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RESEARCH

The Effect of JDP2 and ATF2 on the Epithelial-mesenchymal Transition of Human Pancreatic Cancer Cell Lines

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Abstract Pancreatic cancer is a common malignancy with a bleak outcome due to the early occurrence of micro-metastases and poor prognosis. The epithelial-mesenchymal transition (EMT) is considered to be related to the invasion and metastasis of a variety of malignant tumors. Currently, there is no research regarding the relationship of pancreatic cancer EMT with Jun dimerization protein 2 (JDP2), an inhibitor of the activator protein-1 (AP-1) family, and activating transcription factor-2 (ATF2), an AP-1-family member. In this study, we used western blot analysis and immunofluorescence to detect the protein expression of the epithelial marker E-cadherin and the mesenchymal marker vimentin in the pancreatic cancer cell line BxPC3, which was transfected with JDP2 and induced by Collagen I. Compared with the negative control, the E-cadherin and vimentin expression levels did not change significantly, whereas E-cadherin expression decreased and vimentin expression increased in the control group transfected with empty plasmid, suggesting that JDP2 inhibits the EMT induced by Collagen I. Additionally, we verified that compared with the negative control, the morphology of the Capan2 cell line induced by TGF-β1 after transfection with ATF2 was significantly changed, as was the mRNA expression of E-cadherin, whereas the mRNA expression of vimentin, Snail, and ATF2 was significantly increased. Cell invasiveness was also significantly increased ($P<0.01$), suggesting that ATF2, together with TGF-β1, induced EMT in the Capan2 cell line. The members of the AP-1 family are closely related to EMT and that JDP2, as an AP-1-family inhibitor, inhibits EMT, which could lead to a new direction in molecular-targeted therapy for pancreatic cancer.

Keywords Pancreatic cancer · Epithelial-mesenchymal transition · Jun dimerization protein 2 · Activator protein-1

Introduction

In the United States, pancreatic cancer ranks as the fourth deadliest form of cancer. Its early diagnosis is difficult, and it is relatively insensitive to chemotherapy and other therapies, with a five-year survival rate of only 3%–5% [1]. Further understanding of the molecular mechanisms leading to the occurrence and development of pancreatic cancer will have a profound impact on the diagnosis and treatment of pancreatic cancer.

Jun dimerization protein 2 (JDP2) was isolated as one of the proteins bound to c-Jun, a member of the transcription factor activator protein-1 (AP-1) family [2]. JDP2 consists of 163 amino acids and contains a basic leucine zipper (bZIP) structure. In 53 cases of seven different types of malignant tumor specimens, the expression level of JDP2 decreased by different degrees in 35.8% of the cancer tissue samples but increased in only 5.7% of the samples [3]. Activating transcription factor-2 (ATF2), a member of the AP-1 family, contains the bZIP structure, a binding domain for leucine dimerization at the C-terminus, and a basic domain for binding DNA at the N-terminus [4, 5]. The expression of phosphorylated ATF2 in the TGF-β-mediated epithelial-mesenchymal transition (EMT) in mouse

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NMuMG cells was decreased by SB202190, which is a specific inhibitor of the p38MAPK pathway [6].

Since it was proposed more than 20 years ago, EMT has drawn wide attention. The classic EMT-inducing factor is TGF-β. In addition, epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and other factors induce EMT. EMT causes a loss of cell polarity, leading to the loss of the tight junctions and adhesive connections between cells, thereby allowing invasion and metastasis. This role of EMT has been confirmed in a variety of cancers, including breast cancer, oral squamous cell carcinoma, liver cancer, and pancreatic cancer [7–10].

In this study, we show that after transfection with ATF2, Capan2 cells were more sensitive to the stimulation of TGF-β1 to undergo EMT. Following transfection with JDP2, BxPC3 cells inhibited the EMT induced by Collagen I. We hypothesize that there must be a correlation between JDP2 and EMT and that ATF2 could be synergistic with TGF-β1 to induce the process of EMT.

Materials and Methods

Antibodies and Growth Factors

The rabbit anti-goat monoclonal antibodies for E-cadherin and vimentin and the rabbit anti-goat polyclonal antibody for JDP2 were purchased from Santa Cruz Biotechnology, Inc. TGF-β1 was purchased from Repro Tech Co.

