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Kimoto, Ai ; Kadoi, Yusuke ; Tsuruda, Taisei ; Kim, Yong-Sik ; Miyoshi, Makoto ; Nomoto, Yuna ; Nakata, Yuna ; Miyake, Mutsumi ; Miyashita,…

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Exosomes in ascites from patients with human pancreatic cancer enhance remote metastasis partially through endothelial-mesenchymal transition

Ai Kimoto [a], Yusuke Kadoi [a], Taisei Tsuruda [a], Yong-Sik Kim [b], Makoto Miyoshi [a], Yuna Nomoto [a], Yuna Nakata [a], Mutsumi Miyake [a], Kumiko Miyashita [a], Kazuya Shimizu [a, b], Tetsuo Ajiki [d], Yuichi Hori [a, *]

[a] Department of Biophysics, Kobe University Graduate School of Health Sciences,

[b] Kobe Ohyama Hospital, Kobe, Japan

[c] Department of Internal Medicine, Kobe Medical Center, Kobe, Japan

[d] International Clinical Cancer Research Center, Kobe University School of Medicine, Kobe, Japan

[†] AK, YK and TT contributed equally to this work.

Running title: Exosomes accelerate distant metastasis in pancreatic cancer

*Corresponding author: Department of Biophysics, Kobe University Graduate School of Health Sciences, Tomogaoka 7-10-2, Suma-ku, Kobe, 654-0142, Japan.

Phone & Fax: +81-78-796-4540, E-mail address: horiy@people.kobe-u.ac.jp (Y. Hori).

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ABSTRACT

Background: Despite advances in multidisciplinary treatment, the prognosis of pancreatic cancer remains poor. Since distant metastasis defines prognosis, elucidation of the mechanism of metastasis is important for improving survival. Exosomes are extracellular secretory vesicles and are responsible for intercellular communication. In this study, we investigated whether exosomes secreted by human pancreatic cancer cells are involved in promoting distant metastasis of cancer and the mechanism that underlies the promotion of metastasis.

Methods: Exosomes were isolated from ascites of a patient with pancreatic cancer and a patient with liver cirrhosis as a control. Three days after the administration of exosomes to nude mice, GFP-labeled human pancreatic cancer cells were injected via the spleen or tail vein, and then the liver and lungs were histologically analyzed. To elucidate the mechanism, vascular permeability was estimated using FITC-dextran in place of pancreatic cancer cells *in vivo* and human umbilical vascular endothelial cells (HUVECs) were used to analyze vascular permeability and the induction of endothelial-mesenchymal transition (EndMT) *in vitro*.

Results: Distant metastasis and vascular permeability were significantly enhanced in mice treated with exosomes from pancreatic cancer patients in comparison to exosomes from a control patient *in vivo*. In addition, exosomes from pancreatic cancer patients significantly enhanced vascular permeability and the induction of EndMT in HUVECs *in vitro*.

Conclusion: Exosomes derived from pancreatic cancer cells form a pre-metastatic niche and promote the extravasation and colonization of pancreatic cancer cells to remote organs, partially through endothelial-mesenchymal transition.

1. Introduction

Pancreatic cancer, or pancreatic ductal adenocarcinoma (PDAC), is one of the most aggressive human malignancies, and it is predicted to be the second leading cause of cancer-related death in the United States by 2030 [1, 2]. At the time of discovery, pancreatic cancer has often already progressed, which makes surgical treatment difficult. Pancreatic cancer often causes liver and lung metastasis, and the patient's prognosis depends on the presence or absence of metastasis [3]. However, there are many unclear points concerning the mechanism underlying the development of metastasis, and the control of distant metastasis is an urgent issue for improving prognosis.

In recent years, extracellular nanovesicles, called exosomes, which are secreted from all cells, have been reported to play an important role in cell-cell communication by transmitting the nucleic acids, proteins, and microRNA that they contain [4]. In cancer metastasis research, a paradigm shift has occurred since the role of exosomes in organotropic metastasis was first reported [5]. This group also reported that, after exosomes derived from cancer cells form a metastatic niche, organ-directed cancer metastasis is formed in pancreatic cancer [6].

Peritoneal dissemination and resultant malignant ascites are the most common routes of metastasis from cancer cells, including pancreatic cancer, ovarian cancer, and gastric cancer [7]. We therefore assumed that the ascites of human pancreatic cancer patients would contain abundant exosomes

derived from pancreatic cancer cells in comparison to other samples (e.g., blood or culture supernatant) since pancreatic cancer cells metastasized from primary tumors are present in ascites.

In the present study, we utilized exosomes in ascites collected for therapeutic purposes [8] from patients with human pancreatic cancer and focused on the role of exosomes during the steps of extravasation and colonization of metastasis.

2. Material and methods

Male nude mice of approximately 8 weeks of age (BALB/cAJc1-nu/nu) (CLEA, Tokyo, Japan) were used. All mice were used under approved protocols in accordance with the Kobe University guidelines for the care and use of laboratory animals (permission No: A120905). This study was performed according to the Institutional Review Board-approved guidelines of Kobe Medical Center and Kobe University Graduate School of Health Sciences and we obtained approval from Ethics Committees of Kobe University Graduate School of Health Sciences (Approval No. 152). Written informed consent was obtained from all patients. We used five mice for each *in vivo* experiment and performed three independent experiments for each *in vitro* experiment.

2.1. Cell culture

Human pancreatic cancer cells (KMC34 and KMC26) were isolated and established as described previously [9, 10]. KMC cells were cultured in serum-free Stem medium (DS Pharma Biomedical, Osaka, Japan) containing 0.1 μM 2-mercaptoethanol, 50 U/ml of penicillin and 50 μg/ml of streptomycin (Invitrogen, Carlsbad, CA). The cells were cultured on the confluent PA6 stromal cells (a gift from Dr. Nishikawa [RIKEN, Kobe, Japan]) at 37 °C in a humidified atmosphere containing 5% CO_2. Human umbilical vein endothelial cells (HUVECs) were cultured in EGM BulletKit medium (Lonza, Basel) supplemented with 10% FBS with exosomes removed by ultracentrifugation in a humidified environment of 5% CO_2.

2.2. Lentiviral vector and lentiviral-mediated gene transfer

Enhanced green fluorescent protein (EGFP) gene transfer was conducted as described previously [11]. cDNA for EGFP was amplified by PCR using pCX4ble-EGFP as a template and substituted with puroR (puromycin resistance gene) of the pLKO.1-puro Empty Vector to make a pLKO-EGFP plasmid. Lentiviral-mediated gene transfer was carried out using the ViraPower Lentiviral Packaging Mix (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's directions. Briefly, pLKO-EGFP was cotransfected with the packaging mix into 293FT cells (Thermo Fisher Scientific, Waltham, MA), 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 KMC34 cells. KMC-GFP positive cells were sorted and established by flow cytometry.

