Exosomes Promote Ovarian Cancer Cell Invasion through Transfer of CD44 to Peritoneal Mesothelial Cells

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Abstract

Epithelial ovarian cancer (EOC) cells metastasize within the peritoneal cavity and directly encounter human peritoneal mesothelial cells (HPMC) as the initial step of metastasis. The contact between ovarian cancer cells and the single layer of mesothelial cells involves direct communications that modulate cancer progression but the mechanisms are unclear. One candidate mediating cell–cell communications is exosomes, 30–100 nm membrane vesicles of endocytic origin, through the cell–cell transfer of proteins, mRNAs, or microRNAs. Therefore, the goal was to mechanistically characterize how EOC-derived exosomes modulate metastasis. Exosomes from ovarian cancer cells were fluorescently labeled and cocultured with HPMCs which internalized the exosomes. Upon exosome uptake, HPMCs underwent a change in cellular morphology to a mesenchymal, spindle phenotype. CD44, a cell surface glycoprotein, was found to be enriched in the cancer cell–derived exosomes, transferred, and internalized to HPMCs, leading to high levels of CD44 in HPMCs. This increased CD44 expression in HPMCs promoted cancer invasion by inducing the HPMCs to secrete MMP9 and by cleaning the mesothelial barrier for improved cancer cell invasion. When CD44 expression was knocked down in cancer cells, exosomes had fewer effects on HPMCs. The inhibition of exosome release from cancer cells blocked CD44 internalization in HPMCs and suppressed ovarian cancer invasion. In ovarian cancer omental metastasis, positive CD44 expression was observed in those mesothelial cells that directly interacted with cancer cells, whereas CD44 expression was negative in the mesothelial cells remote from the invading edge. This study indicates that ovarian–derived exosomes transfer CD44 to HPMCs, facilitating cancer invasion.

Implications: Mechanistic insight from the current study suggests that therapeutic targeting of exosomes may be beneficial in treating ovarian cancer. Mol Cancer Res; 15(1); 78–92. ©2016 AACR.

Introduction

Ovarian cancer, which has a cure rate of only 30%, is generally characterized by widespread peritoneal dissemination and ascites (1). During ovarian cancer dissemination, cells detach from the primary site of origin, which was initially thought to be the ovary but is now believed to be the fallopian tube. Subsequently, the cancer cells float in the peritoneal cavity and attach to secondary sites of implantation, most notably the omentum, which is the most common site of ovarian cancer metastasis (2). All of the organs within the peritoneal cavity are lined with a single layer of mesothelial cells which cover an underlying stroma composed of extracellular matrices (ECM) and stromal cells (3). Once ovarian cancer cells attach to this layer, they invade through the barrier of mesothelial cells into the peritoneum, omentum, or bowel serosa. Therefore, inhibiting the initial interaction of cancer with mesothelial cells could be a means of preventing ovarian cancer progression and metastasis.

Mesothelial cells were first thought to function only as a mechanical barrier. This view was supported by histologic studies of ovarian cancer nodules attached to peritoneal organs that showed an absence of mesothelial cells under the tumor, suggesting that the mesothelial cells had failed as a protective barrier and contributed to successful tumor cell implantation (4–7). Recent reports suggest that mesothelial cells actively participate in establishing an ovarian cancer metastatic niche and that they are reprogrammed by ovarian cancer cells to facilitate tumor growth becoming cancer-associated mesothelial cells (2, 8, 9).

Exosomes are 30- to 100-nm membrane vesicles of endocytic origin, mediating diverse biologic functions, including tumor cell invasion, cell–cell communication, and antigen presentation.
through transfer of proteins, mRNAs, and microRNAs (10). The very recently characterized mechanism of cell–cell communication through exosomes raises the possibility that the transfer of genetic information or proteins via exosomes modulates cellular activities in recipient cells (10).

Given that the role of mesothelial cells during ovarian cancer metastasis is unclear and that exosomes are able to transfer genetic information or proteins via exosomes modulates cellular communication between mesothelial cells and ovarian cancer cells.

Materials and Methods

Materials

GM4869 (#32613), 5-[(N-ethyl-N-isopropyl)-amino]-2-(N-methylcarboxamido)benzimidazole (EIPA; #A3058), PKH67 (MIDI67), and Ponceau-S (P3045-10G) were purchased from Sigma-Aldrich. E-Cadherin antibody (#610181) (BioLegend), PKH67 (MIDI67), and Ponceau-S (P3045-10G) were purchased from Thermo Fisher Scientific. Antibodies against integrin-α2 (sc-10728), cytokeratin-18 (sc-6259), and Oct-3/4 (sc-9018) were purchased from Santa Cruz Biotechnology. Antibodies against β-actin (#4967) and CD44 (156-3C11) were obtained from Cell Signaling Biotechnology. Antibodies against CD63 (#11-343-C025) and CD81 (#11-558-01) were purchased from Cell Signaling Technology, Antibodies against CD44 (sc-10728), cytokeratin-18 (sc-6259), and CD81 (sc-10484) were obtained from Cell Signaling Technology. Antibody against CD63 (EXOAB-CD63A-1) was purchased from SBI. Antibodies against RhoA (ab19523), CD44 (ab32926), and antibody against CD63 (ab34045) were from Abcam. Antibody against CD63 (ELOXAB-CD63A-1) was purchased from SBI. Antibody against CD63 (#11-343-C025) and CD81 (#11-558-C025) was purchased from EXBIO. Antibody against vimentin (1:200), calretinin (1:100), or calretinin (1:100). For human peritoneal mesothelial cell (HPMC) staining, cells were plated on 8-well plates and were fixed for 4% paraformaldehyde. Slides were stained with an antibody against vimentin-18 (1:50), vimentin (1:50), or calretinin (1:100).

