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PDCD4 regulates apoptosis in human peritoneal mesothelial cells and promotes gastric cancer peritoneal metastasis

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Summary. Objective. Programmed cell death 4 (PDCD4) is a tumor suppressor gene, however, the function and regulatory mechanism remain to be discovered. The connection between tumorigenesis and apoptosis is one of the most important foci of cancer research. Our study aimed to explore the connections between PDCD4-mediated apoptosis of human peritoneal mesothelial cells (HPMC) and peritoneal metastasis in gastric cancer.

Methods. The PDCD4 expression in 31 pairs of HPMC and tumor tissues was assessed by immunohistochemistry and RT-PCR. In cell experiments, we monitored gastric cancer cell migration with a Transwell chamber assay when PDCD4 was silenced in HPMC. Subsequently, apoptosis of HPMC was detected by a flow cytometric assay and western blotting. After analyzing cytokines in culture supernatants from gastric cancer with enzyme-linked immunosorbent assays (ELISAs), transforming growth factor-beta 1 (TGF-β1) was abundant in the culture supernatants of gastric cancer. Then, PDCD4 expression in HMrSV5 cells was analyzed by western blotting after retreatment with different concentrations of TGF-β1. Moreover, apoptosis of peritoneal mesothelial cells treated with TGF-β1 was detected according to the above methods.

Results. In human metastatic peritoneal tissues, the expression of PDCD4 was significantly lower than that in normal tissues. At the same time, decreased expression of PDCD4 in HPMC was associated with increased migration capacity of gastric cancer cells. Moreover, suppressing the expression of PDCD4 promoted apoptosis in mesothelial cells which may be regulated by TGF-β secreted from gastric cancer cells.

Conclusions. These data suggested that decreased expression of PDCD4 significantly promoted apoptosis in human peritoneal mesothelial cells, thus inducing peritoneal metastasis, and that TGF-β1 secreted from gastric cancer cells may have played a crucial role.

Key words: Programmed cell death 4 (PDCD4), Gastric cancer, Apoptosis, Transforming growth factor-beta 1 (TGF-β1), Peritoneal metastasis

Introduction

According to the most recent annual statistics, for 2018, gastric cancer was the third leading cause of cancer-induced deaths, among all malignant tumors (Bray et al., 2018). Peritoneal metastases are one reason for the poor prognosis. According to the "seed and soil" theory proposed by Paget, a favorable peritoneal microenvironment ("soil") is necessary for gastric cancer cells ("seed") to survive and proliferate rapidly (Paget, 1989); however, the relevant mechanisms have not been specified.

The programmed cell death 4 (PDCD4) gene, located at chromosome 10q24, plays a crucial role as a tumor suppressor; the expression of PDCD4 is frequently altered in many cancer types (Chen et al., 2003; Gonzalez-Villasana et al., 2012; Guo et al., 2013). PDCD4 is a nuclear-cytoplasmic shuttling and RNA-

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Abbreviations. PDCD4, Programmed cell death 4; TGF-β1, Transforming growth factor-beta 1; IL-6, Interleukin-6; VEGF, Vascular endothelial growth factor; TNF-α, Tumor necrosis factor-alpha; EGF, Epidermal growth factor; IL-8, Interleukin-8; MMP-2, Matrix metalloproteinase-2; MMP-7, Matrix metalloproteinase-7; DMEM, Dulbecco"s Modified Eagle Medium

binding protein that is involved in controlling the translation of specific mRNAs; it interacts with the translation initiation factors eIF4A and eIF4G, thus inducing oncogenic pathway suppression (Fassan et al., 2010). The loss or down-regulation of PDCD4 during tumorigenesis dramatically promotes cancer cell proliferation and metastasis (Yang et al., 2006; Li et al., 2014). Moreover, PDCD4 can regulate apoptosis, an important element in tumorigenesis (Miao et al., 2014).

Apoptosis, a fundamental process controlling cell death, plays a critical role in the normal development of multicellular organisms; however, the occurrence of abnormal apoptosis may be associated with cancer (Strasser et al., 1990; Mcdonnell and Korsmeyer, 1991). Some studies have found an interaction between apoptosis and tumorigenesis. Na et al. (2014) have proposed that gastric cancer cell supernatants can induce apoptosis of human peritoneal mesothelial cells, thus resulting in peritoneal carcinomatosis. The integrated HPMC monolayer can protect peritoneal carcinomatosis; however, peritoneal integrity was damaged and submesothelial connective tissue was exposed to the peritoneal cavity when apoptosis occurred in HPMC, then a favorable peritoneal microenvironment promoted peritoneal metastasis (Na et al., 2010; Liu et al., 2013; Zhu et al., 2020). At the same time, apoptotic peritoneal mesothelial cells secreted a number of cytokines and chemokines, thus inducing peritoneal carcinomatosis (Li et al., 2012; Watanabe et al., 2012; Fujita et al., 2015). In addition, apoptotic stimuli have the potential to induce the expression of gene PDCD4 alterations (Eto et al., 2012); however, the mechanism is not clearly understood, especially in gastric cancer, and the related mechanism in peritoneal metastasis of gastric cancer remains to be elucidated.

Gastric cancer cells secrete various cytokines that are important mediators of metastasis. We have analyzed the cytokines in gastric cancer cell supernatants and found that transforming growth factor-beta 1 (TGF-β1) is an abundant cytokine that also mediates gastric cancer metastasis via many pathways (Na et al., 2010). Therefore, we hypothesized that TGF-β1 might promote peritoneal dissemination by regulating the expression of PDCD4 in mesothelial cells (Zhang et al., 2006). Our experiment consisted of two parts: verification of peritoneal tissues from gastric cancer patients and cell experiments. First, we detected the expression of PDCD4 in peritoneal tissues and demonstrated decreased expression in metastatic peritoneal tissue. Then, we determined gastric cancer cell migration capacity after silencing PDCD4 (siPDCD4) in HPMC, and the underlying mechanism was further explored by detecting apoptosis of mesothelial cells. To demonstrate the effects of the peritoneal environment on the expression of PDCD4 in HPMC, we detected cytokines in gastric cancer supernatant, then the effects of abundant cytokines TGF-β1 in gastric cancer on PDCD4 regulating apoptosis in HPMC were initially explored. On the basis of all results, our data demonstrated that decreased expression of PDCD4 led to apoptosis in peritoneal mesothelial cells and promoted peritoneal metastasis of gastric cancer cells, which were probably mediated by TGF-β1 secreted by gastric cancer cells.