2.3. Isolation of exosomes from ascites with human pancreatic cancer (PDAC-1 and PDAC-2) or liver cirrhosis (LC)

The background of each patient is shown in Supplemental Online Table 1. Exosomes were prepared as described previously [12]. In brief, 50 ml of ascites from patients (PDAC-1, PDAC-2, and LC) was centrifuged at 2,000 ×g for 10 min at room temperature. Then, the cell-free supernatant was filtered through a 0.22-μm filter and recentrifuged for 70 min at 100,000 ×g (Beckman Coulter Optima L-70K) at 4 °C to collect the released exosomes and resuspended in phosphate-buffered saline (PBS). The suspension was recentrifuged for 90 min at 100,000 ×g and resuspended in PBS. The presence of exosomes was confirmed using a nanoparticle tracking system (Malvern Panalytical). We stored exosomes at -80 °C for up to 2 weeks before use.

2.4. Liver and lung metastasis studies

KMC-GFP cells were separated from mouse PA6 cells expressing mouse PDGFRβ using a Magnet Activated Cell Sort Separator (Miltenyi Biotech) with a biotin-conjugated anti-mouse PDGFRβ monoclonal antibody (eBioscience, San Diego, CA) and antibiotin Microbeads (Miltenyi Biotech). Male

nude mice (age: 6-8 weeks) were injected with exosomes (5×10^{10} particles in 100 μL of PBS) via the spleen or tail vein. Then, 3 days later, they were injected with 5×10^{5} KMC-GFP cells in 0.1 ml of low glucose DMEM via the same site. Fourteen days later, mice were euthanized and their liver and lungs were removed after whole-body perfusion with 10 ml PBS to remove cancer cells in the vessels, blood and blood cells and analyzed for metastatic lesions by staining and counting GFP^+ cells by light microscopy (OLYMPUS BX53) or fluorescence microscopy (ZEISS Axio Vert.A1). As a control for PDAC exosomes, we used PBS or exosomes derived from a patient with liver cirrhosis (LC).

2.5. Vascular permeability assay and the induction of endothelial-mesenchymal transition (EndMT) by exosomes

In the *in vivo* study, exosomes or control liposomes (5×10^{10} particles in 100 μL of PBS) were injected via the spleen or tail vein. Three days later, 100 mg/kg fluorescein isothiocyanate (FITC)-dextran (average MW ~70,000; Sigma) was administered via injection into the spleen or tail vein. Then, 3 hours later, the liver and lungs were resected and analyzed by immunofluorescence staining as described below. In the *in vitro* study, the permeability of HUVEC monolayers grown on transwell filters (0.4 μm pore size; BD Biosciences) was assessed by the passage of FITC-dextran. Briefly, exosomes or control empty liposomes (5×10^{10} particles in 100 μL of PBS) were added to the top well. Then, 3 hours later, 1 mg/ml FITC-dextran was added to the top well, and the appearance of fluorescence in the bottom well was

measured by an Infinite® F200 microplate reader (TECAN) at 485 nm excitation and 535 nm emission. For the induction of EndMT, 1.0×10^4 HUVECs were seeded in 48-well plates until reaching confluence. Then, exosomes from PDAC-1or 2 patient ascites suspended in PBS or PBS alone as a control were administered. Twenty-four hours later, the cells were fixed in 4% paraformaldehyde for 10 min. The primary antibodies were anti-human VE-cadherin antibody (Abcam, Cambridge, MA, USA) diluted to 1:400 and anti-human Vimentin antibody (BioLegend, San Diego, CA, USA) diluted to 1:400 and reacted for 1 hour at room temperature. The other methods were the same as described below.

2.6. PKH-labeled exosome uptake by HUVECs

Nanoparticles were labeled using a PKH26 Fluorescent Cell Linker Kit (Sigma, Missouri, USA). Exosomes from PDAC or LC were suspended in 50 µL of PBS, and 50 µL of diluent C with 0.2 µL of PKH26 was added. Then the exosomes were incubated for 5 min at room temperature. After centrifugation for 70 min at 10,000 ×g at 4 ºC, labeled exosomes were resuspended in PBS. HUVECs were treated with PKH26-labeled exosomes for 6 h. After washing with PBS, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature.

2.7. Immunohistochemistry

Immunohistochemical staining was performed as previously described [9]. Immunostaining to count GFP^+ cells in human tissues was performed on 4-μm sections from formalin-fixed, paraffin-embedded tissues placed on coated glass slides and dried at room temperature. Sections were dewaxed in xylene and rehydrated. To block peroxidase, the samples were treated with 3% H_2O_2 in methanol for 10 min. The samples were immersed in Tris-buffered saline-Tween 20 (TBST) (25 mM Tris-HCl [pH 7.4], 75 mM NaCl, and 0.1% Tween20) for 5 min and then incubated with a rabbit anti-GFP polyclonal antibody (catalog number 600-401-215, Rockland) diluted to 1:1000 or a mouse anti-CA19-9 antibody (catalog number SIG-3616-26, Covance) in 10％ FBS/TBST at 4 °C overnight. The samples were rinsed 3 times with TBST. Primary antibody detection was performed with rabbit horseradish peroxidase (Histofine® Simple Stain MAX-PO Kit, Rabbit, Nichirei) at room temperature for 60 min, followed by three rinses with TBST. The signal was developed with diaminobenzidine (Dako) for 3 min. The samples were rinsed with distilled water, counterstained with hematoxylin for 1 min, and dehydrated in alcohol solution and xylene.

2.8. Tumorigenicity test

To evaluate the tumorigenicity of cells in ascites from PDAC patients or LC patients, cells were subcutaneously transplanted into immunocompromised mice. Fourteen days later, the tumor was resected and stained.

2.9. Western blotting

HUVECs were seeded on a 96-well plate and cultured to confluence. The cells were collected 48 h after the addition of exosomes and crushed by ultrasonic waves to recover protein. After developing by SDS-PAGE, the proteins were transferred to a nitrocellulose membrane using an iBlot Gel Transfer Device (Life Technologies), and Western blotting was performed with anti-vimentin (BioLegend, San Diego, CA, USA), anti-VE-Cadherin (Abcam, Cambridge, MA, USA), anti-β-actin (Sigma-Aldrich), and anti-Alix (Santa Cruz Biotechnology, Dallas, TX, USA) antibodies. 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 the expression was quantified with Optima Shot CL-420α (Wako Pure Chemical Industries, Osaka, Japan).

