The impact of the ovarian microenvironment on the anti-tumor effect of SPARC on ovarian cancer

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The impact of the ovarian microenvironment on the anti-tumor effect of SPARC on ovarian cancer

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Abstract

A lack of host-derived SPARC promotes disease progression in an intraperitoneal (IP) ID8 mouse model of epithelial ovarian cancer (EOC). Since orthotopic injection (OT) of ID8 cells better recapitulates high-grade serous cancer, we examined the impact of host-derived SPARC following OT injection. Sparc−/− and WT mice were injected with ID8 cells either OT or IP and tumors were analyzed at the moribund stage. Sparc−/− mice had reduced survival and fewer well-defined abdominal lesions compared to WT controls after IP injection, whereas no differences were observed in survival or abdominal lesions between Sparc−/− and WT mice after OT injection. No differences in mass or collagen content were observed in ovarian tumors between OT-injected Sparc−/− and WT mice. The abdominal wall of the IP injected Sparc−/− mice exhibited immature and less abundant collagen fibrils compared to WT mice both in injected and non-injected controls. In contrast to human EOC, SPARC was expressed by the tumor cells but was absent in reactive stroma of WT mice. Exposure to the ovarian microenvironment through OT injections alters the metastatic behaviour of ID8 cells, which is not affected by the absence of host-derived SPARC.

Keywords: SPARC, Epithelial Ovarian Cancer, Mouse model
Background

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy amongst North American women (Canadian Cancer Society's Steering Committee 2010; Jemal et al. 2010). The course of the disease involves initial growth of primary lesions within the adnexal microenvironment followed by metastasis throughout the abdominal cavity. Metastasis involves exfoliation from the primary tumor, dissemination throughout the abdominal cavity, attachment to peritoneal surfaces and invasion of the underlying stroma. From these sites of attachment, secondary lesions develop that further contribute to ascites formation, thereby exacerbating metastasis. Understanding stromal factors that enable or facilitate cancer cell invasion and metastasis is critical to the development of therapeutic targets for disease management.

Several studies have highlighted the potential role of matricellular glycoproteins in cancer progression (Chlenski and Cohn; Song et al. 2008; Wei et al. 2010). These diffusible extracellular matrix (ECM) elements modulate cellular dynamics by binding to transmembrane receptors, growth factors, cytokines, proteases, and the matrix scaffold. A prototypical matricellular glycoprotein implicated in suppression of ovarian cancer progression is SPARC (Secreted Protein, Acidic, Rich in Cysteine)/osteonectin. SPARC expression in EOC cells is decreased compared to non-malignant ovarian epithelial cells (Mok et al. 1996), which has been attributed to methylation-dependent epigenetic silencing of SPARC (Socha et al. 2009). Over-expression of SPARC in SKOV3 cells reduced cell proliferation and inhibited the ability of these cells to form tumors in nude mice (Mok et al. 1996). Similarly, Said et al found that ectopic SPARC expression in these cells induced pleiotropic effects consistent with tumor suppressive activity, including reduction of lysophosphatidic acid (LPA)-induced cell proliferation, chemotaxis, and invasion (Said et al. 2007b). SPARC also attenuated macrophage and
mesothelial cell-induced production of IL-6, urokinase plasminogen activator, matrix metalloproteinases, and prostanoids (Said et al. 2008), and suppressed anchorage dependent AKT and MAPK pathways in response to serum and epidermal growth factors (Said et al. 2007a). Treatment of ovarian cancer cell lines in vitro with exogenous SPARC resulted in decreased proliferation and apoptosis (Bull Phelps et al. 2009; Said and Motamed 2005; Socha et al. 2009; Yiu et al. 2001), indicating that these effects are likely mediated through extracellular effects of SPARC and that the findings can be extended to additional ovarian cancer cell lines. Altogether, these studies constitute compelling evidence for an antitumoral effect of SPARC in EOC.

