Pancreatic cancer cell-derived exosomes induce epithelial-mesenchymal transition in human pancreatic cancer cells themselves partially via transforming growth factor β1

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Abstract
Distant metastasis is a dismal prognostic factor of pancreatic cancer. Metastasis is established in several steps, but the mechanism underlying the very early stages remains unclear. Epithelial-mesenchymal transition (EMT) is involved in these stages. Although signaling molecules have been reported to induce EMT, the mechanism underlying their origin is unclear. In this study, we hypothesized that pancreatic cancer cell-derived exosomes induce EMT in cancer cells themselves, a notion we entertained because we found EMT in in vitro three-dimensional colonies of cancer cells, with vimentin-positive cells observed in some of the budding pancreatic cancer cells and in single cells outside the colony as well. First, we clarified that pancreatic cancer cell-derived exosomes induce EMT in cancer cells themselves. Next, we examined the involvement of transforming growth factor-β1 (TGF-β1), and TGF-β1 knock-down in pancreatic cancer cells with TGF-β1 siRNA significantly suppressed TGF-β1 gene expression in cancer cells, and exosomal TGF-β1 was significantly reduced in the secretory exosomes. Exosomes from TGF-β1 knock-down cells suppressed EMT induction in cancer cells themselves and TGF-β1 protein expression in target cells. Taken together, these findings suggest that TGF-β1 is involved in EMT induction via exosomes, results that may support the production of effective metastasis inhibitors.

Keywords Pancreatic cancer · Exosome · Transforming growth factor-β1 · Epithelial-mesenchymal transition · Metastasis

Introduction
The 5-year survival rate for pancreatic cancer is about ≤ 10%, which is much lower than with other cancers [1]. By the time subjective symptoms appear, distant metastasis is already present, and surgical resection is not indicated, with anticancer drug treatment often performed instead [2, 3]. Although distant metastasis of pancreatic cancer is greatly involved in the prognosis, there are many unclear points concerning the mechanism underlying metastasis, and controlling distant metastasis is an urgent issue to improve the prognosis.

Epithelial-mesenchymal transition (EMT) is involved in the metastasis and infiltration of various epithelial cancers including pancreatic cancer [4, 5]. EMT is a reversible physiological process in which well-polarized epithelial cells lose their apical-basal polarity and cell–cell contacts while acquiring a spindle-shaped mesenchymal-like morphology [6] as well as cancer stem cell properties and therapy resistance [7]. These morphological changes are characterized by repression of epithelial markers (e.g. E-cadherin, claudins, occludins) and production of mesenchymal markers (e.g. vimentin and N-cadherin) [4]. It is also characterized by epithelial-mesenchymal transforming regulatory transcription factors, such as Snail and Zeb1, which are regulated by transforming growth factor-β1 (TGF-β1) and Notch signals [8, 9]. Cancer cells in which EMT is induced acquire migration ability, and distant metastasis becomes possible [10]. Taking the above into consideration, EMT can be a therapeutic target [11].

In recent years, it has been clarified that extracellular vesicles, called exosomes, secreted from all cells play an
important role in cell–cell communication by transmitting the nucleic acids, proteins, and microRNA contained therein [12]. In cancer metastasis research, a paradigm shift has occurred since exosomes were first reported to determine organotropic metastasis [13]. As mentioned above, EMT is important in the early stage of metastasis, but the mechanisms that induce EMT signals are not fully understood.

In the present study, we explored the hypothesis that pancreatic cancer cell-derived exosomes induce their own EMT and establish the initial stage of metastasis.