Immunohistochemistry

Immunohistochemistry of ovarian cancer paraffin-embedded tissue was performed as previously described (14). The slides were incubated with the primary antibody against CD44 (#156-3C11, 1:200), calretinin (1:200), or MMP9 (1:50). For human peritoneal mesothelial cell (HPMC) staining, cells were plated on 8-well chamber slides and were fixed with 4% paraformaldehyde. Slides were stained with an antibody against vimentin-18 (1:50), vimentin (1:50), or calretinin (1:100).

Immunofluorescent analysis

HPMCs were fixed with 4% paraformaldehyde and stained with anti-CD44 antibody (#156-3C11, 1:200) at room temperature for 1 hour (15). After washing, samples were incubated with Alexa Fluor 633–labeled goat anti-mouse IgG (1:100) and stained with DAPI. The samples were imaged using a compound microscope (Olympus).

Plasmid and siRNA transfection

Overexpression of CD44s in HPMCs was performed by transient transfection using the CD44s pBabe-puro retrovector plasmid (Plasmid#19127, Addgene) at a concentration of 1 μg/mL. Twenty-four hours after transfection, HPMCs were used for experiments. The retrovector pBabePuro gene (Plasmid#15570, Addgene) served as a control. CD44 expression in HeyA8 cells was transiently knocked down using a predesigned siRNA duplex directed against CD44 at a concentration of 200 nmol/L. The sequences of the siRNA duplexes (Japan Bioservice) were as follows: CD44 siRNA #1, 5′-GAAGUGUGCUUACUGACGCAU-3′ (sense) and 5′-UUCAUUUUGUGUGAUGGCAU-3′ (antisense); CD44 siRNA #2, 5′-CGUGUUGCAGCUACGUUUT-3′ (sense) and 5′-GAAGUGUGCUUACUGACGCAU-3′ (antisense). MISSION siRNA Universal Negative Control #1 (SIC001, Sigma) was used as a control.
Exosome isolated from cell culture media of cell lines

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Profiling of cellular and exosomal RNA

Total RNA was extracted using TRIzol. RNA isolated from cells and exosomes were analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.).

Exosome labeling and treatment

For exosome tracking, exosomes were fluorescently labeled using the PKH67 dye according to manufacturer's protocol.

HPMC-coated in vitro invasion assay

HPMCs (2 × 10^4) were plated onto 25-μg Matrigel-coated 24-well FluoroBlok cell culture inserts with an 8-μm pore membrane (351152 Corning Incorporated). Because FluoroBlok membranes almost completely block the passage of light from 490 to 700 nm, only fluorescently labeled cells that have invaded are detected by bottom-reading of fluorescence (16). After HPMCs grew to confluence, 5 × 10^4 fluorescently (CMFDA)-labeled epithelial ovarian cancer (EOC) cells (HeyA8 and TYK-nu) cultured in 0.1% BSA/DMEM were incubated on the monolayer of HPMCs and allowed to invade for 48 hours. Cell culture medium consisting of 10%FBS/DMEM was used as a chemoattractant and was kept in the lower chamber. Cell proliferation and exosomes were analyzed using the Agilent 2100 Bioanalyzer (Applied Biosystems: #4309155). The designed primers in this experiment were as follows: CD44: forward primer: 5’-TATGACAACAGCCT-3, reverse primer: 5’-CAATGCCTTT-3, reverse primer: 5’-TTTGCTCAGCCTTCAG-3, GAPDH: forward primer: 5’-CATCGAAG-TATGACACAGGCGT-3, reverse primer: 5’-AGTCCTTCCACGA-TACCAAAGT-3 (Applied Biosystems). PCR was performed in triplicate, and the relative levels of CD44 and MMP9 expression were calculated using the 2−ΔΔCt method.

Matrigel in vitro invasion assay

CMFDA-labeled EOC cells were directly plated onto Matrigel-coated inserts. When exosomes were used, isolated exosomes in 0.1% BSA/DMEM were added to the Matrigel-coated inserts 24 hours before the treatment with cancer cells.

Cell proliferation assay

EOC cells were plated onto 96-well plates (5 × 10^3 cells/well) in 10%FBS/DMEM for 24 hours and incubated in 0.1% BSA/DMEM with or without exosomes (100 μg/ml) or GW4869 for 48 hours. Primary HPMCs were plated onto 96-well plates (1 × 10^4 cells/well) in 20%FBS/RPMI for 24 hours and incubated in 0.1% BSA/RPMI with or without exosomes (1, 10, 100 μg/ml) for 24 hours. Cell proliferation was evaluated by a modified MTS assay as previously described (15).

