CD44 Regulates Formation of Spheroids and Controls Organ-Specific Metastatic Colonization in Epithelial Ovarian Carcinoma

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Abstract

Disseminating epithelial ovarian cancer cells often become assembled into spheroids prior to their arrival at metastatic sites within the peritoneal cavity. Although epithelial ovarian carcinoma (EOC) is the most predominant type, which, in turn, encompasses several different histotypes, such as serous [high-grade (HGSOCS) and low-grade], endometrioid, clear cell, and mucinous, that arise in epithelial cells of the ovary, fallopian tube, or endometrium (2, 3). Common elements between these histotypes include presence of spheroids in ascites, involvement of the ovaries with a short-lived remissions (4–6). Frequent recurrences of chemotherapy-resistant disease are mainly responsible for the high mortality among the ovarian cancers (7, 8). Interaction between CD44 and hyaluronan expressed by mesothelial cells is one of the key regulators of peritoneal adhesion (9). CD44 is a multi-structural single-pass type I transmembrane glycoprotein and a receptor for hyaluronan (10, 11). The isoforms of CD44, such as CD44s (the standard isoform, comprising exons 1–5 and 16–20) and CD44v (variant isoforms, also containing variable exons 6–15) result from alternative splicing of the variable exons (12).

Ovarian carcinoma, the fifth leading cause of death from cancer in women, comprises a number of malignancies of both epithelial and nonepithelial origin (1). Epithelial ovarian carcinoma (EOC) is the most predominant type, which, in turn, encompasses several different histotypes, such as serous [high-grade (HGSOCS) and low-grade], endometrioid, clear cell, and mucinous, that arise in epithelial cells of the ovary, fallopian tube, or endometrium (2, 3). Common elements between these histotypes include presence of spheroids in ascites, involvement of the ovaries with a subsequent locoregional intraperitoneal pattern of metastatic spread, and late presentation. The affected patients undergo debulking surgery and platinum/taxane combination chemotherapy, which allows them to achieve short-lived remissions (4–6). Frequent recurrences of chemotherapy-resistant disease are mainly responsible for the high mortality from this malignancy. Hence, prevention and treatment of the recurrent disease is of paramount importance for increasing patient survival. Prevention of recolonization of the intraabdominal tissues is one of the possible approaches to block rampant expansion of these metastases.

One of the key steps in successful initial colonization and recolonization of the abdomen is implantation of the sloughed off malignant cells on to the mesothelial layers of peritoneal organs and tissues (7, 8). Interaction between CD44 and hyaluronan expressed by mesothelial cells is one of the key regulators of peritoneal adhesion (9). CD44 is a multi-structural single-pass type I transmembrane glycoprotein and a receptor for hyaluronan (10, 11). The isoforms of CD44, such as CD44s (the standard isoform, comprising exons 1–5 and 16–20) and CD44v (variant isoforms, also containing variable exons 6–15) result from alternative splicing of the variable exons (12).

Disseminating EOC cells of all histotypes are often found as multicellular aggregates (MCA) and spheroids (13). Spheroids are defined as aggregates of adherent cells of over 50 μm in diameter that require proteolytic digestion to dissociate them from one another (14). In contrast, multicellular aggregates are composed of cells that attach, but not adhere, to one another, and can be converted into a suspension of individual cells by nonenzymatic methods (i.e., pipetting). Aggregation of cells is important for anchorage-independent cell survival (15), growth (16), and chemotherapy resistance (17, 18), and spheroid formation may represent an important intermediate survival mechanism to facilitate metastatic dissemination. Although mechanisms regulating metastasis from spheroids are less understood, our previous studies indicated that a secreted proteoglycan versican, which is thought to facilitate the interaction between CD44 and hyaluronan (19, 20), is one of the key regulators of peritoneal adhesion and tumor formation in spheroids (21).

CD44 is one of the cancer stem cell markers, and formation of spheroids is one of the features of cancer stem cells (22). By an analogy with the individual cells and based on our previous data, we hypothesized that CD44 could be a key contributing factor in the processes of spheroid formation and their peritoneal adhesion. Here we show that CD44 is important for spheroid formation. Unexpectedly, our studies uncovered a differential role for

Introduction

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CD44 in development of organ-specific metastases. Beyond the predicted reduction of the metastasis affecting tissues predominantly containing mesothelial linings (i.e., the mesenteries), reduction of CD44 resulted in an increase of metastases to other organs, including liver and lungs.

