TGFβ signaling regulates epithelial-mesenchymal plasticity in ovarian cancer ascites-derived spheroids

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Abstract

Epithelial–mesenchymal transition (EMT) serves as a key mechanism driving tumor cell migration, invasion, and metastasis in many carcinomas. Transforming growth factor-beta (TGFβ) signaling is implicated in several steps during cancer pathogenesis and acts as a classical inducer of EMT. Since epithelial ovarian cancer (EOC) cells have the potential to switch between epithelial and mesenchymal states during metastasis, we predicted that modulation of TGFβ signaling would significantly impact EMT and the malignant potential of EOC spheroid cells. Ovarian cancer patient ascites-derived cells naturally underwent an EMT response when aggregating into spheroids, and this was reversed upon spheroid re-attachment to a substratum. CDH1/E-cadherin expression was markedly reduced in spheroids compared with adherent cells, in concert with an up-regulation of several transcriptional repressors, i.e., SNAI1/Snail, TWIST1/2, and ZEB2. Treatment of EOC spheroids with the TGFβ type I receptor inhibitor, SB-431542, potently blocked the endogenous activation of EMT in spheroids. Furthermore, treatment of spheroids with SB-431542 upon re-attachment enhanced the epithelial phenotype of dispersing cells and significantly decreased cell motility and Transwell migration. Spheroid formation was significantly compromised by exposure to SB-431542 that correlated with a reduction in cell viability particularly in combination with carboplatin treatment. Thus, our findings are the first to demonstrate that intact TGFβ signaling is required to control EMT in EOC ascites-derived cell spheroids, and it promotes the malignant characteristics of these structures. As such, we show the therapeutic potential for targeted inhibition of this pathway in ovarian cancer patients with late-stage disease.

Key Words
- ovarian cancer
- TGFβ
- spheroid
- EMT
- metastasis

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Introduction

Epithelial ovarian cancer (EOC) is the most lethal of the gynecologic malignancies in women (Bast et al. 2009, Jemal et al. 2011). Early detection significantly enhances survival; however, more than 75% of patients present at first diagnosis with advanced stage disease when the tumor has already metastasized beyond the primary site (Rustin et al. 2011). EOC is unique among carcinomas in that it very rarely leads to blood-borne metastasis; rather, malignant cells are shed from the primary tumor directly into the peritoneal cavity. Malignant cells aggregate and survive in the peritoneal fluid, often in the form of ascites, as spheroid-like structures. These spheroids then attack and invade the peritoneum, seed metastatic tumor growth, and are resistant to standard chemotherapeutics. Extensive seeding of these spheroids are frequently observed on the uterus, sigmoid colon and omentum in advanced stage and recurrent disease (Shield et al. 2009, Lengyel 2010). Thus, a better understanding of EOC spheroid biology may elucidate important mechanisms involved in the metastasis of this complex disease.

It has been proposed that EOC cells are able to switch between epithelial and mesenchymal states during metastasis (Elloul et al. 2010, Davidson et al. 2014). For most carcinomas, progression toward malignancy is accompanied by loss of epithelial differentiation and a shift towards a mesenchymal phenotype (Hanahan & Weinberg 2011). It is well-established that this occurs through epithelial–mesenchymal transition (EMT), the mechanism believed to help tumor cells in their ability to migrate, invade and metastasize (Turley et al. 2008, Vergara et al. 2010). EMT is an essential morphologic conversion that occurs during embryonic development for complex body patterning. EMT occurs when epithelial cells lose their epithelial cell characteristics, including dissolution of cell–cell junctions (tight junctions, adherens junctions and desmosomes), lose apical–basolateral polarity and acquire a mesenchymal phenotype, characterized by actin reorganization and stress fiber formation, migration and invasion (Ahmed et al. 2007, Do et al. 2008). The loss of cell–cell adhesion is a prerequisite of EMT, a hallmark of which is the functional loss of E-cadherin (Lengyel 2010). Emerging evidence suggests that EMT plays a crucial role in the progression of EOC by increasing cancer cell motility, chemoresistance and cancer stem cell characteristics (Huang et al. 2012).