Mesothelial clearance assay

Primary human HPMCs (5 × 10^4) were plated on a 24-well plate and incubated until confluent (7). Culture media were switched to 0.1% BSA/DMEM, and 100 μg/ml of isolated exosomes was added. Twenty-four hours following the incubation, the cells were fixed and stained with ActiStain 555–conjugated phalloidin (#PHDH1, Cytoskeleton). Mesothelial clearance area was defined as cell-free area, and mesothelial clearance was calculated as the ratio of cell-free area to total area using the ImageJ Software (NIH, Bethesda, MD).

Zymography

Serum-free conditioned medium was collected from 1 × 10^5 HPMCs with or without 100 μg/ml of EOC-derived exosomes, and a gelatin zymogram was performed as previously described (17).

Quantitative real-time PCR

Total RNA was extracted using TRIzol, and cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (FSQ-201; TOYOBO). qRT-PCR was performed using the StepOnePlus Real-Time PCR System. The following probes were used: human matrix metalloproteinase-9 (MMP9: Hs00234579_m1) and human GAPDH (4326317E). Relative expression of CD44 was assayed using the SYBR Green assay (Applied Biosystems: #4309155). The designed primers in this experiment were as follows: CD44: forward primer: 5’-AGTACAGAAGCACCTGGCCCAATGCCTTT-3, reverse primer: 5’-TTTGCTCAGCCTTCAGGACTCCCATG-3; GAPDH: forward primer: 5’-CATCGAAG-TATGACACAGGGCGT-3, reverse primer: 5’-AGTCCTTCCACGATTACCAAAGT-3 (Applied Biosystems). PCR was performed in triplicate, and the relative levels of CD44 and MMP9 expression were calculated using the 2−ΔΔCt method.

ELISA

The concentration of TGFβ in exosomes was analyzed using Human/Mouse TGFβ 1 ELISA Ready-SET-Go! (#88-8350; eBioscience).

GW4869 treatment

GW4869 (18) was stored at −20°C as a 3 mmol/L stock suspension in DMSO. Right before use, the suspension was solubilized by the addition of 5% methane sulfonic acid (MSA; Wako).

Measurement of particle size and concentration distribution

Nanoparticles in exosome suspensions were analyzed using a NanoSight LM10V-HS (Malvern Instruments Ltd.).

**Fig. 1.**

EOC-derived exosomes are transferred to human primary mesothelial cells. A, Electron microscopy: exosomes were immunogold labeled with anti-CD63 antibody and Transmission electron micrographs of purified exosomes secreted from several EOC cell lines taken. B, Exosomes were immunogold labeled with anti-CD63 antibody and Transmission electron micrographs of purified exosomes secreted from several EOC cell lines taken. C, Western blot analysis on isolated exosomes and parental cellular lysates (10 μg/lane). Exosomes were isolated from 2 EOC cell lines (HeyA8 and TYK-nu) and ISOE. D, RNA was extracted from EOC-derived exosomes and analyzed by a Bioanalyzer to detect ribosomal subunits 28S and 18S (left). A profile of cellular RNA is shown as a control (right). Arrows indicate the position of small RNA, 18S and 28S. E, Primary HPMCs isolated from normal human omentum. HPMCs stained for anti-cytokeratin-18, vimentin, and calretinin (negative control omitted primary antibody). Scale bar, 100 μm. F, Confocal microscopy. HPMCs were cultured for 24 hours in the presence of PKH67, a fluorescent dye labeling exosomes. HPMCs are identified by the DAPI-stained, blue nuclei. Scale bar, 20 μm. Data in A, C, D, E, F are representative of 3 experiments with similar results. Data in B are shown as mean ± SEM of 3 independent experiments.
**Tissue samples**

Specimens of human omentum were obtained following written informed consent from patients undergoing gynecological surgeries at Osaka University Hospital (Osaka, Japan). Samples were obtained in accordance with the Declaration of Helsinki (Institutional Review Board of Osaka University Graduate School of Medicine approval no.: 10064). HPMCs were isolated as previously described (19). Briefly, benign omental specimens were minced and incubated with 1:1 solution of PBS and 0.25% trypsin/25 mmol/L EDTA at 37°C for 30 minutes. After the solution containing cells was centrifuged, the pellet was suspended and cultured in 20% FBS/RPMI-1640 with 100 U/mL penicillin/streptomycin. Cells at passage 1 were used for the subsequent experiments. EOC samples were collected from 15 patients with FIGO stage III–IV epithelial serous ovarian cancer consecutively treated at Osaka University Hospital between 2011 and 2015. All cases were histologically confirmed by a gynecologic pathologist as being high-grade serous carcinomas.