Materials and Methods

Cell lines
Normal human mesothelial cell strain LP-3 was obtained from the Coriell Aging Cell Repository and cultured as indicated by the manufacturer for 5–8 passages. EOC cell lines OVCAR4 and SKOV-3 were obtained from the NCI Tumor Cell Repository. EOC cell line ES-2 was obtained from Dr. Stack (University of Notre Dame, Notre Dame, ID). EOC cell line OVASAH0 was obtained from the Japanese Collection of Research Bioresources Cell Bank. All EOC cell lines were cultured as suggested by the manufacturers and routinely assessed by cell morphology and average doubling time; identity of the cell lines; and CRISPR/Cas9 manufacturers and routinely assessed by cell morphology and average doubling time; identity of the cell lines; and CRISPR/Cas9

Mice
Athymic nude–FOXN1NU mice were obtained from Harlan Laboratories. All experimental procedures were performed according to the Institutional Animal Care and Use Committee protocol approved by the Animal Care Committee of University of Illinois (UIC).

Antibodies
Rabbit anti-human CD44 (H-300), mouse anti-human CD44 (DF1485), mouse anti-human β-actin (C4), mouse monoclonal anti-human vimentin (5G3F10) were obtained from Santa Cruz Biotechnology. Rabbit polyclonal anti-human RARRE3 (retinoic acid receptor responder 3) was obtained from Aviva Systems Biology. Rabbit polyclonal anti-human ETS-1 was obtained from Abclonal. Rabbit polyclonal anti-human ACTA2 and ZEB2 were obtained from NeoBioLab.

qRT-PCR was performed as described previously (23). Primers were designed to be specific for CD44 variants (Supplementary Materials and Methods). TissueScan, Ovarian Cancer cDNA Array 1 (catalog no. HORT101) was purchased from OriGene.

Gene editing with CRISPR/Cas9
CRISPR/Cas9 plasmids were designed and created by the UIC Genome Editing Core (Supplementary Materials and Methods). TissueScan, Ovarian Cancer cDNA Array 1 (catalog no. HORT101) was purchased from OriGene.

Live-cell imaging microscopy
The cells were imaged using the Olympus Viva View FL incubator microscope. Images were acquired in the DIC channel every 10 minutes for 2 hours over multiple z-stacks. The acquired images were collapsed and processed using the MetaMorph image analysis software.

Scanning electron microscopy
Spheroids were transferred to fine porous holders and incubated in 4% glutaraldehyde, washed, dehydrated in ethanol, dried with hexamethyldisilazane, mounted on aluminum stubs, and sputter coated with Pt/Pd using a Cressington 208 HR resolution coater. Exterior surfaces were imaged using a Secondary Electron detector with a Hitachi S-3000N variable pressure scanning electron microscope at the Electron Microscopy Service Facility (UIC).

Spheroid formation
Spheroids were generated using an agarose overlay method described previously (21, 28, 29). Briefly, nonadhesive agarose plates were prepared by solidifying agarose solution (0.5% in complete culture media) in 10-cm diameter plastic plates. Cells were released from the monolayers with 0.05% trypsin/EDTA solution, suspended in media containing 2% FBS at the concentration of 125,000 cells/mL. Ten-milliliter of this solution was added atop of the solidified agarose and incubated for 24–72 hours at 37°C and 5% CO2. Spheroids that formed in the suspension were visualized using bright field microscopy, photographed, and their diameters were measured using AxioVision Software (Zeiss). Spheroids were collected using gentle centrifugation for 1 minute at 30 × g. Spheroids were considered as such if proteolytic digestion required to dissociate them into individual cells (14). Conglomerates or clumps of cells that did not require proteolytic digestion, but rather disintegrated into individual cells upon pipetting were not considered "true" spheroids and were further termed "multicellular aggregates".

Procedures for Western blot analysis were conducted as described in (25, 26, 32). Immunofluorescence staining and imaging with AxioVert Zeiss fluorescence microscope was conducted as detailed in (25, 26, 32).

Cell proliferation was performed using WST-1 method as described in (25, 26).

Mesothelial cell adhesion to monolayers of LP-3 was conducted as described previously (25).

Tumor formation
Individual cells (10⁶), spheroids, or MCAs were injected intraperitoneally into athymic nude mice (n = 6). A total of 10⁶ cells were used to generate spheroids or MCAs from parental ES2 and its C7 clone using agarose overlay method for 24 hours. Animals were monitored three times weekly until they reached humane endpoints and sacrificed. Ascites were aspirated. Tumors were excised, weighed, fixed in paraformaldehyde, and paraffin-embedded as described before (21, 25, 33, 34).

