The BHLH transcription factor DEC1 plays an important role in the epithelial-mesenchymal transition of pancreatic cancer

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Abstract. DEC1 (BHLHE40/Stra13/Sharp2) is a basic helix-loop-helix (bHLH) transcription factor that is involved in the regulation of apoptosis and cell proliferation and the response to hypoxia. Epithelial-mesenchymal transition (EMT) is an important step leading to invasion and migration of various tumor cells, and TGF-β treatment has been shown to induce cancer cells to undergo EMT. However, the significance of DEC1 in TGF-β-induced EMT remains unknown. We examined the role of DEC1 in EMT of PANC-1 cells, a human pancreatic cancer cell line. As a result, we found that DEC1 was upregulated by TGF-β in PANC-1 cells, and regulated the expression and the levels of nuclear, cytoplasmic or membrane localization of EMT-related factors, including phosphorylated Smad3 (pSmad3), snail, claudin-4 and N-cadherin. In the presence of TGF-β, DEC1 knockdown by siRNA inhibited morphological changes during EMT processes, while TGF-β induced PANC-1 cells to taken on a spindle-shaped morphology. Furthermore, a combination treatment of DEC1 expression with TGF-β was closely linked to the migration and invasion of PANC-1 cells. Immunohistochemically, DEC1 and pSmad3 were detected within pancreatic cancer tissues, whereas claudin-4 expression was weaker in the cancer tissues compared with the adjacent non-cancer pancreatic tissues. These findings suggest that DEC1 plays an important role in the regulation of these EMT-related factors in pancreatic cancer.

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Abbreviations: DEC1, differentiated embryonic chondrocyte gene 1; TGF-β, transforming growth factor-β; EMT, epithelial-mesenchymal transition; PANC-1, human pancreatic cancer

Key words: differentiated embryonic chondrocyte gene 1, epithelial-mesenchymal transition, transforming growth factor-β, human pancreatic cancer

Introduction

Epithelial-mesenchymal transition (EMT) is a dynamic process that occurs during the tumor progression of various cancers. It is characterized by the loss of epithelial factors, including E-cadherin, claudins, and cytokeratins, and the upregulated expression of mesenchymal markers, including N-cadherin, vimentin and fibronectin. Cancer cells that have undergone EMT are thought to acquire a fibroblast-like motile and an invasive phenotype (1,2).

Transforming growth factor (TGF)-β one of the most potent inducers of EMT, induces spindle-shaped cell morphology, inhibits cell proliferation, and promotes tumor cell motility and invasion (2-4). TGF-β binds to two different serine/threonine kinase receptors; type I (TGF-βRI) and type II receptors (TGF-βRII). The formation of hetero-dimers of TGF-βRI and TGF-βRII leads to the activation of the signaling pathways mediated by TGF-β (5). Human pancreatic cancer (PANC-1) cells express both TGF-βRI and TGF-βRII, whereas MIA PaCa-2 pancreatic cancer cells do not express either of them (6). Smads are key intracellular mediators of the transcriptional responses to TGF-β, and Smad4 mutations are known to be present in more than 50% of pancreatic cancer cases (7,8). In addition, the activation of other Smads plays important roles in the tumor progression of pancreatic cancer. The phosphorylation of Smad2 and Smad3 causes them to translocate from the cytoplasm to the nucleus and associate with Smad4. In the nucleus, the activated Smad complex binds to target gene promoters and regulates the transcriptional responses to TGF-β (5,9,10).

There are several EMT inducing transcription factors, such as snail, slug, twist and ZEB1 (11-14). Since there were some

reports on the roles of snail in PANC-1, we selected snail as a marker of positive control during EMT. Snail is expressed in various tumor cells and regulates the expression of E-cadherin, claudins, and some mesenchymal markers, which are involved in tumor invasion, metastasis, and cell motility (1,15,16). Furthermore, TGF-β directly activates snail transcription through the Smad3 phosphorylation (17).

We have shown that DEC1 (BHLHE40/Stra13/Sharp2) is involved in the regulation of apoptosis and the cell cycle in human breast and oral cancer cells (18-20). DEC1 is also highly expressed in various tumors (20-24). However, the role of DEC1 in EMT is still unknown. In this study, we focused on the role of DEC1 in EMT of pancreatic cancer cells during TGF-β treatment and demonstrated that DEC1 plays important roles in EMT of pancreatic cancer cells.

Materials and methods

Cell culture and treatment. Human pancreatic cancer PANC-1 and MIA PaCa-2 cells were cultured as described previously (25,26). These cells were incubated with recombinant human TGF-β (R&D Systems, Minneapolis, MN, USA) or A-83-01 (Takara, Shiga, Japan) at various concentrations and periods.

Knockdown of DEC1 by RNA interference. Short interference RNA (siRNA) against DEC1 was synthesized by Qiagen (Hilden, Germany). The sequences of the sense and anti-sense DEC1 siRNA and the negative control (scrambled) siRNA, and the siRNA transfection were performed as described previously (18).

DEC1 overexpression. DEC1 overexpression was induced using pcDNA vector as described previously (19). After transfection, the cells were incubated for 24 h and subjected to the invasion assay.

Western blotting. Cells were lysed using M-PER lysis buffer (Thermo Scientific, Rockford, IL, USA) and their protein concentration was determined using the bicinchoninic acid (BCA) assay. Their lysates were subjected to SDS-PAGE and detected the protein expression as described previously (18,19).

Antibodies. The membranes for western blotting were incubated with antibodies specific to DEC1 (1:10,000; Novus Biologicals Inc., Littleton, CO, USA), Smad3 (1:1,000; Epitomics Inc., Burlingame, CA, USA), pSmad3 (1:6,000; Epitomics), phospho extracellular signal-related kinases (p-ERK)½ (1:1,000; Epitomics), pSmad2 (1:5,000; Invitrogen, Carlsbad, CA, USA), slug (1:3,000; Cell Signaling Technology Inc.), snail (1:3,000; Cell Signaling Technology Inc., Danvers, MA, USA), ERK1/2 (1:30,000; Cell Signaling Technology Inc.), α-smooth muscle actin (α-SMA) (1:20,000; Sigma Chemical Co., St. Louis, MO, USA), vimentin (1:10,000; Epitomics), N-cadherin (1:10,000; ECM Biosciences, Versailles, KY, USA), E-cadherin (1:1000; Takara), claudin-1 (1:10,000; Invitrogen), claudin-4 (1:20,000; Invitrogen), claudin-7 (1:1,000; Invitrogen), and actin (1:30,000; Sigma), followed by horseradish peroxidase-conjugated secondary antibody (Immuno-Biological Laboratories, Fujioka, Japan). Can Get Signal immunoreaction enhancer solution (Toyobo Co. Ltd., Osaka, Japan) or Immunoshot immunoreaction enhancer solution (Cosmobio Co. Ltd., Tokyo, Japan) was used to dilute the primary antibody.

