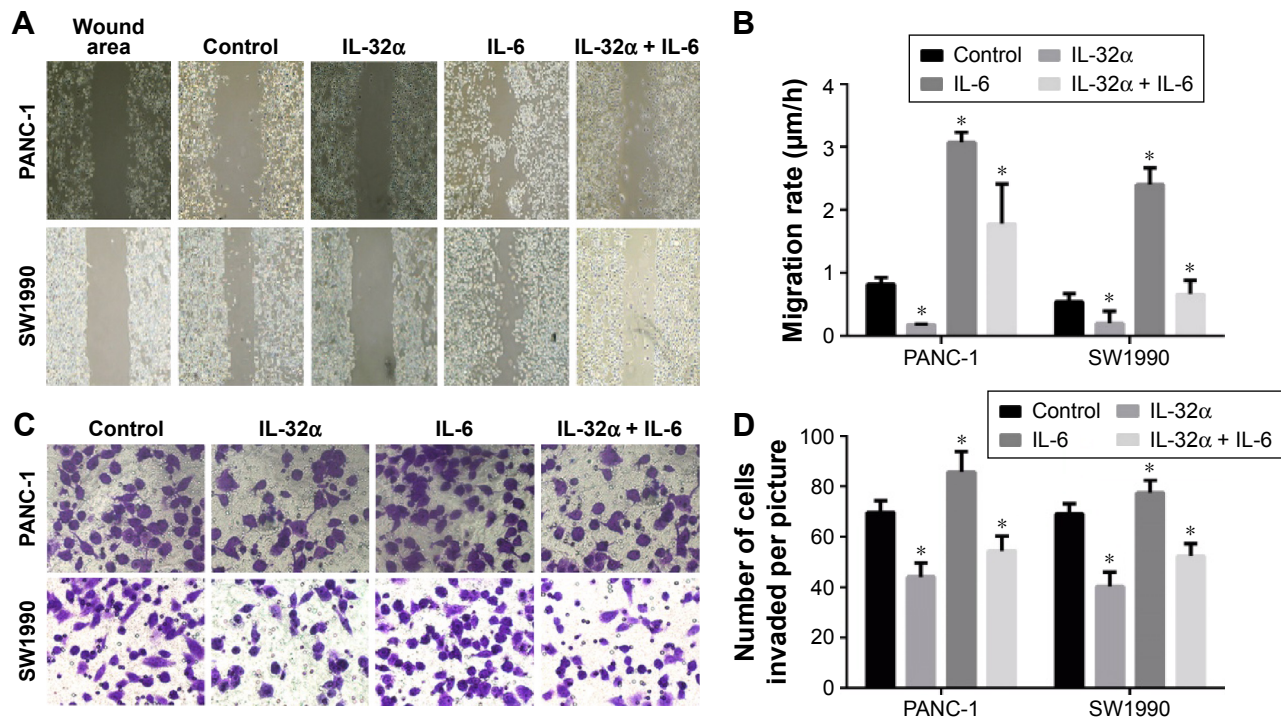


Figure 7 IL-32 α inhibition of IL-6-induced metastasis and invasiveness in PANC-1 and SW1990 cell lines.

Notes: (A) The wound healing assay was performed in various treatment groups of PANC-1 and SW1990 cells. Original magnification: $\times 40$. (B) Analysis of the migration rate after wounding in various treatment groups of PANC-1 and SW1990 cells. (C) The invasion assay was performed in various treatment groups of PANC-1 and SW1990 cells using a specialized invasion chamber. The blue-stained cells were those that invaded the ECMatrix and migrated through the polycarbonate membrane to the lower surface of the membrane (original magnification: $\times 200$). (D) The number of invaded cells per image in various treatment groups of PANC-1 and SW1990 cells. The results are presented as the mean \pm SD of experiments performed in triplicate. Data are represented as the mean \pm SEM, $n=3$, $*P<0.05$ vs $0 \mu\text{M}$ group. Adobe Photoshop CS6 was used to merge the immunofluorescence images. Graph Pad Prism 6 Demo was used to create bar graphs. Western blotting was analyzed using Image Lab. PANC-1 and SW1990 cell lines were treated with IL-6 (100 ng/mL) alone or in combination with IL-32 α (25 $\mu\text{g/mL}$) for 24 hours. $400\times$ magnification.

Abbreviations: ECMatrix, basement membrane matrix; IL, interleukin; SD, standard deviation; SEM, standard error of mean.