Materials and methods

Cell lines and culture conditions

The human pancreatic cancer cell line AsPC-1 was cultured in RPMI medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 µg/mL of streptomycin. PANC-1 and MIA PaCa-2 were cultured in DMEM medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. The human pancreatic tumor-initiating cell line KMC26 was isolated from a pancreatic ductal adenocarcinoma (PDAC) patient as described before [14]. The mouse stromal cell line PA6 (a gift from Dr. Nishikawa [RIKEN, Kobe, Japan]) was maintained in α-minimum essential medium (Invitrogen, Carlsbad, CA, USA) containing 10% FBS [14]. KMC26 was co-cultured with PA6 cells in serum-free Stem medium (DSRK100; DS Pharma Biomedical, Osaka, Japan) containing 0.1 M 2-mercaptoethanol, 50 U/mL of penicillin, and 50 µg/mL of streptomycin (Life Technologies, Kwartsweg, Netherlands) at 37 °C in a humidified atmosphere containing 5% CO₂. To exclude the effect of exosomes from the FBS used in the culture, the exosomes were excluded in advance by ultracentrifugation, as described below.

Lentiviral vector and lentiviral-mediated gene transfer

cDNA for enhanced green fluorescent protein (EGFP) was amplified by polymerase chain reaction (PCR) using pCX4ble-EGFP [15] as a template and substituted with the puromycin-resistant gene (puroR) of pLKO.1-puro Empty Vector to make pLKO-EGFP plasmid. Lentiviral-mediated gene transfer was carried out using the ViraPower Lentiviral Packaging Mix (Thermo Fisher Scientific) according to the manufacturer’s directions. In brief, pLKO-EGFP was co-transfected with the packaging mix into 293FT cells (Thermo Fisher Scientific), and culture supernatants were collected two days after transfection. The supernatants were then filtered, supplemented with 8 µg/ml polybrene, and used for infection to make EGFP-expressing KMC26 and AsPC-1 cells. The EGFP-positive cells were sorted by a flow cytometer, and in all cases, cultures arose from the polyclonal expansion of infected cells.

Colony formation

GFP-AsPC-1 and PA6 were mixed at a ratio of 2:1. A total of 10,000 cells was distributed into 24-well ultra-low attachment plates (Corning, Glendale, AZ, USA) and cultured in RPMI medium containing 10% exosome-depleted FBS for 5 days to form colonies. The colonies were picked and transferred onto PA6 cultured in 24-well plates for 3 h, and then cell staining was performed.

Isolation of exosome from culture supernatant

The culture supernatant from AsPC-1 and PANC-1 cells were collected, centrifuged at 2000g for 10 min at 4 °C, and filtered through a 0.22-µm filter before being ultra-centrifuged at 100,000g for 70 min at 4 °C. The pellet was then resuspended in phosphate-buffered saline (PBS) and ultracentrifuged again at 100,000g for 70 min at 4 °C. The pellets were resuspended in PBS as the exosome fraction. Exosomes were confirmed by Western blotting using the exosome markers CD63, CD81, and Alix. The exosome concentration was analyzed using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific), and the protein concentration was unified to 5 µg/mL for each experiment.

Quantitative reverse transcription PCR (RT-PCR)

AsPC-1 and PANC-1 cells were seeded on a 96-well plate and cultured to confluence. Two days after exosome addition, total RNA was extracted using RNeasy mini Kit (Qiagen Inc., Hilden, Germany), and quantitative real-time PCR was performed using QuantiTect SYBR Green RT-PCR System (Life Technologies). The primer sequences were Snail: 5'-CACAAATCGAAGCGCTAA-3' (forward), 5'-AGGGCTGCTGGAGGTAAAC-3' (reverse) (70 bp), Zeb1: 5'-GCTGGCAATAAGCAACAGT-3' (forward), 5'-CCATTGGGCTGGATCAGTT-3' (reverse) (107 bp), TGF-β1: 5'-GGTGGAGCCGGGAGAAT-3' (forward), 5'-TGAACCCTGGTGTGCCATTGC-3' (reverse) (99 bp), E-cadherin: 5'-CCTATGAGTCTCCCGCTTA-3' (forward), 5'-GAATCAAGCGGCGGCGTG-3' (reverse) (116 bp), β-actin: 5'-AGCACCTGCTTGTGAGCTCCC-3' (forward), 5'-TTGCACATCCCGAGCGGT-3' (reverse) (104 bp).