Statistical analysis
Survival was analyzed with log-rank (Mantel–Cox) test using Kaplan–Meier plotter and GraphPad Prism 7.02 software. Comparisons between two data sets with normal distribution were conducted using Student t test and Microsoft Excel software. Mann–Whitney U test was used to compare two data sets with abnormal distribution. The findings were considered statistically significant at P < 0.05.
Results

High CD44 standard isoform expression correlates with the capability of epithelial ovarian cancer cell lines to form spheroids

Transcoelomic metastatic spread is the major mechanism of formation of the intraperitoneal metastasis from EOC (7). Disseminating ovarian carcinoma cells are often assembled as MCAs and spheroids prior to attachment to the mesothelial layer of visceral and parietal peritoneum outlining organs of the peritoneal cavity (28). Assembly into spheroids may endow metastasizing cells with an increased ability to survive in suspension and avoid exposure to chemotherapeutic drugs, permitting recolonization and disease relapse. We examined spheroid-forming ability in a panel of EOC cell lines, including ES2, SKOV-3, OVCAR-4, and OVSAHO. OVSAHO and OVCAR-4 are representatives of the HGSOC histotype based on their genomic and mutational profiles (35). SKOV-3 is a p53 wild-type cell line originally isolated from a patient with EOC; a recent proteomics analysis placed this cell line into a mesenchymal subtype of HGSOC (36). ES2 was isolated from a patient initially diagnosed with EOC of the clear cell histotype (37), while a more recent study suggested that it closer recapitulates the immune profiles of the endometrioid histotype (38); however, a combination of its unique genomic profile and the presence of a mutation in p53 placed this cell line as “possibly HGSOC” (35). These cell lines displayed various propensities to form spheroids (Fig. 1A–C). Apart from the role of α5- and β1-integrins (14), the molecular mechanisms regulating adhesion of cells within spheroids are not well understood. CD44 came into focus as one of the candidates, as it is a major cell–cell adhesion receptor that also regulates stem cell–like phenotype, one of the features of which is formation of spheroids (39). We compared expression of CD44 in these cell lines using Western blot and found that the standard CD44 isoform was predominately expressed in ES2 and SKOV-3, cell lines with nearly 100% efficiency in spheroid formation, whereas its expression was significantly lower in OVSAHO, the least capable of forming spheroids in vitro (Fig. 1D). Expression of the variant isoforms was at a relatively low level and did not significantly change across all tested cell lines. To better understand the scope of the variant isoform expression, we examined expression of each of the variant domains using real time RT-PCR. We found that ES2 and SKOV-3 expressed the standard isoform of CD44 at the highest level, whereas it was lower in OVCAR-4 and OVSAHO; expression of all variant isoforms was at a comparatively low level for all cell lines (Supplementary Fig. S1), suggesting that the RNA expression of CD44 isoforms in specimens of serous (n = 19), endometrioid (n = 8), and mucinous (n = 2) ovarian carcinoma, we used real time RT-PCR and found that a subset of cases had relatively higher CD44 standard expression and others had lower, mirroring cell line expression data, whereas the expression of the tested variant domains (V2, V6, and V8) was comparatively lower (Supplementary Figs. S2, S3, S4). Thus, the tested cell lines and specimens could be separated in two groups: (i) expressing high CD44s and low CD44v, (ii) expressing low levels of both CD44s and CD44v. As the first group was characterized by strong ability to form spheroids, this suggests that the CD44s may be a key protein regulating cell–cell adhesion within the spheroids. In fact, we examined expression of CD44 in spheroids using immunofluorescence staining and found that CD44 is strongly expressed on the cell surface of the connecting cells in spheroids of ES2 and SKOV-3, while it was very low in spheroids of OVCAR-4 and OVSAHO (Fig. 2A; Supplementary Fig. S5).

These data indicate that CD44s is expressed in cell lines that have a strong capacity in forming spheroids.

