Syngeneic Murine Ovarian Cancer Model Reveals That Ascites Enriches for Ovarian Cancer Stem-Like Cells Expressing Membrane GRP78

Lihong Mo1, Robin E. Bachelder1, Margaret Kennedy1, Po-Han Chen2,3, Jen-Tsan Chi2,3, Andrew Berchuck4, George Cianciolo1, and Salvatore V. Pizzo1

Abstract

Patients with ovarian cancer are generally diagnosed at FIGO (International Federation of Gynecology and Obstetrics) stage III/IV, when ascites is common. The volume of ascites correlates positively with the extent of metastasis and negatively with prognosis. Membrane GRP78, a stress-inducible endoplasmic reticulum chaperone that is also expressed on the plasma membrane (memGRP78) of aggressive cancer cells, plays a crucial role in the embryonic stem cell maintenance. We studied the effects of ascites on ovarian cancer stem-like cells using a syngeneic mouse model. Our study demonstrates that ascites-derived tumor cells from mice injected intraperitoneally with murine ovarian cancer cells (ID8) express increased memGRP78 levels compared with ID8 cells from normal culture. We hypothesized that these ascites-associated memGRP78+ cells are cancer stem-like cells (CSC). Supporting this hypothesis, we show that memGRP78 cells isolated from murine ascites exhibit increased sphere forming and tumor initiating abilities compared with memGRP78− cells. When the tumor microenvironment is recapitated by adding ascites fluid to cell culture, ID8 cells express more memGRP78 and increased self-renewing ability compared with those cultured in medium alone. Moreover, compared with their counterparts cultured in normal medium, ID8 cells cultured in ascites, or isolated from ascites, show increased stem cell marker expression. Antibodies directed against the carboxy-terminal domain of GRP78: (i) reduce self-renewing ability of murine and human ovarian cancer cells preincubated with ascites and (ii) suppress a GSK3α/akt/sna1 signaling axis in these cells. Based on these data, we suggest that memGRP78 is a logical therapeutic target for late-stage ovarian cancer. Mol Cancer Ther; 14(3); 747–56. ©2015 AACR.

Introduction

Ovarian cancer is the most lethal gynecologic malignancy (1). Seventy-five percent of patients with ovarian cancer are diagnosed at stage III/IV, with a long-term survival rate of 10% to 30% (2). Ascites occurs in 17% of patients with FIGO (International Federation of Gynecology and Obstetrics) stage I/II ovarian cancer and 89% of patients at stage III/IV (3). Standard management of ovarian cancer consists of surgical staging, operative tumor debulking, and intravenous chemotherapy (2). Although more than 80% of advanced stage patients benefit from first-line therapy (2), most patients suffer from recurrence within 18 months of diagnosis (4). This high relapse rate may result from failure of conventional therapy to remove cancer stem-like cells (CSC; ref. 5). CSCs are slow-cycling, therapy-resistant tumor cells capable of self-renewal (6, 7). CSCs play a crucial role in tumor initiation and metastasis (8).

Glucose-regulated protein 78 (GRP78) is a stress-inducible endoplasmic reticulum (ER) chaperone that is also expressed on the plasma membrane (memGRP78) of aggressive cancers (9, 10). GRP78 protects cells from stress by activating the PI3K/AKT pathway and inhibiting proapoptotic cascades. However, if the ER is severely stressed, GRP78 can promote cell death (9). Two functional domains of GRP78 have been characterized: an amino-terminal domain that drives PI3K/AKT activity (11, 12), and a carboxy (COOH)-terminal domain that promotes apoptotic signaling (13, 14). Our previous work demonstrates that targeting GRP78 with mouse IgGs against the GRP78 COOH-terminal domain causes tumor cell apoptosis by activating the caspase pathway (13, 14), resulting in delayed tumor growth, and prolonged survival in a mouse melanoma model (15).

