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Original Article

Pancreatic stellate cells promote the epithelial-mesenchymal transition via SDF-1/CXCR4 signaling pathway in pancreatic cancer

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Abstract: Objective: We aimed to investigate the effect of conditioned media from pancreatic stellate cells (PSC-CM) on the epithelial-mesenchymal transition (EMT) of pancreatic cancer cells. Methods: PSC-CM was added into the culture medium of human pancreatic cancer cell lines AsPC-1, BxPC-3, and CXCR4-siRNA-AsPC-1. Cell growth was measured by MTT assay. Snail, Twist, and E-cadherin mRNA and protein expression was examined using real-time-PCR and Western blot. The invasion abilities of AsPC-1 and BxPC-3 cells were determined by cell invasion assays. The changes in related genes were detected by tumorigenesis tests in nude mice. Results: Our results showed that 1.0 μg/mL of PSC-CM could promote the proliferation of human pancreatic cancer cell lines AsPC-1 and BxPC-3. After treatment with PSC-CM, the invasion of the two cell lines was enhanced (27.59±1.2 vs 73.99±2.29, 22.22±1.68 vs 66.01±1.86, all P<0.01). The mRNA expression level of Snail increased (2.89±0.16 vs 1.01±0.18, 1.71±0.24 vs 0.57±0.10, t=23.540, 13.060, all P<0.01), and the mRNA expression level of E-cadherin decreased (0.36±0.06 vs 1.00±0.09, 0.35±0.06 vs 0.6±0.006, t=17.820, 9.747, all P<0.01). The protein expression of Snail increased (3.33±0.20 vs 1.00±0.03, 1.71±0.25 vs 0.67±0.07, t=19.780, 6.885, all P<0.01), and the protein expression level of E-cadherin decreased (0.57±0.06 vs 1.00±0.07, 0.53±0.04 vs 0.73±0.09, t=7.981, 3.707, all P<0.01). Compared with the AsPC-1 and the CXCR4-Con-AsPC-1 groups, the mRNA expression level of Snail decreased (0.28±0.02 vs 1.01±0.18, 0.28±0.02 vs 1.03±0.25, t=12.31, 8.877, all P<0.01), and the mRNA expression level of E-cadherin increased (3.63±0.07 vs 1.00±0.09, 3.63±0.07 vs 1.03±0.21, t=69.040, 35.110, all P<0.01) in the group with silencing of CXCR4 by RNA interference. The protein expression level of Snail also decreased (0.44±0.01 vs 1.00±0.03, 0.44±0.01 vs 0.95±0.02, t=31.770, 36.910, all P<0.01), and the protein expression level of E-cadherin increased (2.21±0.03 vs 1.00±0.07, 2.21±0.03 vs 1.14±0.09, t=26.290, 18.560, all P<0.01) in this group. The positive expression rate of E-cadherin in the CXCR4-siRNA-AsPC-1 group was higher than that in the AsPC-1 group (76.90%±1.69% vs 29.32%±1.18%; t=73.25, P<0.01), and the positive expression rate of Snail in the CXCR4-siRNA-AsPC-1 group was lower than that in the AsPC-1 group (17.62%±1.02% vs 52.58%±1.74%; t=54.80, P<0.01). Conclusion: PSC can promote EMT in pancreatic cancer, and the SDF-1/CXCR4 axis may play a positive regulatory role during this process.

Keywords: Pancreatic stellate cells, pancreatic neoplasms, epithelial to mesenchymal transition, SDF-1/CXCR4

Introduction

Pancreatic cancer is a highly malignant tumor with early local or distant metastasis, which can result in a poor prognosis [1]. Connective tissue hyperplasia is a typical feature of pancreatic cancer, and the connective tissue of pancreatic cancer is made up of the extracellular matrix (ECM), carcinoma-associated fibroblasts (CAFs), immunocytes, and endothelial cells [2]. Pancreatic stellate cells (PSCs) are the most studied CAF subtypes in pancreatic cancer. They are found around the exocrine acinar, vascular, and duct areas of the pancreas and regulated by autocrine and paracrine stimulation [3]. It has been demonstrated that PSCs can produce stromal responses in pancreatic cancer [4]. In some studies on breast cancer and liver cancer, tumor-related fibroblasts were found to be able to promote the epithelial-mesenchymal transition (EMT) of tumor cells [5].