2.10. Statistical analysis

Continuous variables were expressed as the mean ± standard error (SE). Statistically significant differences were determined by Student's *t* test. Significance was defined as * $p < 0.05$ and ** $p < 0.001$.

3. Results

*3.1. Pancreatic cancer ascites-derived exosomes administered via injection into the spleen enhanced liver metastasis of human pancreatic cancer cell*s

Schematic illustrations of the *in vivo* experiments are shown in Fig. 1A and 1B. The presence of exosomes in the ascites of a patient with pancreatic cancer (PDAC) or LC was confirmed by a nanoparticle tracking system (Fig. 1C). In addition, the amounts of exosomes between PDAC and LC were comparable. The administration of vehicle (PBS) (Group 1) or exosomes from LC patient (Group 2), followed by GFP-labeled human pancreatic cancer cells (GFP-KMC34), established previously [9, 10] injection into the spleen was not associated with the development of metastatic lesions in the liver (Fig. 2A and 2B, respectively). On the other hand, the administration of exosomes from PDAC-1 (Fig. 2C) and PDAC-2 (Fig. 2D) significantly accelerated liver metastasis (Fig. 2E). We did not detect any metastatic lesions in the mouse lung in any of the models (data not shown). Collected cells in ascites from PDAC patients had a tumorigenicity in immunocompromised mice, while the cells from ascites from LC patient did not (Fig. 2F). The tumor from cells in ascites from PDAC patients highly expressed CA19-9, a marker of pancreatic cancer, and showed the phenotype of ductal adenocarcinoma (Fig. 2G, H). The phenomenon observed with GFP-KMC34 cells was also confirmed in experiments using GFP-KMC26 cells.

3.2. Pancreatic cancer ascites-derived exosomes administered via injection into the spleen increased vascular permeability in the liver

To evaluate whether human pancreatic cancer ascites-derived exosomes administered via injection into the spleen have effects on vascular permeability in the liver, we injected exosomes (see Figure 1) followed by FITC-dextran (Fig. 3A). In this study, we injected PBS (Group 1) or control liposomes (Group 2) as controls. As a result, we found severe leakage of FITC-dextran in exosomes derived from ascites of PDAC patients in comparison to other groups (Fig. 3B), suggesting that the administration of pancreatic cancer ascites-derived exosomes via injection into the spleen increased vascular permeability in the liver. We did not detect increased permeability in the mouse lung in any of the models (data not shown).

*3.3. Pancreatic cancer ascites-derived exosomes administered via injection into the tail vein enhanced lung metastasis of human pancreatic cancer cell*s

Human pancreatic cancer ascites-derived exosomes administered via injection into the spleen, followed by the injection of GFP-labeled human pancreatic cancer cells via the same site, were associated with significantly accelerated liver metastasis but not lung metastasis, which is the second most common

site of pancreatic cancer metastasis in human patients. We therefore assumed that different organs metastasize depending on the site of exosome injection and that the administration of exosomes via injection into the tail vein would be suitable to reach the lungs without going through the liver. Schematic illustrations of the *in vivo* experiment are shown in Fig. 4A and 4B. As a result, human pancreatic cancer ascites-derived exosomes administered via injection into the tail vein, followed by GFP-labeled human pancreatic cancer cells administered via injection into the tail vein significantly promoted lung metastasis (Fig. 4C). We did not detect any metastatic lesions in the mouse liver in any of the models (data not shown).

3.4. Pancreatic cancer ascites-derived exosomes administered via injection into the tail vein increased vascular permeability in the lung

To evaluate whether human pancreatic cancer ascites-derived exosomes administered via injection into the tail vein increase vascular permeability in the lung, we injected exosomes as shown in Figure 3 and subsequently injected FITC-dextran (Fig. 5A). As a result, we also found severe leakage of FITC-dextran in exosomes derived from ascites of a patient with PDAC in the lung in comparison to other groups (Fig. 5B), suggesting that the administration of pancreatic cancer ascites-derived exosomes

via injection into the tail vein increased vascular permeability in the lung. This increased permeability was not observed in the liver (data not shown).

3.5. Pancreatic cancer ascites-derived exosomes induced ZO-1 protein reduction and endothelial-mesenchymal transition in HUVECs

First, PKH26 was used to confirm that exosomes were incorporated into HUVECs. The incorporation of PKH26-labeled exosomes from PDAC patients into HUVECs was significantly enhanced in comparison to that of PKH26 labeled exosomes from LC patient (Fig. 6A). Next, transwell permeability experiments were conducted to examine the effect of exosomes on the vascular permeability of HUVECs (Fig. 6B). Pancreatic cancer ascites-derived exosomes (PDAC-1 and PDAC-2) significantly increased the level of FITC-dextran passing through HUVECs to the lower part of the chamber in comparison to exosomes in control PBS or exosomes from LC patient (Fig. 6C, 6D). These results were concordant with the suppression of ZO-1 protein (a tight junction marker) expression by the administration of exosomes from PDAC patients (Fig. 6E).

Next, we focused on EndMT a process in which endothelial markers (e.g., VE-cadherin and CD31) decrease and mesenchymal markers (e.g., vimentin) increase. We hypothesized that the increased vascular permeability induced by exosomes derived from ascites is due to EndMT. Immunocytochemistry

showed that VE-cadherin levels were decreased in HUVECs treated with exosomes derived from LC, PDAC-1or PDAC-2 patients compared to HUVECs treated with control PBS (Fig. 7A-D). These findings were also confirmed by Western blotting (Fig. 7E). Surprisingly, regarding the role of exosomes involved in EndMT induction on HUVECs *in vitro*, LC ascites-derived exosomes (LC) showed a phenomenon similar to that observed for pancreatic cancer ascites-derived exosomes (PDAC-1 and PDAC-2). While the reason for this finding is unclear based on the experimental results obtained thus far, this finding may be due to the fact that the molecules in the exosomes involved in metastasis are different from those in the exosomes that induce EndMT.

3.6. Pancreatic cancer ascites-derived exosomes did not show any obvious differences between the groups as in HUVECs, namely regarding ZO-1 protein reduction and the induction of endothelial-mesenchymal transition in vivo

Immunohistochemical analyses *in vivo* for VE-cadherin, ZO-1, and vimentin were performed to confirm the results that were observed in HUVEC cells. Unfortunately, the expressions for VE-cadherin, ZO-1, and vimentin showed no clear difference between the groups. In other words, there was no decrease in Zo-1 expression and the induction of EndMT by pancreatic cancer ascites-derived

exosomes in the liver (Fig.8). Similarly, no obvious differences were observed in the lung as well (not shown).