PANC-1 and SW1990 secrete high levels of MMP2, MMP7, and MMP9 and that these MMPs were downregulated by IL-32 α . Regarding the influence of IL-32 on EMT, migration, and invasion, researchers hold different or even opposite views. Jeong et al³² reported that dysregulation of IL-32 β stimulates migration through the VEGF-STAT3 signaling pathway. IL-32 has also been reported to facilitate MMP2 and MMP9 expression in primary lung adenocarcinoma via nuclear factor- κB activation.³³ Interleukin-32 contributes to invasion and metastasis of primary lung adenocarcinoma via NF- κB induced matrix metalloproteinases 2 and 9 expression. Interleukin-32 β stimulates migration of MDA-MB-231 and MCF-7 cells via the VEGF-STAT3 signaling pathway. First, we thought that different types and amounts of IL-32 receptors are expressed in different types of cancer, which could explain the different effects caused by IL-32 isoforms. Moreover, we accidentally found that high concentrations of IL-32 α can increase the expression of proteins associated with apoptosis in vitro (data not shown). Thus, different concentrations of IL-32 α were considered to be another factor influencing EMT, migration, and invasion.

It has been reported that secretion of IL-6 by pancreatic cancer tissues was obviously higher than that of tissue adjacent to carcinoma.³⁴ Therefore, our research employed IL-6 to induce EMT via the activation of the JAK2/STAT3 signaling pathway in pancreatic cancer cells to simulate the tumor microenvironment in vivo. Numerous studies have indicated that elevated levels of IL-6 protein and mRNA in serum and tumor samples from patients with pancreatic cancer are related to increased tumor size, lymphatic metastasis, distant metastasis, and tumor progression.^{35,36} In our study, IL-6 also induced EMT and enhanced the expression of MMPs, including MMP2, MMP7, and MMP9, in vitro. IL-6, which is secreted by cancer cells and macrophages in the tumor microenvironment, directly binds to its receptor to activate JAKs through certain downstream signaling pathways; in turn, this process increases the activation of STAT3, which is involved in pancreatic cancer initiation and metastasis. Our results showed that IL-6 activation of the JAK2/STAT3 signaling pathway was suppressed by IL-32 α , which was consistent with its effects on the expression of genes associated with metastasis and invasion. Moreover, it has

been confirmed that the IL-6/JAK2/STAT3 pathway plays an important role in pancreatitis-induced, Kras-dependent pancreatic carcinogenesis.^{37,38} As a potent inhibitor of JAK2/STAT3 signaling, IL-32 α also shows the potential ability to block pancreatic cancer initiation.

Moreover, during our study, we assessed the autonomous phosphorylation of STAT3 (p-STAT3) in four pancreatic cancer cell lines: SW1990, PANC-1, AsPC-1, and BxPC-3. All four cell lines showed high levels of autonomous p-STAT3. Although it is not considered to be a classic pathway interacting with EMT, a recent study confirmed that STAT3 was also shown to contribute to EMT through comprehensive alterations of transcription factors, such as Zeb1.^{39,40} Our study, however, found that Zeb1 expression levels were increased by the elevation of p-STAT3 signaling in PANC-1 and SW1990 cells, which was in accordance with previous studies. We thought that the significant suppression of high levels of autonomous p-STAT3 by IL-32 α could explain its effects of inhibiting EMT and invasiveness in pancreatic cancer but promoting these properties in some other types of cancer. High levels of p-STAT3 may play an important role in sustaining the EMT state and invasiveness in pancreatic cancer cells. Finally, we cannot rule out the possibility of other latent mechanisms conducive to the suppression of IL-6-induced EMT, migration, and invasion by IL-32 α . Based on the earlier evidence, the relationship between the effect of IL-32 α on EMT and the effect of IL-32 α on tumor migration and invasion still requires further investigation.

Our study suggested that IL-32 α might have the potential for clinical application as an adjuvant treatment for pancreatic cancer. Clinical pancreatic cancer often shows high invasiveness and a very short course before death. The inhibition of EMT and MMPs by IL-32 α might postpone the progression of pancreatic cancer. As IL-32 α is an endogenous cytokine, we deem that its upregulation would be a potential strategy to suppress invasion, metastasis, and chemoresistance. This approach would have some distinct advantages compared to traditional chemotherapeutic drugs. For example, IL-32 α lacks immunogenicity and has low cytotoxicity, and patients may experience reductions in most side effects and myelosuppression caused by traditional chemotherapeutic agents. Of course, all of these biological functions need further study in vivo.

In conclusion, we found that exogenous IL-32 α deactivates JAK2/STAT3 signaling, reverses the process of EMT, and decreases MMP secretion in pancreatic cancer cells. Additionally, our investigation suggests that IL-6-induced EMT, migration, and invasion can be inhibited by IL-32 α .

Thus, IL-32 α may be a potential drug and the JAK2/STAT3 signaling pathway may represent novel potential targets for pharmacological intervention in the management of pancreatic cancer EMT, metastasis, and invasiveness in the future.

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Disclosure

The authors report no conflicts of interest in this work.

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