GRP78 maintains survival of embryonic (16) and adult mammalian stem cells (17), and is highly expressed in hematopoietic stem cells (18). Overexpression of GRP78 correlates with malignant transformation in epithelial ovarian tumor cells (19), where as inducible knockout of GRP78 in the hematopoietic system suppresses Pten-null leukemogenesis (20). MemGRP78 is associated with increased cancer stemness in head and neck cancer (21). Based on these findings, in addition to our laboratory’s studies of memGRP78 functions in cancer (11–15, 22), in the current work, we investigated functions for memGRP78 in in ovarian cancer stemness.
To test the hypothesis that memGRP78 is an ovarian CSC marker, we utilized a syngeneic, immunocompetent ovarian cancer model (23). We chose this model because GRP78 auto-antibodies circulating in patients with cancer activate memGRP78 (11, 12, 24). We now demonstrate that memGRP78 expression is increased in ovarian cancer cells isolated from ascites in vivo and ovarian cancer cells treated with ascites in vitro. We also show that memGRP78 cells possess higher tumorigenic potential and stemness properties than memGRP78 cells. Neutralizing memGRP78 with anti-COOH terminal domain antibodies suppresses ovarian cancer cell sphere-forming ability. Our work is the first to demonstrate that memGRP78 CSCs are enriched by ascites and that memGRP78 is a logical target for eliminating ovarian CSCs.

Materials and Methods

Cell culture

ID8 cells (mouse epithelial ovarian cancer cell line obtained in 2006 from Dr. Kathy Roby, Kansas University Medical Center) were maintained in DMEM (high glucose, Gibco-Life Technologies) containing 4% FBS, 1% penicillin streptomycin. Comparison of these cells with the original published ID8 cell line is described (Supplementary Fig. S1). ID8-GFP was a gift from Dr. Brent Berwin (Dartmouth Medical School; 2007). Both ID8 and ID8-GFP cells were shown to be mycoplasma-free (March 20, 2010) and tested to be identical by STR DNA profiling (November 05, 2014). Human ovarian cancer cell lines [OvCar3 (January 20, 2014) and ES2 (June 06, 2013)] were purchased from Duke Cell Culture Facility (CCF) and cultured under ATCC-recommended conditions. OvCar3 (December 06, 2013) and ES2 (June 20, 2009) cells were shown to be mycoplasma-free and authenticated using STR DNA profiling before being frozen by Duke CCF. Fifty percent ascites treatment conditions were adjusted to normal culture conditions with regard to FBS and glucose concentrations.

Antibodies

Antibody sources were as follows: GRP78 N20 and C20 (Santa Cruz Biotechnology); OCT4, SOX9, and GSK3 (Millipore); CD133 and SCA1 (Biolegend); SNAI1, p-GSK3, AKT, and p-AKT (Cell Signaling Technology). GRP78 C38 and C107 antibodies were produced in our laboratory (15).

Mouse studies

Animal experimentation was performed according to the regulations of the Duke Institutional Animal Care and Use Committee. ID8 cells were injected into the mouse peritoneum of female 6- to 8-week-old C57BL/6 mice (Charles River). End points for euthanasia included lethargy, decreased motility, or cross-sectional abdominal diameter increase greater than 1/3.

Ascites

De-identified patient ascites samples (Ov476, Ov480) were obtained from informed subjects with FIGO stage IIIC grade 2 ovarian serous adenocarcinoma (Duke University IRB approved protocol Pro00013710). For murine ascites, mice were euthanized and peritoneal fluid was collected and centrifuged twice at 500 × g for 5 minutes to separate cellular and acellular fractions. Acellular fractions from multiple mice were pooled and filtered through 0.22 μm sterile filters.

Microarray analysis

Total RNA was isolated (Qiagen RNeasy Micro kit) according to manufacturer’s protocol and analyzed by Mouse Genome 430 2.0 Array (Affymetrix, GEO accession number GSE61285). Array data were then normalized by Robust Multiarray Averaging (RMA) for further analysis. RMA is a normalization procedure for microarrays that background corrects, normalizes, and summarizes the probe level information from the Affymetrix microarray data. To identify differentially expressed genes, we applied SAM 4.01 Excel Add-In (25) that provides the estimate of FDR for multiple testing. Using the FDR threshold of 2.5%, we identified 1,257 probesets whose expression values were extracted, mean-centered and clustered by Cluster 3.0, and viewed by Treeview v1.6 (26). These selected genes (Supplementary Table S1) were then deposited into Gather (gather.genome.duke; ref. 27) to determine the enrichment for the Gene Ontology (GO) and KEGG. For the stemness score, the mean expression level of 38 stemness-related genes was calculated as “stemness score” in a multi-gene signature approach (28, 29) and compared by t test to avoid quantitative bias from any single gene.