Real-time polymerase chain reaction (PCR). We prepared three independent RNA samples (n=3). Total-RNA was isolated and first-strand cDNA was synthesized as described previously (19). The real-time PCR was performed using SYBR-Green Master Mix (Life Technologies, Carlsbad, CA, USA). The sequences of the primers and the sizes of products are shown in Table I.

Immunofluorescent staining. Immunofluorescent staining was performed as described previously (18). The permeabilized cells were incubated with anti-DEC1 (1:300), pSmad3 (1:300), snail (1:300), N-cadherin (1:300), E-cadherin (1:300), claudin-1 (1:300), claudin-4 (1:300), or claudin-7 (1:300) antibodies at 4˚C overnight. The cells were then incubated for 1 h with goat anti-rabbit IgG antibody conjugated to Alexa 488 dye (Molecular Probes Inc., Tokyo, Japan), while nuclear staining was performed using 4',6-diamidino-2-phenylindole (DAPI). These cells were visualized using confocal laser scanning microscopy LSM 710 (Zeiss, Wetzlar, Germany).

Cell stain was carried out using the CnT-ST-100 stain kit (CellnTEC Advanced Cell Systems AG, Bern, Switzerland), in accordance with the manufacturer's instructions.

Invasion assay and migration assay. The invasion assay was performed using a BD BioCoat Matrigel invasion chamber kit (Becton Dickinson, Franklin Lakes, NJ, USA). PANC-1 cells were separated using cell dissociation solution (Sigma) and then ($5x10^4$ cells/600 μl) were added to the top chamber of a cell culture insert in a 24-well companion plate. After overnight incubation, the cells that had invaded the lower surface of the membrane were fixed with methanol and subjected to Giemsa staining. The number of cells that had migrated was quantified by counting them in ten random distinct fields using a light microscope.

For the migration assay, PANC-1 cells were seeded in a 4-chamber slide glass, and an artificial 'wound' was carefully created at 0 h by scratching the confluent cell monolayer with the tip of a P-200 pipette. Microphotographs were taken at 0, 24 and 48 h.

Human pancreatic tissues. We examined immunohistochemical analyses of surgically resected pancreatic cancers (n=17), which have been filed in Hirosaki University Hospital, Japan (Table II). All of the 17 cases examined were invasive ductal carcinoma of pancreas. Histological specimens were retrieved from the archives of our hospital according to the guidelines produced by the Japanese Society of Pathology.

Immunohistochemistry. Immunohistochemistry was performed as described previously (27). The sections were incubated overnight at 4˚C with anti-DEC1 (1:100), pSmad3 (1:200), or claudin-4 (1:400) antibodies diluted in Can Get Signal Immunostain Solution (Toyobo Co.). Finally, the sections were counterstained with Mayer's hematoxylin.

Results

DEC1 expression was induced by TGF-β in PANC-1 cells. TGF-β treatment induced Smad3 phosphorylation, and upregu-

Table I. Sequences of the primer sets and the product sizes of real-time PCR.

Gene	Product size (bp)	Primer sequences
DEC1	76	F: 5'-GAAAGGATCGGCGCAATTAA-3' R: 5'-CATCATCCGAAAGCTGCATC-3'
Snail	73	F: 5'-CTTCAACTGCAAATACTGCAACAAG-3' R: 5'-GCGTGTGGCTTCGGATGT-3'
E-cadherin	75	F: 5'-ACAGTCACTGACACCAACGATAATC-3' R: 5'-ACTGCTGCTTGGCCTCAAA-3'
Claudin-4	64	F: 5'-TGGGAGGGCCTATGGATGA-3' R: 5'-TCGTACACCTTGCACTGCATCT-3'
N-cadherin	77	F: 5'-TGATCGAGAAAAAGTGCAACAGTAT-3' R: 5'-GGCTGTGTTTGAAAGGCCATA-3'
TGF-βRI	105	F: 5'-CTGAAGTTCTCGATGATTCCATAAATAT-3' R: 5'-GAACATCGTCGAGCAATTTCC-3'
18S rRNA	150	F: 5'-GTAACCCGTTGAACCCCATT-3' R: 5'-CCATCCAATCGGTAGTAGCG-3'

F, forward primer; R, reverse primer.

Table II. Immunohistochemical expression of DEC1, pSmad3, and claudin-4 proteins in human pancreatic cancer tissues.

			DEC1		pSmad3		claudin-4	
C	A/S	D	T	N	T	N	T	N
1	66/F	Moderately	Strong	Weak	Strong	Weak	Weak	Strong
2	62/M	Moderately	Strong	Weak	Strong	Weak	Strong	Weak
3	66/F	Moderately	Strong	Weak	Strong	Weak	Weak	Strong
4	66/M	Moderately	Strong	Weak	Strong	Weak	Weak	Strong
5	67/F	Moderately	Strong	Weak	Strong	Weak	Weak	Strong
6	62/F	Moderately	Strong	Weak	Strong	Weak	Weak	Strong
7	75/M	Moderately	Strong	Weak	Strong	Strong	Weak	Strong
8	58/M	Moderately	Strong	Strong	Strong	Weak	Weak	Strong
9	65/M	Moderately	Strong	Weak	Strong	Weak	Weak	Strong
10	72/F	Well	Strong	Weak	Strong	Weak	Strong	Weak
11	67/M	Poorly	Strong	Weak	Strong	Strong	Weak	Strong
12	71/F	Well	Strong	Weak	Strong	Weak	Weak	Weak
13	74/M	Moderately	Strong	Strong	Strong	Weak	Weak	Strong
14	72/M	Poorly	Strong	Weak	Strong	Weak	Strong	Weak
15	55/M	Moderately	Strong	Weak	Strong	Weak	Weak	Weak
16	61/F	Moderately	Strong	Strong	Strong	Strong	Weak	Strong
17	50/F	Moderately	Strong	Weak	Strong	Weak	Strong	Weak