CD44 is required for spheroid formation and cell adhesion in vitro

To determine the role of CD44 in formation of EOC spheroids in vitro, we silenced its expression using CRISPR/Cas9 in ES2 and SKOV-3 (Supplementary Fig. S6A–S6C). Clones of both cell lines with silenced CD44 were deficient in forming spheroids: all ES2 clones formed MCAs, but failed to form spheroids (Fig. 2B), and only 35% of CD44-deficient SKOV-3 cells formed spheroids that were significantly smaller in diameter (Supplementary Fig. S6D and S6E). Consistent with previous reports, silencing of CD44 resulted in reduction of cell proliferation in all ES2 clones (Supplementary Fig. S6F). Because 100% of ES2 reproducibly form spheroids, this cell line and its CD44−/− clone C7 were selected for further validation of the role of CD44 in spheroids and their metastatic potential in a xenograft model. ES2 spheroids and C7 MCAs were examined using scanning electron microscopy demonstrating formation of tight intercellular connections between the parental cells within spheroids that were lacking in the case of C7 MCAs, further supporting that CD44 is required for spheroid formation (Fig. 2C).

CD44 was demonstrated to regulate cell–cell adhesion between EOC and mesothelial cells (40). In our experiments, presence of CD44 function blocking antibodies, as well as CD44 silencing significantly affected adhesion of spheroids and MCAs to monolayer of peritoneal mesothelial cell line LP-3 (Supplementary Fig. S7A and S7B), suggesting that CD44 is one of the key molecules facilitating mesothelial adhesion of not only the individual cells, but also spheroids and MCAs.

In addition, we examined early dynamics of adhesion of the parental and CD44−/− cells to solid supports using time-lapse imaging. Hundred-percent of parental ES2 completely adhered and spread on to the solid supports within 2 hours of culture, whereas 85% of C7 remained largely unattached at this timepoint; furthermore, consistent with previous findings regarding the role of CD44 in formation of podosomes and invadopodia (41–43), C7 clone cells displayed significantly reduced membrane dynamics compared with the parental cells (Supplementary Fig. S7C, Supplementary Videos S1 and S2).

Collectively, these data confirm the role of CD44 in cell–cell adhesion, which appears to be an important contributing factor for spheroid formation in vitro.

CD44 differentially regulates organ-specific metastatic dissemination of EOC cells

On the basis of the published reports (9, 40) and our in vitro data, we posited that CD44 is important for peritoneal adhesion of spheroids and that its loss would impair metastatic colonization of intraperitoneal organs. We tested this premise using a xenograft model wherein ES2 or C7 were intraperitoneally injected either as individual cells (ES2 or C7) or as spheroids (ES2) or MCAs (C7; Fig. 3A). Animals were monitored until they became moribund and then sacrificed. A comparison of the overall survival between the groups revealed that there were no statistically significant differences in the survival between indi-
individual cell and spheroid or MCA groups within each cell line (Fig. 3B), thus the individual cell and spheroid (or MCA) groups were combined for further analysis. The latter is also more biologically relevant, as it would not be possible to separately assess spheroids and individual cells in the *in vivo* situation.

Expression of CD44 and lack thereof in ES2 and C7, respectively, was confirmed in tumor tissues at the end of the experiment (Fig. 3C).

The overall survival in the CD44−/− group was statistically significantly higher than that in the parental group (Fig. 3D).

Similarly, analysis of the postprogression survival (PPS) in patients with metastatic EOC revealed significant increase in the cohort with low CD44 expression (Supplementary Fig. S8A). We delved deeper into patients subpopulations and analyzed PPS of suboptimally debulked patients, which showed that patients with low CD44 lived longer; however, for optimally debulked patients with low CD44 PPS was significantly shorter (Supplementary Fig. S8B). In patients treated with platinum therapies, low CD44 significantly correlated with longer PPS; however, for those treated with a combination of platinum and taxol low CD44...
Figure 2.
CD44 is required for spheroid formation. A, Expression of CD44 in spheroids of ES2 was examined with immunofluorescence staining. ES2 was allowed to form spheroids when seeded over a nonadhesive layer of agarose, collected, paraffin-preserved, sectioned, and immunostained with CD44-specific antibodies (1:1,000 dilution in 1% goat serum); nuclear DNA was stained with DAPI. Images were acquired using Zeiss fluorescence microscope. CD44 (in black and white, left), DAPI (in black and white, center). Images of CD44 and DAPI superimposed using Zeiss Axiovert software: green, CD44; blue, DAPI (right). Scale bar, 20 μm.

B, Parental ES2 cells and clones with silenced CD44 expression were used to create spheroids. Spheroids were separated from individual cells by gentle centrifugation and trypsinized. Cells released from spheroids were counted and plotted as a percentage of the seeded cells (left). Bright field images demonstrate spheroids of ES2 as well as MCAs formed using ES2 clone C7, which disintegrated into individual cells after gentle pipetting, as indicated.