*In situ* hybridization studies of patient samples revealed strong expression of SPARC transcripts confined to the reactive stroma of primary EOC tumors (Brown et al. 1999). Subsequent studies have shown a similar upregulation of SPARC protein in reactive stroma in EOC (Paley et al. 2000); however, this upregulation was not observed in another immunohistochemical study (Yiu et al. 2001). High levels of SPARC in tumor-associated stroma have been described for several solid tissue cancers including pancreatic, breast, and lung cancers. While the role of increased SPARC in these cancers is unclear, several studies in breast carcinoma indicate that increased stromal SPARC expression is associated with increased patient survival (Barth et al. 2005; Beck et al. 2008; Bergamaschi et al. 2008). Increased stromal SPARC expression appears to represent a response intended to contain the tumor. Data from *Sparc*−/− mice are consistent with a tumor suppressive role for SPARC in EOC. *Sparc*−/− mice injected intraperitoneally (IP) with spontaneously transformed ID8 murine EOC cells progressed to a moribund state more rapidly than wild-type control animals (Said and Motamed 2005).
We have previously reported that orthotopic (OT) injection of ID8 cells into the ovarian bursa of mice models high-grade serous ovarian cancer as demonstrated by the formation of numerous peritoneal lesions, ascites, and formation of large ovarian lesions with histology consistent with high-grade serous carcinoma (Greenaway et al. 2008).

Since OT administration of ID8 cells better reflects human EOC than IP injection, we compared the impact of an absence of stromal SPARC on disease progression when mice were injected OT and IP with ID8 cells. Our findings indicate that EOC progression is unaffected by host-derived SPARC when ID8 cells are orthotopically administered; and, we identify limitations regarding the use of this mouse model to test and interpret the effects of stromal SPARC expression on EOC progression.

**Methods**

**Animal Model**

C57Bl6 X 129SvJ *Sparc*−/− and wild-type (WT) mice were provided by Dr. Helene Sage (Benaroya Research Institute, Seattle, WA) and housed at the Division of Comparative Medicine, University of Toronto, under the guidelines of the Canadian Council for Animal Care. Mice were back-crossed against wild-type C57Bl6 mice for at least 10 generations before experimental use. ID8 cells, a spontaneously transformed mouse ovarian surface epithelial cell line (MOSEC), were provided by Drs. Katherine Roby and Paul Terranova (University of Kansas) and cultured in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. *Sparc*−/+ mice (n=10) and aged-matched WT controls (2-4 months of age, n=10) were anesthetized with isoflurane and a single dorsal midline incision was made to allow for ovarian access. ID8 cells (1.0X10⁶ in 5 µl of PBS) were injected into the ovarian bursa with a Hamilton syringe and a 30-gauge needle. All
mice were allowed to recover and monitored closely for the next 24 hrs. A second experimental arm consisted of Sparc−/− (n=10) and WT (n=10) mice that were injected with 1.0×10^6 ID8 cells in 200 µl PBS by intraperitoneal (IP). All animals were weighed and given health assessments three times a week. Moribund animals were identified based on ascites accumulation that led to increased abdominal girth and weight gain of 30%. Moribund mice were subsequently euthanized by CO₂ asphyxiation. Ascites fluid volume, ovarian tumor weight, contralateral tumor weight and girth measurement were quantified at necropsy. Following an abdominal flush with PBS, abdominal wall hemorrhage (petechiae) was scored: (0) no petechiae evident, (1) petechiae barely visible and (2) petechiae prevalent.