C, cases; A/S, age (yr)/sex; D, differentiation of pancreatic cancer; T, tumor cells; N, Adjacent non-tumor cells.

lated the expression of DEC1, snail, α-SMA, vimentin and N-cadherin, whereas it downregulated E-cadherin, claudin-1 and claudin-4 in PANC-1 cells (Fig. 1A and B). The highest level of DEC1 expression was observed in the cells treated with 2 ng/ml of TGF-β for 24 h. TGF-β had little effect on the Smad2 phosphorylation and the expression of Smad3, slug and claudin-7 in PANC-1 cells. On the other hand, no apparent effects were observed on the expression of the aforementioned molecules after TGF-β treatment in MIA PaCa-2 cells. Next, we investigated the endogenous mRNA expression of these proteins in PANC-1 cells treated with TGF-β. The expression of DEC1, snail and N-cadherin was upregulated by TGF-β, whereas the expression of E-cadherin and claudin-4 was downregulated (Fig. 1C). A-83-01-an inhibitor of the TGF-β signaling pathway prevents Smad2/3 phosphorylation. We therefore investigated whether A-83-01 affects the Smad3 phosphorylation and

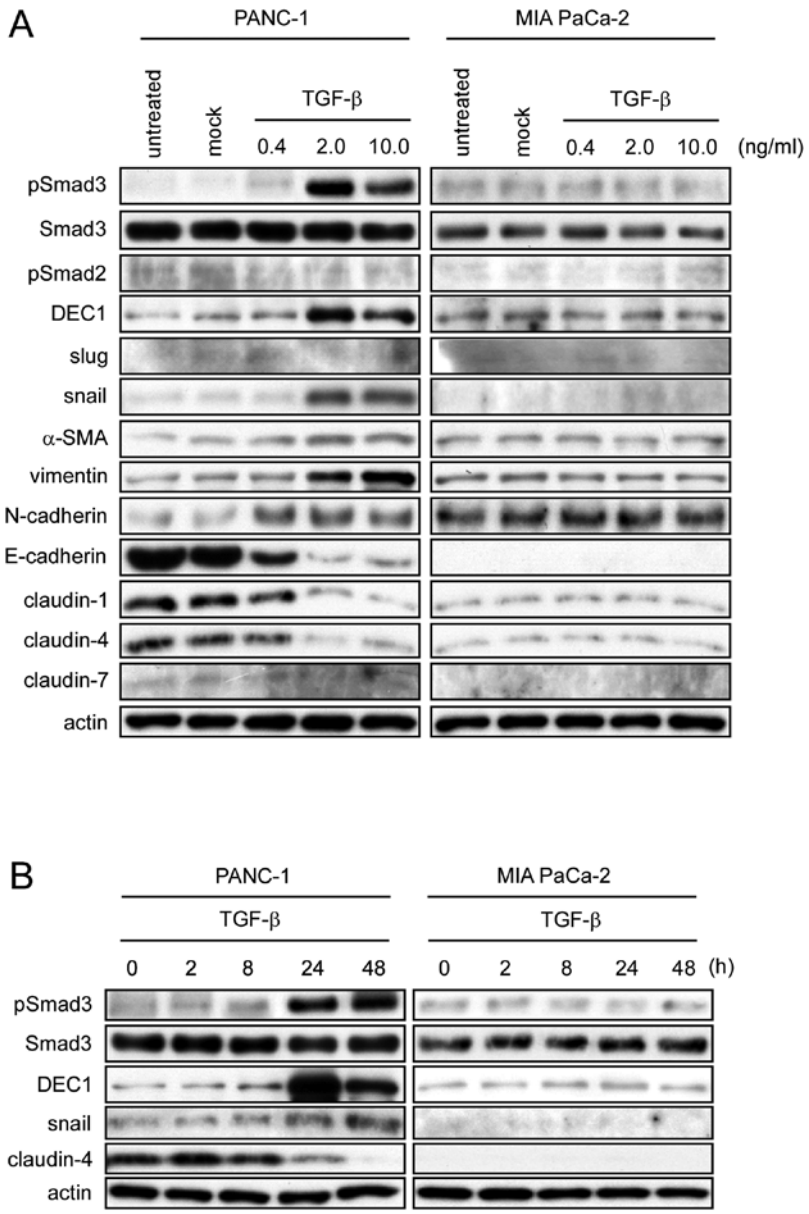


Figure 1. DEC1 expression is upregulated by TGF-β in human pancreatic cancer PANC-1 cells. (A) PANC-1 and MIA PaCa-2 cells were treated with various concentrations of TGF-β for 24 h. The control cells (mock) were treated with TGF-β-diluted buffer. These cells were then lysed and the lysates were subjected to western blot analyses of pSmad3, Smad3, pSmad2, DEC1, slug, snail, α-SMA, vimentin, N-cadherin, E-cadherin, claudin-1, claudin-4, claudin-7 and actin. One representative of at least three independent experiments with similar results is shown. (B) PANC-1 and MIA PaCa-2 cells were treated with TGF-β (2 ng/ml) for various periods. The cells were lysed, and the lysates were subjected to western blot analyses of pSmad3, Smad3, DEC1, snail, claudin-4 and actin. One representative of at least three independent experiments with similar results is shown.

DEC1 expression induced by TGF-β in PANC-1 cells. A-83-01 suppressed the Smad3 phosphorylation, and the expression of DEC1 and snail. On the other hand, claudin-4 expression was upregulated by A-83-01 (Fig. 1D).

DEC1 knockdown suppressed EMT induced by TGF-β. The cells that were transfected with the control siRNA showed a spindle-shaped morphology after 24 h treatment with TGF-β, while the cells transfected with DEC1 siRNA displayed no morphological changes after the same treatment (Fig. 2A). As shown in Fig. 2B, in the absence of TGF-β, DEC1 siRNA upregulated the expression of claudin-4, claudin-7 and E-cadherin, and downregulated the expression of N-cadherin, whereas it had little effect on the Smad3 phosphorylation, and the expression of snail and claudin-1. In the presence of TGF-β, DEC1 siRNA decreased the Smad3 phosphorylation, and the expression of snail and N-cadherin, whereas it upregulated the expression of claudin-1, claudin-4, claudin-7, and E-cadherin. In the presence or absence of TGF-β, DEC1 siRNA had little effect on the ERK1/2 phosphorylation, and the expression of total-ERK1/2, α-SMA and vimentin. Another DEC1 siRNA oligonucleotide yielded similar results (data not shown). In order to examine whether DEC1 regulates EMT-related factors at the transcriptional level, we performed real-time PCR analysis. The altered mRNA expression patterns of snail, E-cadherin, claudin-4