Flow cytometric analysis

ID8 cells (95% confluence) were harvested using dissociation buffer (Gibco). C20 was used for memGRP78 staining. 7-AAD (BD Biosciences) was used to exclude dead cells. Cells were fixed and permeabilized using Cytofix/Cytoperm Kit (BD Biosciences) before OCT4 staining. Antibody binding was assessed using a Guava EasyCyte Plus flow cytometer (Millipore).

Cell sorting

Red blood cells were removed from the cellular fraction of murine ascites using RBC lysis buffer (BD Biosciences). Remaining cells were prepared with Fc block and anti-CD11b antibody coated magnetic beads (558013; BD Biosciences) and macrophages were removed with a BD magnet. The remaining sample was stained with F4/80, 7AAD, and GRP78 N20 and sorted by Duke Cancer Institute Flow Cytometry Shared Resource to select for the F4/80−, 7AAD−, and memGRP78− or F4/80+, 7AAD+ and memGRP78+ populations.

Sphere formation assay

Single-cell suspensions were plated in 24-well ultraslow attachment plates at 6 × 10^3 cells per well (ID8 and OvCar3) and 1 × 10^3 cells per well (ES2) in primary assay. Cells were grown in serum-free DMEM supplemented with 1% methyl cellulose (Sigma-Aldrich), B27 (Invitrogen), 20 ng/ml EGF, 20 ng/ml bFGF (BD Biosciences), and 4 μg/ml bovine insulin (Invitrogen). Spheres were cultured for 7 to 14 days, after which all spheres (diameter > 50 μm) were counted in the well. For serial passages, spheres were dissociated to single cells with trypsin, and 6 × 10^3 cells plated and cultured in ultraslow attachment plates for 14 days.

DID retention

ID8 cells were labeled with DiD dye (Life Technologies) using the manufacturer’s protocol. Cells were split into two groups being cultured in either medium or 50% acellular ascites. DiD intensity was measured on day 7. Similar experiments were performed with DiO, Dil (Life Technologies), and Cyto-ID Red (Enzo Life Sciences).

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Statistical analysis
Prism (version 5.0; GraphPad Software) was used to perform statistical analyses. Two-tailed Student \( t \) test was used to test differences in sample means for data with normally distributed means. The \( \chi^2 \)-test was used to evaluate the survival difference in animal studies. The likelihood ratio test of single-hit model was performed as a standard statistical analysis method for limiting dilution studies. \( P \) values less than 0.05 were considered statistically significant.

Results
Acellular ascites increases ovarian cancer cell stemness
A syngeneic mouse model was established by injecting murine epithelial ovarian cancer cells (ID8 cells) intraperitoneally into C57BL/6 mice. We observed a strong linear regression correlation between the volume of ascites and tumor burden (\( R^2 = 0.49; \) Supplementary Fig. S2A). Mice had shortened survival when injected with ID8 cells isolated from ascites or with ID8 cells plus ascites supernatant from ID8 tumor–bearing mice as compared with mice receiving ID8 cells from normal culture (Supplementary Fig. S2B). These results indicate that acellular ascites derived from ID8 tumor–bearing mice is capable of driving ovarian cancer progression.

We next tested the effect of ascites on ID8 cell self-renewing ability. Equal numbers of ID8 cells from normal culture, ID8 cells isolated from ascites, or ID8 cells preincubated with ascites for 7 days were seeded into sphere culture. Increasing concentrations of ascites were associated with enhanced sphere-forming ability (Supplementary Fig. S3A and S3B). We chose 50% ascites for further studies because it effectively increased \( \text{memGRP78} \) expression and sphere-forming ability of these cells. For all conditions, glucose and FBS concentration was normalized to that of control medium.

ID8 cells isolated from ascites and ascites-preincubated ID8 cells exhibited increased sphere-forming ability compared with ID8 cells in regular medium (Fig. 1A). Of note, our sphere assay includes methylcellulose to avoid nonspecific cell aggregation (30). As shown in Fig. 1A, primary spheres, when dissociated, effused into well-defined secondary spheres, thus displaying CSC behaviors. Protein concentration was normalized with albumin as a control for the 50% ascites treatment, and no significant difference from normal cell culture was detected (Supplementary Fig. S3C).