Materials and methods

Patients and tissue samples

The specimens of human omentum and carcinoma were obtained from 31 gastric cancer patients treated in the Department of Surgical Oncology and Department of Pathology at Shengjing Hospital of China Medical University (Shenyang, China) in 2020. According to the pathological examination of peritoneal tissues, samples were divided into two groups (22 gastric cancer patients without peritoneal metastasis and nine gastric cancer patients with peritoneal metastases). All gastric cancer patients underwent gastrectomies, and standard resections were 5 cm away from the tumor margin for all peritoneal samples. This study was approved by the Institutional Research Ethics Board of China Medical University.

Isolation of human peritoneal mesothelial cells

Specimens of human omentum were obtained from consenting patients undergoing gastrectomy. Blunt dissection removed excess fat and provided predominantly transparent samples of tissue. The omentum was washed in several changes of sterile phosphate-buffered saline (PBS), and finely divided into approximately 5 mm^2 segments. These segments were thrice-washed in PBS to remove any contaminating red blood cells. Explants were seeded into collagen-coated 25-cm^2 tissue culture flasks at a density of approximately 1/cm^2 prior to experimentation. HPMC (human peritoneal mesothelial cell line [HMrSV5 cells]) monolayers were growth-arrested for 48 h in serum-free culture medium and stimulated for 24 h. Then, the cells were harvested and total RNA was isolated with TRIzol.

Immunohistochemistry and assessment of PDCD4 expression

Following deparaffinization and dehydration of the cells, 2–3 μm sections of the tissues were treated with 1 mM EDTA for 5 min in a pressure cooker. These specimens were then blocked and incubated with the primary antibody overnight at 4°C. The primary antibody against PDCD4 (Abcam, Cambridge, MA, USA) was diluted at a 1:500 ratio. Antibody binding was detected by a horseradish peroxidase kit (Dako Cytomation, Glostrup, Denmark), and all sections were counterstained with hematoxylin. The expression of PDCD4 was first assessed by scanning the entire tissue specimen under low power magnification (×40), then confirmed under high power (×200 and ×400). PDCD4 expression was evaluated semi-quantitatively (Dos Reis

et al., 2008), considering staining intensity (0 = absent, 1 = low, 2 = moderate, 3 = strong) and percentage of positively-stained cells (1 = immunostaining in ≤ 10% of cells, 2 = 11-30%, 3 = 31–60%, 4 = 61-100%). The final score was calculated by adding the intensity and percentage scores. Scores 1-4 represented weak expression and scores 5-7 represented higher expression.

Real time (RT)-PCR

TRIzol reagent was used to isolate total RNA from mesothelial cells in peritoneal tissues. Total RNA was subsequently reverse-transcribed to first-strand cDNA with random primers and reverse transcriptase (Invitrogen, Carlsbad. CA, USA), and quantitative real time polymerase chain reaction (qRT-PCRs). were performed with SYBR Green PCR master mix (Tiangen Biotech, Shanghai, China), and 25–50 ng of subsequent cDNA was used to determine mRNA expression by TaqMan analysis on the StepOnePlus system (Life Technologies, Grand Island, NY, USA). The primer sequences used for PCR were as follows: PDCD4, 5'-ACTGTGCCAACCAGTCCAAAGG-3' and 5'-CCTCCACATCATACACCTGTCC-3'; and GAPDH, 5'-CCACCACCCTGTTGCTGTAG-3' and 5'-CACCCACTCCTCCACCTTTG-3'. PCRs were performed with the following cycling conditions: (i) 1 cycle at 95°C for 10 min; (ii) 40 cycles, at 95°C 15 s, 60°C for 1 min; and (iii) 1 cycle at 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s using an Applied Biosystems ABI 7900 HT machine (Carlsbad, CA, USA). Baseline and threshold for Ct values were determined using the Sequence Detection System 2.3 software (Applied Biosystems). The PDCD4 expression was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as a log2ratio of PDCD4 versus GAPDH or fold increase CT (PDCD4/GAPDH2).

Cell culture

A human peritoneal mesothelial cell line (HMrSV5 cells) and gastric cancer cells (BGC-823 and MGC-803 gastric cells) were placed in a humidified 5% CO_2 incubator at 37°C. HMrSV5 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal bovine serum (GIBCO BRL, Grand Island, NY, USA), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 4 mmol/L L-glutamine, and gastric cancer cells were cultured at an atmosphere in low glucose DMEM (GIBCO BRL) containing 10% fetal bovine serum (GIBCO BRL).

Silencing of PDCD4 by siRNA

The target sequence for PDCD4 mRNA was 5'-TTGCATACTCCGACAGGCT-3', and the sense sequence of 5'-UUCUCCGAACGUGUCAGGUTT-3' was used as the negative control (siNC). Both silencing PDCD4 (siPDCD4) and siNC were synthesized by GeneChem, Ltd. (Shanghai, China). According to the manufacturer's protocol, the siRNA was transfected into the cell line HMrSV5 with Lipofectamine 2000 reagent, and induction was performed with 1 mg/ml of tetracycline for 48 h (Invitrogen). The transfection efficiency was then detected according to green fluorescence indicating the expression of the siRNA and western blotting. All assays were performed 24 h after transfection.