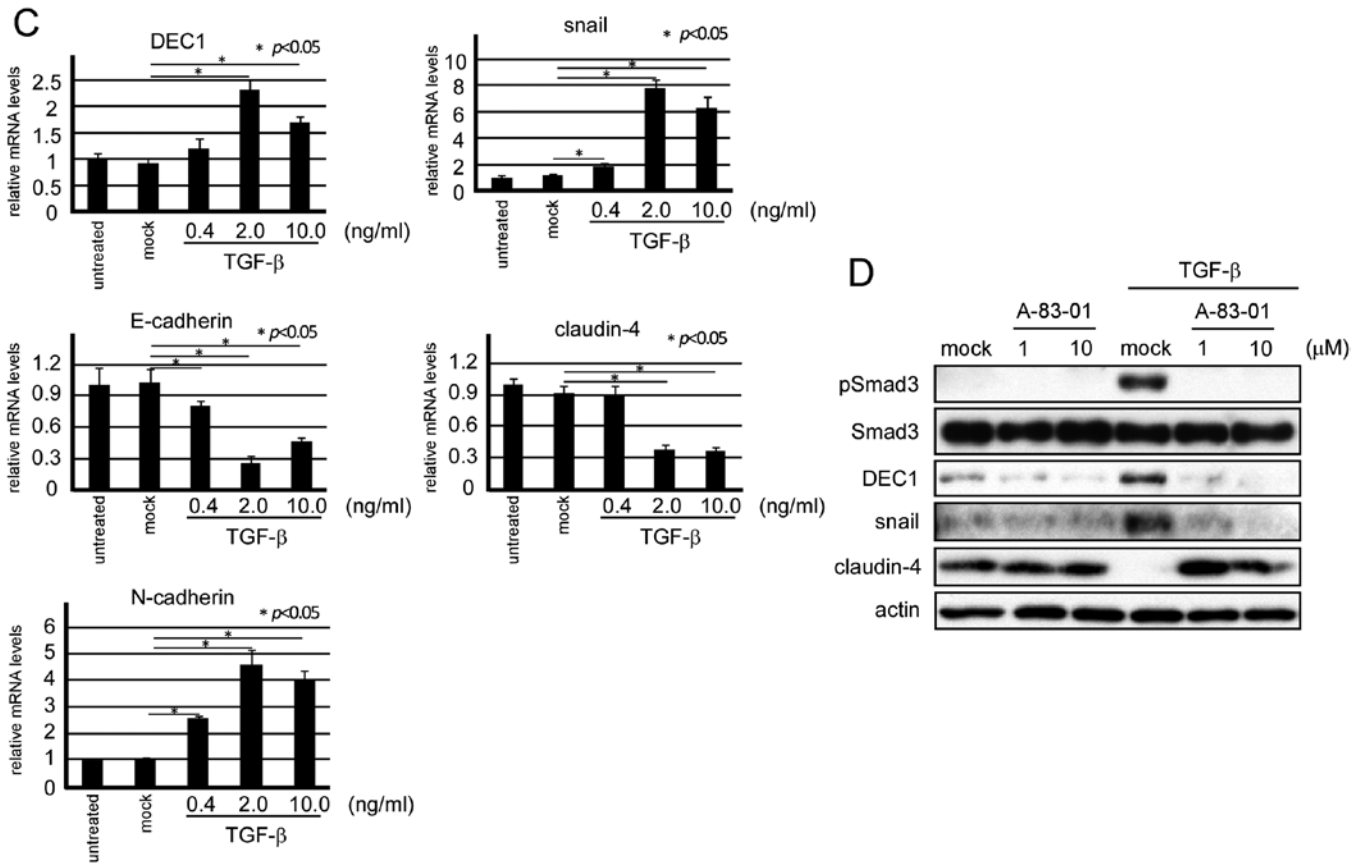
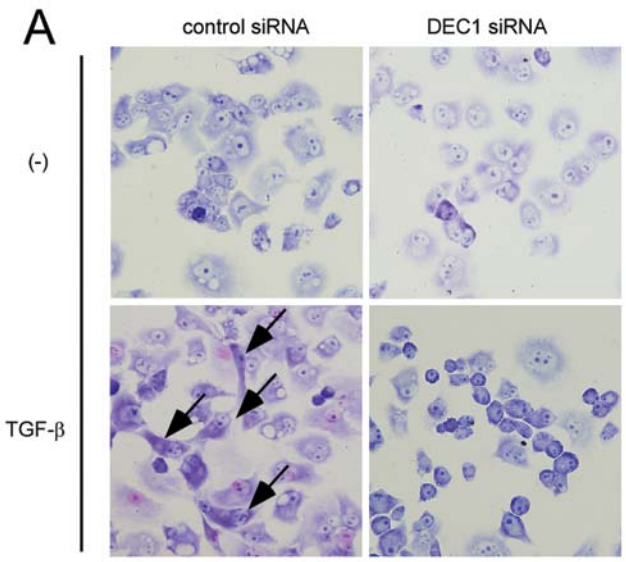


Figure 1. Continued. (C) PANC-1 cells were treated as above, and total-RNA was prepared and subjected to real-time PCR of DEC1, snail, E-cadherin, claudin-4 and N-cadherin. Each value represents the mean ± SE (bars) of three independent experiments $^{*}p<0.05$, according to the t-test. (D) PANC-1 cells were treated with the TGF-β inhibitor A-83-01 (1 or 10 μM) for 90 min before being treated with or without TGF-β (2 ng/ml) for 24 h, and the lysates were subjected to western blot analyses of pSmad3, Smad3, DEC1, snail, claudin-4 and actin. One representative of at least two independent experiments with similar results is shown.

and N-cadherin were compatible with the above protein results (Fig. 2C). In the presence of TGF-β, DEC1 siRNA also significantly decreased the expression of TGF-βRI, although DEC1 siRNA without TGF-β slightly decreased the expression of TGF-βRI. DEC1 siRNA regardless of TGF-β had little effect on the expression of TGF-βRII (data not shown).