To confirm that ascites increases sphere-forming ability of ovarian cancer cells, we employed a competition strategy between ascites-preincubated and -untreated cells. ID8-GFP cells, which share the same proliferation rate as ID8 cells (data not shown), were preincubated with acellular ascites for 7 days and then mixed 1:1 with untreated ID8 cells. The cell mixture was seeded into a sphere assay. Serial passage of primary sphere cells into a secondary sphere assay was also performed. Pictures were taken from 5 different fields (Fig. 1B, left plot) and the percentages of ID8-GFP and ID8 cells from sphere assays were quantified. As shown in Fig. 1B, spheres are composed mostly of ascites-preincubated ID8-GFP cells.

To test whether increased sphere-forming ability was reversible by removing ascites, we recultured ID8 cells isolated from ascites in ascites-free medium or removed ascites from ascites-treated ID8 cells. In both situations, sphere-forming ability of ID8 cells was decreased significantly (Fig. 1C).

Increased sphere-forming ability of ascites-preincubated ID8 cells could reflect either ascites stimulation of CSC signaling or ascites enrichment of a stem cell population. To differentiate between these possibilities, we included ID8 cells exposed to acellular ascites for 4 hours, a short incubation promoting signaling but not sufficient for enrichment of a tumor cell subpopulation. Sphere-forming ability of ID8 cells exposed to ascites for 4 hours was similar to that of untreated ID8 cells (Fig. 1A), supporting the enrichment hypothesis. After 7 days ascites treatment, 34.5% ID8 ovarian cancer cells were Annexin V positive compared with 7.2% ID8 cells in normal medium (Fig. 1D). Collectively, our findings suggest that ID8 ovarian cancer cells are heterogeneous. Although bulk tumor cells do not survive in an ascites microenvironment, a subpopulation of cells with cancer-stem-like behavior survives ascites exposure.

To provide further evidence for ascites enrichment of a slow-cycling CSC population, we labeled ID8 cells with a lipophilic tracer (DiD) that is diluted in proliferating cells, but maintained in nonproliferating/slow-proliferating cells. We detected 0% DiD\(^+\) cells in ID8 cells cultured for 7 days in normal medium. In contrast, 66.7% of cells treated with 50% acellular ascites for 7 days were DiD\(^+\) (Fig. 1E). Similar results were observed using 3 other lipophilic dyes (data not shown).

To begin to validate our findings in human ovarian cancer, human ovarian cancer cell lines were preincubated with either of two patient ascites samples. Notably, these human ascites samples increased sphere-forming ability of both human ovarian cancer lines compared with untreated cells (Fig. 1F and G).

Microarray analysis of stemness-related genes in ascites-treated ID8 cells
We performed microarray analysis on untreated and asites-preincubated ID8 cells (Fig. 2A). Gene expression profiles were interrogated with Affymetrix mouse 430A2 arrays (GEO accession number GSE61285) and normalized by RMA. To identify differentially expressed genes while considering FDR, we used SAM (25) to identify 1,257 probesets (707 induced and 550 repressed; Supplementary Table S1) with 2.5% of FDR. Expression of these genes was extracted, mean-centered, and clustered to generate the overview heatmap (Fig. 2A). GO and KEGG enrichments of these selected genes were performed (GEO accession number GSE61285).

We next investigated how ascites treatment of ovarian cancer cells affected expression of 38 genes that were previously reported to associate with cancer stemness (31). To avoid the bias of any individual stem-related gene, we took a multigene "signature" approach that provides a quantitative measurement of the "stemness" of each sample (32). When the mean expression values of all 38 genes were calculated as a "stemness-score," ascites-treated samples had a significantly higher stemness-score than control cells (Fig. 2B; Supplementary Table S2). The expression of 11 stemness genes (Sca-1/Ly6a, Albhu1a/b, Vegfa, Sna1, Sox9, Krt14, Cdh4, Kit, Cad43, Kitl, and K671) was upregulated by ascites (Fig. 2C). Sca-1 (Ly6a), stem cell antigen 1, is a murine CSC marker (32, 33). Sna1 contributes to a stem-like phenotype in ovarian cancer (34–36). Sox9 converts differentiated breast cancer cells to CSCs (36).