4. Discussion

Elucidation of the mechanism underlying distant metastasis is important for improving the prognosis of patients with pancreatic cancer. Many processes are involved in the formation of cancer metastasis. Distant metastasis consists of several steps including angiogenesis, intravasation of tumor cells, extravasation of tumor cells distant organs, and colonization [13]. In recent years, many studies have identified the roles of exosomes in cancer metastasis [14-16]. Exosomes have also been reported to play a role in pancreatic cancer metastasis [17-19]. In the present study, we wanted to examine the roles of exosomes from human pancreatic cancer patients and focus on extravascular leakage and colonization of pancreatic cancer cells in distant organs in the process of metastasis. The injection of exosomes followed by the injection of cancer cells into the spleen and tail vein was used to induce liver and lung metastasis, respectively. In addition to cancer cells, various cells, including omental adipose cells, are present in ascites from patients with pancreatic cancer. Qu et al. recently reported that exosomes from omental adipose-derived mesenchymal stem cells in ascites also promote peritoneal metastasis [20]. Since experiments using exosomes from ascites of LC patients instead of exosomes from ascites of pancreatic

cancer patients showed no evidence of distant metastasis or increased vascular permeability, we hypothesized that exosomes derived from pancreatic cancer cells in ascites are primarily responsible for the promotion of distant metastasis and increased vascular permeability.

The majority of exosomes injected into the spleen that reach the liver via the portal vein formed premetastatic niches in the liver, causing increased vascular permeability and subsequent colonization of pancreatic cancer cells. On the other hand, cancer cells reaching the lungs via the hepatic vein through the liver did not show this phenomenon, suggesting that exosomes from this pathway may have some affinity to the liver where they first arrive. To confirm this hypothesis, exosomes and subsequent cancer cells were administered by injection into the tail vein instead of through the liver by injection from the spleen. Interestingly, this time only the lungs showed vascular permeability and subsequent colonization, which was confirmed by the absence of this phenomenon in the liver. It remains to be elucidated in detail why exosomes administered by injection into the spleen were associated with liver metastasis, which is the first site of delivery, but no vascular permeability or lung metastasis, and why exosomes administered by injection into the tail vein are associated with increased lung metastasis but not liver metastasis. Costa-Silva et al. previously reported that pancreatic cancer cells transfer macrophage migration inhibitory factors into Kupffer cells in the liver through secreted exosomes and activate extracellular matrix remodeling [6]. The same process might be induced by alveolar macrophages by exosomes administered by injection into the tail vein; however, future studies are needed to validate

this hypothesis. Another possibility is that the increased uptake of exosomes by vascular endothelial cells in the first organs reached may induce vascular permeability through EndMT, causing enhanced metastasis. Nevertheless, from a drug delivery system perspective, we believe that exosomes can be an effective tool for the organ-specific delivery of inhibitors.

The extravasation of cancer cells normally occurs in blood vessels, and then cancer cells attach to endothelial cells and pass the endothelial barrier. Interaction between cancer cells-derived exosomes and the capillary wall was found to cause vascular permeability [21]. Similarly, breast cancer cell-derived exosomes carrying miR-105 can induce vascular permeability by breaking tight junctions, and the tight junction protein ZO-1 has been shown to be the key target of exosomal miR-105 [22]. Yokota et al. found that exosomal miR-638 is a prognostic marker of HCC via the downregulation of VE-cadherin and ZO-1 in endothelial cells [23]. In the present study, we also found that the expression of ZO-1 protein in HUVECs was suppressed by pancreatic cancer-derived exosomes.

Epithelial-to-mesenchymal transition (EMT) is a critical process in the initiation phase of metastasis. Very recently, we demonstrated that pancreatic cancer cell-derived exosomes induce EMT in human pancreatic cancer cells themselves, partially via TGF-β1 [12]. This finding might be involved in the early stages of cancer metastasis before the abundant stromal formation, which is a characteristic of pancreatic cancer, occurs. In addition to EMT, the EndMT is a process that promotes loss of intercellular adhesion, angiogenesis, and tumor migration out of the endothelium [24-26]. For pancreatic cancer,

EndMT has also been reported to be involved in vascular permeability [27, 28]. However, there are few reports on the role of pancreatic cancer-derived exosomes in EndMT. We therefore hypothesized that the induction of EndMT by exosomes causes vascular permeability. The present study is associated with some limitations. First, the process through which pancreatic cancer cells migrate in the vascular endothelial cell junctions cannot be accurately understood; thus, it is necessary to perform live imaging. Second, at the current time we have no definite data to support the specific molecules on the exosomes that were involved in these mechanisms.

The elucidation of the mechanism by which exosomes lead to EndMT of vascular endothelial cells may lead to drug discovery research on the suppression of metastasis by selectively inhibiting exosomes from pancreatic cancer cells, which could improve the prognosis of patients with pancreatic cancer. The idea for the present study came to us because we thought it would be a good model to investigate the roles in the mechanisms underlying extravasation, vascular permeability, and colonization. We would like to further study the molecular mechanism through which exosomes promote EndMT and tumor angiogenesis in metastatic organs.

Conclusions:

Exosomes derived from pancreatic cancer cells form a premetastatic niche and promote the extravasation and colonization of pancreatic cancer cells to remote organs, partially through EndMT.

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Declaration of competing interest

The authors declare no conflicts of interest in association with the present study.

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Figure legends

Fig. 1. Exosomes in ascites from patients with human pancreatic cancer promoted remote metastasis. (A, B) A schematic illustration of the *in vivo* experiment. We injected exosomes into the spleens of immunocompromised mice. Then, after three days, we administered human GFP-labeled PDAC cells (GFP-KMC34), established in our lab, via injection into the spleen. After 2 weeks, the liver and lungs were extracted. In this study, we used exosomes in ascites from patients with human pancreatic cancer (PDAC-1, PDAC-2) or a patient with liver cirrhosis (LC) as a control. The presence of exosomes was confirmed using a nanoparticle tracking system (C).