Effects of DEC1 knockdown on the amounts of nuclear/cytoplasmic EMT-related factors. As shown in Fig. 2D, DEC1 siRNA without TGF-β decreased the amount of N-cadherin in the cell membrane, while it increased the cell membrane levels of E-cadherin, claudin-4 and claudin-7. On the other hand, DEC1 siRNA without TGF-β had little effect on the amounts of nuclear/cytoplasmic pSmad3, snail and claudin-1. In the presence of TGF-β, control siRNA increased the levels of pSmad3 and snail in the nucleus compared with the absence of TGF-β, and slightly increased the level of N-cadherin in the cell membrane, whereas it decreased the amounts of E-cadherin and claudin-1 in the cell membrane, and decreased the amount of claudin-4 in the cytoplasm. However, it had little effect on the amount of claudin-7 in the cytoplasm or membrane. In the presence of TGF-β, DEC1 siRNA decreased the levels of pSmad3 and snail in the nucleus compared with control siRNA, and it also decreased the amount of N-cadherin in the cell membrane. On the other hand, a combination treatment of DEC1 siRNA with TGF-β significantly increased the levels of E-cadherin, claudin-1 and claudin-4 in the cell membrane compared with control siRNA. A combination treatment of DEC1 siRNA with TGF-β also slightly increased the amount of claudin-7 in the cell membrane. These findings demonstrated that DEC1 has inducible effects on EMT in PANC-1 cells during TGF-β treatment, which involved alterations in the cellular amounts of EMT-related factors.

In the presence of TGF-β, DEC1 expression was closely involved in the migration and invasion. We examined whether DEC1 expression was involved in the migration and invasion of PANC-1 cells. In the presence of TGF-β, DEC1 siRNA delayed cell migration for 24-48 h in comparison with those transfected with the control siRNA (Fig. 3). We performed an invasion assay in which we transiently transfected the cells with a DEC1 expressing plasmid. As a result, the number of invasive PANC-1 cells with a spindle-shaped morphology was increased in the presence of TGF-β (Fig. 4). The invasion assay also demonstrated that there were significantly more invasive spindle-shaped cells among the cells transfected with DEC1 than among those transfected with the control pcDNA vector.



DEC1, pSmad3 and claudin-4 protein expression in human pancreatic cancer tissues and the adjacent non-cancerous pancreatic tissues. We examined the immunohistochemical expression of DEC1, pSmad3 and claudin-4 in human pancreatic cancer tissues. Photographs of DEC1, pSmad3 and claudin-4 expression in representative cases are shown in Fig. 5. Significant DEC1 immunoreactivity was detected in the cancer tissues (100%, 17/17 cases) compared with the adjacent non-cancerous pancreatic tissues, and it was predominantly located within the cytoplasm of the cancer cells, although very weak DEC1 immunoreactivity was found in the non-cancerous pancreatic ductal cells of all cases. DEC1 immunoreactivity was detected in parts of the adjacent non-cancerous tissues (17%, 3/17 cases).

It is often difficult to distinguish between spindle-shaped cells of the cancer invasive front and fibroblasts in stroma. We distinguish them by dyskaryosis and size. Firstly, dyskaryosis was shown in spindle-shaped cells, whereas it was not shown