The results are presented as the parameter threshold cycle (C_T) values. ΔΔC_T was the difference in the C_T values derived from the specific gene being assayed and β-actin, whereas ΔΔC_T represented the difference between the paired
samples, as calculated by the formula $\Delta \Delta C_T = \Delta C_T$ of a sample-$\Delta C_T$ of a reference.

**Western blotting**

AsPC-1 and PANC-1 cells were seeded on a 96-well plate and cultured to confluent. The cells were collected 48 h after exosomes addition and crushed by ultrasonic waves to recover the protein. After developing by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), gel was transferred to a nitrocellulose membrane using an iBlot Gel Transfer Device (Life Technologies), and Western blotting was performed with anti-vimentin antibody (BioLegend, San Diego, CA, USA), anti-TGF-β1 antibody, anti-CD63 antibody, anti-CD81 antibody (Abcam, Cambridge, MA, USA), anti-β-actin antibody (Sigma-Aldrich), and anti-Alix antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Using human-specific β-actin expression as an endogenous control, color was developed with Clarity Max Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA), and expression was quantified with Optima Shot CL-420α (Wako Pure Chemical Industries, Osaka, Japan).

**Immunostaining**

The cells and the colony grown on plates were fixed in 4% paraformaldehyde for 10 min at room temperature and washed three times with PBS. Next, non-specific binding of antibodies was blocked with Blocking One Histo (Nacalai Tesque, Kyoto, Japan) for 5 min, and the cells and colony were incubated with primary antibodies diluted in 10% FBS/Tris-buffered saline-Tween20 (TBST) (25 mM Tris–HCl [pH 7.4], 75 mM NaCl, and 0.1% Tween20) for 1 h at room temperature. Primary antibodies were used at the following dilutions: mouse anti-E-cadherin monoclonal antibody, 1:1000 (BioLegend); mouse anti-vimentin monoclonal antibody, 1:1000 (BioLegend); and chicken anti-GFP polyclonal antibody, 1:500 (Abcam). One hour later, the cells and colony were washed three times with PBS and incubated with secondary antibodies for one hour at room temperature. Secondary antibodies were used at the following dilutions: goat anti-mouse-IgG Alexa Fluor 555, 1:200 (Thermo Fisher Scientific); goat anti-chicken-IgY Alexa Fluor 488, 1:200 (Thermo Fisher Scientific). One hour later, the cells and colony were washed and incubated with DAPI (Thermo Fisher Scientific) for 10 min. Immunoperoxidase staining for human normal pancreas and mouse tumors from human pancreatic cancer cells was performed as described previously [16]. Each slide was incubated with rabbit anti-TGF-β1 monoclonal antibody (Abcam) diluted 1:100 in Dako Real antibody diluent (Dako, Glostrup, Denmark) at 4 °C overnight. The primary antibody detection was performed with anti-rabbit horseradish peroxidase polymer (Dako) at room temperature for 1 h, followed by three rinses with TBST. The signal was developed with diaminobenzidine solution (Dako) for 3 min. The samples were rinsed with distilled water three times, counterstained with hematoxylin for 3 min, dehydrated in alcohol solution and xylene, and mounted in Entellan (Merck, Darmstadt, Germany).

**Transfection of siRNA**

AsPC-1 or PANC-1 cells ($1 \times 10^5$ cells) were plated 24 h before transfection in RPMI or DMEM supplemented with 10% FBS. Transfection was performed in serum-free Opti-MEM medium (Invitrogen, Carlsbad, CA, USA) with 150 pmol Stealth RNAi mixture for TGF-β1 (Invitrogen; HSS110683 and HSS110684) and 5 µL Lipofectamine (Invitrogen) for 24 h. The transfection medium was then replaced with RPMI or DMEM supplemented with 10% exosome-deplete FBS for 48 h. Negative controls (NCs) were transfected with the scrambled siRNA. After 48 h, the culture supernatant was collected, and exosomes were collected by the same method as described above.