To show that these stemness genes were upregulated at the protein level in ascites-treated ovarian cancer cells, we next performed flow cytometry and Western blotting. By flow cytometry, we detected increased SCA1 expression in ascites-treated...
**Figure 1.**

Ascites enriches for CSCs. A, ID8 cells were cultured for the indicated times in medium or 50% acellular ascites from ID8-bearing mice. A total of $6 \times 10^3$ cells per well were seeded in a primary sphere assay, and then harvested, trypsinized, and passaged to form secondary spheres. Y axis represents spheres $/C^2/50 \mu m$ and SD. B, competition between ascites-pretreated and -untreated ID8-GFP cells in sphere formation. Left, ID8-GFP cells were pretreated with acellular ascites derived from tumor-bearing mice for 7 days and then mixed 1:1 with untreated ID8 cells. The mixture was seeded into a sphere assay (primary spheres). On day 7, primary spheres were trypsinized and seeded into a sphere assay (secondary spheres). Pictures were taken from 5 random fields. A representative field is shown for primary spheres at 2.5x, secondary spheres at 2.5x, primary spheres at 20x, and secondary spheres at 20x. Right, quantification of percentage of ID8-GFP and ID8 cells in 5 fields at 20x magnification from primary and secondary sphere assays. Red arrows, GFP$^+$ spheres; white arrows, GFP$^-$ spheres. C, culturing in vivo ascites cells in vitro for 7 days (recultured; left plot) or reculturing ID8 cells pretreated with ascites for 7 days (ascites treated 7 days) in culture for 9 days (ascites off 9 days; right plot) decreases their sphere-forming ability. Error bars, SD from 3 trials in triplicate. D, after 7-day ascites treatment, 34.5% of ID8 cells became Annexin V positive, whereas 7.7% ID8 cells were positive in normal culture. E, ID8 cells were labeled with DiD on day 0 and split into two groups, receiving either medium or 50% ascites for 7 days. The majority of ascites-treated ID8 cells maintained DiD label on day 7, whereas most ID8 cells in medium lost the dye. F and G, OvCar3 or ES2 cells were pretreated with 50% ascites from either of two patients with ovarian cancer (Ov476, Ov480) for 7 days, and their ability to form spheres was determined. Error bars, SD from 3 different trials in triplicate for this figure.
Figure 2. Incubating murine ovarian cancer cells with ascites increases stemness gene expression. A, hierarchy cluster of 1,257 probesets identified by SAM to be differentially expressed between ID8 cells (n = 3) and ascites-treated ID8 cells (n = 2). The heatmap showed the mean-centered expression values of the selected genes (red, induced; green, repressed). B, expression levels of 38 stemness-associated genes were extracted, mean-centered, and arranged by hierarchical clustering (Supplementary Table S2). C, stemness-associated genes significantly upregulated after ascites pretreatment of tumor cells for 7 days. D, SCA1 expression on ID8 cells (Medium), ID8 cells treated with ascites for 7 days (Ascites treated), and ID8 cells from ascites (In vivo). Representative of 3 different experiments. E, Western blotting for SNAI1 and SOX9 in ID8 cells and ID8 cells treated with ascites for 7 days. Quantification was by densitometry. Error bars, SD from 3 trials in triplicate.
ID8 cells, as well as in ID8 cells harvested from ascites in vivo compared with that in control ID8 cells (Fig. 2D). Compared with normal culture, ascites treatment also increased expression of SNAI1 and SOX9 significantly (Fig. 2E).