C, Scanning electron microscopy was used to investigate the structure and integrity of the parental ES2 spheroids (imaged at 1,200 × magnification) and MCAs (imaged at 1,500 × magnification) of clone C7. Dashed lines outline areas imaged at higher resolution, that is, 4,000 × magnification for ES2 spheroids and 4,500 × magnification for C7 MCAs.
Figure 3.
Effect of CD44 silencing on ES2-athymic nude mice xenograft model. A, The scheme representing experimental design wherein either 10^6 individual cells or spheroids created using 10^6 cells were intraperitoneally injected into athymic nude mice (n = 6) and sacrificed when the animals reached humane endpoints. B, Median overall survival of animals in the parental single cell (SC) and spheroid (Sph), as well as CD44^−/− single cell and MCA groups was calculated using Kaplan–Meier estimator, analyzed using log-rank Mantel–Cox test, and plotted. Median survival in each group is indicated in the table. C, Expression of CD44 was examined in animal tumor tissues (shown: metastasis to omentum) using immunofluorescence staining and CD44-specific antibodies. CD44, green; DAPI, blue.
D, Median overall survival of animals in the parental [combined single cell (SC) and spheroid (Sph) groups] and CD44^−/− [combined single cell (SC) and multicellular aggregate (MCA) groups] groups was calculated using Kaplan–Meier estimator, analyzed using log-rank Mantel–Cox test, and plotted using GraphPad software. Median survival in parental and CD44^−/− groups is indicated in the table.
E, Volumes of ascites collected from animals in the parental and CD44^−/− groups were plotted and statistically analyzed with Mann–Whitney U test (**, P < 0.01). Images of animals in each group demonstrate the typical presentation at the time of sacrifice. F, Tumor burden at intraperitoneal sites (mesenteries, peritoneal wall, diaphragm, liver, omentum, stomach, ovary/uterus, and abdominal fat) for each animal in the parental and CD44^−/− groups was found by weighing the excised tumor specimens, then plotted, and statistically analyzed with Mann–Whitney U test (**, P < 0.05).
predicted shorter PPS (Supplementary Fig. S8C). These data indicate that the role of CD44 in disease progression and treatment response could be more complex than previously thought.

To better understand the biology behind CD44-driven metastases we further analyzed our xenograft animal model. Loss of CD44 resulted in twice lower volumes of ascites, suggesting that these cells may have been less efficient in blocking the lymphatic drainage compared with the parental cells (Fig. 3E). However, in sharp contrast with the expected, the overall tumor burden at intraperitoneal sites in the CD44<sup>-/-</sup> group was significantly higher than that in the parental group (Fig. 3F). We further analyzed colonization of individual metastatic sites and found that only the tumor weight of lesions colonizing the mesenteries was twice lower in the CD44<sup>-/-</sup> group (Fig. 4A), well in line with the <em>in vitro</em> data. In contrast, a subset of intraperitoneal sites, including liver, omentum, and stomach, as well as a retroperitoneal site, kidney, developed 2- to 3-fold greater tumor burden in the CD44<sup>-/-</sup> group (Fig. 4B). On the other hand, the burden of lesions colonizing the remaining intraperitoneal sites (peritoneal wall and diaphragm, ovary/uterus, and abdominal fat) and retroperitoneal sites (inguinal lymph node and pancreas) did not correlate with CD44 expression (Fig. 4C). More detailed analysis between the individual cell and spheroid groups within the parental group revealed that the efficiency of colonization of most of the intra- and retro-peritoneal sites was similar. Omentum and ovary/uterus were the exception, as these sites were more avidly colonized by spheroids in comparison with individual cell group (Supplementary Fig. S9). Most medically fit patients undergo salpingo-oophorectomy in the process of debulking surgery (44). Thus, more aggressive behavior of spheroids during recolonization would only affect those who did not undergo debulking surgery. The omentum may be partially or completely surgically resected depending on the degree of its involvement with metastasis; however, when the omentum is tumor free, typically, it is not surgically removed (45). Hence, patients with remaining omentum could be potentially at higher risk of recurrent tumors seeded by metastasizing cells organized into spheroids.