EMT is a key step in embryonic development and is closely related to the development of

organs such as the neural crest, heart, musculoskeletal system, craniofacial system, and peripheral nervous system. EMT is also associated with intraembryonic transformation and archenteron formation and is involved in metastasis of pancreatic cancer [6, 7]. The essence of EMT is the process of cell reprogramming. In this process, the highly differentiated epithelial cells lose their epithelial characteristics and begin to show the characteristics of poorly differentiated mesenchymal cells. These manifestation includes loss of cell adhesion, increase of mesenchymal markers, strengthening of cell vitality, cell invasion into local tissue, cell migration along a certain path, and the synthesis of extracellular matrix proteins particular to mesenchymal cells [8]. EMT is regulated by a complex network of epigenetic modifications and transcriptional regulation, including EMT-inducing transcription factors, such as Snail, Zeb, and Twist and transcription regulators, such as microRNAs [9]. E-cadherin is an adhesive protein that maintains the integrity of epithelial cells and keeps the conglutination between different epithelial cells. Its low expression in cancer cells may weaken the adhesion of cancer cells and make the cancer cells become detached from the tumor more easily. The transcription factor Snail is an epithelial suppressor of EMT. By inhibiting the expression of E-cadherin, Snail can reduce adhesion between cells, enhance the movement of cells, promote the EMT of tumor cells, and improve the invasion and metastasis abilities of the tumor cells [10]. Twist is a key gene of EMT, which plays an important role in the differentiation of myoblasts, osteoblasts and mesoblasts. The transcription factor Twist is a major regulator of embryonic morphogenesis and has an important role in tumor metastasis. Twist is highly expressed in breast cancer, liver cancer, and other tumors, but is less expressed in pancreatic ductal adenocarcinoma and the tissues of chronic pancreatitis. Twist can inhibit E-cadherin expression and promote EMT and tumor cell invasion and metastasis [11]. In conclusion, EMT plays a key role in the invasion and metastasis of malignant tumors. Therefore, it is of great clinical significance to investigate the mechanism of EMT in tumor growth and metastasis for the diagnosis and treatment of cancer [5].

The tumor microenvironment is also a key element in tumor progression. It is an extremely complex internal environmental network made up of chemokines. Chemokines are 8-12 kDa polypeptides, which participate in cell activation, differentiation and transportation by chemokine cytokines [12]. SDF-1 is a small chemokine that promotes the migration and metastasis of tumor cells by stimulating tumor cells and tumor mesenchymal cells in the form of paracrine factors. SDF-1 is expressed in various tumors such as breast cancer, liver cancer and pancreatic cancer [13]. The formation of the microenvironment before tumor metastasis is the prodromal stage of metastatic cancer, and the recruitment of tumor-related macrophages to tissues in response to the SDF-1/CXCR4 axis is a basic process of tumor formation, development, and migration. Recent studies have shown that the SDF-1/CXCR4 axis plays a key role in the dissemination and organ-specific metastasis of multiple tumors [14, 15]. CXCR4 can be expressed in epithelial cells, mesenchymal cells, and hematopoietic system. In solid tumors, CXCR4 expression is found in cancers including breast cancer, prostate cancer, pancreatic cancer, small-cell lung cancer, and colon cancer. SDF-1 is highly expressed in many malignant tumor tissues, and organs with high expression of SDF-1 can attract tumor cells by specific binding to the ligands, which results in targeted metastasis of tumor cells [16].

In our previous studies, we found that the SDF-1/CXCR4 axis is an important signal transduction pathway for the function of PSC [17]. In this study, we used the pancreatic cancer cell line as the target cells and observed the effects of PSC-CM on the expression of genes related to EMT in pancreatic cancer cells to further explore the role and mechanism of PSCs in the EMT in pancreatic cancer cells.

Materials and methods

Materials

DMEM and fetal bovine serum were purchased from Gibco, USA. Antibodies to CXCR4, E-cadherin, Twist, and Snail were purchased from Abcam, UK. Trizol, MTT proliferation kit, and SYBR Green Realtime PCR Master Mix were purchased from TOYOBO, Japan. The nude mice were provided by the animal experiment center of Shanghai First People's Hospital, China.