Fig. 2. The administration of exosomes in ascites from patients with human pancreatic cancer via injection into the spleen promoted liver metastasis. (A, B) The administration of vehicle (PBS) or exosomes from a patient with liver cirrhosis (LC) via injection into the spleen did not induce metastasis in the mouse liver. (C-E) On the other hand, exosomes from PDAC-1 and PDAC-2 cells significantly accelerated liver metastasis. (n = 5; mean ± SEM; ** $P < 0.01$) We enlarged the tissue and consider liver tubeculae as an insert. However, we could not detect lung metastasis in any of the groups. (F) Cells in ascites from PDAC-1 patients, but not cells in ascites from LC patient, showed tumorigenicity. (G, H) Tumor tissue from cells in ascites from PDAC-1 patients in (F) highly expressed CA19-9, a marker of pancreatic cancer, and showed the histological phenotype of ductal adenocarcinoma. Scale bar = 100 μm.

Fig. 3. The administration of exosomes in ascites from patients with human pancreatic cancer via injection into the spleen increased vascular permeability in the liver. (A) A schematic illustration of the *in vivo* experiment. We injected exosomes into the spleens of immunocompromised mice as shown in Figure 1. After three days, we subsequently administered FITC-dextran via injection into the spleen. Then, after 3 hours, the liver and lungs were extracted. In this study, we utilized exosomes in ascites from PDAC patients, and liposomes or exosomes in ascites from a patient with LC as a control. (B) The administration of exosomes from PDAC patients via injection into the spleen significantly increased vascular permeability in the liver. Scale bar = 100 μm.

Fig. 4. The administration of exosomes in ascites from patients with human pancreatic cancer via injection into the tail vein promoted lung metastasis. (A, B) Instead of injection into the spleen (as seen in Figure 1), we injected exosomes into the tail vein of immunocompromised mice. (C) Exosomes from PDAC-1 and PDAC-2 significantly accelerated lung metastasis, while PBS (not shown) and exosomes from a patient with liver cirrhosis (LC) injected into the spleen did not induce metastasis in the lungs (n = 5; mean ± SEM; ** $P < 0.01$) However, we could not detect liver metastasis in any of the groups. Scale bar = 100 μm.

Fig. 5. The administration of exosomes in ascites from patients with human pancreatic cancer via injection into the tail vein increased vascular permeability in the lung. (A) The experimental protocol was the same as Figure 3, except that exosomes and FITC-dextran were injected into the tail vein. (B) The administration of exosomes from PDAC patients via injection into the tail vein significantly increased vascular permeability in the lung. Scale bar = 100 μm. Extravascular deposition of FITC-dextran is indicated by arrows (d).

Fig. 6. Exosomes in ascites from patients with human pancreatic cancer recapitulated permeability in HUVECs *in vitro*. (A) The uptake of PKH26-labeled exosomes from PDAC patients into HUVECs was significantly enhanced in comparison to the uptake of PKH26 labeled exosomes from LC patient. (B-D) In a transwell permeability assay using HUVECs, FITC-dextran permeability was significantly increased by the administration of exosomes from PDAC patients in comparison to PBS or exosomes from LC patient. (E) On the other hand, the expression of ZO-1 protein was suppressed by the administration of exosomes from PDAC patients. The intensity of the ZO-1 band relative to β-actin was calculated using densitometry, and the ratio to the control liposome was tested by statistical analyses (n = 3; mean ± SEM; * $P < 0.05$)

Fig. 7. Induction of the endothelial-mesenchymal transition in HUVECs by exosomes from human patients with pancreatic cancer. (A-D) Immunohistochemistry demonstrated that the expression of VE-cadherin was markedly decreased in exosomes from LC, PDAC-1 and PDAC-2 patients in comparison to control vehicle (PBS). (E) The expression of VE-cadherin protein was significantly decreased, and the expression of vimentin protein was increased in exosomes from LC, PDAC-1 and PDAC-2 patients in

comparison to control vehicle (PBS) on Western blotting (n = 3; mean ± SEM; * $P < 0.05$)

Fig. 8. Induction of endothelial-mesenchymal transition *in vivo* by exosomes from human patients with pancreatic cancer. (A) Immunohistochemical staining of VE-cadherin, ZO-1, and vimentin in the liver did not show any obvious differences among the groups, as *in vitro*.

Supplemental online Table 1 Summary of patients and their clinical characteristics

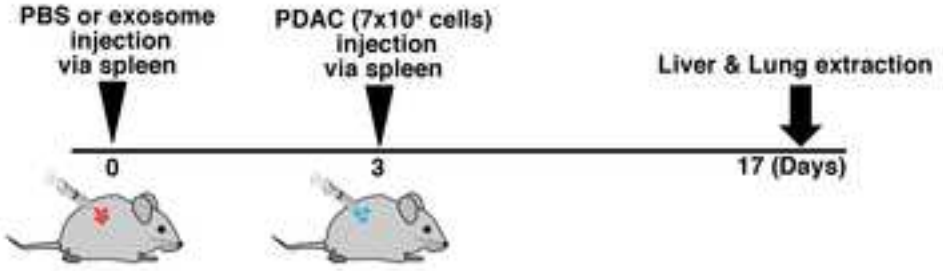
Patient #	Sex	Age	Chemotherapy	Origin of exosome
#1 Liver cirrhosis	F	50	none	Peritoneal effusion
#2 PDAC1	F	80	none	Peritoneal effusion
#3 PDAC2	M	60	FOLFIRINOX, GEM + nab-PTX	Peritoneal effusion

Supplemental online Table 2 Antibodies used in the present study

Antigen	Species	Source	Dilution
Green Fluorescent Protein	Rabbit	Rockland	1:1000
Green Fluorescent Protein	Chicken	Abcam	1:500
Carbohydrate antigen19-9	Mouse	Novus	1:100
Beta-Actin	Mouse	SIGMA	1:5000
Heat Shock Protein70	Rabbit	Santa Cruz	1:1000
Alix	Mouse	Santa Cruz	1:200
VE-cadherin	Rabbit	Abcam	1:400
Vimentin	Mouse	BioLegend	1:400

Figure1

A



B

Group	PBS or exosome	Cancer cells
1	PBS	human PDAC (GFP-KMC34)
2	Exosome in ascites (LC)	human PDAC (GFP-KMC34)
3	Exosome in ascites (PDAC-1)	human PDAC (GFP-KMC34)
4	Exosome in ascites (PDAC-2)	human PDAC (GFP-KMC34)