**Animal experiments**

Female athymic BALB/c nude mice were purchased from CLEA Japan, Inc. All animal experiments were approved by the Institutional Animal Care and Use Committee of Osaka University. HeyA8 cells indicated in the figures were injected intraperitoneally. Seven days after the inoculation, the mice were sacrificed and omental tissues were carefully dissected. No statistical methods were used to predetermine sample size. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

**Statistical analysis**

JMP software version 10.0.2 (SAS Institute Japan Ltd.) was used for statistical analysis. All data had normal distribution and equal variances were tested by F-test. Data are expressed as mean ± SEM. Differences were analyzed using one-way ANOVA and Bonferroni correction for multiple comparisons. Differences were considered statistically significant at $P < 0.05$.

**Results**

**Ovarian cancer–derived exosomes can be transferred to mesothelial cells**

To elucidate the effects of exosomes derived from EOC cells on peritoneal dissemination, exosomes were characterized by electron microscopy, Western blotting, and capillary electrophoresis of RNA. Electron microscopy revealed that exosomes had a rounded morphology and were approximately 50 to 100 nm in size and stained positive for CD63, an exosome marker (ref. 20; Fig. 1A). Exosome secretion was measured in several EOC cell lines used in these experiments. CD81 (21), another exosome marker, was also enriched in exosomes from HeyA8 cells and IOSEs, whereas whole-cell lysates expressed minimal CD63 and CD81 (Fig. 1C). In contrast, β-actin was expressed in whole-cell lysates, whereas exosomes showed low expression (Fig. 1C). Total RNAs extracted from whole cells using capillary electrophoresis expressed the 18S and 28S ribosomal subunits, whereas they were not detected in exosomes which expressed small RNAs, such as miRNAs (ref 22; Fig. 1D).

We then studied the internalization of EOC-derived exosomes by HPMCs. Primary HPMCs displayed a cobblestone-like morphology and expressed cytokeratin-18, vimentin (19), and calretinin, a discriminatory marker for mesothelial cells (Fig., 1E; ref. 23). Exosomes from EOC cell lines and IOSE were internalized by HPMC and detected in the cytoplasm of the HPMCs using confocal laser microscopy (Fig. 1F), indicating that EOC-derived exosomes can be internalized by HPMCs.

**EOC-derived exosomes promote ovarian cancer invasion through increased mesothelial clearance and MMP induction**

The role of EOC-derived exosomes in ovarian cancer invasion was studied using an in vitro invasion assay. To mimic the mesothelial layer covering the omentum and other peritoneal organs, a monolayer of HPMCs was plated onto growth factor-reduced Matrigel (Fig. 2A). Pretreatment with HeyA8- or TYK-nu-derived exosomes on HPMCs significantly stimulated the invasion of ovarian cancer cells, whereas pretreatment with IOSE-derived exosomes did not affect EOC invasion. EOC exosomes did not affect proliferation of HPMCs (Fig. 2B and C). In the absence of HPMCs (Matrigel-only coating), exosomes derived from either HeyA8 or TYK-nu cells did not promote invasion (Fig. 2D), nor did treatment with exosomes alone promote ovarian cancer cell proliferation (Fig. 2E), indicating that exogenous treatment of EOC-derived exosomes affected HPMCs but not cancer cells.

Next, we aimed to identify the changes in HPMC surface receptors induced by exosomes (Fig. 3A). The expression pattern of key proteins related to tumor cell adhesion or invasion (24) was examined by Western blotting (Fig. 3B). Uptake of EOC-derived exosomes into HPMCs induced expression of the standard form of CD44 (CD44s) and downregulation of E-cadherin, whereas the expression patterns of integrin-α1, -α2, -α5, -αv, -β1, -β3 were unchanged (Fig. 3B). CD44 has several isoforms generated by alternative mRNA splicing (25). Only the expression of CD44s (80 kDa) was increased in HPMCs (but not in IOSE), whereas the expression of other CD44 variants (CD44v, 120–200 kDa) was very low (Fig. 3B). Western blotting as well as electron microscopy showed that CD44s was expressed by ovarian cancer–derived exosomes but not IOSE-derived exosomes (Fig. 3C and D). Three minutes after the uptake of PKH67-labeled exosomes, HPMCs were immunostained with Alexa Fluor 555–labeled CD44 antibody. CD44 was colocalized with exosomes, indicating that CD44 derived from ovarian cancer cells was actually internalized in HPMCs via exosomes (Fig. 3E). There was no significant change in CD44 mRNA levels in HPMCs following treatment with EOC-derived exosomes, indicating that exosomes do not regulate CD44 expression on the transcriptional level (Fig. 3F). Given that E-cadherin expression was decreased in HPMCs, we considered the possibility that mesothelial cells undergo epithelial–mesenchymal transition (EMT; ref. 2). Confocal microscopy revealed that HPMCs treated with EOC-derived exosomes become elongated and spindle-shaped, whereas HPMCs treated with IOSE-derived exosomes retained their cobblestone-like morphology (Fig. 1F). Because EOC-derived exosomes induced the morphologic transition to a mesenchymal phenotype, we determined HPMC cell clearance (6). Twenty-four...
hours after treatment with EOC-derived exosomes, cell–cell contact between HPMCs was reduced and the cell-free areas (i.e., mesothelial clearance) were significantly greater than those found with mesothelial cells treated with IOSE-derived exosomes (Fig. 3G). Because CD44 is associated with a proteolytic form of the MMP9 on the cancer cell membrane and CD44-