Table 1. PCR primers

Target gene	Primer sequence	Target segment
CXCR4		228 bp
Forward	5'-ATCATCTTCTTAACTGGCATTGTG-3'	
Reverse	5'-GCTGTAGAGGTTGACTGTGTAGA-3'	
E-cadherin		420 bp
Forward	5'-CGAGAGCTACACGTTCACGG-3'	
Reverse	5'-GGGTGTCGAGGGAAAAATAGG-3'	
Twist		527 bp
Forward	5'-GTCCGCAGTCTTACGAGGAG-3'	
Reverse	5'-GCTTGAGGGTCTGAATCTTGCT-3'	
Snail		557 bp
Forward	5'-TCGGAAGCCTAACTACAGCGA-3'	
Reverse	5'-AGATGAGCATTGGCAGCGAG-3'	
β-actin		302 bp
Forward	5'-GTGGACATCCGCAAAGAC-3'	
Reverse	5'-AAAGGGTGTAACGCAACT-3'	

Methods

Cell culture: Human pancreatic cancer cell lines AsPC-1 and BxPC-3 were purchased from ATCC, USA. PSCs were purchased from ScienCell, USA and immortalized by Shanghai Genechem Co., Ltd, China. The AsPC-1 cell line used in this study was the previous cell line retained from our previous studies. The silencing of the CXCR4 gene by RNA interference (RNAi) and lentiviral vector was performed by Shanghai Genechem Co., Ltd., China [7]. AsPC-1 and BxPC-3 cell lines were cultured in DMEM medium containing 10% fetal bovine serum, PSCs were cultured in F-12/1640 medium containing 10% fetal bovine serum, and all the cell lines were cultured in incubators with saturated humidity at 37°C in 5% CO_2.

Cell proliferation assay: Single-cell suspension of BxPC-1 and AsPC-3 was prepared when the cells were in the logarithmic growth phase, and the suspension was inoculated into 96-well plates ($5*10^3$/well). The serum-free medium was replaced after the cells adhered to the wall, followed by addition of PSC-CM (1.0 μg/mL). At the same time, the serum-free medium was added to the control group. Each group included five replicate wells, and 20 μL of MTT (5 mg/mL) was added into each well after 24 h, 48 h, and 72 h. After 4 hours of incubation, the supernatant was discarded, and 150 μL DMSO was added into each well followed by agitation for 10 min. The absorbance value of each well at the wavelength of 490 nm was measured with a microplate reader, and the growth curve was plotted accordingly.

Cell invasion assay: The pancreatic cancer cells in the logarithmic growth phase were digested by trypsin and washed with PBS twice. Then the cells were suspended in serum-free medium, and the cell concentration was adjusted to $1*10^6$/mL. Cell suspension (100 μL) was added into the upper compartment of each group. The lower compartment of the experimental group (24-well plate) had culture medium containing 1.0 μg/mL PSC-CM, while the lower compartment of the control group contained serum-free culture medium. The invading cells were counted in 400-fold microscopic fields after 48 hours of culture. The experiment was repeated three times.

qRT-PCR: The total RNA in each group was extracted with Trizol, and mRNA was reversely transcribed into cDNA using a reverse transcription kit. β-actin was selected as the internal reference. The primers of human CXCR4, E-cadherin, Twist and Snail are shown in **Table 1**. According to the instructions of the SYBR Green Realtime PCR Master Mix kit, the reaction system included 2.5 μL of cDNA as template, 1 μL of forward primer (12.5 pmol/μL), 1 μL of reverse primer (12.5 pmol/μL), 25 μL of Realtime PCR Master Mix, and 20.5 μL sterile ddH_2O. Eppendorf real-time fluorescent quantitative PCR was used for amplification. Reaction conditions were as follows: pre-denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Each reaction was done in triplicate for each group. After PCR, standard curve analysis was conducted.