C

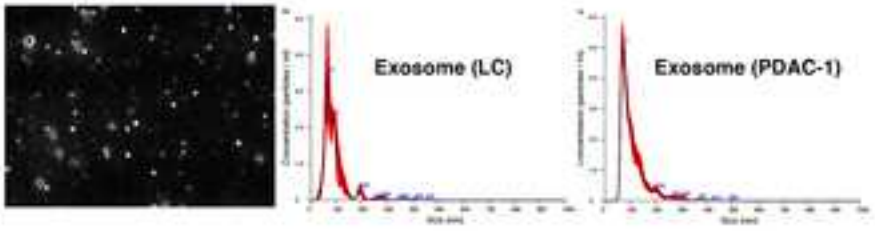
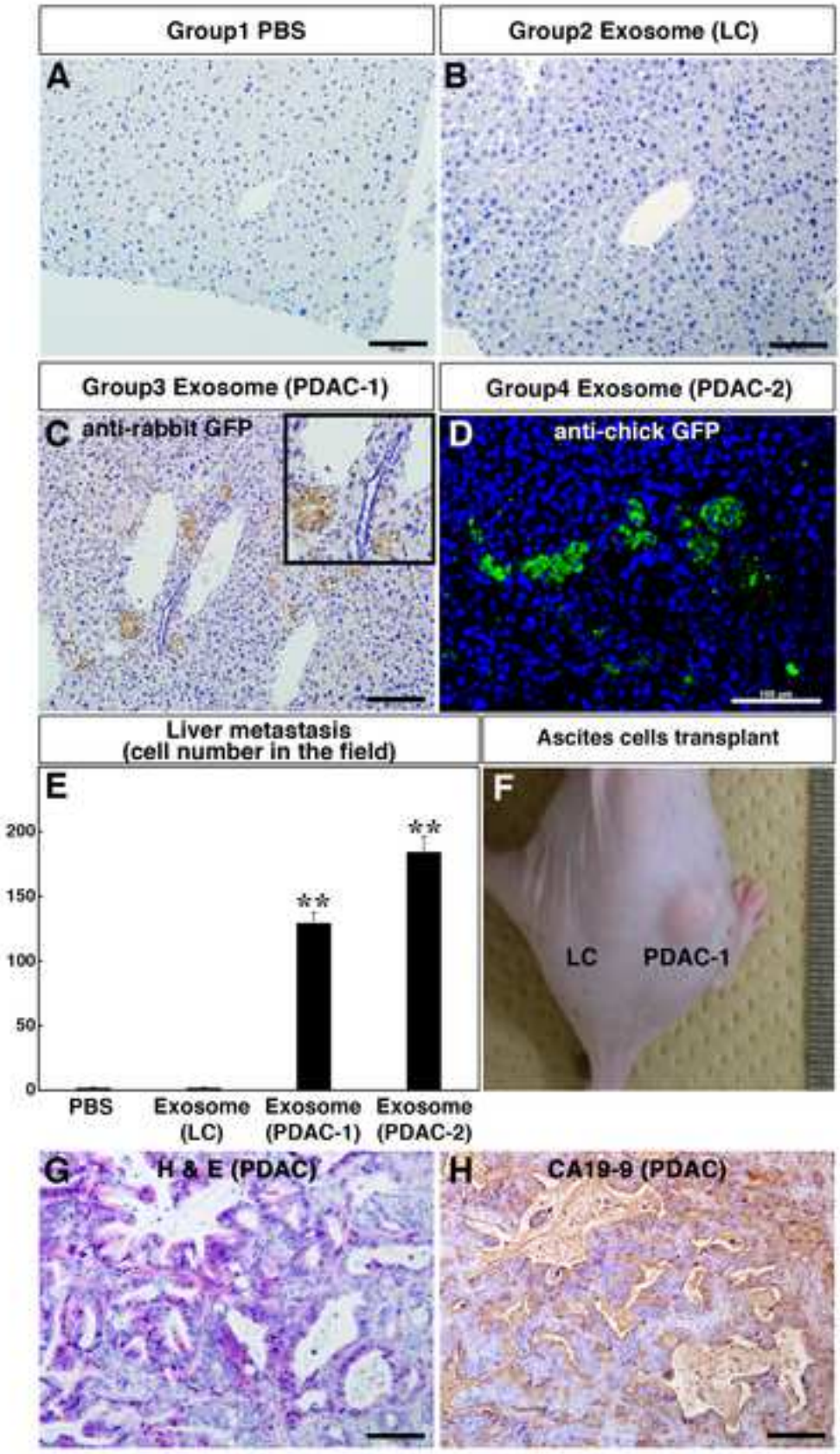


Figure2



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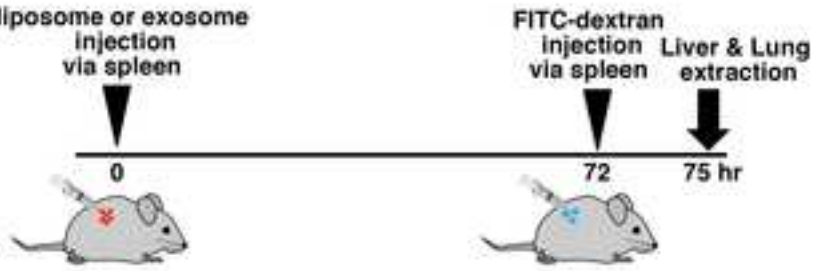
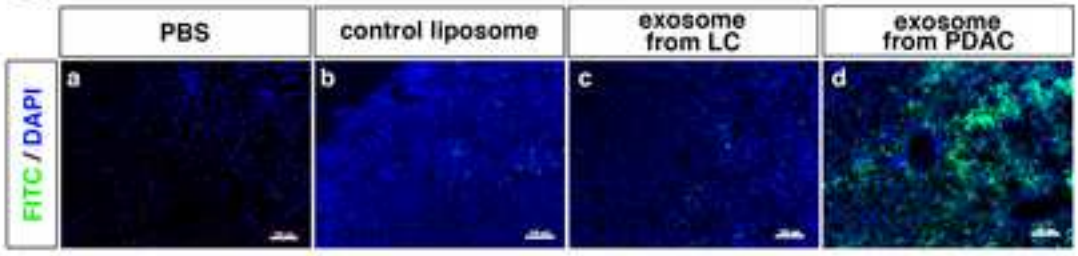
A



group	PBS or liposome or exosome	fluorochrome
1	PBS	FITC-dextran
2	control liposome	FITC-dextran
3	exosome from LC	FITC-dextran
4	exosome from PDAC	FITC-dextran