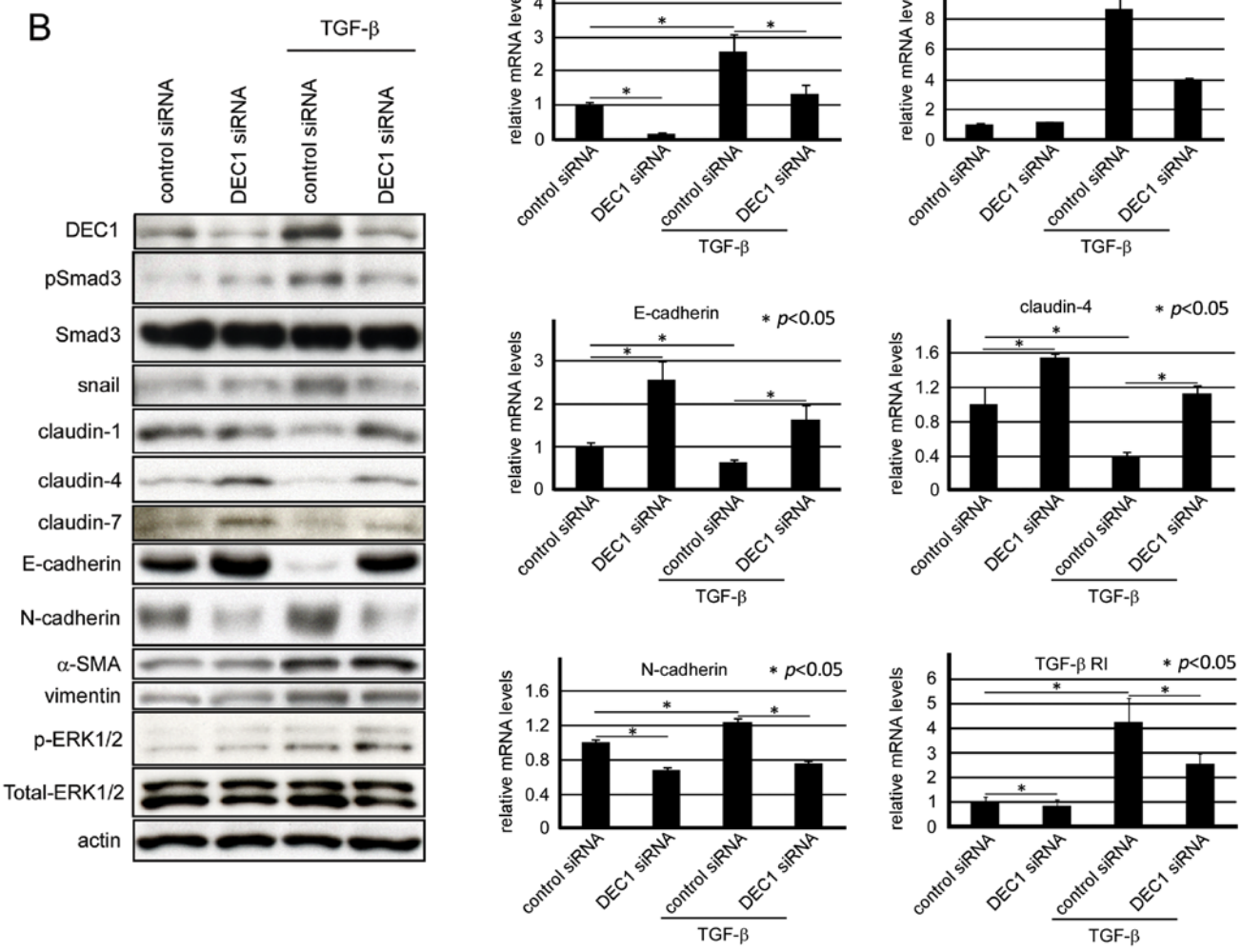


Figure 2. DEC1 knockdown inhibits TGF-β-induced EMT in PANC-1 cells. (A) PANC-1 cells were transfected with control siRNA or siRNA against DEC1. At 24 h post-transfection, the cells were treated with or without TGF-β (2 ng/ml) and incubated for 24 h. The cells were then fixed, stained and observed in bright field. The black arrows show spindle-shaped cancer cells. One representative of at least two independent experiments with similar results is shown. (B) PANC-1 cells were treated with control siRNA or DEC1 siRNA in the presence or absence of TGF-β (2 ng/ml) for 24 h, and the lysates were subjected to western blot analyses of DEC1, pSmad3, Smad3, snail, claudin-1, claudin-4, claudin-7, E-cadherin, N-cadherin, α-SMA, vimentin, pERK1/2, total-ERK1/2 and actin. One representative of at least three independent experiments with similar results is shown. (C) PANC-1 cells were treated as above, and total-RNA was prepared and subjected to real-time PCR of DEC1, snail, E-cadherin, claudin-4, N-cadherin, and TGF-βRI. Each value represents the mean ± SE (bars) of three independent experiments $^{*}p<0.05$, according to the t-test.

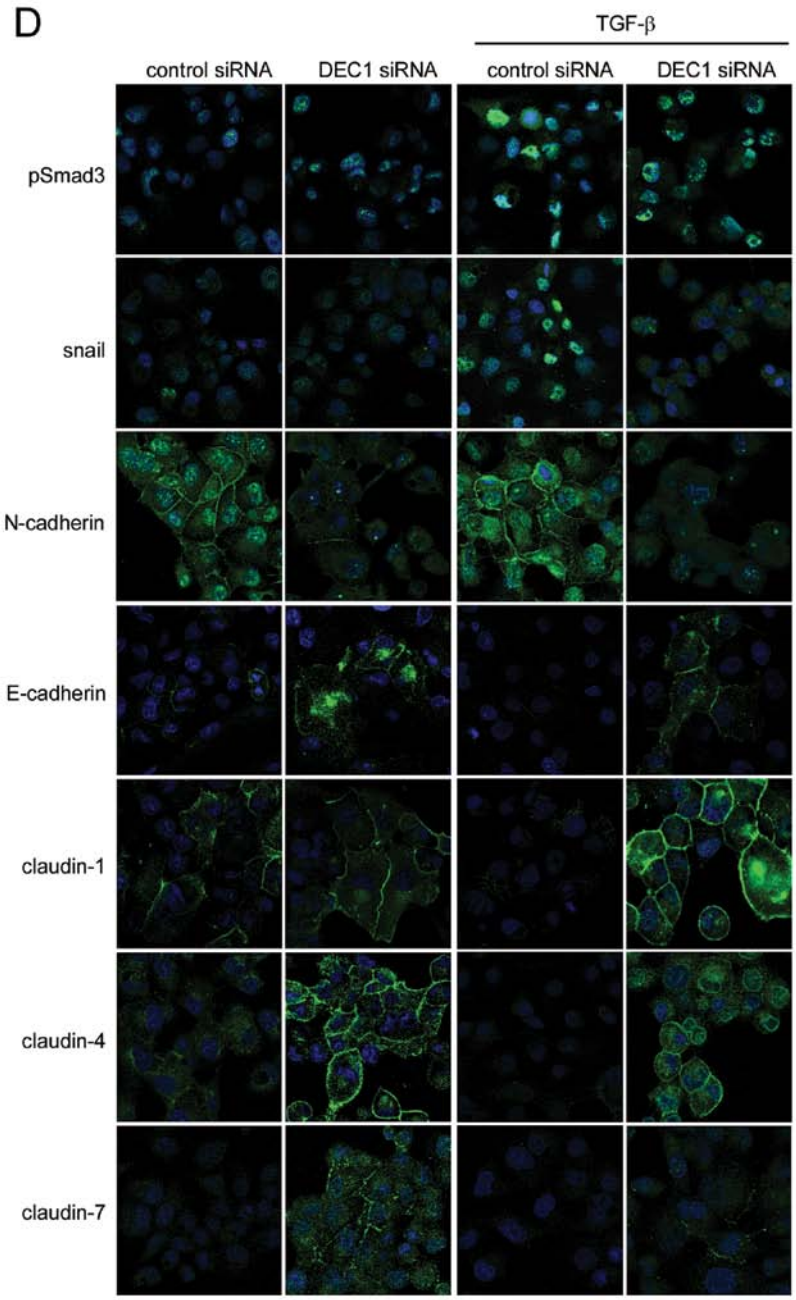


Figure 2. Continued. (D) PANC-1 cells were transfected with control siRNA or siRNA against DEC1. At 24 h post-transfection, the cells were treated with or without TGF-β (2 ng/ml) and incubated for 24 h. The cells were then fixed, incubated with anti-pSmad3, snail, N-cadherin, E-cadherin, claudin-1, claudin-4, or claudin-7 antibodies, and visualized using Alexa488-conjugated secondary antibody (green). The cells were also counterstained with DAPI (blue) in order to detect their nucleus. A merged image that is representative of at least two independent experiments with similar results is shown.

in fibroblasts in stroma. Secondary, the sizes of spindle-shaped cells were larger than fibroblasts. The spindle-shaped cells of the cancer invasive front showed strong DEC1 immunoreactivity similar to that seen in the other cancer regions (case 9, right panel). Marked claudin-4 immunoreactivity was detected in the membrane and/or cytoplasm of non-cancerous pancreatic ductal cells, whereas that in the cancer cells was weak, except for 4 cases in which strong claudin-4 expression was detected in cancer cells. Significant pSmad3 expression was found in the nucleus of spindle-shaped cancer cells, whereas it was detected in non-cancerous ductal cells in 3 cases. The changes in the immunohistochemical expression of DEC1, pSmad3, and claudin-4 were found to be independent of the cancer grade and the patient's age and gender.