Western blot: The total protein in each group was extracted, and the protein in each group was mixed with an equal volume of SDS loading buffer (2X). The mixture was boiled in a water bath for 5 min. After electrophoresis, the protein was transferred to a nitrocellulose membrane, and the membrane was blocked in TBST solution containing 5% skim milk for 1 h. Then the membrane was incubated with primary antibody diluent (1:500) overnight at 4°C. The

Table 2. Effects of PSC-CM on proliferation abilities of AsPC-1 and BxPC-3 cells ($\overline{x}$±sd)

Group	AsPC-1	AsPC-1+PSC-CM	BxPC-3	BxPC-3+PSC-CM
Cell proliferation level				
0 h	0.225±0.004	0.278±0.014	0.213±0.009	0.236±0.002
24 h	0.356±0.014	0.446±0.031*	0.249±0.004	0.324±0.002#
48 h	0.540±0.012	0.626±0.013*	0.303±0.007	0.405±0.003##
72 h	1.039±0.033	1.189±0.079**	0.537±0.049	0.904±0.048##

Note: Compared with AsPC-1 group, *P<0.05, **P<0.01; compared with the BxPC-3 group, #P<0.05, ##P<0.01. PSC-CM, conditioned medium from pancreatic stellate cells.

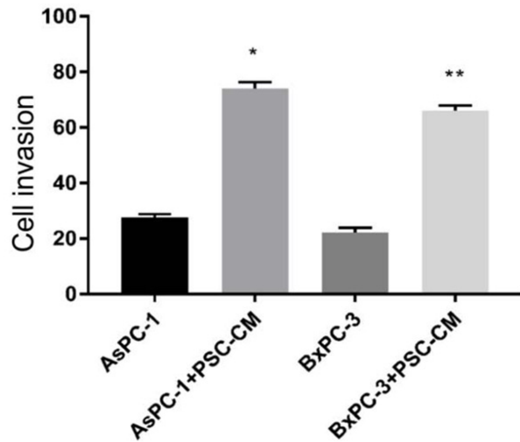

Figure 1. Effect of PSC-CM on the invasion abilities of AsPC-1 and BxPC-3 cells. Compared with the AsPC-1 group, *P<0.01; compared with the BxPC-3 group, **P<0.01. PSC-CM, conditioned medium from pancreatic stellate cells.

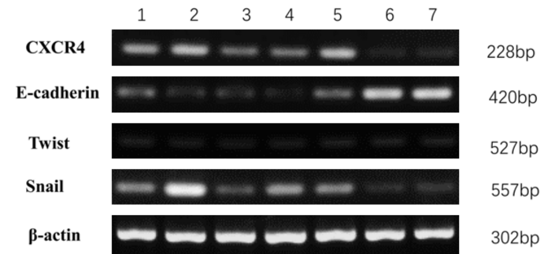

Figure 2. Electrophoretogram of CXCR4, E-cadherin, Twist, and Snail mRNA expressions in each group detected by RT-PCR. CXCR4, CXC chemokine receptor 4; Snail, a zinc finger transcription factor; Twist, a transcription regulator. 1: the AsPC-1 group; 2: the AsPC-1+PSC-CM group; 3: the BxPC-3 group; 4: the BxPC-3+PSC-CM group; 5: the CXCR4-Con-AsPC-1 group (AsPC-1 transfected with the empty lentiviral vectors); 6: the CXCR4-siRNA-AsPC-1 group (AsPC-1c transfected with lentiviral vectors for silencing CXCR4); 7: the CXCR4-siRNA-AsPC-1+PSC-CM group.

membrane was washed in TBST 3 times and then incubated with secondary antibody diluent (1:1,000) at room temperature for 1 h. The membrane was washed in TBST, the luminescence was enhanced by ECL, and the image was developed through X-ray. β-actin was used as the internal reference.

Tumorigenesis in nude mice: Nude mice were raised in a specific pathogen free (SPF) sterile room, and the operation procedures for SPF experimental animals were strictly followed. Under sterile conditions, 8 nude mice were randomly divided into two groups: the control group (mouse was subcutaneously inoculated with AsPC-1, $2*10^6$ cells per mouse) and the experimental group (mouse was inoculated with CXCR4-siRNA-AsPC-1, $2*10^6$ cells per mouse). The standard for tumorigenesis was that the tumor diameter exceeded 0.5 mm. After 24 days, the animals were sacrificed, and the tumors were taken out and weighed. Then, the tumor was fixed in 10% formaldehyde solution and embedded in paraffin. The tumor was sectioned at a thickness of 4 mm for immunohistochemical staining. The whole procedure was carried out according to the instructions.

Statistical analysis

SPSS 21.0 was used for the statistical analysis. The measurement data are expressed as mean ± standard deviation. All the experiments were repeated three times. One-way analysis of variance and t-test were used for intergroup comparison. P<0.05 indicated that the difference was statistically significant.