Migration assays

Transwell chambers were used to monitor gastric cancer cell migration capacity changes after silencing PDCD4 of HPMC. Briefly, MGC-803 cells (2×10^5) were seeded into the upper chamber, which was precoated with 100 μL of Matrigel. The lower chamber contained the following: (i) HMrSV5 cell suspension in serum-free medium; (ii) HMrSV5 cell suspension in siNC-RNA medium; and (iii) HMrSV5 cells with silenced PDCD4. After the HMrSV5 cells reached 95% confluence, MGC-803 cells were seeded in the upper chamber with serum-free medium and allowed to migrate for 20 h after induction with medium from the lower chamber. Methanol was used to fix invading cells, which were then stained with Trypan Blue, and the number of penetrating cells was counted under a microscope at ×200 magnification in ten random fields.

ELISA

The human gastric cancer cell lines BGC-823 and MGC-803 were cultured with DMEM supplemented with 10% FBS until reaching 70-80% confluency. The cells were incubated in serum-free medium for 48 h. The culture supernatants were collected, and the concentrations of human transforming growth factor-beta 1 (TGF-β1), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-α), epidermal growth factor (EGF), interleukin-8 (IL-8), matrix metalloproteinase-2 (MMP-2), and matrix metalloproteinase-7 (MMP-7) were analyzed with ELISA kits (R&D Systems, Minneapolis, MN, USA). An absorbance microplate reader was used to read the absorbance at 450 nm.

Investigation of the direct effects of TGF-β1 on HMrSV5 cell expression of PDCD4

To study the effects of TGF-β1 (obtained from Sigma-Aldrich, St. Louis, MO, USA) on HMrSV5 cell expression of PDCD4, we assigned HMrSV5 cells to four groups as follows: (1) a control group free from intervention; (2) a 1 ng/mL TGF-β group; (3) a 10 ng/mL TGF-β group; and (4) a 100 ng/mL TGF-β group. Sub-confluent cells were starved in serum free DMEM medium for 12 h. The cells were subsequently washed twice with phosphate-buffered saline (PBS) and

processed according to the treatments for each experimental group for up to 72 h. After the culture period, HMrSV5 cells were analyzed for PDCD4 expression and cell apoptosis.

Flow cytometric assays of cell apoptosis

The apoptosis ratio of mesothelial cells was analyzed with an Annexin V-FITC/PI double staining kit (Tiangen Biotech) according to the manufacturer's instructions. Briefly, HMrSV5 cells were exposed to different apoptotic stimuli, including different concentrations of TGF-β1 (0, 10, and 100 ng/mL) and to PDCD4 silencing for 48 h. The cells were trypsinized, pelleted, and gently washed in PBS. HMrSV5 cells were rinsed with binding buffer, then resuspended in binding buffer. The mixture was incubated in 10 μL of propidium iodide (PI, 50 μg/mL) and 5 μL of Annexin V-FITC (20 μg/mL) at room temperature for 10 min in the dark. Cell apoptosis was examined under a BD LSR Fortessa II analyzer (BD Biosciences, San Jose, CA, USA) and analyzed using flowJo cytometer software.

Western blotting

Cells treated with different concentrations of recombinant human TGF-β and siPDCD4 were harvested with a lysis buffer containing protease inhibitors (50 mmol/L of HEPES [pH 7.4], 250 mmol/L of NaCl, 1 mmol/L of DTT, 1 mmol/L of EDTA, 1 mmol/L of NaF, and 1% Triton X-100). SDS-polyacrylamide gels were used to separate total protein (30-50 μg) under reducing conditions, and the protein was then transferred to polyvinylidene fluoride membranes (Millipore). Chemiluminescence reagent (Tiangen Biotech) was used to visualize the immunoblots by autoradiography (BioMaxfilm; Kodak). The concentrations of primary antibodies to the following proteins were used: PDCD4 and GAPDH (Abcam, Cambridge, MA, USA) diluted 1:1,000, and Bcl2 and Bax (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:500. The secondary antibody was horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology, Inc.).

Statistical analysis

All experiments were repeated three times. All data are presented as mean±standard deviation. Comparisons between two groups were performed with the Student's t-test. A P-value<0.05 (two-tailed) was considered statistically significant. All analyses were performed in SPSS software (version 13.00; SPSS, Inc., Chicago, IL, USA).

Results

Expression patterns of PDCD4 in gastric carcinoma and HPMC

The clinicopathological characteristics of the 31 subjects enrolled in this study are summarized in Table

Table 1. Clinicopathological variables and PDCD4 protein expression in gastric cancer patients.

Variable	n	Tumor			Peritoneal tissue		
		High (n=16)	Low (n=15)	p value	High (n=19)	Low (n=12)	p value
Sex							
Male	21	12	9	0.371	11	10	0.14
Female	10	4	6		8	2	
Age (y)							
<70	15	9	6	0.365	7	8	0.105
≥70	16	7	9		12	4	
Histological type							
Intestinal type	17	11	6	0.108	8	9	0.07
Diffuse type	14	5	9		11	3	
T stage							
pT1/2	12	7	5	0.422	8	4	0.121
pT3/4	19	9	10		11	8	
N stage							
N0	7	5	2	0.553	6	1	0.168
N1	2	2	0		1	1	
N2	14	7	7		9	5	
N3	8	2	6		3	5	
Peritoneal metastasis							
Present	9	3	6	0.192	2	7	0.004*
Absent	22	13	9		17	5	
Histological grade							
Well /Moderate	14	10	4	0.035*	9	5	0.756
	Poor	17	6	11		10	7