B



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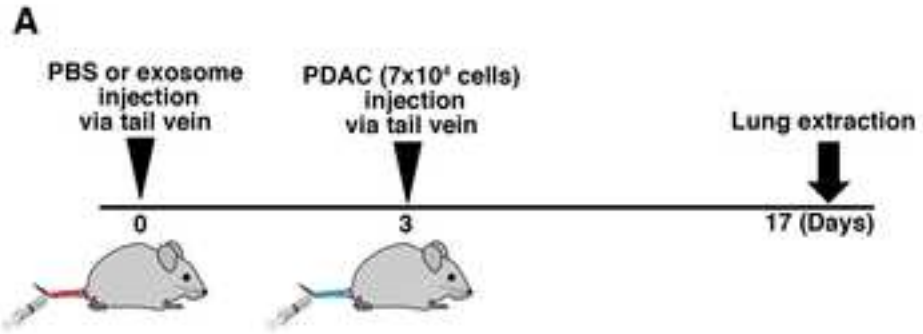


B

Group	PBS or exosome	Cancer cells
1	PBS	human PDAC (GFP-KMC34)
2	Exosome in ascites (LC)	human PDAC (GFP-KMC34)
3	Exosome in ascites (PDAC-1)	human PDAC (GFP-KMC34)
4	Exosome in ascites (PDAC-2)	human PDAC (GFP-KMC34)

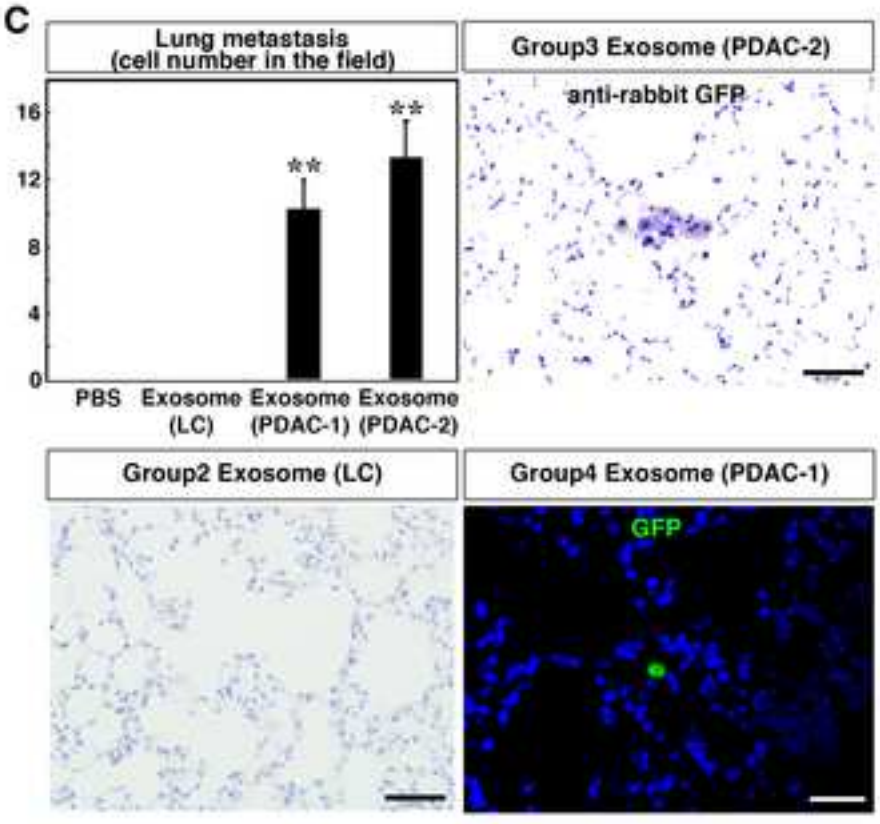
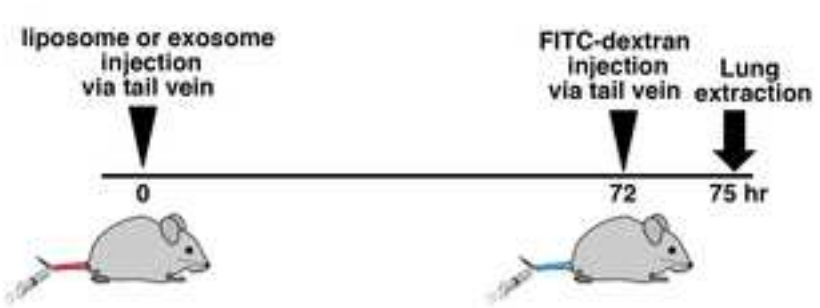


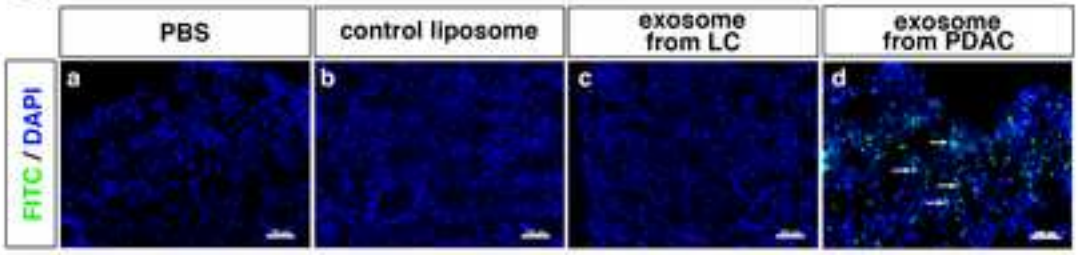
Figure5 Click here to access/download;Figure;Fig5(Kimoto)R1.tif

A



group	PBS or liposome or exosome	fluorochrome
1	PBS	FITC-dextran
2	control liposome	FITC-dextran
3	exosome from LC	FITC-dextran
4	exosome from PDAC	FITC-dextran