**Histology**

At sacrifice, ovarian and abdominal wall tissues were fixed with 10% formalin, embedded in paraffin and 5 µm sections were prepared. Sections were stained with hematoxylin and eosin (H&E) (Sigma) or Masson’s trichrome (blue collagen) (Fisher Scientific) or picrosirius red (Sigma). Masson’s trichrome kit was used as according to manufacturer’s instructions (Fisher Scientific). Images were collected at low magnification (20X) to visualize the maximal area of tumor tissue. Collagen content following Masson’s trichrome staining was quantified by colour thresholding and reported as collagen positive area relative to total tumor area using Northern Eclipse Software (Empix Imaging). Collagen fiber maturity was determined using picrosirius red staining followed by polarizing light microscopy (Pickering and Boughner 1991).
Immunohistochemistry

For immunohistochemical analyses, antigen retrieval was performed by treatment with citrate buffer heated to 90°C (SPARC, Ki67) or enzymatic retrieval with 0.2% bovine hyaluronidase at 37°C (fibronectin). Sections were incubated overnight with primary antibodies against SPARC (R&D systems; goat polyclonal), Ki67 (Abcam; rabbit polyclonal) or fibronectin (Abcam; rabbit polyclonal) followed by species-appropriate biotinylated secondary antibodies and finally peroxidise-conjugated streptavidin. Immunocomplexes were detected by 3’,3’-diaminobenzidine treatment and counterstaining with hematoxylin. For immunofluorescent staining, endogenous autofluorescence was quenched using 0.2% sodium borohydride and sections were blocked in 5% normal serum. Sections were incubated with the endothelial-specific fluorescent conjugate isolectin IB4 (Sahagun et al. 1989) for four hours at room temperature (Invitrogen) followed by DAPI counterstaining. Photomicrographs were obtained at 200X magnification with an Olympus BX-61 fluorescent microscope. Blood vessels were identified as single endothelial cells or cluster of cells with or without visible red blood cells. Microvessel area was measured relative to the total tissue area using integrated morphometry software (Metamorph). A minimum of 5 random fields of view were evaluated for each tissue (n=10 for each genotype).

Western Blotting

ID8 cells were cultured in serum-containing (10% FBS) or serum-free conditions. Cells were lysed using ice-cold RIPA buffer (25 mM Tris-HCl; pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Protein concentrations were determined by BCA reaction kit (Pierce) and equal amounts of total protein lysates were resolved on 10% SDS-PAGE gels and transferred to Immobilon polyvinylidene...
difluoride (PVDF) membranes. Membranes were blocked in 3% (wt/vol) skim milk for 1 hr and incubated overnight with SPARC primary antibody (AF941, R&D Systems; goat polyclonal. Peroxidase-conjugated secondary antibody was applied for one hour at room temperature. Samples were also probed using a GAPDH antibody (MAB374, Millipore) to enable loading of equal protein. Proteins were visualized with ECL chemiluminescence (GE Healthcare) and Konica X-ray film and the amount of SPARC protein was normalized against the intensity of the GAPDH signal.

**Statistical Analysis**

Survival data were plotted using the Kaplan-Meier method and differences between genotypes were determined using the Gehan-Wilcoxon test (Jager et al. 2008). Differences between genotypes with regards to presence of abdominal wall metastasis and petechial-like hemorrhages were analyzed by Fisher’s exact test. Results for collagen wall thickness, endothelial immunofluorescence and immunohistochemical experiments were assessed with Student t test. Statistical analyses were performed using GraphPad Prism software. All statistical tests were two-sided and p> 0.05 was considered statistically significant.

**Results**

**SPARC Increases Survival in an IP, but not OT model of EOC**

To examine the effects of an absence of host-derived SPARC on ovarian tumor formation and cancer progression, ID8 cells were injected into the bursa surrounding the left ovary or into the peritoneal cavity of WT and Sparc−/− mice. Mice were injected with 1.0X10⁶ ID8 cells in a 5 µl volume into the ovarian bursa or peritoneal cavity of...
Sparc−/− and age-matched control mice (n=10/group). Mice were monitored and euthanized when moribund. As previously reported (Said and Motamed 2005), IP injection of ID8 cells resulted in a more rapid progression to a moribund state in Sparc−/− mice compared to WT mice (p<0.05, Figure 1A). In contrast, progression to a moribund state was not altered by SPARC expression in host animals when ID8 cells were administered OT (Figure 1A).