Results

Effect of PSC-CM on the proliferation of pancreatic cancer cells

After the application of 1.0 μg/mL PSC-CM on the cells in the two groups for 24 h, 48 h and, 72 h, the cell proliferation abilities in the AsPC-1 and the BxPC-3 groups were enhanced

Table 3. Effects of PSC-CM on mRNA expression levels of CXCR4, E-cadherin, Snail, and Twist in pancreatic cancer cells ($\bar{x}$±sd)

Group	CXCR4	E-cadherin	Snail	Twist
AsPC-1	1.00±0.08	1.00±0.09	1.01±0.18	0.03±0.02
AsPC-1+PSC-CM	1.20±0.08*	0.36±0.06*	2.89±0.16*	0.03±0.02
BxPC-3	0.55±0.02	0.61±0.06	0.57±0.10	0.03±0.04
BxPC-3+PSC-CM	0.77±0.03**	0.35±0.06**	1.71±0.24**	0.03±0.03
CXCR4-Con-AsPC-1	1.01±0.03	1.03±0.21	1.03±0.25	0.03±0.01
CXCR4-siRNA-AsPC-1	0.17±0.03#	3.63±0.07#	0.28±0.02#	0.02±0.01
CXCR4-siRNA-AsPC-1+PSC-CM	0.17±0.02#	3.60±0.17#	0.30±0.02#	0.02±0.01

Note: Compared with the AsPC-1 group, *P<0.01; compared with the BxPC-3 group, **P<0.01; compared with the CXCR4-Con-AsPC-1, #P<0.01. PSC-CM, conditioned medium from pancreatic stellate cells; CXCR4, CXC chemokine receptor 4; Snail, a zinc finger transcription factor; Twist, a transcription regulator; CXCR4-Con-AsPC-1, AsPC-1 transfected with empty lentiviral vector; CXCR4-siRNA-AsPC-1, AsPC-1 transfected with lentiviral vectors for silencing CXCR4.

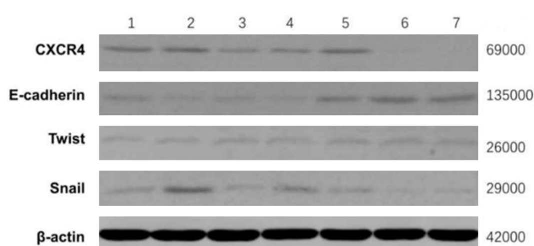

Figure 3. CXCR4, E-cadherin, Twist and Snail protein expressions in each group. CXCR4, CXC chemokine receptor 4; Snail, a zinc finger transcription factor; Twist, a transcription regulator. 1: the AsPC-1 group; 2: the AsPC-1+PSC-CM group; 3: the BxPC-3 group; 4: the BxPC-3+PSC-CM group; 5: the CXCR4-Con-AsPC-1 group (AsPC-1 transfected with empty vector); 6: the CXCR4-siRNA-AsPC-1 group (AsPC-1c transfected with lentiviral vectors for silencing CXCR4); 7: the CXCR4-siRNA-AsPC-1+PSC-CM group.

(t=3.118, 3.003, 5.191, P=0.021, 0.029, 0.000; t=2.991, 4.045, 14.52, P=0.030, 0.002, 0.000). But the effect of PSC-CM on AsPC-1 cell proliferation at 48 h and 72 h was more significant (both P<0.05). See **Table 2**.

Effect of PSC-CM on the invasion of pancreatic cancer cells

After the application of 1.0 μg/mL PSC-CM on cells of each group for 24 h, the numbers of the invading cells in the AsPC-1 and the AsPC-1+PSC-CM groups were 27.59±1.21 and 73.99±2.29, respectively; which showed intergroup differences (t=56.68, P<0.001). The numbers of the invading cells in the BxPC-3 and the BxPC-3+PSC-CM groups were 22.22±1.68 and 66.01±1.86, respectively; and the intergroup difference was also statistically significant (t=55.34, P<0.001). See **Figure 1**.