At advanced stages, metastasizing cells could be found in the thoracic cavity, and they are thought to arrive at this distant site via direct extension of the metastasis from the peritoneal cavity or by means of lymphatic invasion (46, 47). Analysis of the thoracic cavity revealed that all but 1 animal in the CD44<sup>-/-</sup> group had lesions within the superior mediastinum (average tumor weight 80 mg) in a striking contrast to the parental group, in which only 1 animal developed a small lesion (Fig. 5A). Furthermore, the lungs in animals from the parental group were largely tumor free at the time when they reached humane endpoints; in contrast, most of the animals in the CD44<sup>-/-</sup> group contained up to 50% of tumor cells in the field of view (Fig. 5B and C).

Collectively, these data suggest that the <em>in vitro</em> studies closely correlate only with the outcomes of mesenteric metastasis, as this tissue mostly consists of mesothelial cells. However, <em>in vitro</em> models have failed to recapitulate metastatic success of CD44<sup>-/-</sup> cells at the remaining frequently colonized local peritoneal, as well as less frequently colonized distant sites. Our <em>in vivo</em> data suggest that the role of CD44 in formation and growth of metastatic lesions is more complex than previously appreciated and may depend on the cancer cell–microenvironment interactions specific for each site so that at some sites loss of CD44 is detrimental (mesenteries), whereas at others it is either beneficial for tumor growth (liver, omentum, kidney, and stomach), or it does not play a role (peritoneal wall/diaphragm, lymph node, oviduct, pancreas, and fat).

**Loss of CD44 induces expression of RARRES3, a gene predisposing to lung metastasis**

Our data indicate that loss of CD44 promotes lung metastasis (Fig. 5B and C). As ovarian cancer cells could arrive to the thoracic cavity by direct extension from the peritoneal cavity or through lymphatics, we postulated that one of the possible mechanisms could be related to increase of cell migration or degradation of extracellular matrix and invasion. However, neither the cell morphology, nor expression of mesenchymal state markers, nor cell migration, nor Matrigel invasion changed in CD44-deficient cells (Supplementary Fig. S10A–S10E).

More recent studies indicated a role for hematogenous route of metastatic ovarian carcinoma (48, 49). However, lower levels of CD44 protein significantly correlated with reduced vascular invasion in patients with serous ovarian cystadenocarcinoma (Supplementary Fig. S10F). We therefore hypothesized that loss of CD44 could be regulated through CD44-dependent signaling pathways. Thus, we hypothesized that loss of CD44 induced gene expression change that permitted formation and growth of the lung metastasis. Downregulation of RARRES3 in breast cancer (50, 51) was associated with increased metastasis to the lung. Expression of CD44 mRNA significantly associated with expression of RARRES3 mRNA in specimens of serous ovarian cystadenocarcinoma (Supplementary Fig. S10G), suggesting that some of these proteins could be regulated through CD44-dependent signaling pathways. Thus, we hypothesized that loss of CD44 induced gene expression change that permitted formation and growth of the lung metastasis. Downregulation of RARRES3 in breast cancer (50, 51) was associated with increased metastasis to the lung. Expression of CD44 mRNA significantly associated with expression of RARRES3 mRNA in specimens of serous ovarian cystadenocarcinoma (52, 53; Fig. 6A). In our model, protein expression of RARRES3 was significantly reduced in CD44-deficient cells (Fig. 6B) and in tumors formed in the mouse model (Fig. 6C). These data suggest that CD44 could serve as lung metastasis suppressor gene in EOC (Fig. 6D), and its loss may induce expression of genes associated with “lung metastasis gene signature”.

**Loss of CD44 induces loss of FAS, a gene associated with liver metastasis—specific gene signature**

Loss of CD44 expression resulted in a 3-fold increase in the metastasis to the liver (Fig. 4B). We hypothesized that loss of CD44 induced gene expression changes that enabled metastasizing cells to gain the ability to thrive in the liver microenvironment. Several studies have addressed the differential expression of genes, which predispose to liver metastasis from other cancers. Among these, downregulation of Fas cell surface death receptor (FAS) was associated with liver metastasis from colorectal and breast cancers (54–57). Using The Cancer Genome Atlas database we found that CD44 mRNA and protein significantly correlated with MRNA and protein for FAS (Fig. 7A), suggesting that loss of CD44 induced gene expression changes consistent with the phenotype highly adaptable to survival and proliferation within the host liver tissue. We further validated expression of FAS and found that it was downregulated in CD44<sup>-/-</sup> cells and tumor tissues formed by these cells, consistent with the expected pattern of expression in liver metastasis (Fig. 7B and C). These data suggest that CD44 is a liver metastasis suppressor in EOC (Fig. 7D), and its downregulation results in promoting the liver metastasis.
**Discussion**