**Statistical analyses**

All values were expressed as the means± standard error of the mean. Statistical significance was determined using the two-tailed unpaired Student’s $t$ test, and differences were considered to be statistically significant when $P<0.05$.

**Results**

**EMT in the colonies of human pancreatic cancer cells and epithelial phenotypes of human pancreatic cancer cell lines**

First, we examined whether or not EMT could be induced in pancreatic cancer cells with the epithelial phenotype. In the colonies of human pancreatic cancer cells (KMC26) established in our laboratory [14], vimentin-positive cells were observed in some of the budded pancreatic cancer cells (arrowheads). Vimentin-positive cells were also observed in single cells outside the colony (arrows) (Fig. 1a). Similar results were also obtained in colonies generated by culturing GFP-AsPC-1 cells, a human pancreatic cancer cell line with epithelial cell phenotypic characteristics (Fig. 1b). These phenomena are similar to how pancreatic cancer cells metastasize away from neighboring cells by EMT.

We focused on the exosomes in the supernatant secreted by pancreatic cancer cells. We thus performed E-cadherin staining on AsPC-1, PANC-1, and MIA PaCa-2 cells and confirmed that E-cadherin staining was positive in AsPC-1 and PANC-1 cells and negative in MIA PaCa-2 cells, as
previously reported [17] (Fig. 1c–e), indicating that AsPC-1 and PANC-1 have an epithelial phenotype while MIA PaCa-2 has a mesenchymal one. Thereafter, we considered the induction of EMT by exosomes using AsPC-1 and PANC-1 cells.

**Exosomes derived from pancreatic cancer cells with epithelial phenotype induce EMT in pancreatic cancer cells themselves**

Exosomes from AsPC-1 and PANC-1 cells were confirmed by Western blotting using exosome markers, such as CD63, CD81, and Alix (data not shown). The expression of epithelial-mesenchymal transforming regulatory transcription factors, such as the Snail and Zeb1 genes, was significantly increased in exosome-treated cells compared to PBS-treated control cells in both cancer cell lines (Fig. 2a, b). Vimentin gene product was also significantly increased in exosome-treated cells (Fig. 2c, d). These results indicate that exosomes derived from pancreatic cancer cells with epithelial phenotype induce EMT in pancreatic cancer cells themselves.

**The TGF-β1 expression in human normal pancreatic ducts and pancreatic cancer tissue, human pancreatic cancer cell lines, and mouse subcutaneous tumors from human pancreatic cancer cells**

We next focused on TGF-β1, a typical EMT-inducing molecule, to investigate the mechanism underlying EMT induction by exosomes from pancreatic cancer cells. First, we examined the TGF-β1 expression in human normal pancreatic tissue and pancreatic cancer tissue, human pancreatic cancer cell lines (AsPC-1, PANC-1, and MiaPaCa-2), and mouse subcutaneous tumors from human pancreatic cancer cells AsPC-1, PANC-1, and KMC26 by immunohistochemistry. As a result, we noted the accumulation of TGF-β1-positive cells in the islets of normal pancreas, while the pancreatic duct, which is the origin of human pancreatic ductal adenocarcinoma, was negative (Fig. 3a). TGF-β1 was strongly expressed in both stromal and pancreatic cancer cells in human PDAC tissue (Fig. 3b). The comparison of the TGF-β1 expression among the three cell lines revealed that TGF-β1 was predominantly expressed at both the gene and protein levels in AsPC-1 and PANC-1 cells in comparison to MiaPaCa-2 cells (Fig. 3c–f). These results are consistent with a previous report [18]. In contrast, in AsPC-1-, PANC-1-, and KMC26-derived mouse subcutaneous tumors, TGF-β1 was strongly expressed in stromal cells and weakly expressed in pancreatic cancer cells (arrowheads) (Fig. 3g–i). Furthermore, in the colonies of KMC26 and AsPC-1 cells, TGF-β1-positive cells were observed in pancreatic cancer cells on the outer surface, in addition to vimentin-positive cells (arrowheads) (Fig. 3j, k).