Ovarian tumor weight, and ascites volume were not statistically different at time of sacrifice between the groups (Figure 1B,C). However, a striking decrease in the presence of discrete abdominal lesions was observed in Sparc−/− mice relative to WT control mice (Figure 2A). This difference was statistically significant in mice injected IP (p<0.001) and approached statistical significance in mice treated OT (p=0.056).

Discrete abdominal lesions were virtually absent in Sparc−/− mice when ID8 cells were injected IP. A greater number of Sparc−/− mice that were injected OT exhibited discrete abdominal lesions (p=0.056) compared to IP injected Sparc−/− mice, suggesting that the exposure of cells to the ovarian microenvironment may alter the behaviour of the cancer cells (Figure 2A).

Compared to WT controls, there was an increase in the presence and grade of hemorrhagic petechiae in the abdominal wall of Sparc−/− mice injected IP with ID8 cells (p<0.05, Figure 2B) but this difference was not as pronounced with OT injections (Figure 2C).

Fibrillar Collagen Content of Tumors in Sparc−/− and WT mice

SPARC has been implicated in regulating collagen fibrillogenesis and turnover, which could contribute to the differences observed in abdominal implant formation (Bradshaw et al. 2010). We examined the fibrillar collagen content of abdominal wall
sections from Sparc−/− and wild-type mice using Masson’s trichrome and Picrosirius red, which stain mature fibrillar collagens blue and red, respectively, under brightfield microscopy. Picosirius red also differentiates thinner mature collagen fibers (red) from thicker immature collagen fibers (yellow/green) under polarizing light microscopy (Coleman 2011). Differences in collagen staining in abdominal wall sections were evident between Sparc−/− and WT mice. Regardless of the injection route, Sparc−/− mice exhibited a thinner layer of collagen fibers compared to WT mice, in the peritoneum. A similar reduction in collagen fiber content in Sparc−/− mice was also observed in non-injected littermate controls. Under polarizing light microscopy, picrosirius red staining revealed predominantly immature collagen fibers in abdominal walls from Sparc−/− mice whereas mature collagen fibers were predominant in WT mice (Figure 3). In contrast to the abdominal wall, picrosirius red staining of ovarian lesions from Sparc−/− and WT mice injected OT revealed no difference in fibrillar collagen content under brightfield or polarizing light microscopy (Table 1).

**SPARC Expression in OT Tumors**

Ovarian tumors were only observed in mice injected OT with ID8 cells. Since differences in survival and ovarian tumor weight were not observed between WT and Sparc−/− mice injected OT with ID8 cells, the expression of SPARC was examined in the ovarian tumors to confirm the absence of stromal SPARC. SPARC was detected by immunohistochemistry in epithelial cells (ID8 cells) within the ovarian tumors regardless of genotype (Figure 4E and F) and Western blot analysis of ID8 cell lysates confirmed these cells express SPARC (Figure 4G). Surprisingly, stromal cells associated with the ovarian tumors did not express SPARC. While this was expected for the Sparc−/− mice, we and others have demonstrated pronounced activation of
SPARC expression in the reactive stroma associated with human EOC primary tumors (Brown et al. 1999; Paley et al. 2000). The reactive stroma associated with abdominal lesions also did not contain SPARC protein when ID8 cells were injected OT; however, positive SPARC staining was observed in abdominal tumors formed in wild-type mice after IP injection (Figure 4A-D). ID8 cells exhibited SPARC expression regardless of injection route and genotype. Collectively, these observations indicate that the injection of ID8 cells by the OT route is not a suitable model to assess the impact of SPARC on cancer progression. Further, these data suggest that exposure to the ovarian microenvironment inhibits the ability of the cancer cells to activate SPARC expression in the reactive stroma within peritoneal implants.