1. We investigated the associations between clinicopathological factors and PDCD4 expression in the tumor and HPMC using immunohistochemical analysis. PDCD4 protein expression was primarily expressed in the nuclei of the cells. In the tumor group, the PDCD4 expression was significantly associated with histologic grade (P=0.035). In the HPMC group, it was significantly associated with the presence of peritoneal metastasis (P=0.004; Table I). For the early stage gastric cancer patients without peritoneal metastasis, PDCD4 was strongly expressed in the nuclei of the normal HPMC (Fig. 1A) and strongly expressed in the matched moderately differentiated gastric adenocarcinoma (Fig. 1B). However, PDCD4 protein was weakly localized in the nuclei of metastatic HPMC, mild expression in the metastatic tumor site (Fig. 1C), and primary tumor (Fig. 1D). A comparison of the staining patterns in the differentiated types of gastric carcinoma (Fig. 1E-G) revealed that the frequencies of nuclear staining of grade 2 or more were highest in the well- and moderately-differentiated tissues (10/14 [71.4%]) and decreased in the poorly-differentiated tissues (11/17 [64.7%],

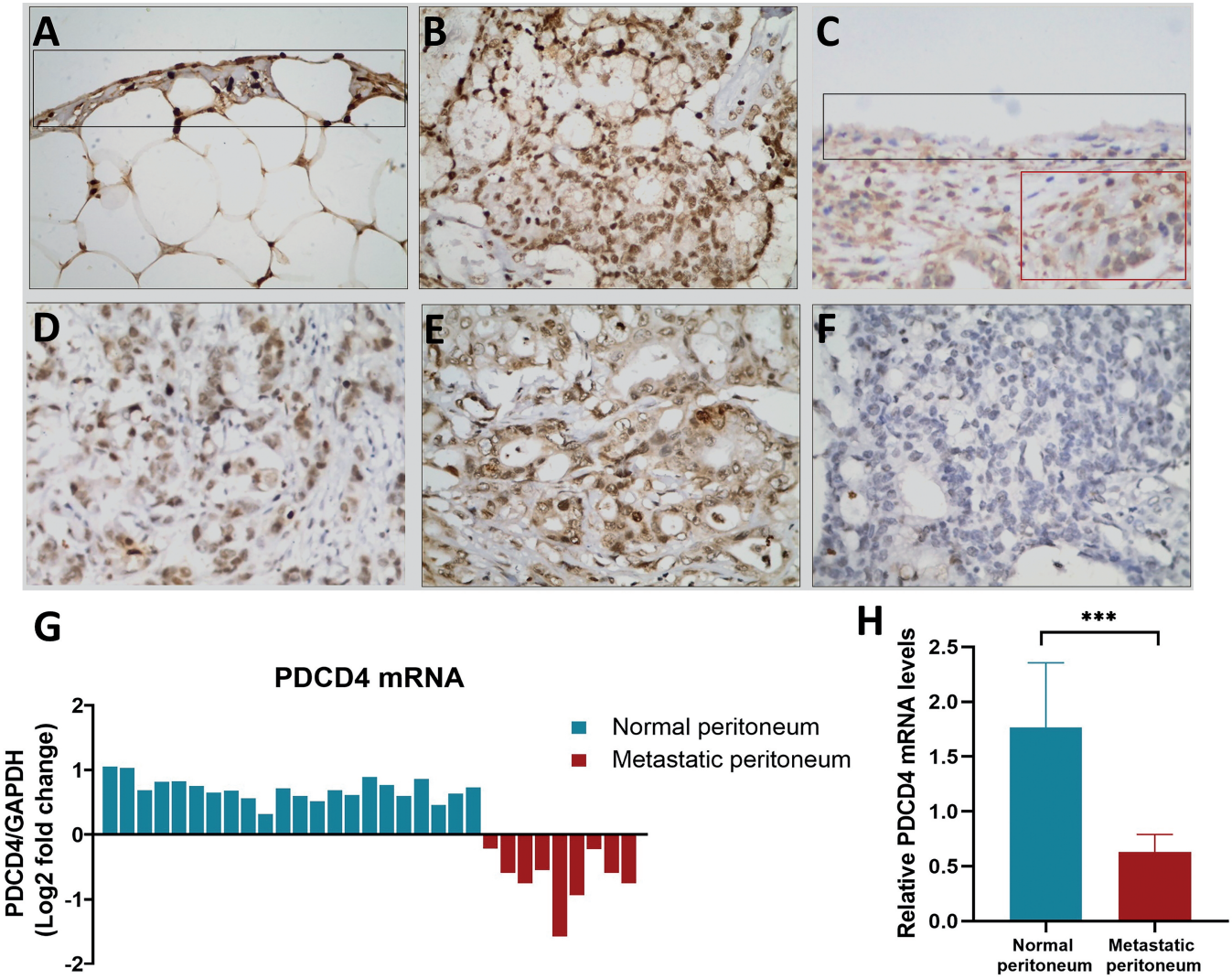

Fig. 1. Expression patterns of PDCD4 in the HPMC and gastric carcinoma. The PDCD4 showed strong positive expression in the nuclei of the mesothelial cells **(A)** (indicated by square) and tumor cells **(B)** of a well-differentiated gastric carcinoma patient (T1N0M0). The mesothelial cells showed poor expression **(C)** (indicated by square) in the metastatic HPMC of a moderately differentiated gastric carcinoma patient (T4aN3aM1), but the metastatic and primary tumor sites showed higher expression of PDCD4 **(D)**. PDCD4 expression in the different differentiated types of gastric carcinoma, well- and moderately-differentiated **(E)** and poorly-differentiated **(F)**, which revealed that the frequencies of nuclear staining were lowest in the poorly-differentiated tissues and increased according to the differentiation grade. **G.** PDCD4 mRNA levels in the HPMC of 31 patients were determined by quantitative RT-PCR analyses. The results are presented as relative fold changes (The log2 ratio of PDCD4 in metastatic peritoneum versus normal peritoneum). **H.** The relative levels of PDCD4 expression were also calculated as fold increase CT (PDCD4/GAPDH2) method, data are expressed as the mean ± standard deviation. Comparisons between two groups were performed using a Student's t test, ***P<0.001. x 400.