MemGRP78 expression in ascites-associated ovarian cancer cells

We next studied ascites effects on memGRP78 expression. MemGRP78 levels were significantly higher in ID8 cells isolated from ascites compared with that in ID8 cells cultured in normal medium (Fig. 3, top plot). When in vivo cells were cultured in normal medium for 7 days, the memGRP78 level shifted back to parental cell levels, leading us to hypothesize that survival of a memGRP78-expressing ovarian cancer cell subpopulation is supported by soluble ascites factors. To test this hypothesis, we cultured ID8 cells with acellular ascites for 7 days and found that the % memGRP78 cells increased significantly from 7.5% (parental) to 43.2% (ascites 7 days), almost reaching the level of in vivo tumor cells (Fig. 3, bottom). Removal of ascites from these cells for 9 days restored memGRP78 expression to baseline levels (ascites off 9 days). Notably, memGRP78 levels remained at baseline following short-term ascites exposure (Fig. 3, bottom). The reversibility in memGRP78 induction by ascites correlates with the reversibility of ascites-enhanced sphere-forming ability of ID8 cells (Fig. 1C), supporting the hypothesis that memGRP78 is an ovarian CSC marker.

MemGRP78 is associated with stemness

To further test whether memGRP78 is a stem cell marker in murine ovarian cancer cells, we sorted ascites-derived tumor cells into memGRP78+ and memGRP78− cells and characterized their self-renewing activity in a sphere assay. 7AAD− dead cells and F4/80+ macrophages were excluded, and the gate for memGRP78+ and memGRP78− was set at the extreme ends to insure purity (Fig. 4A; ref. 37). Although memGRP78− cells proliferated slower than memGRP78+ cells and unsorted tumor cells (Supplementary Fig. S4), memGRP78+ cells formed more spheres than memGRP78− cells (Fig. 4B). These studies suggest that memGRP78+ ovarian cancer cells are similar to CSCs, which are characterized by their slow-cycling cells capable of sphere formation (6–8).

We then performed double staining of memGRP78 and two stem cell markers [Octamer-binding transcription factor 4 (OCT4; ref. 38) and CD133 (Prom1; ref. 39)]. Acellular ascites treatment for 7 days increased the OCT4/memGRP78 and CD133/memGRP78 double-positive populations. ID8 cells isolated from ascites had 4.9% memGRP78+/OCT4+ cells (Fig. 4C) and 7.8% CD133+/memGRP78+ cells (Fig. 4C).

Limiting dilution transplantation assay is a standard method for assessing tumor-initiating activity associated with cancer stemness. We investigated the ability of memGRP78+ and memGRP78− cells to initiate tumor growth when injected at cell numbers ranging from 10³ to 10⁶. More mice developed tumors or tumor-associated ascites in memGRP78+ cells injected groups than those injected with memGRP78− cells at 10³ to 10⁵ injection numbers (Table 1). The likelihood ratio test of single-hit model was performed as a standard statistical analysis method for limiting dilution studies of stem cells (40) and detected P < 0.05 between memGRP78+ versus memGRP78− cells. Moreover, mice bearing 10⁶ and 10⁵ memGRP78− cells died sooner than those bearing memGRP78+ cells (Fig. 4D).

GRP78 antibodies suppress sphere formation

Monoclonal antibodies developed by our laboratory (C38 and C107), and a commercial antibody (C20) directed against the COOH-terminal domain of GRP78, suppress tumor growth in
Melanoma and prostate models (13, 15). We studied effects of these antibodies on ovarian cancer cells. ID8 cells were treated for 7 days with 50% acellular ascites. Antibodies (C38, C107, isotype control at 10 μg/mL; C20 or goat IgG at 5 μg/mL) were added on day 5. On day 7, each group was harvested and sphere-forming ability was determined. Ascites treatment significantly increased sphere number. Notably, C38 and C20 antibodies, but not C107, decreased sphere numbers (Fig. 5A).

To test antibody activity in vivo, 400 μg antibody per mouse was injected intraperitoneally 5 days before implanting 10^5 ascites tumor cells. Following implantation, 200 μg antibody was delivered intraperitoneally every other week until the endpoint. Mice receiving C38 or C107 antibodies had significantly lengthened survival (C38 median = 64 days, C107 median = 64 days) compared with those receiving IgG2b (median = 55 days; Fig. 5B).

We next investigated signaling pathways associated with memGRP78-expressing ovarian CSCs. Ascites treatment of ID8 cells significantly increased AKT and GSK3α phosphorylation (Fig. 5C), and GSK3α phosphorylation/inactivation led to increased SNAI1 levels (41, 42), associated with enhanced CSC behaviors (35, 43). Notably, C20 blocked these signaling events by binding to memGRP78 (Fig. 5C and D; refs. 13, 14). These data suggest that a memGRP78–AKT–GSK3–SNAI1 signaling axis is associated with stemness of ascites-derived murine ovarian cancer cells.