**Adipocyte Content is Altered in SPARC−/− Ovarian Lesions**

SPARC has been reported to exhibit anti-proliferative and anti-angiogenic effects (Kupprion et al. 1998). Accordingly we examined the ovarian tumors for differences in micro-vessel density (MVD) and Ki67 immunostaining. Despite the lack of SPARC expression in the stroma in both Sparc−/− and WT mice, IB4 staining revealed an increase in ovarian tumor blood vessel density in Sparc−/− mice compared to WT controls (p<0.05) (Figure 5). The ratio of Ki67 positive cells to negative cells revealed no significant difference between genotypes (p = 0.6307).

Histological staining of ovarian tumor sections revealed the presence of adipocytes within ovarian tumors from OT-injected mice. The number and size of adipocytes in ovarian tumors from Sparc−/− mice was increased compared to WT controls (Figure 6). SPARC has been shown to inhibit adipogenesis by enhancing laminin expression over fibronectin in the ECM (Bradshaw et al. 2003, Nie and Sage.
Discussion

This study investigated the survival of WT and Sparc⁻/⁻ mice following administration of malignant murine ID8 cells into either the ovarian microenvironment or the peritoneal cavity. The OT administration of these cells better models human ovarian cancer as these tumors initially form within the ovarian cortex or distal fallopian tube and subsequently disseminate throughout the peritoneal cavity. The presence of the ovarian bursa in mice provides a barrier to the seeding of the ovaries by ID8 cells; therefore, administration of these malignant cells into the peritoneal cavity bypasses the ovarian microenvironment. Consistent with previous studies (Said and Motamed 2005), direct injection of ID8 cells into the peritoneal cavity of Sparc⁻/⁻ mice compared to WT mice led to a more rapid progression to a moribund state, characterized most prominently by accumulation of ascites. In contrast, no difference in survival was observed between these genotypes when ID8 cells were placed into the ovarian bursa. These data suggests that a lack of SPARC within the peritoneum alters disease progression, whereas an absence of SPARC within the ovarian microenvironment has little or no significant impact.

The pattern of peritoneal seeding was dramatically different in Sparc⁻/⁻ mice compared to WT mice. Discrete peritoneal implants, reminiscent of advanced EOC in humans, were observed in WT mice, whereas a diffuse spread of the ID8 cells with increased petechial-like hemorrhages on the abdominal wall was observed in the Sparc⁻/⁻ mice. This difference in peritoneal seeding was most prominent in mice administered ID8 cells IP, where ID8 cells appeared to form diffuse layers along the peritoneum.
Said and Motamed (2005) also reported that IP-injected Sparc−/− mice had a more diffuse peritoneal spread of the ID8 cells than wild-type mice, which is consistent with our findings; however, they also reported an increased number of distinct peritoneal lesions in the Sparc−/− mice, which differs from our results. This is likely due to the difference in the number of cells injected. In our studies, we injected the same number of cells IP as were injected OT, which was 50-fold fewer cells than injected in the studies by Said and Motamed (2005). The greater number of cells injected likely enabled distinct lesions to form, whereas with fewer cells, the lesions had not yet grown to form distinct lesions prior to the development of massive ascites. The increased number of petechial-like hemorrhages that we observed in the Sparc−/− mice supports this interpretation.