Effect of PSC-CM on mRNA expression of CXCR4, E-cadherin, Snail, and Twist in pancreatic cancer cells

After PSC-CM was applied to the AsPC-1 and the BxPC-3 groups for 24 h, the mRNA expression level of E-cadherin became lower than that in the corresponding control group (t=17.820, P<0.001; t=9.747, P<0.001), and snail mRNA expression level became higher than that in the control group (t=23.54, P<0.001; t=13.06, P<0.001). After CXCR4 was silenced by RNAi, the mRNA expression level of E-cadherin in the AsPC-1 group was increased compared with that in the CXCR4-con-AsPC-1 group (t=69.04, P<0.001; t=35.11, P<0.001). Compared with the group control of AsPC-1, Snail mRNA expression levels decreased in the CXCR4-siRNA-AsPC-1 and the CXCR4-siRNA-AsPC-1+PSC-CM groups (t=12.31, P<0.001; t=8.877, P<0.001). There mRNA expression levels of Twist were low in each group with no intergroup differences, which may be related to the low content of Twist in pancreatic cancer. mRNA expression of E-cadherin, Twist, and Snail in CXCR4-siRNA-AsPC-1 cells treated with PSC-CM for 24 h had no significant change compared with that of CXCR4-siRNA-AsPC-1 cells. See **Figure 2** and **Table 3**.

Effect of PSC-CM on protein expression of CXCR4, E-cadherin, Snail, and Twist in pancreatic cancer cells

After PSC-CM was applied to the AsPC-1 and the BxPC-3 groups for 24 h, E-cadherin protein expression level became lower than that of the corresponding control group (t=7.981, P=0.001; t=3.707, P=0.021), and Snail protein

Table 4. Effects of PSC-CM on protein expressions of CXCR4, cE-cadherin, Snail, and Twist in pancreatic cancer cells ($\bar{x}\pm$sd)

Group	CXCR4	E-cadherin	Snail	Twist
AsPC-1	1.00±0.04	1.00±0.07	1.00±0.03	0.05±0.01
AsPC-1+PSC-CM	1.22±0.04*	0.57±0.06*	3.33±0.20*	0.06±0.01
BxPC-3	0.58±0.01	0.73±0.09	0.67±0.07	0.05±0.01
BxPC-3+PSC-CM	0.66±0.02**	0.53±0.04**	1.71±0.25**	0.04±0.01
CXCR4-Con-AsPC-1	1.04±0.04	1.14±0.09	0.95±0.02	0.06±0.01
CXCR4-siRNA-AsPC-1	0.14±0.01#	2.21±0.03#	0.44±0.01#	0.06±0.01
CXCR4-siRNA-AsPC-1+PSC-CM	0.11±0.01	2.29±0.16	0.43±0.01	0.06±0.01

Note: Compared with the AsPC-1 group, *P<0.01; compared with the BxPC-3 group, **P<0.01; compared with the CXCR4-Con-AsPC-1, #P<0.01. PSC-CM, conditioned medium of pancreatic stellate cells; CXCR4, CXC chemokine receptor 4; Snail, a zinc finger transcription factor; Twist, a transcription regulator; CXCR4-Con-AsPC-1, AsPC-1 transfected with empty lentiviral vector; CXCR4-siRNA-AsPC-1, AsPC-1 transfected with lentiviral vectors for silencing CXCR4.

expression level became higher than that of the corresponding control group (t=19.780, P<0.001, t=6.885, P=0.002). After the CXCR4 was silenced by RNAi, E-cadherin protein expression level in the AsPC-1 group was increased compared with that of the CXCR4-con-AsPC-1 group (t=26.290, P<0.001; t=18.560, P<0.001), and the expression level of Snail protein was decreased (t=31.770, P<0.001; t=36.910, P<0.001). The expression level of Twist protein was low in each group. The expression levels of E-cadherin, Twist, and Snail proteins in CXCR4-siRNA-AsPC-1 cells showed no significant change after PSC-CM treat for 24 h compared with CXCR4-siRNA-AsPC-1 cells. See **Figure 3** and **Table 4**.