Cell Culture and Transfection

The human pancreatic cancer cell lines Capan2 and BxPC3 were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences. Cells were cultured in DMEM medium containing 10% FBS in a 37°C incubator with 5% CO2. The culture medium was changed every two days, and cells were passaged every 3 to 4 days. The JDP2 expression plasmid pCEFL-HA-JDP2 was a gift from Professor Ami Aroheim (The Rappaport Family Institute for Research in the Medical Sciences, Technion-Israel Institute of Technology, Israel), and the ATF2 expression plasmid pGEX-ATF2 was a gift from Professor Jin C (Center for Cancer and Stem Cell Biology, Texas A & M Health Science Center, Institute of Biosciences and Technology, Houston, TX, USA). When cultured cells in the 6-well plate reached 70%-80% confluence, the pCEFL-HA-JDP2 or pCEFL vector plasmids were transiently transfected into BxPC3 cells and the pGEX-ATF2 or pGEX vector plasmids were transiently transfected into Capan2 cells using Lipofectamine 2000 according to the manufacturer's instructions. After 48 hours, the BxPC3 cells transfected with pCEFL-HA-JDP2 and pCEFL vector were passaged onto 6-well plates pre-coated with collagen I using enzymatic digestion. Capan2 cells transfected with pGEX-ATF2 and pGEX vector were continuously cultured in DMEM culture medium containing 10 ng/ml TGF-β1 with the same amount of PBS in the negative control group. After another 48 hours, cell morphology was observed under an inverted phase contrast microscope.

Reverse Transcription-PCR and Quantitative Real-time PCR

After harvesting the cells in each experimental group, total RNA was extracted with the RNAiso plus kit from TAKARA Co., the RNA quality was tested by electrophoretic analysis, and the concentration was detected with A260. The primer sequences used were as follows: JDP2 sense, 5′-AGA CCC AGA TTG AGG AGC TG-3′; JDP2 antisense, 5′-AGT GGG TTG CCT TCT GAC CTC-3′ (124 bp); ATF2 sense, 5′-ATG GTA GCG GAT TGG TTA-3′; ATF 2 antisense, 5′-TCG GCA CTG AAA TGT CTT-3′ (440 bp); Snail sense, 5′-CCG TGG TAC TTC TTG ACA TC-3′; Snail antisense, 5′-TAT GCT GCC TTC CCA GGC TT-3′ (335 bp); E-cadherin sense, 5′-TCC CAT CAG CTG CCC AGA AA-3′; E-cadherin antisense, 5′-TGA CTC CTG TGT TCC TGT TA-3′ (500 bp); Vimentin sense, 5′-CCA GTG CGT GAA ATG GAA G-3′; vimentin antisense, 5′-TCA AGG TCA TCG TGA TGC TG-3′ (366 bp); GAPDH sense, 5′-TGA AGG TCG GAG TCA ACG GAT TTG G-3′; and GAPDH antisense, 5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′ (982 bp). The PCR amplification conditions were 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min. PCR products were detected by electrophoresis on a 2% agarose gel. All experiments were repeated 3 times.

Western Blot Analysis

The expression levels of JDP2, E-cadherin, and vimentin were detected by western blot. Proteins in each group were extracted in strict accordance with the instructions of the RIPA buffer protein lysis kit. The protein samples were mixed with 5× protein loading buffer in a ratio of 4:1, denatured by boiling for 5 minutes, and then applied onto a 10% SDS-polyacrylamide gel for electrophoresis. The proteins separated on the gel were transferred onto a PVDF membrane and then blocked in TBST with 5% skim milk at room temperature for 2 hours. The primary antibodies against JDP2 (1:600), E-cadherin (1:800), vimentin (1:1000), and GAPDH (1:1500) were added and incubated at 4°C overnight, and then the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody at room temperature for 2 hours. After the

membranes were washed with TBST 4 times for 5 minutes, they were visualized with electrochemiluminescence (ECL). All the experiments were repeated 3 times.

Transwell Motility Assays

The upper chamber of a Transwell was coated with 50 μl of 100 mg/l Matrigel matrix, and 600 μl DMEM medium containing 15% FBS was added into the lower chamber. The cell density for all experiments was adjusted to 2×105/well, which was seeded on the upper chamber and placed in the incubator for 24 hours. The chambers were then removed, and the cells were rinsed with PBS, fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and counted under a 200× microscope. Five fields were randomly selected, and the average number of cells was considered to be the number of cells that invaded the membrane. Each experiment was repeated 3 times, and the result is presented as the average.