Figure 2.
Ovarian cancer–derived exosomes promote ovarian cancer invasion through mesothelial cells. A, Schematic of the human peritoneal mesothelial cells in vitro invasion assay. HPMCs were plated onto 24-well cell culture inserts coated with Matrigel. Twenty-four hours after exosomes were added to culture medium, fluorescently (CMFDA) labeled ovarian cancer cells were incubated on HPMCs and allowed to invade for 48 hours. Cells that migrated to the trans-side of the membrane were quantitated using immunofluorescent microscopy. B, Cell proliferation assay. Primary HPMCs were plated onto 96-well plates for 24 hours with or without exosomes (1, 10, 100 µg/mL). C, HPMC-coated in vitro invasion assay. Exosomes were added to culture medium at the indicated concentrations and invasion of CMFDA-labeled HeyA8 or TYK-nu cells was evaluated. D, Invasion assay. Exosomes (100 µg/mL) were added to a Matrigel-coated insert. Twenty-four hours later, 5 × 10⁴ fluorescently (CMFDA) labeled ovarian cancer cells (HeyA8 and TYK-nu) were added. E, Cell proliferation assay. HeyA8 or TYK-nu cells (5 × 10³ cells) plated onto 96-well plates were treated with 100 µg/mL exosomes. Forty-eight hours later, proliferation was assessed by an MTS assay. B and C, cancer cell invasion was measured relative to control (PBS). Data in B and C are shown as mean ± SEM of 3 independent experiments. *, P < 0.05; **, P < 0.01.
associated cell surface MMP9 is known to promote cell-mediated degradation of ECM (26). MMP9 expression in HPMCs was assessed after exosome uptake. Quantitative real-time PCR confirmed that MMP9 mRNA expression was significantly increased in HPMCs treated with EOC-derived exosomes as compared with those treated with PBS only or IOSE-derived exosomes (HeyA-8; 5-fold, TKY-nu; 6.3-fold; Fig. 3H). Gelatin zymography was performed to analyze whether EOC-derived exosomes induce HPMCs to secrete MMP9. Conditioned medium from HPMCs treated with EOC-derived exosomes increased the gelatinolytic activity of pro-MMP9, whereas they did not affect gelatinolytic activity of MMP-2 (Fig. 3I). The involvement of MMP9 activity in ECM invasion was analyzed using an anti-MMP9 antibody after its inhibitory effect against MMP9 activity was confirmed by gelatin zymography (Supplementary Fig. S1A). EOC invasion was significantly decreased by MMP9 inhibition, especially when HPMCs were pretreated with EOC-derived exosomes (Supplementary Fig. S1B). Collectively, these data show that EOC-derived exosomes promote ovarian cancer invasion by affecting HPMCs through the increase of mesothelial clearance and the induction of CD44-mediated MMP9 secretion by HPMCs.

Inhibition of exosome secretion attenuated CD44 acquisition by HPMCs and blocked ovarian cancer invasion

GW4869, a neutral sphingomyelinase inhibitor, inhibits exosome secretion (18). Addition of GW4869 to HeyA8 (Fig. 4A and B) and TKY-nu (Supplementary Fig. S2A) ovarian cancer cells blocked exosome secretion in a dose-dependent manner, without affecting cell viability (Fig. 4C, Supplementary Fig. S2B). GW4869 treatment significantly inhibited (49%) the invasion of both cell lines in the presence of HPMC coating, whereas in the absence of HPMCs, invasion was unchanged (Fig. 4D, Supplementary Fig. S2C). Consistent with a regulation of CD44 by exosomes, GW4869 attenuated CD44 uptake in HPMCs cocultured with EOC cells in a dose-dependent manner. E-cadherin expression was inversely correlated with CD44, suggesting that the inhibitor blocked EMT in the HPMCs (Fig. 4E, Supplementary Fig. S2D) which was also confirmed by phase-contrast microscopy (Fig. 4F, Supplementary Fig. S2E). When EIPA, a known exosome uptake inhibitor that blocks macropinocytosis (27), was treated with HPMCs cocultured with EOC cells (Supplementary Fig. S3), CD44 expression in HPMCs was similarly inhibited.

In addition, GW4869 restored the epithelial appearance of HPMC in a dose-dependent manner (Fig. 4F). These results indicate that the inhibition of exosomes released from EOC cells inhibit ovarian cancer invasion by attenuating CD44 expression and reversing EMT in HPMCs.