In EOC, signals from the neoplastic microenvironment, including a variety of cytokines and growth factors, function to control EMT. Although several growth factors participate in EMT, transforming growth factor-beta (TGFβ) has been the most studied. The TGFβ superfamily consists of a large number of structurally related polypeptide growth factors, including TGFβ, bone morphogenetic protein (BMP), inhibin/activin and Mullerian inhibiting substance (MIS) families; each is capable of regulating a broad spectrum of cellular processes, including cell proliferation, lineage determination, differentiation, motility, adhesion, embryogenesis, fibrosis, immunosuppression and apoptosis (Jakowlew 2006). TGFβ signaling has been implicated in the pathogenesis of many different cancers, including EOC (Polyak & Weinberg 2009, Vergara et al. 2010). Indeed, TGFβ is thought to play a role in the pathobiological progression of EOC because this cytokine is overexpressed in cancer tissue, plasma and peritoneal fluid of ovarian cancer patients (Dunfield & Nachtigal 2003).

We have discovered that patient ascites-derived EOC cells naturally undergo a robust EMT response by simply aggregating into spheroids, and this was reversed upon spheroid re-attachment to a substratum. Given that TGFβ is a key regulator of EMT in carcinomas (Zavadil & Bottinger 2005), we investigated its role in spheroid-induced EMT and found that treatment with the TβRI inhibitor, SB-431542, potently blocked endogenous EMT in spheroids. Additionally, SB-431542 treatment upon spheroid re-attachment further enhanced the epithelial phenotype of dispersing cells while decreasing cell motility and migration. In fact, spheroid formation was compromised by exposure to SB-431542, and this rendered cells susceptible to carboplatin-induced cell death.

Materials and methods

Cell culture

Ascites fluid obtained from ovarian cancer patients at the time of debulking surgery or paracentesis was used to generate primary cell cultures as described previously (Shepherd et al. 2006). Briefly, bulk ascites containing cells was mixed 1:1 with growth medium (MCDB105 (Sigma)/M199 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Wisent, St Bruno, Quebec, Canada) and 50 μg/ml penicillin–streptomycin). Cells were grown in a 37 °C humidified atmosphere of 95% air and 5% CO2. To account for inter-patient variability and the finite lifespan of primary ascites-derived cells, many independent samples collected from patients with high-grade EOC.
were used throughout the study (Supplementary Table S1, see section on supplementary data given at the end of this article). Experiments were performed using cells between passages 3 and 5 and performed with at least four independent patient samples and at least three experimental replicates. Samples have been confirmed independently to have >90% EpCAM-positive cells beyond passage 2. All patient-derived cells were used in accordance with institutional human research ethics board approval (UWO HSREB 12668E).

Adherent cells were maintained on tissue culture-treated polystyrene (Sarstedt, Newton, NC, USA). Non-adherent cells (spheroids) were maintained for 72 h on ultra-low attachment (ULA) cultureware (Corning, Corning, NY, USA) which is coated with a hydrophilic, neutrally charged hydrogel to prevent cell attachment. For spheroid re-attachment assays, spheroids were removed from ULA cultureware and re-introduced to tissue culture-treated polystyrene for up to 72 h to allow for cell dispersion.

Quantitative RT-PCR
Total RNA was isolated from primary adherent and spheroid EOC cells using Qiagen RNeasy Mini Kit (Qiagen). RNA was isolated from reattached spheroids by first trypsinizing cells and spheroids from the tissue culture plastic. Purified RNA was quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RT was performed using total RNA isolated from independent patient samples (adherent and/or spheroid, treated and untreated) and Superscript II reverse transcriptase (Invitrogen) as per manufacturer’s instructions. PCRs were carried out using Brilliant SYBR Green QPCR Master Mix (Agilent Technologies/Stratagene, Wilmington, DE, USA). RT was performed using total RNA isolated from independent patient samples and at least three experimental replicates. Samples have been confirmed independently to have >90% EpCAM-positive cells beyond passage 2. All patient-derived cells were used in accordance with institutional human research ethics board approval (UWO HSREB 12668E).

Antibodies and other reagents
Antibodies against E-cadherin (#3195), Snail (#3879), β-Catenin (#9852P), Claudin-1 (#4933), Smad2 (#3122) and phospho-Smad2 (#3108S) were purchased from Cell Signaling Technologies (Danvers, MA). Anti-human Lamin A+C antibody (MAB3211) was purchased from Millipore. Anti-actin antibody (A2066) and anti-α-tubulin (T5168) were purchased from Sigma. Recombinant human TGFb1 was purchased from Millipore and prepared in distilled water, and used at a concentration of 10 ng/ml (Vergara et al. 2010). The TGFb receptor I (TβR1) inhibitor, SB-431542, was purchased from Sigma and prepared in DMSO according to manufacturer’s instructions, and used at a concentration of 5 μM (Inman et al. 2002).