Immunofluorescence Analysis

The cells in each group were washed with PBS three times, fixed with 4% paraformaldehyde at room temperature for 30 minutes, blocked with 5% BSA at room temperature for 1 hour, and then incubated with the appropriate primary antibody (E-cadherin or vimentin) at 4°C overnight. After washing with PBS three times, the cells were incubated with the secondary antibody labeled with FITC or TRITC at room temperature for 30 minutes. The fluorescent signal was observed under a fluorescence microscope.

Statistics and Data Analyses

All measured data are presented as the mean ± standard deviation $(x \pm s)$. SPSS13.0 software was used for data analysis using the Student's t-test. Values of $P<0.05$ were considered to be statistically significant.

Results

Transfection of JDP2 and ATF2 Respectively Inhibited and Promoted Changes in Cell Morphology

Compared with the negative control, only the BxPC3 cells transfected with empty plasmid showed significant morphological changes 48 hours after Collagen I induction. Most cells changed to a spindle shape, and the tight junctions between cells were lost (Fig. 1a, C). Forty-eight hours after Collagen I re-induction, only a few BxPC3 cells transfected with JDP2 changed into a spindle shape, and the loss of tight junctions between cells was not obvious (Fig. 1a, B and 2). The Capan2 cells transfected with ATF2 were more easily stimulated by TGF-β1. Compared with the negative control group, most cells were spindle-shaped, showing an obvious morphology of mesenchymal cells (Fig. 3a, D). The control groups of ATF2 transfection alone or TGF-β1 stimulation alone did not show clear morphological changes (Fig. 3a; B, C). Hereafter, the following nomenclature will be used: B-V-C, cells transfected with empty plasmid and induced with Collagen I; B-J-C, cells transfected with JDP2 and induced with Collagen I; C-A-T, cells transfected with ATF2 and stimulated with TGF-β1; C-A, cells transfected with ATF2 only; and C-T, cells stimulated with TGF-β1.

In C-A-T Cells, Vimentin Expression Significantly Increased, E-cadherin Expression was Significantly Reduced, and Cell Invasiveness was significantly Enhanced

To determine if C-A-T cells had changes in the EMT-related markers, we measured the mRNA and protein expression of E-cadherin, an epithelial associated marker, and vimentin, a mesenchymal-related marker, using RT-PCR and western blot methods. Additionally, mRNA expression of ATF2 and Snail were detected with RT-PCR. Compared with the C, C-A, and C-T cells, the C-A-T cells had significantly increased expression of vimentin, Snail, and ATF2, whereas the expression of E-cadherin was significantly reduced (Fig. 3b, c), suggesting a clear trend of EMT. Cell invasion is one of the hallmarks of EMT, so the Transwell cell invasion assay was used in this study to verify the invasiveness of cells in each group. The invasiveness of the C-A-T cells was significantly greater than those of other cells (Fig. 4).

Compared to B-J-C Cells, B-V-C Cells had Increased Vimentin Expression and Increased Cell Invasiveness

To determine if the BxPC3 cells transfected with JDP2 were able to change the expression of EMT-related markers, the protein expression levels of vimentin and E-cadherin were detected by western blot. The results showed that compared with the negative control, the expression of E-cadherin and vimentin in B-J-C cells did not change significantly (Fig. 2). In the B-V-C cells, vimentin protein expression increased, and E-cadherin protein expression did not change significantly (Fig. 2). To verify that E-cadherin expression changed, we also tested the expression of vimentin and E-cadherin by immunofluorescence. The results indicated that in B-V-C cells, vimentin expression significantly increased and E-cadherin expression was significantly reduced (Fig. 1b). These data are similar to previously reported results [11]. The Transwell assay proved that the invasiveness of the B-J-C cells was also