Picrosirius red and Masson’s trichrome staining of abdominal wall tissue sections showed an alteration of collagen content in these tissues that existed even in the absence of injected cancer cells. Consistent with previous reports (Rentz et al. 2007; Said and Motamed 2005), Masson’s trichrome staining of peritoneum from non-injected Sparc−/− and WT mice demonstrated a significant decrease in the fibrillar collagen layer, raising the possibility that the differences observed in peritoneal seeding in Sparc−/− mice are a result of more receptive stromal ECM rather than a direct effect of tumor-associated SPARC. Seeding of the peritoneum by EOC cells initially involves the attachment of cells to stromal collagen that is exposed near lymphatic ports referred to as milky spots (Cui et al. 2002; Khan et al. 2010). The ensuing inflammatory environment results in retraction of the protective mesothelium, further exposing the adhesive collagen-rich stroma (Freedman et al. 2004). SPARC has been shown to inhibit expression of macrophage chemoattractants and proinflammatory mediators (Said et al. 2008), thus a peritoneal inflammatory response to IL8 injection would be enhanced in Sparc−/− mice. Moreover, it is increasingly evident that alterations in the
ECM microenvironment has potent effects on metastasis (Egeblad et al. 2010). Hence the lack of mature collagen fibrils and diminished expression of other ECM components such as collagen IV, laminin, and fibronectin (Said and Motamed 2005) in Sparc-/- mice may promote the observed phenotype of widespread attachment of ID8 cells rather than the formation of discrete lesions.

In contrast to the peritoneum, ovarian lesions from mice injected OT with ID8 cells did not exhibit differences between genotypes in content and maturity of collagen fibrils. Similarly, no difference in fibronectin content was evident in these ovarian tumors. Since fibronectin serves as a provisional matrix to promote collagen fibrillogenesis (Schwarzbauer 1991), the abundance of fibronectin may overcome the impact of a lack of SPARC on collagen processing. In addition, ID8 cells produce significant amounts of SPARC which may augment fibronectin-mediated fibrillogenesis (Schwarzbauer 1991). Given the marked activation of SPARC expression by the reactive stroma in human EOCs (Brown et al. 1999), it was surprising that SPARC staining was not detected in the ovarian reactive stroma of WT as well as Sparc-/- mice. The similarity in the collagen and fibronectin content of the ovarian tumor ECM between Sparc-/- and wild-type mice likely accounts for the lack of difference in disease progression when ID8 cells are injected OT. In light of the evidence that SPARC expression is activated in the ovarian reactive stroma associated with human EOC, the lack of stromal SPARC expression in the murine model limits the use of the OT model in addressing the role of SPARC in ovarian cancer progression. Moreover, in contrast to ID8 cells, human primary tumor EOC cells typically do not express SPARC (Brown et al. 1999; Paley et al. 2000), due to hypermethylation of the SPARC promoter (Socha et al. 2009).
The peritoneal reactive stroma associated with abdominal lesions also lacked SPARC protein when ID8 cells were injected OT; however, positive SPARC staining was observed in abdominal tumors formed in WT mice after IP injection. A possible explanation for this difference is that the ID8 cells were reprogrammed by the ovarian microenvironment, and as a result, do not signal to the peritoneal stroma to activate SPARC expression. ID8 cells injected IP, on the other hand, would trigger the SPARC expression since they have not been reprogrammed by interactions within the ovarian bursa. We have previously shown that cells derived from ascites or ovarian tumors following OT injection of ID8 cells are more aggressive than cells derived from ascites or implants following IP injection (Greenaway et al. 2008). These data are consistent with the concept that interaction of the cells with the ovarian microenvironment results in a reprogramming of the cells.

Analysis of the proliferative marker, Ki67, in ovarian lesions indicated a similar expression between genotypes. This is consistent with the similarities in ovarian weight and survival time. However, an increased vascular area was observed in tumors from Sparc-/- mice as indicated by staining of endothelial cell with IB4. The functional significance of this increased vascular area is not clear as ovarian lesion weight, ascites volume and survival were similar in the OT injected groups. SPARC binds to VEGF and inhibits its interaction with its receptors, thereby exerting an anti-angiogenic effect (Kupprion et al. 1998). Increased VEGF bioactivity could account for the increased vascular area, but since rapidly growing angiogenic vessels are tortuous and malformed, this increased area likely did not confer a growth advantage to ovarian tumors in the Sparc-/- mouse.