To begin to validate our findings in human ovarian cancer cell lines, we incubated OvCar3 or ES2 cells with acellular human ascites and treated them with either C38, C107, C20, or isotype control IgG. C38, C107, and C20 suppressed sphere-forming ability of ascites-pretreated OvCar3 and ES2 cells (Fig. 5E and F).

**Discussion**

We demonstrate that treatment of murine ovarian cancer cells with acellular ascites enriches for memGRP78^+ cells with CSC properties. We also show an ability of human ascites to enrich for human ovarian CSCs in vitro. Further studies are needed to show that ascites enriches for a CSC population relevant to human ovarian cancer disease. Collectively, our findings raise the important question of whether blocking the occurrence of ascites and/or memGRP78

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**Table 1.** memGRP78^+ or memGRP78^- tumor cells were sorted from ascites generated in ID8 cell-bearing mice.

<table>
<thead>
<tr>
<th>Cells injected</th>
<th>memGRP78 positive</th>
<th>memGRP78 negative</th>
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<tbody>
<tr>
<td></td>
<td>Tumor detected</td>
<td>Tumor take rate</td>
</tr>
<tr>
<td>10^6</td>
<td>9/9</td>
<td>100%</td>
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<td>10^4</td>
<td>7/10</td>
<td>70%</td>
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<td>10^3</td>
<td>2/10</td>
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NOTE: Mice were injected intraperitoneally with 10^6 to 10^8 of these sorted cell populations, and tumor take rate was determined. The likelihood ratio test of single-hit model was performed and showed P < 0.05.

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Figure 4. memGRP78^+ cells exhibit increased sphere-forming ability/tumor-initiating activity compared with memGRP78^- cells. A, gating for memGRP78^+ and memGRP78^- cells from ascites cells generated in ID8 cell-bearing mice. B, sphere-forming ability of memGRP78^+ and memGRP78^- gated tumor cells. 10^5 cells per well were seeded in a sphere assay. Primary spheres were trypsinized and passaged to form secondary spheres. Y axis represents spheres ≥ 50 μm in diameter.
Figure 5.
GRP78 antibodies suppress ovarian cancer sphere-forming ability and prolong mouse survival in a syngeneic model of ovarian cancer. A, ID8 cells were pretreated for 7 days with ascites obtained from ID8-bearing mice plus 10 μg/mL C38, mouse isotype control (IgG2b), 5 μg/mL C20, goat IgG (Goat), or no added antibody (Blank) for 2 days. Y axis represents spheres ≥50 μm. Error bars, SD from 3 trials in triplicate. B, mouse study was performed on 4 groups of mice (n = 10/group) receiving C38, C107, IgG2b control, or PBS. We injected 400 μg antibody or PBS per mouse 5 days before implanting 10⁵ ID8 cells isolated from ascites. Following implantation, 200 μg antibody or 200 μL PBS was delivered intraperitoneally every other week until the endpoint. C, C20 or goat IgG was added to ID8 cells from medium or 7-day ascites pretreatment conditions. After 2 days, cells were lysed and extracts probed for phospho-AKT, phospho-GSK3, GSK-3, and SNAI1 by Western blotting. A representative blot is shown. D, densitometry of three independent experiments (Western blotting). E and F, OvCar3 or ES2 cells were incubated with Ov480 ascites for 7 days plus 10 μg/mL C38, C107, mouse isotype control (IgG2b), 5 μg/mL C20, goat IgG, or no antibody (Blank) for 2 days. Y axis represents spheres ≥50 μm. Error bars represent SD from 3 trials in triplicate.

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clinical removal of ascites may be a useful adjuvant to other ovarian cancer therapies.

We are currently investigating which ascites soluble factor(s) maintain ovarian CSCs. GRP78 ligands are potential candidates. We identified in murine ascites several GRP78 ligands [anti-GRP78 antibodies, alpha-2-macroglobulin (α2M) and murinoglobulin] that activate the amino terminal domain of GRP78 (Supplementary Fig. 5S; ref. 11). Further studies are needed to determine the importance of these ascites factors in the maintenance of ascites-enriched ovarian CSC.