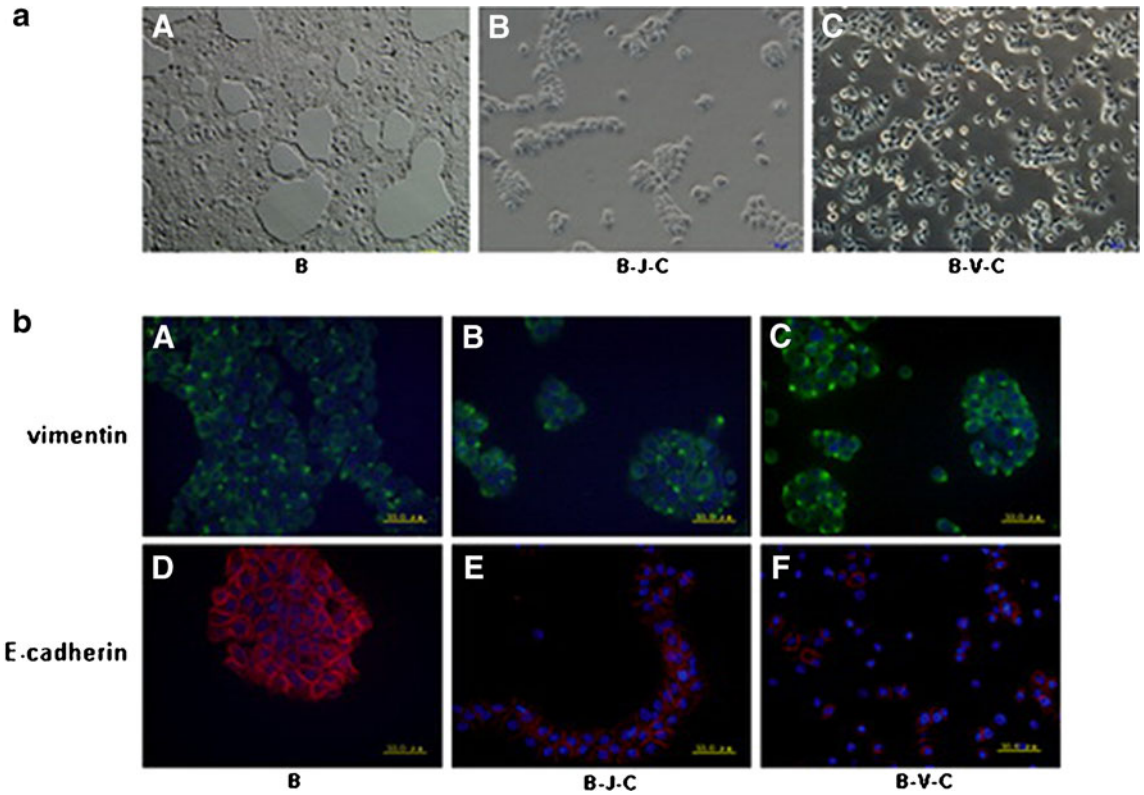

Fig. 1 The morphological differences, as detected by 200× light microscopy, and vimentin and E-cadherin expression, as detected by immunofluorescence and 400× fluorescent microscopy, in BxPC3 cells in the blank control group, the JDP2 transfection group, and the empty plasmid transfection group. **a:** Compared with the blank control group, cells transfected with JDP2 showed slight dispersion between cells, some cells became spindle-shaped (B). Cells transfected with empty plasmid had significant changes in morphology, and most of the cells become spindle-shaped, forming a typical EMT (C). **b:** Compared with the blank control group, the cells transfected with empty plasmid had a significant increase in vimentin expression and a significant decrease in E-cadherin expression, whereas no significant change was observed in the expression of vimentin and E-cadherin in cells transfected with JDP2

significantly less than that of B-V-C cells ($P<0.05$) (Fig. 4). Together, these data show that cells with high JDP2 expression could inhibit the EMT induced by Collagen I.

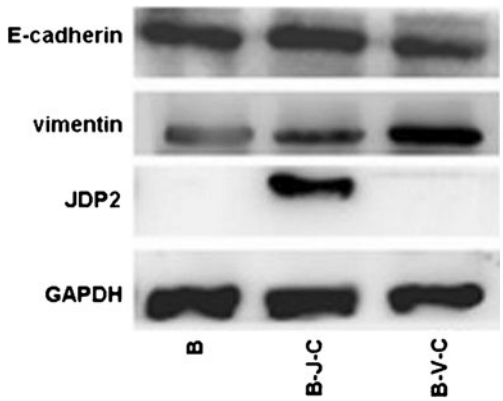

Fig. 2 Protein expression of E-cadherin, vimentin, and JDP2 detected by western blot in BxPC3 cells in the blank control group, the JDP2 transfection group, and the empty plasmid transfection group. Compared with the cells of blank control group, vimentin expression significantly increased in the cells transfected with empty plasmid, with no significant change in the cells transfected with JDP2. Only the cells transfected with JDP2 had a detectable amount of JDP2 protein