Our data demonstrate that CD44 is a major regulator of spheroid formation from individual cells. Those with high expression of CD44 avidly formed spheroids, and loss of CD44 expression almost completely blocked spheroid formation. Cell lines with low CD44 expression tended to be less successful in forming spheroids, mostly remaining as individual cells in suspension. Our data show that either loss or hindering of CD44 with adhesion-blocking antibodies not only prevents cell–cell adhesion in the context of spheroid formation, but also impairs spheroid adhesion to mesothelial cells. Electron microscopy and

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**Figure 4.**
The parental and CD44+/− ES2 cells colonize intra- and retro-peritoneal sites with different efficiency. A, Silencing of CD44 significantly reduced colonization of the mesenteries. B, Silencing of CD44 increased colonization of the liver, omentum, kidneys, and stomach. C, Silencing of CD44 did not affect tumor burden formed at peritoneal wall and diaphragm, inguinal lymph node, pancreas, ovary/uterus, and abdominal fat. Both parental and CD44−/− demonstrate data for combined single cell (SC) and spheroid (Sph) groups. Tumor weights in each group were plotted and analyzed with Mann-Whitney U test. *, P < 0.05; **, P < 0.01; ***, P < 0.005. For lymph nodes involved with tumor, plotted weights reflect the total weight of the lymph node(s) and tumor within. For other organs, tumors were excised from the surface, weighted, and plotted. Typical images of the hematoxylin and eosin stained tumor tissues from the single-cell groups generated by Aperio ScanScope. Scale bar, 200 μm.
time-lapse imaging results suggest that loss of CD44 impairs membrane dynamics of the cells, evidenced by loss of pseudopodia and podosome formation, which precludes successful adhesion. However, it is likely that there are other molecular interactions, albeit rather minor, that allow covalent cell attachment in the absence of CD44, as evidenced in the case of CD44−/− SKOV-3 clones.

Our data indicate that CD44 is a key contributor to several aspects of ovarian cancer metastasis where this protein works either to promote or to suppress organ-specific metastatic spread. Among all parental tumors formed within the peritoneal cavity, lesions colonizing the mesentery, a fold of the peritoneum that attaches the stomach, small intestine, pancreas, spleen, and other organs to the posterior wall of the abdomen, in our models were, by far, the largest. Loss of CD44 reduced mesenteric tumor burden by half. It appears that disruption of CD44 could be beneficial in reducing mesenteric lesions formed by both spheroids.

The single layer of mesothelium outlines all other frequently colonized intraperitoneal organs. In striking contrast, loss of CD44 either did not affect their colonization or increased it several fold. Given that survival of the CD44−/− group was only

Figure 5.
Silencing of CD44 promotes metastases to the thoracic cavity in the ES2/athymic nude xenograft model. A, Tumors colonizing the superior mediastinum in the parental and CD44−/− groups were excised, weighted, plotted, and analyzed using Mann-Whitney U test. *** P < 0.005. B, Lungs from the parental and CD44−/− groups were removed from the sacrificed animals, paraffin preserved, sectioned, subjected to hematoxylin and eosin (H&E) staining, and examined by the pathologists. Percent of tumor cells within the area was assessed in five random fields (20× magnification on the objective), averaged, and plotted. C, Typical images of the H&E-stained lung tissues from the parental and CD44−/− groups generated by Aperio ScanScope. Scale bar, 200 μm. SC, single cell; Sph, spheroid. Dashed yellow line outlines areas involved by the tumor.
Figure 6.
Silencing of CD44 induces gene expression change promoting metastases to the lung. **A**, Correlation of expression of CD44 and RARRES3 in specimen from patients with serous ovarian cystadenocarcinoma (cBioportal.org, date of access 02-20-2019). **B**, Expression of RARRES3 was examined with Western blot (1:1,000 dilution) in the parental ES2 and C7 cells, analyzed with digital densitometry, plotted, and statistically analyzed using Mann–Whitney U test. **B**, *P < 0.01. β-Actin as used as loading control. **C**, Expression of RARRES3 was examined in the specimen of omental and lung metastases using immunostaining and RARRES3-specific antibodies (10 µg/mL, overnight, 4°C). Brown, RARRES3; blue, hematoxylin. The images were generated by Aperio ScanScope. Scale bar, 300 µm. Dashed lines outline tumor areas on the images of lung sections. **D**, Scheme depicting lung metastasis burden as a function of CD44 expression.
Figure 7. Silencing of CD44 induces gene expression change promoting metastases to the liver. **A**, Correlation of expression of FAS with CD44 in specimens from patients with serous ovarian cystadenocarcinoma (cBioportal.org; date of access 02-20-2019). **B**, Expression of FAS was examined with Western blot (1:500 dilution in 5% BSA) in the parental ES2 and C7 cells, analyzed with digital densitometry, plotted, and statistically analyzed using Mann-Whitney U test. **,**, \( P < 0.01 \). β-Actin was used as loading control. **C**, Expression of FAS was examined in the specimens of omental and liver metastases using immunostaining and FAS-specific antibodies (10 μg/mL, overnight, 4°C). Brown, FAS; blue, hematoxylin. The images were generated by Aperio ScanScope. Scale bar, 300 μm. **D**, Scheme depicting liver metastasis burden as a function of CD44 expression.