Discussion

DEC1 is expressed in various tumors and regulates the responses to hypoxia, apoptosis and the cell cycle (19,20,23,24,28). Recent studies have shown that the expression of DEC1 is related to apoptosis resistance in pancreatic cancer cells (29). However, the significance of DEC1 in pancreatic cancer is poorly understood. In the present study, we showed that DEC1 was upregulated by TGF-β in PANC-1 cells. DEC1 expression was highest in the

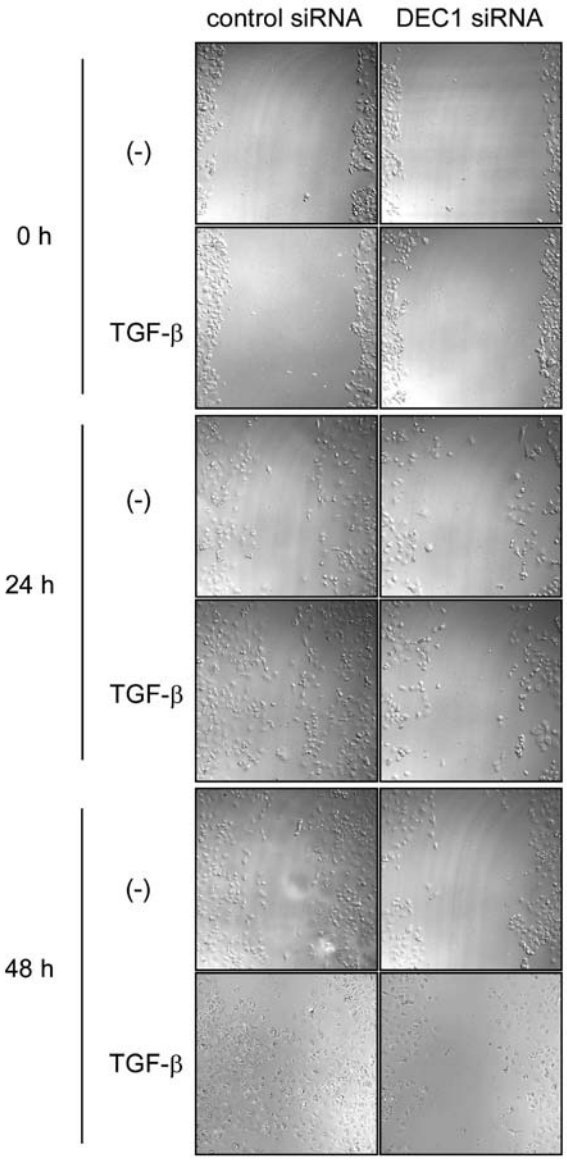


Figure 3. In the presence of TGF-β, DEC1 siRNA decreased the migration of PANC-1 cells. PANC-1 cells were transfected with control siRNA or siRNA against DEC1. After 24 h transfection, a wound was made with a pipette tip and photographs of the wounded area were taken periodically.

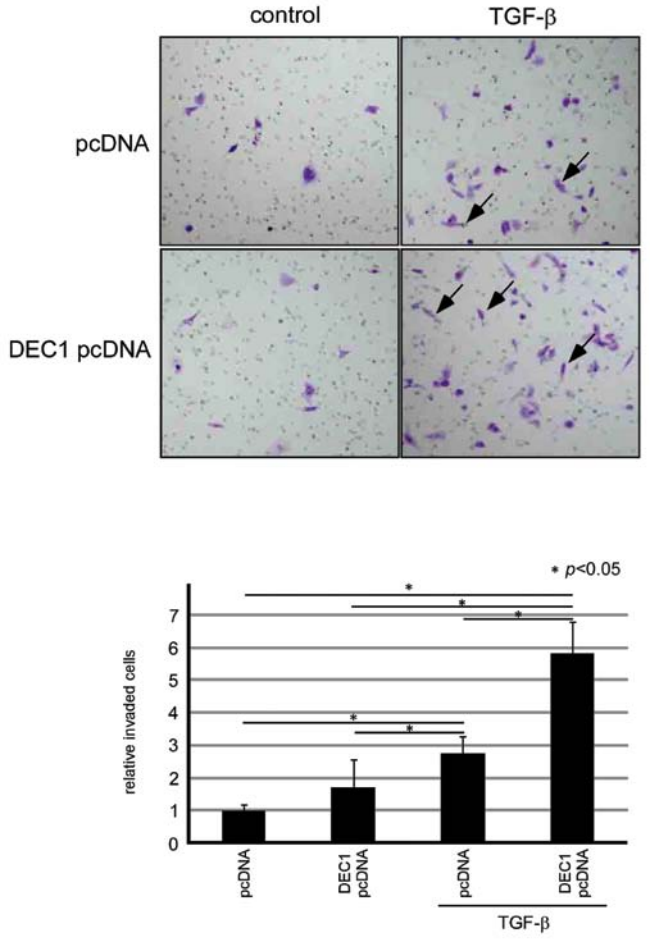


Figure 4. DEC1 overexpression increased the invasion induced by TGF-β in PANC-1 cells. PANC-1 cells were transfected with pcDNA or DEC1 pcDNA. At 24 h post-transfection, the cells were treated with or without TGF-β (2 ng/ml) for 24 h and $5x10^4$ cells each were seeded in the invasion chamber. At 24 h after cell seeding, the chambers were stained and the numbers of invasive cells was counted. The top panel shows a representative photograph. The black arrows show spindle-shaped cancer cells. The bottom panel shows the quantitative data. Each value represents the mean ± SE (bars) of three independent experiments $^*p<0.05$, according to the t-test.

cells cultured in the presence of TGF-β for 24 h, whereas snail expression was highest in the cells cultured in the presence of TGF-β for 48 h. PANC-1 cells showed the highest level of Smad3 phosphorylation when cultured in the presence of TGF-β for 24-48 h, which also induced a spindle-shaped morphology and enhanced migration and invasiveness. These findings indicate that EMT of PANC-1 is induced by 24 h TGF-β treatment and that TGF-β-induced DEC1 expression is closely related to EMT phenomena. Previous studies have reported that TGF-β affects circadian phase shifts in rat fibroblasts, as well as the immediate-early induction of DEC1, and Smad binding sites (SBE) were presented in the DEC1 promoter (30). The differences in DEC1 induction time between the previous report and our present study are probably related to cell type. It was reported that TGF-β increased the protein expression of Smad4 in PANC-1 cells (31). We performed a chip assay whether Smad3 or Smad4 bound to the DEC1 promoter in PANC-1 cells, and found that in the presence of TGF-β, Smad3 or Smad4 bound to the SBE in the DEC1 promoter (data not shown). Thus, we thought, at least, Smad3 or Smad4 might have activities for binding to the DEC1 promoter in PANC-1 cells.