**Effect of TGF-β1 knock-down cells on TGF-β1 gene expression in pancreatic cancer cells and pancreatic cancer cell-derived exosomes**

Next, we examined the effect of TGF-β1 knock-down on TGF-β1 gene expression in pancreatic cancer cells and pancreatic cancer cell-derived exosomes using real-time PCR. In AsPC-1 (Fig. 4a) and PANC-1 (Fig. 4b), the expression of TGF-β1 mRNA was significantly decreased in pancreatic cancer cells, and exosomal TGF-β1 was significantly reduced in the secretory exosomes. The exosomes utilized in this study were the same ones used in the experiment in Fig. 2. Furthermore, in the presence of NC siRNA-exosome, some cells in both cell lines showed transformation to
spindle-shaped cells. On the other hand, these transformations were not observed in the presence of TGF-β1 siRNA-exosome (Fig. 4c, d).

**Exosomes from TGF-β1 knock-down cells suppressed EMT induction in pancreatic cancer cells themselves and TGF-β1 protein expression in target cells**

We investigated the effect of exosomes from TGF-β1 knock-down pancreatic cancer cells (hereafter, TGF-β1 siRNA-exosome) on EMT induction in pancreatic cancer cells. The Snail and Zeb1 gene expression (Fig. 5a, b) and vimentin gene product (Fig. 5c, d) were significantly suppressed in TGF-β1 siRNA-exosome treated cells compared to NC siRNA-exosome treated cells. In contrast, the E-cadherin gene expression was significantly downregulated in NC siRNA-exosome-treated cells and these changes were reversed in TGF-β1 siRNA-exosome-treated cells (Fig. 5c, d). Taken together, these findings suggested that exosomes from TGF-β1 knock-down cells suppressed EMT induction in pancreatic cancer cells themselves. Importantly, TGF-β1 siRNA-exosomes suppressed the expression of TGF-β1 protein in target cancer cells (Fig. 5e, f).

**Discussion**

Distant metastasis from an early stage is a factor that worsens the prognosis of pancreatic cancer. In the present study, we analyzed the mechanism underlying metastasis in the early stage of pancreatic cancer development, as the inhibition of metastasis would greatly contribute to the improvement of the prognosis. EMT, which is an early event in distant metastasis, is a process by which cancer cells lose intercellular adhesion and acquire migratory and invasive abilities, and then leave the primary tumor and metastasize [19]. In other words, activation of EMT induces metastasis, which correlates with poor clinical prognosis [20]. However, although there have been reports on signaling molecules that induce EMT in cancer cells, the mechanism concerning their origin remains unclear. The idea for the present study came to us because we noted EMT in three-dimensional colonies in vitro in our human pancreatic cancer strain, KMC [14], and thought it would be a good model to investigate the
mechanism underlying the induction of EMT in the early stage of metastasis.

In this study, we demonstrated that exosomes derived from AsPC-1 and PANC-1, which have a strong epithelial phenotype, induced EMT in the pancreatic cancer cells themselves. In addition, in TGF-β1 knock-down pancreatic cancer cells, the TGF-β1 gene expression was suppressed in the cancer cells, and exosomal TGF-β1 was significantly reduced in the secretory exosomes, thereby inhibiting the induction of EMT by exosomes and indicating the partial involvement of TGF-β1. Although the process by which TGF mRNA in exosomes is incorporated into target pancreatic cancer cells and the subsequent fate of TGF mRNA has not been clarified, the mechanism of action of recent mRNA vaccines against COVID-19 may serve as a reference. Lipid nanoparticles, including mRNA, encoding spike protein of COVID-19 are taken up into target cell by endocytosis, the mRNA is translated into protein, and the protein is released [21]. Our experimental results confirmed the validity of this hypothesis, as TGF-β1 protein expression was upregulated in target pancreatic cancer cells that had themselves been exposed to exosomes, and the amount of protein was downregulated by TGF-β1 knock-down in host cells. Regarding the relationship between exosomes and metastasis, metastasis is reportedly promoted by cleavage of vascular endothelial cells [22]. We also confirmed this fact in in vivo experiments, but that was not the very early stage of metastasis, as in the present study.