TGFb signaling modulation
To test TGFb responsiveness of primary EOC cells, adherent cells were treated with recombinant human TGFβ1 at 2, 24

pyrophosphate, 10 mM NaF, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF and 1× protease inhibitor cocktail (Roche), clarified by centrifugation (20 min at 15 000 g), and quantified by Bradford analysis (Bio-Rad Laboratories). Nuclear fractions were isolated from primary adherent and spheroid EOC cells by washing cells in PBS, resuspending the cell pellet in a hypotonic lysis buffer (20 mM HEPES (pH 7.4), 1 mM EGTA, 1 mM EDTA and 1 mM DTT) for 15 min on ice. Lysates were centrifuged, supernatant removed and nuclear pellet washed twice with wash buffer (10 mM HEPES (pH 7.4), 10 mM KCl, 0.1 mM EGTA and 0.1 mM EDTA). The washed nuclear pellet was dissolved in lysis buffer and clarified by centrifugation as described above for total cellular protein isolation. Forty to fifty micrograms of protein extract per lane were separated by SDS–PAGE in the presence of 1% β-mercaptoethanol using 8% or 10% gels. Proteins were then transferred to a PVDF membrane (Roche), blocked with 5% BSA in Tris-buffered saline with Tween-20 (TBST; 10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.1% Tween-20). Membranes were washed in TBST and incubated (overnight, 4°C) with appropriate antibodies (1:1000 in 5% BSA/TBST). Immunoreactive bands were visualized by incubating (1 h, room temperature) with peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:10 000 in 1% skim milk/TBST; GE Healthcare, Mississauga, ON, Canada) followed by exposure to enhanced chemiluminescence reagent (Luminata Forte, Millipore, Temecula, CA, USA).

Western blot analysis
Total cellular protein was isolated from primary adherent and spheroid EOC cells. Cells were washed once in cold PBS, dissolved in lysis buffer (50 mM HEPES pH7.4, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl2, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium
and 72 h in reduced serum media (0.5% FBS). Cells were harvested for analysis at each time point and images were captured using a Leica DMI 4000B inverted microscope. Spheroids were treated with SB-431542 or DMSO vehicle control at the time of initial seeding to ULA culture ware. Seventy-two hours later, spheroids were imaged and harvested for analysis. For spheroid re-attachment assays, native (untreated) spheroids were removed from ULA cultureware and re-introduced to tissue culture-treated polystyrene for 72 h to allow for cell dispersion. SB-431542 treatment or DMSO control was given at the time of re-attachment to tissue culture-treated plates and harvested 72 h later for analysis.

**Spheroid re-attachment assay**

Macroscopically-visible spheroids (formed over 3 days) were transferred individually into single wells of 48-well tissue culture-treated polystyrene plates (12 replicates per treatment per EOC sample) and treated with DMSO or SB-431542 at the time of re-attachment. Phase contrast images of each well were captured using Leica DMI 4000B inverted microscope at 24 h and dispersion area was quantified using ImageJ (NIH, Bethesda, MD, USA), as described previously (Peart et al. 2012) Area of dispersion for each re-attached spheroid was determined as total dispersion area minus spheroid (core) area at 24 h. At 72 h, re-attached spheroids were fixed and stained using Hema-3 Stain kit (Fisher, Kalamazoo, MI, USA).

**Transwell migration assay**

Primary EOC cells were seeded at $5 \times 10^4$ cells/well in 24-well ULA plates and treated with either DMSO or SB-431542 at the time of seeding. EOC spheroids were washed in PBS and transferred individually to 100 µl of reduced serum media (0.5% FBS) and added to the top chamber of a Transwell (6.5 mm in diameter; 8 µm pore size) (Costar), with 600 µl of medium containing 10% FBS added to the bottom chamber of well. After a 12 h incubation, Transwell membranes were fixed and stained with Hema-3 Stain kit and non-migrated cells on the upper side of the membrane were removed completely using a cotton swab. Migrated cells on the underside of the membrane were imaged with a Leica DMI 4000B inverted microscope. Images in five different fields per membrane were captured and cells were counted with ImageJ software. The mean number of migrated cells was determined for each Transwell.

**Immunofluorescence**

Analysis of F-actin and β-catenin protein by fluorescent staining was performed on re-attached EOC spheroids seeded to glass coverslips and treated with SB-431542 or DMSO vehicle control at the time of spheroid re-attachment. After 72 h, cells were fixed in a buffered 10% formalin solution and spheroid cores were removed leaving only the cells that have dispersed out of the spheroids attached onto the coverslips for analysis. Cells were washed with PBS and permeabilized with 0.1% Triton X-100 in PBS. Overnight incubation with anti-β-catenin antibody (1:500) was followed by 1 h incubation with anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (1:500; Vector Laboratories, Burlington, ON, Canada) and subsequently a 1 h stain with Alexa Fluor 568-conjugated phalloidin (A12380, Invitrogen). Coverslips were mounted in VectaShield mounting medium (Vector Laboratories) containing 4',6-diamidino-2-phenylindole (1:1000; Sigma). Fluorescence images of mounted coverslips were captured using a Leica DMI 4000B inverted microscope.