P=0.035; Table 1).

We further evaluated PDCD4 gene expression using fresh frozen specimens. The relative mRNA levels of PDCD4 in peritoneal tissues from 31 patients were examined by RT-PCR. We calculated the log2ratio of PDCD4 in metastatic tissues versus normal tissues. As shown in Fig. 1H, the positive log2 indicated decreased expression of PDCD4 in the tested metastatic peritoneal tissues. The mRNA expression of PDCD4 was significantly lower in the metastatic peritoneal tissues than the normal peritoneal tissues (1.75±0.63 vs. 0.62± 0.21, n=31; P<0.001; Fig. 1I).

Migration capacity of gastric cancer cells increased after silencing PDCD4 in mesothelial cells.

When we obtained the result of decreased expression of PDCD4 in the tested metastatic peritoneal tissues, the migration capacity of gastric cancer cells was monitored in control, siNC, and silenced PDCD4 groups (Fig. 3A-C), in which the transfection efficiency was detected according to green fluorescence indicating the expression of the siRNA and western blotting (Fig. 2). As shown in Fig. 3D, the migration capacity of gastric cancer cells clearly increased after silencing PDCD4 in HPMC by siRNA (Fig. 2B).

Increased invasion of gastric cancer cells was associated with PDCD4 expression of HPMC regulated apoptosis.

To further explore the mechanism underlying increased migration capacity of gastric cancer cells induced by PDCD4 expression decreased in HPMC, we detected the apoptosis in mesothelial cells after silencing PDCD4. To reveal the effects of PDCD4 expression on apoptosis in mesothelial cells, we detected apoptosis rates with flow cytometry using Annexin V-FITC/PI double staining. As shown in Fig. 4A, B, compared with that in the control group, the apoptotic rates in HMrSV5 cells were significantly higher in the siPDCD4 mesothelial cells. The percentage of apoptotic cells in the siRNA control groups was 14.99%; however, when the HMrSV5 cells were treated with siPDCD4, the

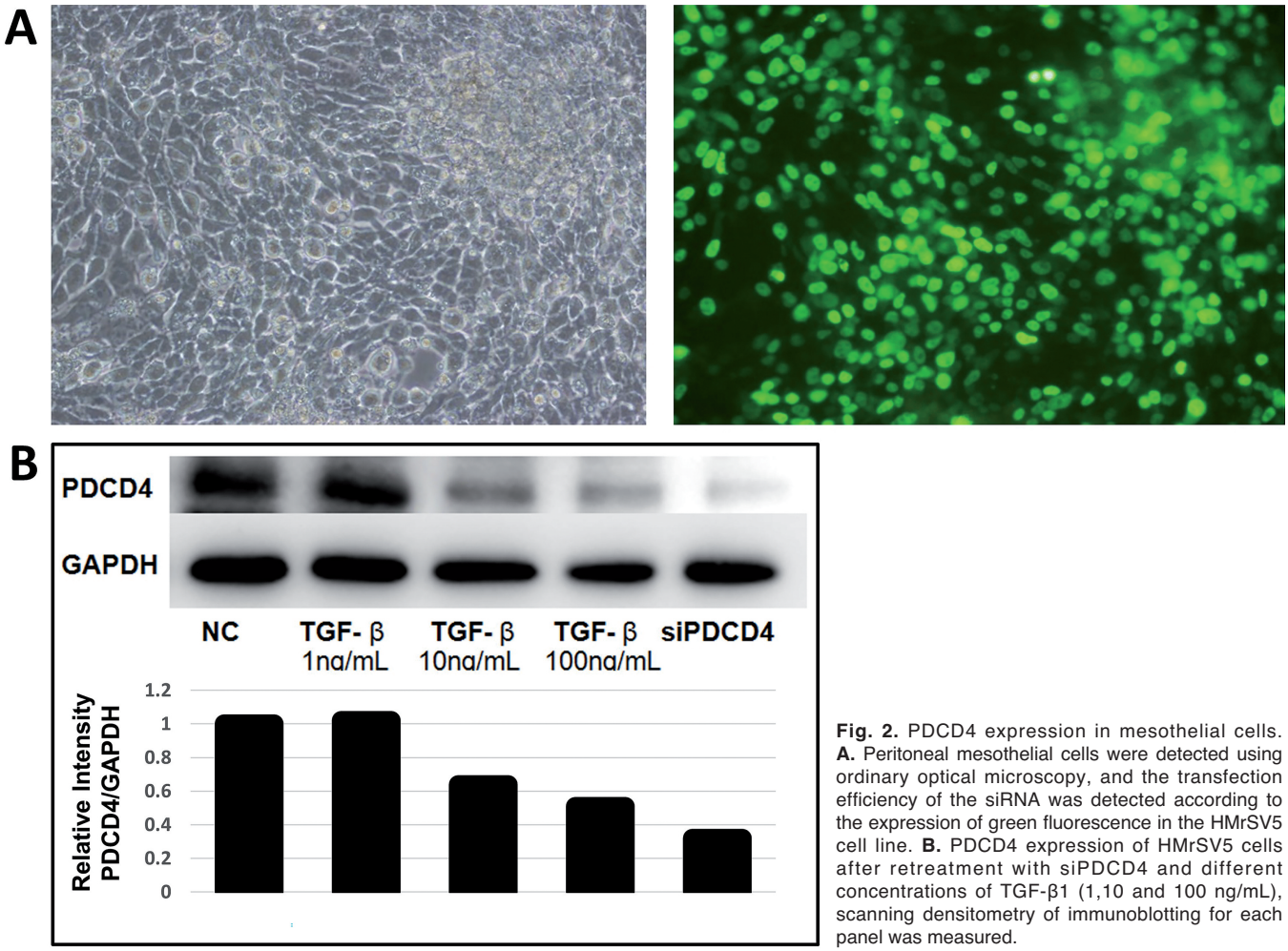

Fig. 2. PDCD4 expression in mesothelial cells. **A.** Peritoneal mesothelial cells were detected using ordinary optical microscopy, and the transfection efficiency of the siRNA was detected according to the expression of green fluorescence in the HMrSV5 cell line. **B.** PDCD4 expression of HMrSV5 cells after retreatment with siPDCD4 and different concentrations of TGF-β1 (1,10 and 100 ng/mL), scanning densitometry of immunoblotting for each panel was measured.