Discussion

In this study, Collagen I induction was first applied to the pancreatic cancer cell line BxPC3 transfected with the pCEFL-HA-JDP2 plasmid or the empty pCEFL plasmid. We found that compared with the negative control group, cells transfected with the empty plasmid significantly changed into a long spindle shape, lost tight junctions between cells, and had increased vimentin expression. Furthermore, according to the immunofluorescence data, E-cadherin protein expression was significantly reduced, and the Transwell invasion assay showed a significant increase in invasiveness. These changes were not obvious in the BxPC3 cells transfected with JDP2, indicating that after the transfection of JDP2, the BxPC3 cells could inhibit the Collagen I-induced EMT. JDP2 is an inhibitor of the AP-1 protein family. Because JDP2 inhibited EMT, we hypothesized that ATF2, as one of the members of the AP-1 family, could promote EMT. To this end, Capan2 cells transfected with ATF2 were cultured with TGF-β1 (10 ng/μl) added into the medium. After 48 hours, significant morphological changes were observed in most cells. RT-PCR and

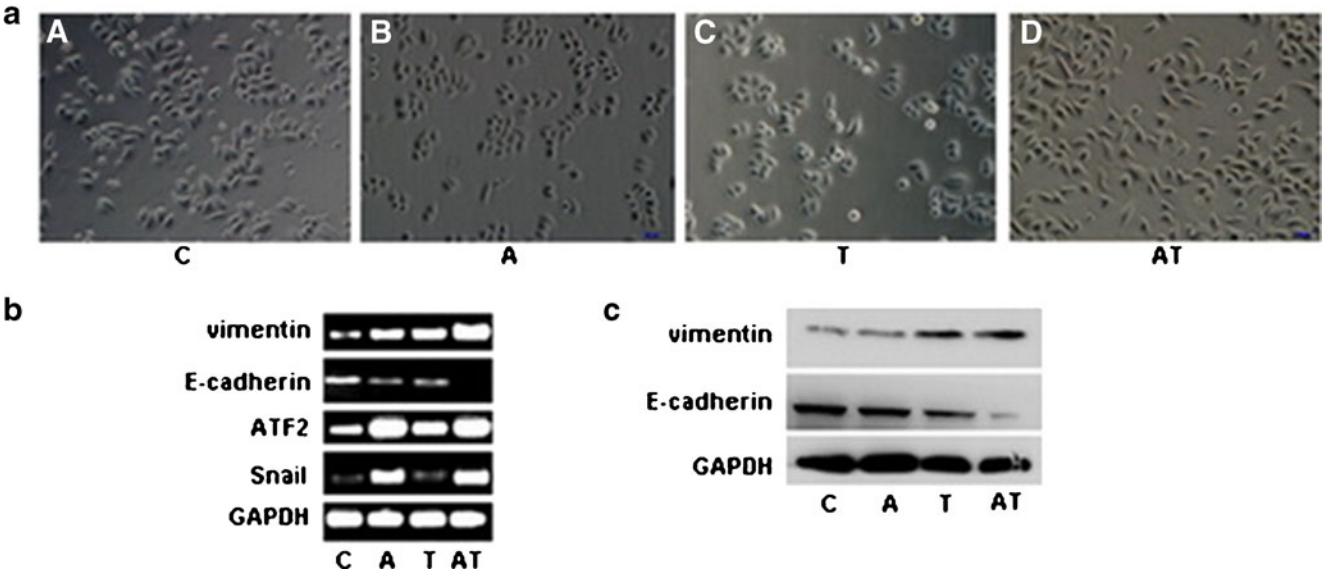

Fig. 3 Morphological changes and mRNA and protein expression of EMT-related factors in Capan2 cells in the blank control group, the ATF2 transfection group, the TGF-β1 stimulation group, and the ATF2 transfection and TGF-β1 stimulation group. **a:** Morphological changes were most significant in the cells transfected with ATF2 and stimulated with TGF-β1, and most of the cells became spindle-shaped (D). **b:** RT-PCR results indicated that the expression of vimentin, ATF2, and Snail was the highest in the cells transfected with ATF2 and stimulated with TGF-β1, whereas the expression of E-cadherin is the lowest in these cells. **c:** The results of the western blot analysis were similar to those of RT-PCR

western blot analysis showed the increased expression of vimentin, Snail, and ATF2 and the decreased expression of E-cadherin, which were not significant in the C-A or C-T cells. These data suggested that JDP2 could inhibit the Collagen I-induced EMT in BxPC3 cells and that ATF2 could be synergistic with TGF-β1 to produce EMT in Capan2 cells.