**Cell viability**

Cells were seeded to either 24-well tissue culture plastic at a density of $1 \times 10^4$ per well to form adherent cultures, or ULA plates at a density of $5 \times 10^4$ per well to form spheroids. SB-431542 treatment was initiated at the time of seeding for cells in suspension, while cells under adherent conditions were given 12 h to adhere before commencing treatment. At 72 h post-treatment, spheroids were collected, pelleted and left in media (100 µl), at which point CellTiter-Glo reagent (Promega) was added (1:1 v/v ratio). All samples were subjected to a freeze/thaw cycle prior to analysis. Approximately 100 µl of the mixture was added to a white-walled 96-well microplate and luminescence signal was detected using a Wallac 1420 Victor 2 spectrophotometer (PerkinElmer, Waltham, MA, USA) and normalized to vehicle-treated cells. Under adherent conditions, cells were kept in original 24-well plates and cell viability was assessed using alamarBlue (Invitrogen) as per manufacturer’s instructions. Briefly, medium was replaced with alamarBlue reagent diluted 1:20 in complete medium, incubated for 4 h at 37°C in a humidified atmosphere of 95% air and 5% CO2, and then fluorescence was quantified using 560/590 nm excitation/emission filter settings.

For measuring spheroid cell viability in the presence of carboplatin, spheroids were treated first with SB-431542 or DMSO for 72 h, followed by 100 µM carboplatin (LHSC Pharmacy, London, ON, USA); CyQUANT NF (Invitrogen).
assays were performed 72 h later. Briefly, spheroids were collected and pelleted, and medium was removed. After a single freeze–thaw, spheroids were resuspended in 1X Hank's balanced salt solution (HBSS), and 50 µl aliquots were dispensed into microplate wells. Fifty microlitres of 2X dye binding solution was added to each microplate well, and fluorescence signal was detected with the Wallac plate reader using 485/530 nm excitation/emission filter settings.

**Statistical analysis**

Data were expressed as mean ± S.E.M. All statistical analyses were performed using a two-tailed Student’s t-test or one-way ANOVA with Tukey’s Multiple Comparison test. Significance levels were set at $P<0.05$.

**Results**

**EMT is induced during EOC spheroid formation**

To examine EMT in EOC ascites-derived cells, we followed the expression profile of the epithelial marker E-cadherin and mesenchymal markers known to repress E-cadherin (Snail, Twist1, Twist2 and Zeb2) during spheroid formation and reattachment (Pon et al. 2008, Micalizzi et al. 2010). We used an *in vitro* 3D culture system whereby primary ascites-derived human EOC cells are maintained in suspension on ULA plates for 3 days, followed by re-introduction to standard tissue culture-treated plates where they rapidly re-attach and disperse (Correa et al. 2012, Peart et al. 2012). Accordingly, we performed quantitative RT-PCR analysis on RNA isolated from several independent patient samples ($n$=6) that were grown in adherent, spheroid and re-attached spheroid culture conditions. Compared to their matched adherent counterparts, primary EOC ascites cells were found to naturally undergo an EMT response during spheroid formation based on mRNA upregulation of SNAI1, TWIST1, TWIST2, ZEB2 and a coordinate down-regulation of CDH1 (Fig. 1a).

Of these mesenchymal markers, SNAI1 was the most robustly and consistently up-regulated EMT marker in spheroids (11.7–42.5 fold increase). Several of these trends were strongly reversed upon EOC spheroid re-attachment (after 3 days re-attachment), as the mRNA levels of SNAI1, CDH1, TWIST1, TWIST2 and ZEB2 were significantly decreased.

**Figure 1**

EMT is induced during EOC ascites cell spheroid formation and reversed upon re-attachment. (a) Quantitative RT-PCR analysis of CDH1, SNAI1, TWIST1, TWIST2 and ZEB2 mRNA in monolayer/adherent cells, spheroid cells, and 72 h re-attached spheroid cells using primary ascites-derived EOC patient samples ($n=6$). (b) Western blot analysis of E-cadherin and Snail protein in adherent (A), spheroid (S) and 72 h re-attached spheroid samples (R) samples in primary ascites-derived EOC patient samples ($n=7$). Tubulin was used as a loading control.
TWIST1, TWIST2 and ZEB2 return to levels comparable to those of matched adherent cells. Although CDH1 transcript levels begin to recover, they did not fully attain expression levels of original adherent cells in this model system.