B



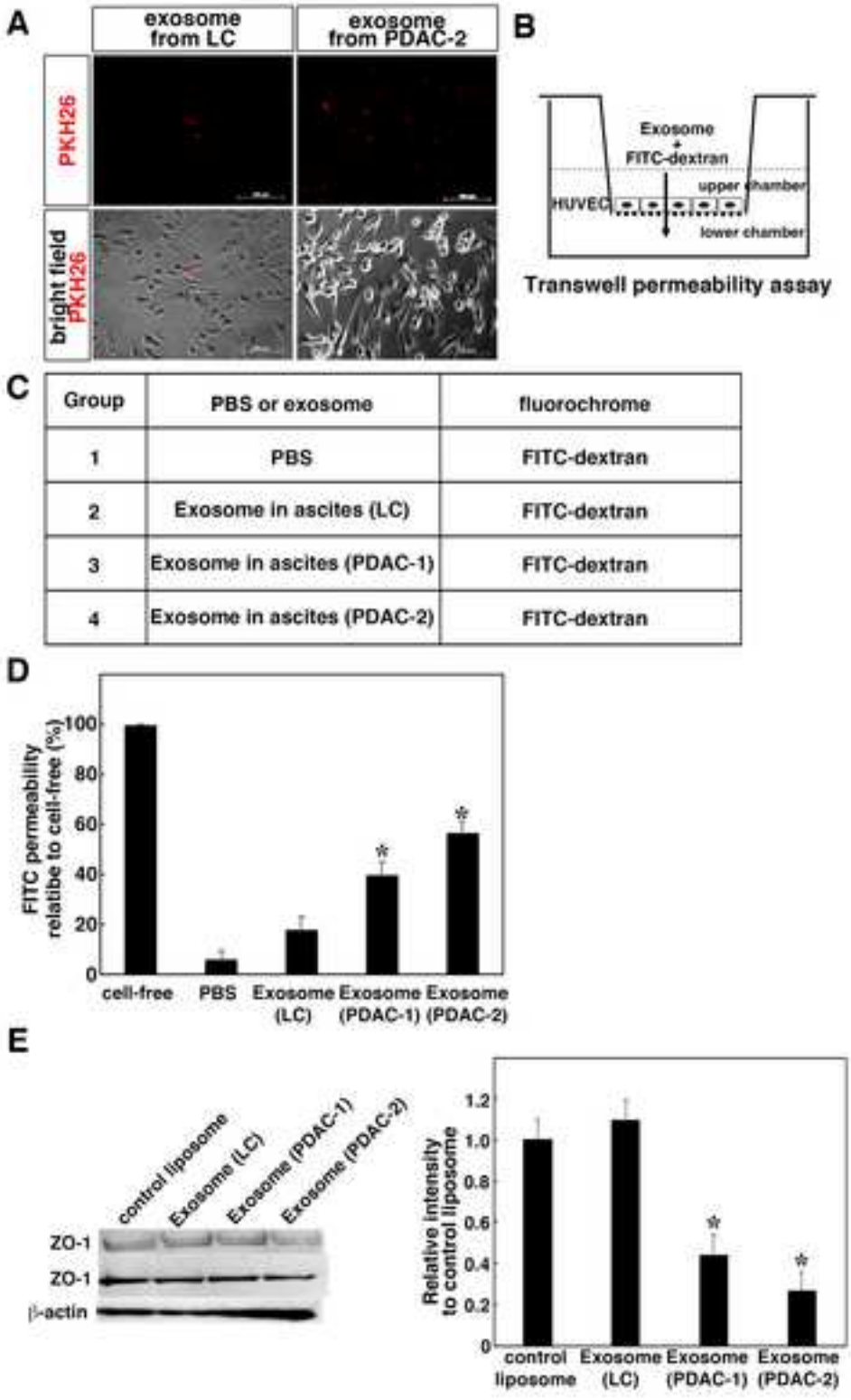


Figure7

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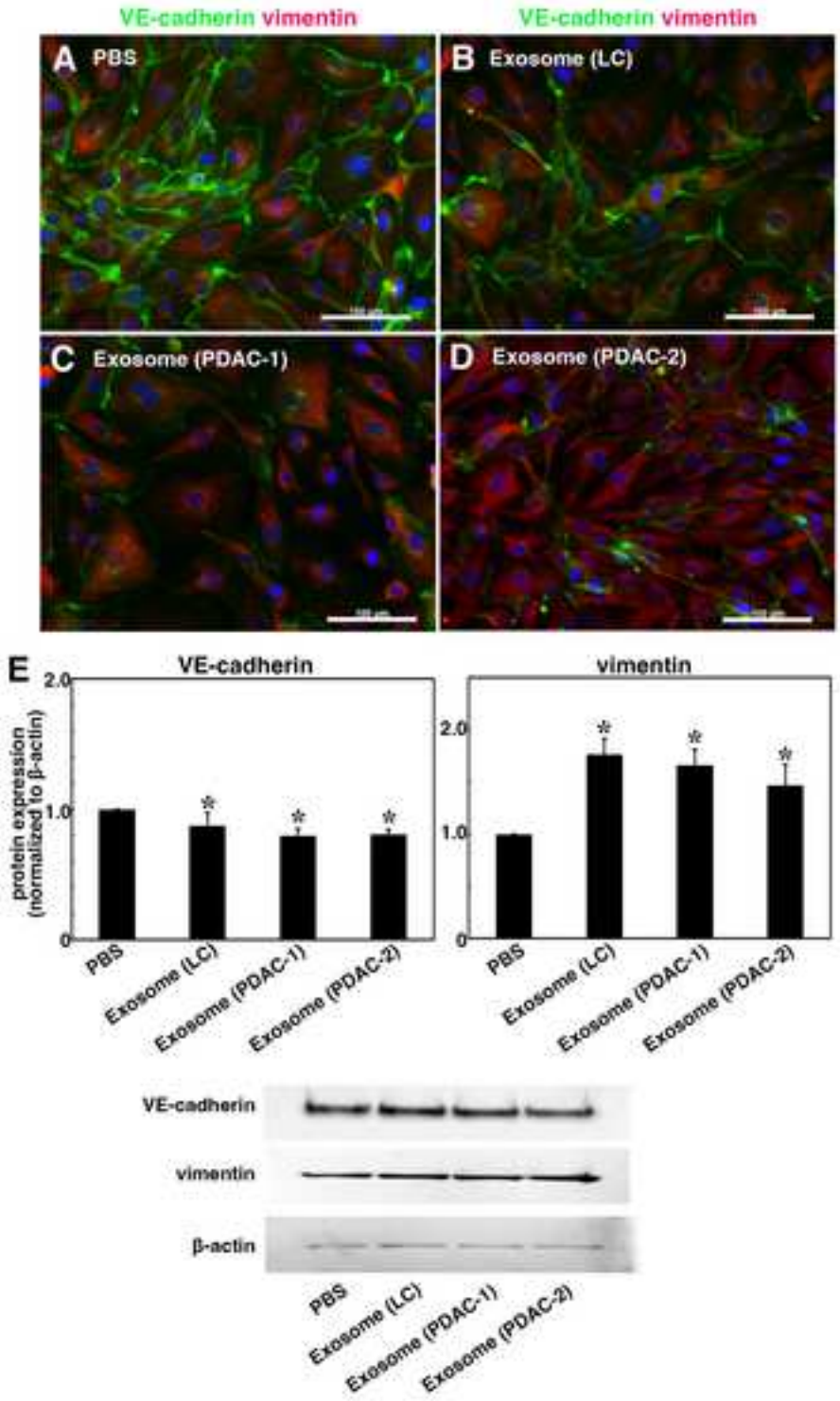

Figure8

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