EOC-derived exosomes induce morphologic change in HPMCs independent of TGFβ signaling

Because TGFβ signaling is one of the key regulators of EMT (28) and is also correlated with enhanced CD44 expression (29), we studied the involvement of TGFβ in the acquisition of CD44 by HPMCs. At 10 ng/mL, TGFβ induced CD44 expression and downregulated E-cadherin expression, effects which were abrogated by use of the potent TGFβ receptor 1 kinase inhibitor LY2157299 (Fig. 5A). In addition, TGFβ induced the morphologic change of HPMCs to a spindle shape, an effect which was also inhibited by LY2157299 (Fig. 5B). The TGFβ receptor 1 kinase inhibitor did not interfere with the ability of EOC-derived exosomes to upregulate CD44s and downregulate E-cadherin in HPMCs (Fig. 5C). ELISA assays revealed that each exosome contains a low level of TGFβ (6–162 pg/100 μg exosomes) and that this concentration was similar between EOC cells and IOSEs (Fig. 5D). Coculture with EOC cells not only induced CD44 expression in HPMCs but also resulted in acquisition of a spindle cell, EMT-type morphology, despite LY2157299 treatment (Fig. 5E and F). These experiments suggest that CD44 acquisition by HPMCs via exosomes is independent of TGFβ signaling.

Downregulation of CD44 expression in EOC-derived exosomes attenuated ovarian cancer invasion

To further understand the role of exosomal CD44 in ovarian cancer progression, CD44 expression in HeyA8 cells was knocked down using siRNAs (Fig. 6A and B), which resulted in a reduction of CD44 expression in the HeyA8-derived exosomes (Fig. 6C). Treatment of HPMCs with exosomes derived from HeyA8 CD44 knockdown (KD) cells abrogated the upregulation of CD44s and the downregulation of E-cadherin in HPMCs (Fig. 6D). The pretreatment of HPMCs with exosomes from HeyA8 CD44 KD cells significantly blocked invasion (Fig. 6E) and mesothelial cell clearance (Fig. 6F) and reduced the gelatinolytic activity of MMP9 (Fig. 6G). In the opposite experiment, HPMCs were transfected with a CD44 expression vector and their effect on EOC invasion was determined. Immunoblotting showed that CD44 was overexpressed in HPMC, leading to the downregulation of E-cadherin expression (Fig. 6H). CD44-overexpressing HPMCs displayed a spindle cell morphology and lost cell–cell contact, whereas control vector–transfected HPMCs retained a cobblestone-like appearance (Fig. 6I). CD44-overexpressing HPMCs promoted invasion of EOC cells when compared with control vector–transfected HPMCs (Fig. 6J). The mesothelial clearance area was significantly increased in CD44-overexpressing HPMCs (Fig. 6K), and the conditioned medium of CD44-overexpressing HPMCs increased the gelatinolytic activity of MMP9 (Fig. 6L).
Inhibiting exosome secretion attenuates CD44 uptake by HPMCs and suppresses ovarian cancer invasion. A, HeyA8 cells (5 x 10⁶) were treated with the sphingomyelinase inhibitor GW4869 (48 hours), exosomes collected, and exosomal proteins concentration quantified (Lowry). B, Nanoparticle analysis. Concentration and size distribution of nano-sized particles in exosome suspension were measured using NanoSight system. Concentration was indicated by the number of nano-sized particles per 1-mL culture medium. C, Proliferation assay. HeyA8 cells (5 x 10⁶) were treated with GW4869. Cell viability was assessed 48 hours after incubation. D, Left, invasion assay. After an HPMC monolayer was formed on Matrigel-coated inserts, CMFDA-labeled HeyA8 cells were plated onto Matrigel and were allowed to invade for 48 hours in the presence of GW4869. Right, Matrigel in vitro invasion assay without HPMC. E, Western blotting. HPMC growing as monolayer on the bottom was cocultured with HeyA8 cells in an insert in the presence or absence of GW4869 (24 hours). Thereafter, HPMC cell lysates were collected, lysed and immunoblotting was performed. F, Phase contrast microscopy. HPMCs were treated with GW4869 and morphologic changes evaluated. Scale bar, 100 μm. Data in A (left), B, C are shown as mean ± SEM of 3 independent experiments. Data in A (right) are shown as mean of 5 measurements. Images in D, E are representative of 3 experiments with similar results. **, P < 0.01.

Taken together, the data in Fig. 6 show that exogenous expression of CD44 in HPMCs promotes ovarian cancer invasion by increasing mesothelial clearance and inducing MMP9 secretion.

CD44 is overexpressed in mesothelial cells in omental metastasis

Next, we investigated whether the results found using cell lines have correlates in human tissue from patients with EOC; specifically whether CD44 expression is increased in HPMCs from omental metastasis. Omental tissue harboring tumor was obtained from 15 patients and immunostained with anti-CD44 antibody (Supplementary Table S1). Normal HPMCs lining the omentum surface were identified using positive calretinin staining (Fig. 7A). In HPMCs from normal omentum, CD44 expression was mostly negative (Fig. 7A). In contrast, in 9 of 15 (60%) tumors, strong CD44 expression was seen in the primary ovarian high-grade serous performed (Supplementary Fig. S4) and showed CD44 to be colocalized with CD63 carcinomas (Fig. 7B). To confirm exosomes contain CD44 in these primary ovarian high-grade serous carcinomas, double immunostaining with CD44 and CD63, a representative exosome marker, was conducted. In omental tissue harboring disseminated ovarian cancer cells, CD44 was strongly expressed in metastatic implants including HPMCs. However, HPMCs did not express CD44 in areas further away from the tumor cells (Fig. 7C). HPMCs intertwined with metastatic cancer cells displayed spindle-like morphology, and the mesothelial cell barrier was disrupted (Fig. 7C). In serial sections, CD44-positive HPMCs harboring tumors expressed MMP9, whereas normal HPMCs were mostly MMP9-negative (Supplementary Fig. S5).