While our data support a role for SPARC in inhibition of tumor progression after IP cancer cell injection, the mean difference in survival was less than that reported in
previous studies. This survival difference may be attributed to variations in methodology between studies. As previously mentioned, the present study used a 50-fold lower number of cells than used in previous studies (Said and Motamed 2005; Said et al. 2007a; Said et al. 2007b; Said et al. 2008). This was necessary to enable an equal number of cells injected into the ovarian bursa and peritoneal cavity. In addition, ascites volume was a principal factor contributing to our animals attaining a moribund state. In fact, the rate of ascites accumulation in mice injected IP with ID8 cells reported by Said and Motamed (Said and Motamed 2005) closely resembled the survival curves we report. VEGF plays a primary role in ascites production by increasing vascular permeability (Weis and Cheresh 2005). Since SPARC inhibits VEGF activity, a more rapid accumulation of ascites would be expected in the absence of host-derived SPARC.

The observed increase in petechial-like hemorrhages in the peritoneum of Sparc−/− mice is also consistent with an excess of VEGF signaling. To our knowledge, our finding of peritoneal petechiae have not been previously reported in Sparc−/− mice; however, thrombocytopenia has been reported (Lehmann et al. 2007). Since platelets play an important role in maintaining endothelial cell tight junctions, thrombocytopenia increases the occurrence of petechiae (Nachman 2008): This predilection for destabilization of vascular endothelial cell adherens junctions in the peritoneum of Sparc−/− mice, as reflected by the finding of petechiae, could facilitate the production of ascites and progression to the moribund state.

A striking feature of ovarian lesions in Sparc−/− mice was an increased number and size of adipocytes. Adipogenesis is accompanied by a shift from a fibronectin- to a laminin-1 rich ECM (Nie and Sage 2009) and SPARC inhibits the differentiation of preadipocytes to adipocytes. Interestingly, fibronectin immunostaining in ovarian lesions was not reduced in SPARC−/− mice, thus it is possible that these do not represent
terminally differentiated adipocytes or that fibronectin production by myofibroblasts or infiltrating leukocytes to the tumor microenvironment masks this decrease. This is an interesting observation further highlighting the role of SPARC in adipogenesis.

The observations in this study have identified caveats regarding the use of the \textit{Sparc}\(^{-/-}\) mice to address the role of SPARC in ovarian cancer progression for several reasons: 1. The increased progression observed in \textit{Sparc}\(^{-/-}\) mice when ID8 cells are injected IP may reflect differences in ascites formation rather than a direct effect in the stroma. 2. The seeding of the peritoneum observed following this route of administration is not reflective of the pattern observed in EOC patients. Rather, pre-existing differences within the peritoneal ECM may contribute to differences in tumor spread. 3. While an orthotopic approach provides many attractive features to this model, most notably its ability to phenocopy high-grade serous carcinoma, an up-regulation of SPARC expression in the ovarian reactive stroma does not occur. 4. The ovarian ECM of the \textit{Sparc}\(^{-/-}\) mouse responds to ID8 cells with increased adipogenesis, an activity that is not observed in human EOC. Moreover, ID8 cells express endogenous SPARC whereas SPARC expression by malignant human ovarian epithelial cells is strongly down-regulated or silenced.

There are considerable data to support an inhibitory influence of SPARC on ovarian cancer progression. Principal among these are the antiproliferative and anti-angiogenic activities of SPARC. While we did not observe overt differences in survival between \textit{Sparc}\(^{-/-}\) and WT mice following OT injection of ID8 cells, our data suggest that exposure to the ovarian microenvironment alters the ability of the cancer cells to activate stromal SPARC expression in the peritoneum. Further studies examining the role of SPARC in EOC requires the use of models that better reflect the reported
expression of SPARC in human disease and that avoid the confounding developmental
effects on the ECM created by the genetic absence of host SPARC expression.

**Competing interests**
The authors declare that they have no competing interests.

**Authors' contributions**
JBG, AK, JP, TJB and MJR performed the experiments. CAM, JP, TJB and MJR
conceived, designed and supervised experimental work. JBG, TJB and MJR drafted the
manuscript. All authors read and approved the final manuscript.