GRP78 is generally associated with proproliferative activities (11, 12). However, CSCs are slow-cycling cells (6, 7). Although forming tumors faster in vivo, memGRP78+ cells have a slower proliferation rate than memGRP78− cells (Supplementary Fig. S4). Interestingly, the DiD retention study showed that most of the ascites-pretreated ID8 cells were slow-cycling cells (Fig. 1E), correlating with the finding that memGRP78+ ovarian cancer cells are slowly proliferating.

Our studies show that memGRP78+ murine ovarian cancer cells exhibit increased tumorigenicity compared with memGRP78− cells (Table 1). These results confirm similar findings in head and neck cancer (21), but differ from a study showing that GRP78+ colon cancer cells exhibit reduced tumorigenicity compared with GRP78− cells (44). We attribute differences between these studies to the fact that our work, but not the study of Hardy and colleagues, excluded dead cells during sorting. Dead cells expose their endoplasmic reticulum, which is a major source of GRP78. The findings of Hardy and colleagues (44) are likely complicated by their inclusion of a large percentage of dead GRP78+ cells in their tumor injection study.

Cancer stem cells are a heterogeneous population consisting of cells in different differentiation stages, each stage expressing distinct CSC markers (45). The difference in % SCA1+ cells found in vivo versus in vitro likely reflects the fact that (i) SCA1 is expressed only during specific CSC differentiation stages and (ii) this SCA1-expressing stem cell subpopulation is represented more frequently in our in vitro ascites enrichment model than in the in vivo model. This phenomenon may be attributable to microenvironmental regulation of CSC differentiation state. In contrast, memGRP78 is expressed equally on cancer cells from our in vitro model and from ascites cells in vitro, suggesting that memGRP78 is a universal murine CSC marker.

We demonstrated that ascites increased cancer stem cell markers (SOX9 and SNAI1) in ID8 cells (Fig. 2D). Activation of GSK3, a differentiation-related gene that is inactivated by AKT (46), reduces Snail-1 mRNA and SNAI1 protein levels (41, 42). Antibodies against the COOH-terminal GRP78 domain blocked AKT and GSK3β phosphorylation, thus reducing SNAI1 expression level and stem-cell activities. These data demonstrate efficacy of these GRP78 antibodies in reducing CSC markers in murine ovarian cancer cells.

Our in vivo study, showing that a GRP78+ COOH terminal domain antibody (C38) prolonged survival of ovarian cancer-bearing mice, indicates a potential clinical application of targeting memGRP78. Although C107 only showed a modest effect on sphere formation in vitro (Fig. 5A), mice receiving this antibody survived longer than control mice. This finding may be attributable to different conformations of memGRP78 on tumor cells in vitro versus in vivo, with the epitope for C107 being more accessible in vivo. We believe that efficacy of GRP78 antibodies will be increased when combined with chemotherapy. According to our model, chemotherapy should target the fast proliferating tumor bulk cells, whereas anti-COOH terminal domain GRP78 antibodies will target CSCs. Combination studies are currently under investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: L. Mo, R.E. Bachelder, A. Berchuck, S.V. Pizzo

Development of methodology: L. Mo, R.E. Bachelder, S.V. Pizzo

 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Mo, A. Berchuck

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Mo, R.E. Bachelder, M. Kennedy, P-H. Chen, J-T. Chi, S.V. Pizzo

Writing, review, and/or revision of the manuscript: L. Mo, R.E. Bachelder, M. Kennedy, A. Berchuck, G. Cianciolo, S.V. Pizzo

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Mo, R.E. Bachelder, M. Kennedy, P-H. Chen, J-T. Chi, A. Berchuck, G. Cianciolo, S.V. Pizzo

Study supervision: R.E. Bachelder, S.V. Pizzo

Acknowledgments

The authors thank the Duke Microarray Core facility for technical support and the generation of microarray data. They also thank Dr. Xiao-fan Wang, Dr. Gustaf de Ridder, Dr. Rupa Ray, and Sturgis Payne for providing comments. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 3, 2014; revised December 23, 2014; accepted December 29, 2014; published OnlineFirst January 14, 2015.

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Lihong Mo, Robin E. Bachelder, Margaret Kennedy, et al.


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