CD44 expression in mesothelial cells was also studied using an ovarian cancer xenograft model (30). To mimic early ovarian cancer metastasis to omental tissue, HeyA8 cells transfected with control siRNA or CD44 siRNA were injected intraperitoneally into female BALB/c nu/nu mice (31). One week after inoculation, mice showed microscopic tumor dissemination on the omental surface. Human CD44 expression was seen at the leading edge of invading HeyA8 cells transfected with control siRNA and mouse mesothelial cells harboring cancer cells...
displayed positive human CD44 expression (Fig. 7D, top). However, HeyA8 cells transfected with CD44 siRNA showed faint CD44 expression and CD44 was negative in mouse omental tissues harboring cancer cells (Fig. 7D, bottom). To further quantify the induction of CD44 expression, we injected either 1 × 10⁵ or 1 × 10⁶ HeyA8 cells intraperitoneally into female athymic BALB/c nude mice and compared human CD44 expression in omental tissues from the 2 groups of mice (Supplementary Fig. S6A–S6C). In the tissue of mice injected with 1 × 10⁵ HeyA8 cells, strong CD44 expression was seen (Supplementary Fig. S6D) as compared with tissue from mice injected with 1 × 10⁶ HeyA8 cells. These data support our in vitro findings indicating that CD44 acquisition by mesothelial cells is induced by direct contact with EOC cells.

**Discussion**

Cancer-derived exosomes can act as mediators of metastasis, and, as a result, a minority population of cancer cells can reprogram stromal cells through the intercellular transfer of proteins, mRNAs, and miRNAs (32). Here, we show that EOC-derived exosomes promoted ovarian cancer invasion by transferring
Exosomes Promote Ovarian Cancer Cell Invasion

Exosomes containing CD44 to HPMCs which reprograms them to a more EMT phenotype, thereby promoting ovarian cancer cell invasion and metastasis. EOC cells, by releasing exosomes, reprogram HPMCs, which then facilitate EOC invasion supporting a model of bidirectional communication (Fig. 7E).

Several studies have shown that cancer cell–derived exosomes reprogram or educate other cells to aid tumor survival and promote metastasis (33); however, to date, only a limited number of reports have investigated the cancer-promoting effect of a particular protein or miRNA in cancer-derived exosomes. For instance, melanoma-derived exosomes reprogram bone marrow progenitor cells to support tumor growth and metastasis through MET (34) and breast cancer–derived exosomal miR-105 promotes metastasis by destroying endothelial cell barriers (35). Recently, Singh and colleagues revealed that integrin-β3 is transferred from tumorigenic to nontumorigenic cancer cells via exosomes and its downregulation in recipient cells promotes cell migration on its ligand (36).

It is well-known that the tumor microenvironment, including stromal cells, plays an important role in cancer progression (37). Under normal conditions, mesothelial cells act as a mechanical barrier to protect intra-abdominal organs (3). However, reports have shown that mesothelial cells can also serve as a metastatic niche, promoting cancer cell adhesion and invasion, which are essential steps of the peritoneal dissemination of ovarian cancer (2, 38). Since several previous reports have focused on TGFβ signaling as a means of communication between EOC cells and HPMCs, we inhibited TGFβ signaling using a potent inhibitor, LY2157299. While this TGFβ inhibitor completely abrogated the morphologic changes to a mesenchymal phenotype and the downregulation of E-cadherin induced by TGFβ, treatment with EOC-derived exosomes induced these phenomena in the presence of LY2157299, indicating that the mechanism is TGFβ-independent.

Several adhesion molecules such as integrins or ECM components have been identified as important mediators of cancer metastasis to the mesothelium (39). Among these, hyaluronan (HA) and its receptor, CD44, have been shown to play critical roles in ovarian cancer metastasis (40). It is reported that ovarian cancer cell adhesion to mesothelial cell monolayers is mediated, at least in part, by the interaction between HA and CD44, which further mediates ovarian cancer progression (41).

A recent immunohistochemical study showed that CD44 expression was seen in 42 of 72 (58.3%) ovarian tumors and that CD44 (+)/E-cadherin (−) ovarian tumors have a more aggressive phenotype and are associated with poor patient survival (42). We found a similar strong expression of CD44 in tumors analyzed. CD44 is also known to be a key regulator of EMT in several cancer types (43–45). Consistent with these reports, our findings show that HPMCs treated with EOC-derived exosomes displayed a mesenchymal phenotype and reduced E-cadherin expression. CD44 has also been shown to recruit MMP9 to the cell surface and mediate tumor cell invasion (25), which is consistent with our zymographic analyses (Fig. 6). Given the important role of CD44 in cancer invasion, the molecule holds promise as a therapeutic target in ovarian cancer treatment. However, clinical trials of systemic treatment with CD44 neutralizing antibodies have been terminated because of unacceptable levels of toxicity (46). Nontoxic alternative therapies need to be developed for future trials. Capturing CD44-expressing exosomes in body fluid appears to be less toxic and has the potential to be a possible anticancer therapy.