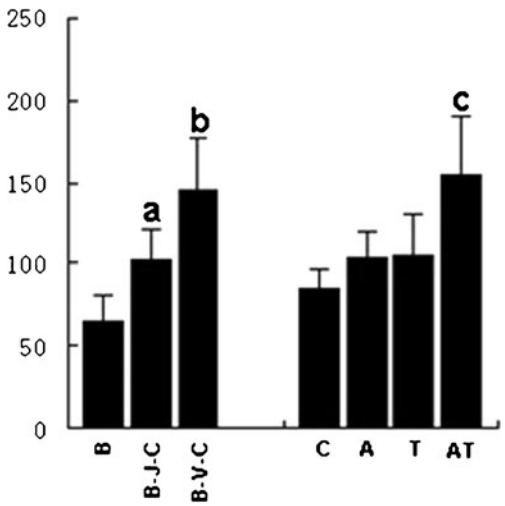

Fig. 4 Comparison of cells in each group using the Transwell invasion assay. Compared with the blank control group, invasiveness was significantly reduced in B-J-C cells and significantly increased in AT cells. **a** $P < 0.05$, B-J-C versus B; **b** $P < 0.01$, B-V-C versus B and $P < 0.05$, B-V-C versus B-J-C; **c** $P < 0.01$, AT versus C, AT versus A, and AT versus T

EMT is currently the focus of much attention. EMT is closely related to tumor invasion and metastasis, especially early stage metastasis, and can be induced by multiple pathways. After binding to its receptor, TGF-β activates the intracellular Smad signaling pathway and induces EMT by inhibiting the expression of E-cadherin [12, 13]. In human keratinocytes, EMT induced by TGF-β requires the activation of the Ras gene [14]. In human pancreatic cancer, Collagen I can upregulate the expression of N-cadherin in pancreatic cancer by activating JNK1, thereby increasing cell invasiveness [11]. Transfection with a plasmid containing Snail alone induces EMT in BxPC3 cells [15]. Mutation of the oncogene k-Ras is one of the key events in the early stages of pancreatic cancer. Approximately 30% of early pancreatic cancers and nearly 100% of advanced pancreatic cancers are associated with mutations in this gene [16–18]. The activated k-Ras can induce the transformation of cells through a variety of pathways, including the MEKK/SEK/JNK/AP-1 (ATF2/Jun) pathway [16–18]. c-Jun expression is increased in pancreatic cancer, confirming that the Ras/AP-1 pathway may be involved in pancreatic cancer. JDP2, a common protein inhibitor of AP-1, can form dimers with itself and with c-Jun, JunB, JunD, or ATF2 and inhibit the transcriptional activity of c-Jun, c-Fos, and ATF2 [19], suggesting that JDP2 may block the Ras signaling pathway by inhibiting AP-1. JDP2 can recruit histone deacetylase 3 (HDAC3) to the regulation area of the proto-oncogene c-jun, thereby suppressing its expression [20]. JDP2 can also directly inhibit histone acetyltransferase

(HAT) activity. As a DNA-binding protein, JDP2 functions in nucleosome assembly [21]. Recently, our laboratory found that compared with the surrounding tissue, JDP protein expression is significantly decreased in pancreatic cancer tissues [22], and JDP2 can reverse the EMT co-induced by EGF and TGF-β1 in BxPC3 cells (data not shown). ATF2, a member of the AP-1 family, can form dimers with c-Jun or CREB [23, 24]. In melanoma cells, the expression of ATF2 is closely related to the prognosis of patients [25]. In breast cancer, the stable expression of ATF2 is related to the DNA damage tolerance of the tumor cells [26]. ATF2 is critical for the normal development of the brain, as brain development of ATF2-knockout mice is abnormal, resulting in structural defects of the inner ear, cerebellum, and ventricles [27]. In a human colorectal cancer cell line stimulated with Tolfenamic acid (TA), ATF3 and phosphorylated ATF2 expression increased; the increased expression of ATF2 was achieved by the activation of the P38 MAPK, JNK, and ERK pathways [28]. Together, these data suggest that JDP2 and ATF2 is most likely associated with the EMT of pancreatic cancer induced by the ERK and JNK pathways, but studies on the exact mechanism are still not available.

In summary, in this study, we found that the pancreatic cancer cell line BxPC3 transfected with JDP2 inhibited Collagen I-induced EMT, and the pancreatic cancer cell line Capan2 transfected with ATF2 became sensitive to TGF-β1 induction of, resulting in EMT. Thus, ATF2 is closely related to the invasion and metastasis of pancreatic cancer. JDP2, as a tumor suppressor, provides a new direction in molecular-targeted therapy for pancreatic cancer.

Disclosure The authors declare no conflicts of interest.

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