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technical assistance.
References


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Figure Captions

Figure 1 - Survival analysis of SPARC knockout mice and WT age-matched controls

A) Kaplan-Meier survival curve of Sparc^-/- mice (n=10) compared to WT age-matched controls (n=10) injected with $1.0 \times 10^6$ ID8 cells into the peritoneum (A, p<0.05) or ovarian bursa (B, p=0.2069). End points were determined by presence of ascites accompanied by an increase of 30% initial body weight. Statistical significance was determined by the Gehan-Breslow-Wilcoxon test. No statistical differences were detected in injected ovarian weights (B) or ascites volume (C) between genotypes in a specific injection route (statistical differences were determined by one-way ANOVA followed by Newman-Keuls multiple comparison test). Bars with different letters are statistically different from one another.

Figure 2 - Metastatic spread and abdominal wall bleeding is dependent upon genotype in IP, but not OT injected mice

(A) Percentage of mice with gross abdominal tumors. Abdominal wall petechial hemorrhaging in IP (B) and OT (C) injection routes.

Figure 3 - Abdominal wall fibrillar collagen content is reduced in Sparc^-/- mice

Masson’s trichrome staining of abdominal wall sections in OT, IP and uninjected controls revealed a reduction in fibrillar collagen content in Sparc^-/- mice. Picrosirius red staining for collagen maturity revealed no differences between the genotypes.

Figure 4 - SPARC expression is restricted to ID8 cells in ovarian lesions
Images of SPARC immunostaining in Sparc−/− mice (panels A, C, and E) and WT mice (panels B, D, and F) injected IP or OT as indicated. Panels A-D are of abdominal lesions. Panels E and F are of ovarian tumors. SPARC was expressed at high levels in ID8 cells regardless of genotype and injection route. SPARC was localized to the reactive stroma (arrow in panel B) in WT mice injected IP, but not OT (panel D). Western blotting confirmed SPARC expression in ID8 cells under serum and serum free conditions. Recombinant mouse SPARC (rm) was used as a positive control.

Figure 5 - SPARC−/− ovarian lesions exhibit increased microvessel density.

(A) Density of IB4 staining was assessed by immunofluorescent microscopy. Increased staining density reflecting microvessels was observed in ovarian tumors from Sparc−/− mice compared to WT mice (*p<0.05, Student's t-test). (B) The mitotic index, as assessed by Ki67 staining, was similar between genotypes.

Figure 6 - Sparc−/− ovarian lesions have increased adipocyte content

(A) The number and average cellular area of individual adipocytes are increased in Sparc−/− ovarian tumors compared to tumors in WT controls. (B) Fibronectin immunostaining of ovarian tumors indicated alterations in adipocyte size and numbers are independent of fibrinogen content.
Table 1 – Fibrillar collagen content of ovarian tumors

Percent fibrillar content area of ovarian lesions stained with Masson’s trichrome and picrosirius red.

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<th>Trichrome Staining</th>
<th>Picrosirius Red Staining</th>
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<tr>
<td>Sparc^{-/-}</td>
<td>10.9±7.8%</td>
<td>43.7±18.9%</td>
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<tr>
<td>WT</td>
<td>8.3±3.6%</td>
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Figure 1
156x125mm (300 x 300 DPI)
A

Percentage of Mice with Abdominal Lesions Present

WT-IP
SPARC⁻ IP
WT-OT
SPARC⁻ OT

B

Intraperitoneal (IP)

Percentage of Mice

WT
SPARC⁻

C

Orthotopic (OT)

Percentage of Mice

WT
SPARC⁻

Figure 2
131x179mm (300 x 300 DPI)
Figure 3
190x247mm (300 x 300 DPI)
Figure 4
120x163mm (300 x 300 DPI)
Figure 5
163x272mm (300 x 300 DPI)
Figure 6

165x270mm (300 x 300 DPI)