We demonstrated that a combination treatment of DEC1 siRNA with TGF-β downregulated the Smad3 phosphorylation, and the expression of TGF-βRI and snail, and it also decreased the amounts of pSmad3 and snail in the nucleus. These findings suggest that DEC1 is an upstream factor of these genes. It has been reported that DEC1 upregulates and downregulates target genes by binding to sp1 and E-box sites, respectively (21,32,33). The promoter region of TGF-βRI contains sp1 sites (34). Therefore, we speculate that DEC1 binds to sp1 sites of the TGF-βRI promoter and regulates EMT-related factors through pSmad3/snail signaling. TGF-β also affects the ERK1/2 phosphorylation independent of Smad pathway (35). However, DEC1 siRNA regardless of TGF-β had little effect on the ERK1/2 phosphorylation. These results suggest that DEC1 specifically regulates the pSmad3/snail pathway induced by TGF-β. In MIA

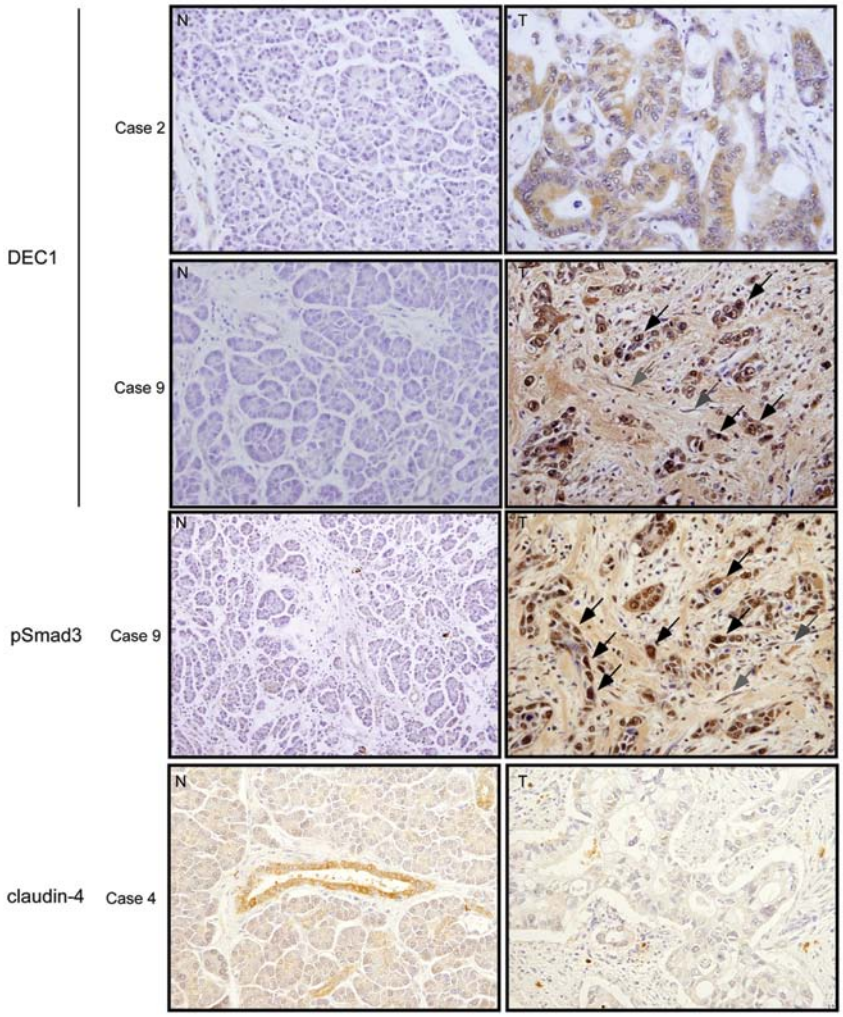


Figure 5. DEC1 and pSmad3 expression were increased in human pancreatic cancer tissues. Significant DEC1 immunoreactivity was detected in the pancreatic cancer tissues (case 2 involved moderately differentiated adenocarcinoma; right panel, labeled T), while it was weak in the non-cancerous pancreatic tissues (N, left panel). Strong DEC1 and pSmad3 immunoreactivities ware found in the spindle-shaped cancer cells (case 9 involved moderately differentiated adenocarcinoma; the black arrows show spindle-shaped cancer cells, whereas the gray arrows show fibroblasts; right panel, labeled T). Claudin-4 expression was strongly detected in the cytoplasm and the membrane of non-cancerous pancreatic ductal cells, while it was weak in the cancer tissues (case 4).

PaCa-2 cells lacking the TGF-β receptor, TGF-β treatment had little effect on the expression of DEC1 and snail, and the Smad3 phosphorylation, and cell morphology. These findings suggest that DEC1 regulates pSmad3 by positive and negative feedback systems during EMT of pancreatic cancer.

Claudin-4 expression has been shown to decrease the invasiveness and metastatic potential of pancreatic cancer (36). Claudin and E-cadherin expression were found to be down-regulated in breast, esophageal, and head and neck cancer tissue (37-39). The promoter regions of claudins and E-cadherin contain E-boxes (15,40,41). We showed that DEC1 had effect on the expression and the amounts of claudin-4, claudin-7, and E-cadherin in the cell membrane, while DEC1 and claudin-4 were immunohistochemically detected in cancer tissues and non-cancerous ducts, respectively. Based on the above findings, DEC1 is considered to negatively regulate the expression of E-cadherin and claudins.

Our study is the first report to demonstrate marked pSmad3 immunoreactivity in pancreatic cancer cells compared with that in the adjacent non-cancerous pancreatic tissues. In particular, strong pSmad3 immunoreactivity was found in the nucleus of the spindle-shaped cancer cells, which were located at the cancer invasive front. A recent study reported that Smad3, but not Smad2, increased the expression of matrix metalloproteinase-9 in lung cancer cells and contributed to EMT through TGF-β (42) which suggests that pSmad3 expression is closely involved in EMT of cancer cells.

In the present study, we demonstrated that DEC1 has inducible effects on EMT, which are mediated through the Smad3 phosphorylation, and plays an important role in EMT of pancreatic cancer; i.e., it alters the expression of EMT-related factors and affects the morphology, migration, and invasion of cancer cells.

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