Interestingly, inhibition of exosomes released from EOC cells using GW4869 attenuated CD44 acquisition in HPMCs and accordingly suppressed ovarian cancer invasion. Kosaka and colleagues suggested that cancer cell–derived exosomes circulating in the blood not only promote cancer malignancy through the formation of peritoneal nests but also interfere with treatment (47). Indeed, Ciravolo and colleagues reported that the exosomes released by the HER2-overexpressing breast cancer cell lines express a full-length HER2 molecule and that these exosomes bound to trastuzumab and inhibited its anti-cancer cell proliferative activity (48). On the basis of this preclinical data, Aethlon Medical Inc. has developed HER2ost, as a therapeutic strategy to combat HER2-positive breast cancer through the capture of circulating HER2-positive exosomes (49).

There are some limitations to this study. First, as a large amount of exosomes were needed to stably reproduce the experimental data, HeyA8 and TYK-nu cells, which express exosomes most abundantly, were used. However, a recent genomic profiling of ovarian cancer cell lines (50) suggests that HeyA8 seems not to be typical of high-grade serous ovarian

Figure 6. CD44 expression in HPMC promotes ovarian cancer invasion. A–G, Downregulation of exosomal CD44 suppresses ovarian cancer invasion. A, Schematic of siRNA transfection and exosome treatment of HPMCs. Exosomes were isolated from HeyA8 cells transfected with control siRNA or 2 different CD44 siRNAs. B–D, Western blot analyses. B, HeyA8 cells transfected with control siRNA (ctrl) or 2 different CD44 siRNAs (#1 and #2). C, Exosomes described in A, D, HPMCs treated with exosomes described in A. E, Left, HPMC-coated in vitro invasion assay. After the HPMC monolayer was formed, Matrigel-coated culture inserts and exosomes were added followed by CMFDA-labeled HeyA8 cells 24 hours later. Right, Matrigel in vitro invasion assay. Culture medium with 100 μg/mL of exosomes was added to Matrigel-coated inserts. Twenty-four hours later, 5 × 10^4 fluorescence (CMFDA)-labeled HeyA8 cells were incubated in a 24-well Fluoroblok cell culture insert and allowed to invade for 48 hours. F, Mesothelial clearance assay. HPMCs were treated with exosomes and the percentage of cell-free area induced by exosome treatment (indicated by white dashed line) was measured using ImageJ software (left). Representitive images of HPMCs (right). G, Gelatin zymography. HPMC supernatant treated with exosomes was added to a gelatin-containing gel. Proteolysis was detected as a white band. H–L, Overexpression of CD44 in HPMCs promotes ovarian cancer invasion. H, Western blotting. HPMCs were transfected with wild-type CD44 plasmid (CD44+) or control vector (ctrl). L, Confocal microscopy. HPMCs were stained with DAPI (blue nuclei) and anti-CD44 antibody followed by Alexa Fluor 633-conjugated secondary antibody (red). Bars represent 20 μm. J, Invasion assay. HPMCs transfected with control or CD44 plasmid were plated onto cell culture inserts. Thereafter, an in vitro invasion assay was performed as described in E, K, Mesothelial clearance assay. HPMCs were transfected with control or CD44 plasmid and incubated for 24 hours. Cells were added to the assay as described above. The percentage of cell-free area is shown (left). Representitive images of HPMCs (right). Bars indicate 50 μm. L, Gelatin zymography. HPMC supernatants from HPMCs transfected with control or CD44 plasmid were used for gelatin zymography. Images in B–D, F–I, K, L are representative of 3 experiments with similar results. Data in F, J, K are mean ± SEM of 3 experiments. * P < 0.05; ** P < 0.01.
cancer (HGSC). While TYK-nu conforms more closely to HGSC, this cell line was not transplantable to mice and, therefore, we could use it in our in vivo experiments. Second, it was not conclusively demonstrated that EOC-derived exosomes contain enriched CD44. Ultracentrifugation-based exosome isolation is commonly used; however, it is not possible to completely eliminate contamination pulled down from other sources in the culture medium (51).

In conclusion, we propose a novel role for EOC-derived exosomes in targeting adjacent peritoneal mesothelial cells (Fig. 7E). Our data identified exosome-mediated transfer of CD44 as a key regulator of mesothelial cell reprogramming and the metastatic progression of ovarian cancer. In the light
of several publications highlighting the importance of exosomes in cancer biology and the results described here, we hypothesize that targeting exosomes might have a therapeutic application in ovarian cancer treatment and recommend that it should be explored further for future clinical use.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Nakamura, E. Nakatsuka, E. Lengyel, T. Kimura
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References

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