number of apoptotic cells dramatically increased to 75.2% (P<0.001) in the siPDCD4 group. Moreover, western blotting was used to estimate the expression of important makers of apoptosis at the protein level to further explore the mechanism underlying PDCD4's effects on apoptosis. As shown in Fig. 4C, siPDCD4 significantly decreased the expression of Bcl2 (2.73±0.27-fold, P<0.01) and increased the expression of Bax, as compared with that in the siRNA control groups (2.58±0.22-fold, P<0.05), thus demonstrating that the Bcl2/Bax signaling pathway may play an important role in the induction of gastric cancer cell apoptosis. These results suggested that increased invasion of gastric cancer cells was associated with PDCD4 expression of HPMC-regulated apoptosis.

Expression of PDCD4-induced apoptosis in HPMC was regulated by TGF-β secreted from gastric cancer cells.

To demonstrate the effects of the peritoneal environment on the expression of PDCD4 in HPMC, some soluble mediators, including human TGF-β1, IL-6, IL-8, TNF-α, VEGF, EGF, MMP-2, and MMP-7, were assessed with specific ELISA kits. TGF-β1 was abundant in the culture supernatants of MGC-803 (Fig. 3E) and BGC-823 cells (Fig. 3F). Therefore, we evaluated the effects of TGF-β on the expression of PDCD4 at the protein level. TGF-β decreased the expression of PDCD4 with increasing concentrations of TGF-β (Fig. 2B). After detecting apoptosis using flow cytometry and western blotting, TGF-β1 significantly decreased the expression of Bcl2 (P<0.01) and increased the expression of Bax (P<0.05; Fig. 4C), and the number of apoptotic cells increased to 50% (P<0.01) and 61.8% (P<0.001; Fig. 4A) with 10 and 100 ng/mL of TGF-β1, respectively, compared with control groups (14.99%). The expression of PDCD4-induced apoptosis in HPMC may be associated with TGF-β secreted from gastric cancer cells.

Discussion

Peritoneal dissemination is one of the most important reasons for the poor prognosis of gastric cancer patients, even after successful surgical treatment (Yuan et al., 2017). An appropriate peritoneal environment favors adhesion and proliferation of gastric cancer cells, and promotes peritoneal carcinomatosis (Yashiro et al., 1996). Mesothelial cells have been shown to prevent cancer invasion; however, their alterations are involved in tumor metastasis (Kiyasu et al., 1981). In recent years, several investigations have been conducted on mesothelial cells. The mechanisms preventing cancer invasion are still not clearly understood.