Based on the present results, several hypotheses can be formulated regarding the source of TGF-β1 that causes the EMT in pancreatic cancer cells: (1) an autocrine or paracrine pathway by TGF-β1 directly secreted by pancreatic cancer...
cells or by secreted exosomes; and (2) stromal cells that strongly express TGF-β1 in human PDAC and mouse subcutaneous tumors. Regarding (1), the TGF-β1 expression in the outer layer of the colony was observed in in vitro colony formation, along with the expression of vimentin, suggesting that TGF-β1 may be derived from cancer cells themselves. Another possibility in case (2) is that the expression of TGF-β1 is only observed in islets in the normal human pancreas, but once the pancreatic tumor is formed, the expression of TGF-β1 is strongly observed in the surrounding stromal cells, suggesting that the expression of TGF-β1 is strongly observed in the surrounding stromal cells, suggesting that the expression of TGF-β1 may be induced by the formation of a microenvironment through the interaction of stromal cells and pancreatic cancer cells. Further studies are needed to determine the mechanism underlying the induction of the expression of TGF-β1 on stromal cells in stromal formation, which is a characteristic of pancreatic cancer. Thus far, we do not know the extent to which these sources are involved in inducing the EMT in pancreatic cancer.

Recently, highly differentiated pancreatic ductal adenocarcinoma was reported to undergo partial EMT, in which cancer cells acquire mesenchymal characteristics while retaining some epithelial ones, thereby resulting in collective dissemination, while poorly differentiated pancreatic ductal adenocarcinoma undergoes complete EMT, in which cancer cells undergo single-cell dissemination [23]. Considering the observation that tumor cell clusters display enhanced metastatic potential compared to single cells, it is speculated that tumors exhibiting partial EMT phenotype might exhibit an increased metastatic rate compared to tumors with a complete EMT phenotype. As AsPC-1 is a highly to moderately differentiated pancreatic ductal adenocarcinoma while PANC-1 is a moderately to poorly differentiated pancreatic ductal adenocarcinoma [24], AsPC-1 may cause partial EMT while PANC-1 causes complete EMT in this study. It will be interesting to see if there is a difference in the EMT of AsPC-1 and PANC-1 cells and if this affects how they metastasize.
In the present study, we used a homogeneous human pancreatic cancer cell line. However, since cancer tissues are composed of heterogeneous cell populations, the induction of EMT by exosomes secreted by cells with different characteristics will require a further investigation. To develop novel therapeutic agents to inhibit metastasis, we would like to propose two strategies. The first involves the use of inhibitors for TGF-β or EGF receptor, which are signaling molecules of EMT and have already been studied as metastasis inhibitors [25]. Second, since exosomes secreted by cancer cells circulate from tumor vessels to normal blood vessels in the bloodstream, the inhibition of exosomes secretion by antibodies targeting CD9 and CD63 in the outer membrane of exosomes might suppress metastasis [26].
If therapies based on these strategies are developed in the future, distant metastasis of pancreatic cancer will likely be suppressed, and the prognosis will be improved.

**Conclusion**

The development of metastasis inhibitors in the early stage of pancreatic cancer, which has an extremely poor prognosis, is an urgent issue. Although signaling molecules have been reported to induce EMT in the early stage of cancer metastasis, the mechanism by which they are supplied remains unclear. In this study, we demonstrated that pancreatic cancer cell-derived exosomes induce EMT in human pancreatic cancer cells themselves, partially via TGF-β1.

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**Declarations**

**Conflict of interest** The authors have no conflicts of interest to declare.

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