Effect of CXCR4 gene silencing on E-cadherin, Twist, and Snail expression in subcutaneous tumorigenesis of nude mice

During the tumorigenesis, the nude mice were active and did not die. The tumor size in the CXCR4-siRNA-AsPC-1 group was significantly reduced compared with that in the AsPC-1 group (t=3.817, P=0.009). Immunohistochemical staining showed that the positive expression rate of E-cadherin in the tumor tissue sections of the CXCR4-siRNA-AsPC-1 group was higher than that in the AsPC-1 group (76.90%±1.69% vs 29.32%±1.18%, t=73.25, P<0.001), and the positive expression level of Snail in this group was lower than that in the AsPC-1 group (17.62%±1.02% vs 52.58%±1.74%, t=54.80, P<0.001). The positive expression rate of Twist was low. See **Figures 4** and **5**.

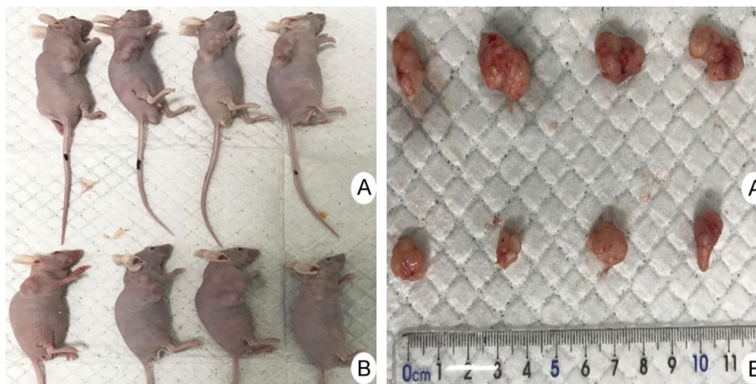

Figure 4. The nude mouse model (left) and the overview of the transplanted tumors *in vitro* (right). A: The AsPC-1 group; B: The CXCR4-siRNA-AsPC-1 group.

Discussion

Pancreatic cancer is a highly malignant tumor with early local or distant metastasis, which can result in a poor prognosis [1]. The reason for this cancer can metastasize locally or to distant tissues and organs is that there is a large amount of tumor stroma in the tumor tissue, and connective tissue hyperplasia is a typical feature of pancreatic cancer, which may change the tumor microenvironment. EMT is one of the important mechanisms in the cancer metastasis. The tumor microenvironment changes during tumor development and inflammatory process, resulting in various transformations of cell phenotypes, including EMT. The matrix cells in an activated tumor microenvironment can secrete different protein factors and cytokines, causing many biological effects on tumor cells

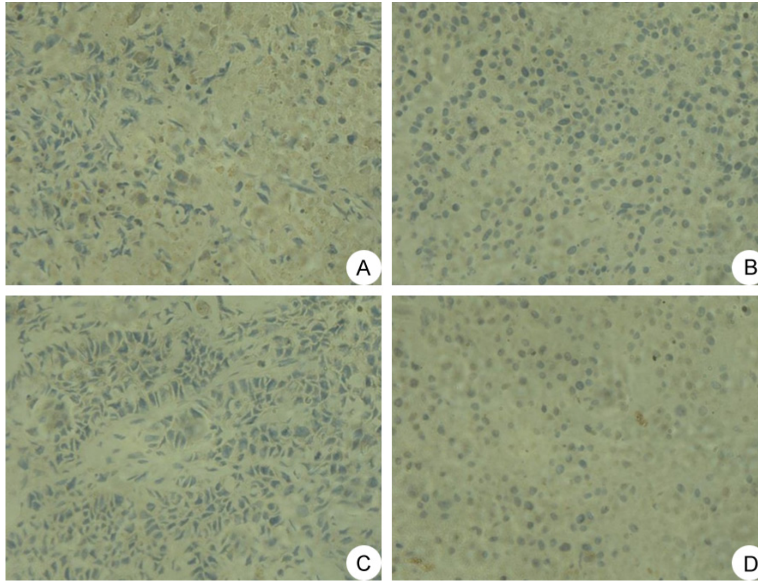

Figure 5. Expressions of E-cadherin and Snail in tumor tissues of nude mice detected by immunohistochemical staining at high magnification. A: E-cadherin expression in the AsPC-1 group; B: E-cadherin expression in the CXCR4-siRNA-AsPC-1 group; C: Snail expression in the AsPC-1 group; D: Snail expression in the CXCR4-siRNA-AsPC-1 group.

[8]. Pancreatic stellate cells (PSCs) can produce stromal responses in pancreatic cancer [8]. In studies on breast cancer and liver cancer, tumor-related fibroblasts were found to promote EMT in tumor cells [5]. In this study, we aimed to investigate the effects of PSCs on the metastasis of pancreatic cancer through the tumor microenvironment.