<table>
<thead>
<tr>
<th>Gene A</th>
<th>Gene B</th>
<th>Pearson</th>
<th>( P )</th>
<th>Spearman</th>
<th>( P )</th>
<th>Dataset</th>
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<tr>
<td>CD44</td>
<td>FAS</td>
<td>0.27</td>
<td>6.71e-10</td>
<td>0.32</td>
<td>5.57e-13</td>
<td>TCGA, Nature 2011, ( n = 563 ), mRNA</td>
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<td>CD44</td>
<td>FAS</td>
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<td>1.399e-6</td>
<td>0.38</td>
<td>2.84e-12</td>
<td>TCGA, Provisional, ( n = 606 ), mRNA</td>
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<tr>
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<td>FAS</td>
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<td>8.156e-5</td>
<td>0.29</td>
<td>2.691e-4</td>
<td>TCGA, Provisional, ( n = 174 ), protein</td>
</tr>
</tbody>
</table>
loss of FAS function was both necessary and sufficient (59, 60). It was reported in a mouse model of melanoma that the FAS/FASL receptor-ligand system is one of the key regulators shown to correlate with the propensity to colonize liver. The chyma may secrete factors that specifically impair differentiation (51). In addition, lung metastasis suppressor gene, reduction of which facilitates lung metastasis (63). In another breast cancer study the isoform switch from CD44v4 to CD44s suppressed lung metastasis (64). Mechanistically, yet another breast cancer study suggested that impairment of metastasis protective CD44-hyaluronan interactions could play a role in development of lung metastasis upon loss of CD44 (65). In pancreatic cancer, expression of CD44v6 and CD44v8 correlated with liver metastasis, whereas loss of CD44s also promoted liver metastasis (66). Taken together, these data may suggest that CD44s serves as a metastasis suppressor gene in many cancer types in which it commonly precludes colonization of liver and lung, and, additionally, omentum, kidney, and stomach in our model of EOC.

Studies in other cancer models suggest the existence of different clonal subpopulations of metastasizing cells with propensities to colonize different organs (67–69). It is not understood how metastatic colonization evolved in our model, whether the cells disseminated independently to different sites or first established themselves at one site and further colonized other organs. Nonetheless, it is very striking that a single genomic change was able to shift the balance of the usual route of metastatic development and could ultimately be responsible for formation and growth of metastases at different organ sites.

Our data suggest that CD44 plays a dual role in progression of EOC as follows: (i) it is a major regulator of spheroid formation and promoter of metastasis to mesenteries, (ii) it serves as a suppressor of metastatic growth at liver, omentum, kidney, and stomach, as well as a suppressor of formation and growth of thoracic metastases. It remains to be tested whether impairment of CD44 by other approaches, such as function-blocking antibodies (70), also results in unlocking the tumor suppressor function of CD44. Meanwhile, our studies indicate that silencing of CD44 as a therapeutic strategy in EOC may result in unintended consequences manifesting themselves as development and growth of metastasis at several peritoneal and distant organ sites.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Sacks Suarez, M.V. Barbolina
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Sacks Suarez, O. Elturri, H.-L. Xu, A.A. Kajdacy-Balla, M.V. Barbolina
Writing, review, and/or revision of the manuscript: J. Sacks Suarez, O. Elturri, M.V. Barbolina
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Sacks Suarez, O. Elturri, M.V. Barbolina
Study supervision: M.V. Barbolina

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References


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