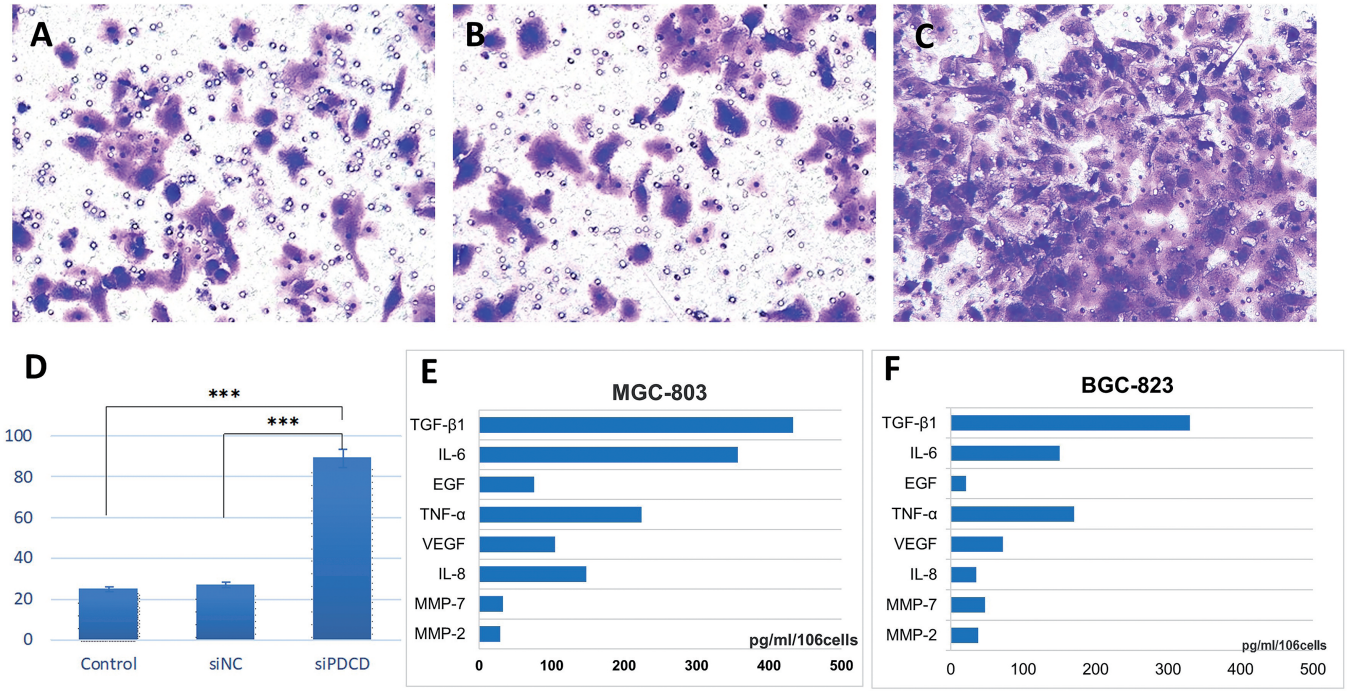

Fig. 3. Suppression of PDCD4 in mesothelial cells promotes invasion of gastric cancer cells. The Transwell chamber assay was used to detect cell migration. MGC-803 cells were seeded into the upper chamber and induced to migrate to the lower chamber containing different treated HMrSV5 cells, over the course of 20 h. **A.** HMrSV5 cell suspension in serum-free medium. **B.** HMrSV5 cells with siNC medium. **C.** HMrSV5 cells with silenced PDCD4. **D.** The number of invasive cancer cells was significantly higher in HMrSV5 cells with silenced PDCD4 than in controls. Expression of TGF-β1, IL-6, IL-8, TNF-α, VEGF, EGF, MMP-2, and MMP-7 in gastric cancer cell lines (MGC803 and BGC823), as determined with ELISA kits. **E.** Soluble mediators in culture supernatants of MGC-803. **F.** Soluble mediators in culture supernatants of BGC823.

As a tumor suppressor gene, PDCD4 expression is significantly correlated with the development and progression of several human tumors, such as cancer of the stomach, lung, colon, liver, and breast, as well as glioblastomas (Chen et al., 2003; Afonja et al., 2004; Zhang et al., 2006; Mudduluru et al., 2007; Wang and Sun, 2008; Park et al., 2012; Liang et al., 2016). The expression and function of PDCD4 protein during apoptosis also play important roles in tumorigenesis. In our study, a series of related experiments were conducted to elucidate the influence of PDCD4 expression in gastric cancer peritoneal metastasis. The clinicopathological exploration for primary tumor and peritoneal tissue of gastric cancer patients showed that PDCD4 expression was significantly associated with histologic grade of the primary tumor and peritoneal metastasis. In contrast, using peritoneal tissues from early and advanced gastric cancer patients and cell experiments, we found that the down-regulated expression of PDCD4 protein by TGF-β1 induced substantial apoptosis in human peritoneal mesothelial cells. In addition, apoptotic stimuli have the potential to induce the expression of PDCD4 to decrease (Eto et al., 2012), which increased apoptosis expression in HPMC. Finally, a more favorable peritoneal microenvironment promoted peritoneal carcinomatosis.

TGF-β1, a 25 kD homodimeric polypeptide (Attisano and Wrana, 1996), participates in the alteration of mesothelial cells through a broad array of pathways, including the epithelial mesenchymal transition (EMT), which is characterized by repression of E-cadherin expression and enhancement of α-SMA expression, thus promoting fibrosis and inducing apoptosis (Zhang et al., 2006; Tsukada et al., 2012; Jiang et al., 2013). Notably, apoptotic stimuli induced by TGF-β1 may be a crucial mechanism involved in PDCD4-mediated apoptosis. Eto et al. (Lazebnik et al., 1994; Eto et al., 2012) have reported that PDCD4 protein is decreased in cells treated