We added PSC-CM into the medium of AsPC-1 and BxPC-3 cells. As a result, the proliferation and invasion abilities of the two cell lines were improved, indicating that the microenvironments in which PSCs were present may promote the proliferation and invasion abilities of the pancreatic cancer cells. At the same time, after the treatment with PSC-CM, the mRNA and protein expression levels of E-cadherin were downregulated and those of Snail were upregulated. But there was no significant change in Twist expression, which may be related to the low expression level of Twist in pancreatic cancer. The connection between pancreatic cancer cells is mainly realized through E-cadherin. The process of EMT can decrease the E-cadherin expression in the pancreatic cancer cells, causing the cancer cells to be detached from the peripheral cells more easily and enhancing the cancer cell activity. On the contrary, Snail is an epithelial suppressor of EMT. By inhibiting the expression of E-cadherin, Snail can reduce the adhesion between cells, enhance the movement of cells, promote the EMT in tumor cells, and improve the invasion and metastasis abilities of tumor cells [10]. The cancer cells in the central part of the tumor often express E-cadherin normally, but the cancer cells at the edge of the tumor have the typical characteristics of EMT, which makes these cells be more likely to lose their cell adhesion ability and to invade the surrounding tissues. Therefore, EMT can make the cancer cells obtain a mesenchymal cell phenotype and participate in and promote the transfer of pancreatic cancer. Pancreatic cancer cells also show a similar movement to that of EMT cells as cancer cells infiltrate surrounding tissues [18]. Our study showed that the PSC-CM can promote the EMT of pancreatic cancer cells through downregulating E-cadherin expression and upregulating Snail expression.

After silencing CXCR4 of AsPC-1 by RNAi, mRNA and protein expression levels of E-cadherin were increased, whereas Snail expression levels were decreased as compared with those of the AsPC-1 parental cells. The expressions of E-cadherin and Snail were not significantly changed in CXCR4-siRNA-AsPC-1 cells treated with PSC-CM, suggesting that blocking the SDF-1/CXCR4 axis can inhibit the effect of PSCs on EMT in pancreatic cancer cells. SDF-1 is a small chemokine that promotes the migration and metastasis of tumor cells by stimulating tumor cells and tumor mesenchymal cells in the form of paracrine factors, and SDF-1 is expressed in various tumors such as breast cancer, liver cancer, and pancreatic cancer [19]. The SDF-1/CXCR4 axis plays a key role in tumor progression. Our previous studies found that PSC-derived SDF-1 can promote invasion and metastasis of pancreatic cancer cells through the SDF-1/CXCR4 axis in pancreatic cancer [20, 21]. In light of the findings in this

study, we speculated that PSC-CM can regulate the expression of E-cadherin and Snail through the SDF-1/CXCR-4 axis, thus promoting the EMT of pancreatic cancer cells and allowing pancreatic cancer metastasize to other tissues and organs more easily.

With *in vivo* studies, we subcutaneously inoculated nude mice with an equal number of cancer cells, AsPC-1, or CXCR4-siRNA-AsPC-1. After 24 days, the animals were sacrificed, and the tumors were weighed. The tumor size in the CXCR4-siRNA-AsPC-1 group was much smaller compared with that in the AsPC-1 group. The results of the immunohistochemical staining showed that the positive expression rate of E-cadherin was increased, whereas Snail was decreased, which was consistent with the *in vitro* studies. These results indicate that PSCs can promote EMT in pancreatic cancer, and the SDF-1/CXCR4 axis may play a positive regulatory role in the process of EMT.

In conclusion, EMT serves an essential role in tumor invasion and metastasis, and PSCs can promote the EMT in pancreatic cancer. This study demonstrated that RNAi silencing of CXCR4 can upregulate the expression of E-cadherin and downregulate the expression of Snail to inhibit EMT in pancreatic cancer. Targeting the SDF-1/CXCR4 signaling pathway may be a new approach for the treatment of pancreatic cancer. However, EMT is also involved in many other regulatory mechanisms. Therefore, more studies need to be carried out on the regulatory mechanism of EMT in the future in order to find new therapeutic targets for tumor therapy.

Disclosure of conflict of interest

None.

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