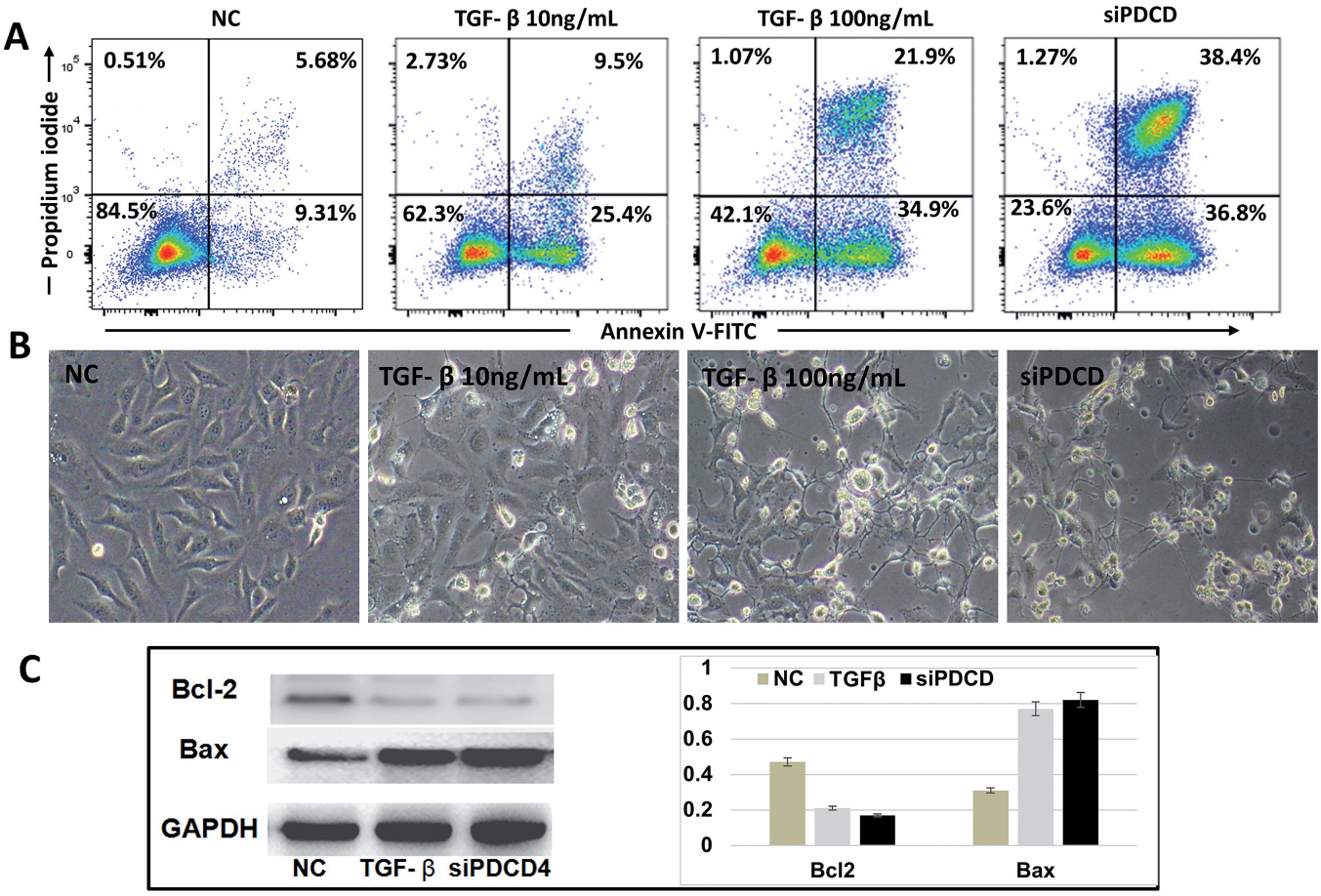

Fig. 4. Expression of PDCD4 was associated with apoptosis of HMrSV5 cells. **A.** Apoptotic rates were measured by flow cytometry. Increasing TGF-β concentrations increased apoptosis in HMrSV5 cells. Silencing of PDCD4 in HMrSV5 cells dramatically increased apoptosis. **B.** The cellular morphology of HMrSV5 cells after retreatment with different concentrations of TGF-β1 (0, 10, and 100 ng/mL). **C.** PDCD4 knockdown significantly decreased the expression of Bcl2 (2.73±0.27 fold, **P<0.01) and increased the expression of Bax, as compared with that in the siRNA control groups (2.58±0.22-fold, *P<0.05). × 400.

with apoptosis inducers, and the loss of PDCD4 increases procaspase-3 expression, thus leading to activate the expression of caspase-3, the final executioner, which cleaves poly(ADP)ribose polymerase, one of the substrates. Subsequently, proteolysis of cellular constituents, nuclear fragmentation, and DNA degradation finally induce apoptosis (Strasser et al., 2000).

In addition to cytokines, microRNAs play an important role in cell apoptosis. MicroRNAs are short non-coding RNAs that regulate target gene expression at the post-transcriptional level (Bartel, 2004). Some microRNAs directly bind the 3'-untranslated region of the PDCD4 transcript, thus blocking its translation to protein and consequently preventing Pdcd4 protein from regulating apoptosis (Eto et al., 2012). Interestingly, some microRNAs, including miR-21, miR-181b, miR-183, miR-23a/b, and miR-93, like oncomiRs, negatively regulate the expression of PDCD4. The downregulation of PDCD4 has been reported to suppress apoptosis, contradicting our results (Miao et al., 2014; Liang et al., 2016; Liu et al., 2016; Hu et al., 2017; Nicole et al., 2018; Shimizu et al., 2018). These discrepant results may be due to PDCD4 regulating apoptosis through separate mechanisms. For example, Wang et al. have reported that knockdown of PDCD4 expression decreases the sensitivity of tumor cells to tumor necrosis factor-related apoptosis-induced ligand, an important apoptosis inducer in a variety of tumor cells, thus increasing expression of FLICE-inhibiting protein, a negative regulator of apoptosis (Wang et al., 2010). Beyond microRNAs, RNA-binding proteins are additional host factors mediating post-transcriptional processing of mRNA transcripts. Wigington et al. have reported that the RNA-binding proteins human antigen R and T-cell Intracellular antigen 1, which bind the 3′-untranslated (3'-UTR) region of PDCD4, also play an important role in post-transcriptional regulation of PDCD4 (Wigington et al., 2015).

To date, no PDCD4-mediated specific mechanism controlling apoptosis has been clearly elucidated, especially for some regulations which were in opposition to our study. Indeed, PDCD4 has been reported to be a pro-apoptotic molecule involved in TGF-β1-induced apoptosis in human hepatocellular carcinoma, findings completely different from our results (Zhang et al., 2006). These discrepant results may be associated with potential biological and molecular differences in different cells or pathways, or another unknown reason. Although our experimental results showed that TGF-β induced apoptosis by decreasing PDCD4 expression of peritoneal mesothelial cells, thereby preventing peritoneal metastasis in gastric cancer, the specific mechanism remains to be explored. We found that PDCD4 plays a critical role in peritoneal metastasis of gastric cancer, because low expression of PDCD4 promotes tumor growth and suppresses apoptosis in gastric cancer cells (Liang et al., 2016; Hu et al., 2017), while promoting mesothelial cell apoptosis, thus providing a favorable peritoneal microenvironment for gastric cancer cell metastasis. In addition, PDCD4 knockdown significantly decreases the expression of Bcl2 and increases the expression of Bax (Mohan et al., 2012), thus suggesting that silencing of PDCD4 may contribute to the induction of mesothelial cell apoptosis via the Bcl2/Bax signaling pathway.

There were some limitations to our study. Although we demonstrated low PDCD4 expression in pathological tissues probably related to poor prognosis, and the relationship between the expression of PDCD4 and mesothelial apoptosis in gastric cancer peritoneal metastasis, the effects of TGF-β on PDCD4 in peritoneal mesothelial cells require further exploration. Moreover, the specific mechanism underlying the induction of apoptosis by PDCD4 remains unclear. PDCD4 plays an important role in regulating apoptosis in peritoneal mesothelial cells. Overall, PDCD4 has the potential to serve as a regulatory maker for therapeutic strategies, and the effects of TGF-β1 on PDCD4 in peritoneal mesothelial cells warrant further exploration.

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Conflict of interest statement. All authors have no competing interests.

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