Western blotting was performed using protein lysates isolated from independent EOC ascites-derived human cells grown in the same culture conditions to verify the trends seen at the transcript level. In EOC spheroids, E-cadherin protein levels were down-regulated and this was associated with a robust up-regulation of Snail. These effects started to reverse upon EOC spheroid re-attachment, although E-cadherin protein expression was not restored in most patient samples by 3 days after re-attachment (Fig. 1b). Thus, RNA and protein expression data demonstrate EOC ascites-derived cells undergo endogenous EMT during spheroid formation, and this phenomenon is triggered to reverse upon spheroid re-attachment.

**TGFβ signaling regulates EMT in primary ascites-derived EOC spheroids**

To address the potential mechanism by which EOC cells undergo endogenous EMT upon spheroid formation, we chose to assess the role of the TGFβ signaling pathway in mediating the pathobiological characteristics of EOC ascites spheroids. Previous microarray data from our lab (Peart et al. 2012) revealed increased TGFβ1 expression in EOC spheroids compared with adherent cells in five independent patient ascites samples (mean 4.36-fold, \( p < 0.001 \))

First, we tested the responsivity of primary EOC cells in adherent culture to TGFβ1 treatment. In doing so, patient ascites samples (\( n = 4 \)) were treated with 10 ng/ml recombinant TGFβ1 ligand for 2, 24 and 72 h (Fig. 2a) and quantitative RT-PCR analysis was performed. Treatment of adherent cells with TGFβ1 ligand resulted in a significant decrease of CDH1 mRNA at 24 h but returned to near basal levels by 72 h. However, we observed significant increases in mesenchymal marker transcript levels for SNAI1, ZEB2, TWIST2 and VIM at 24 and 72 h. Moreover, transition of cells from a typical cobblestone epithelial morphology to a distinctly fibroblast-like phenotype was observed after 72 h of TGFβ1 treatment (Fig. 2b), indicating induction of EMT and responsiveness of EOC ascites-derived cells to TGFβ.

EMT was shown to be induced endogenously upon spheroid formation in primary ascites-derived EOC cells (Fig. 1). Thus, we wanted to assess whether further treatment with exogenous TGFβ1 ligand would potentiate EMT phenotype induced in spheroids, and compare endogenous EMT induction in EOC spheroids to TGFβ1-induced EMT in adherent EOC cells. Quantitative RT-PCR analysis revealed no significant changes in CDH1, SNAI1, VIM, TWIST2 or ZEB2 mRNA levels in TGFβ1-treated EOC ascites spheroids relative to untreated spheroids

**Figure 2**

TGFβ induces EMT in EOC ascites-derived cells but spheroid formation alone is a more potent inducer of EMT. (a) Quantitative RT-PCR analysis of CDH1, SNAI1, TWIST2, ZEB2 and VIM mRNA in control and TGFβ1-treated adherent EOC ascites-derived cells (\( n = 4 \)) at 2 h, 24 h and 72 h \( ( \* p < 0.05 \) as determined by Student’s t-test), (b) TGFβ1 treatment of EOC ascites-derived cells induces transformation of epithelial cells towards a fibroblastic mesenchymal morphology. Representative image from EOC57 patient sample at 72 h post treatment (scale bar = 200 \( \mu \)m). (c) Western blot analysis of E-cadherin, Claudin1 and Snail protein in adherent (A) and spheroid (S) samples treated with TGFβ1 or not in ascites-derived EOC patient samples (\( n = 2 \)). Tubulin was used as a loading control. Panels represent separate lanes from the same blot and same length of exposure.
Western blot analysis confirmed that treatment of spheroids with TGFβ1 does not further increase Snail protein levels (Fig. 2c). Furthermore, there were higher levels of Snail protein in untreated spheroids compared with TGFβ1-treated EOC ascites cells indicating that spheroid formation alone may act as a potent inducer of EMT. We also observed that TGFβ1 treatment of adherent cells results in sustained reduction in E-cadherin protein as compared with the slight recovery in CDH1 mRNA level at 72 h.

Because we have observed increased TGFβ1 expression in EOC ascites-derived spheroids, we propose that endogenous TGFβ signaling activity regulates the natural induction of EMT observed in spheroids. In support of this, we have observed increased nuclear phosphorylated Smad2 and total Smad2 protein in spheroid cells as compared with adherent ascites cells (Supplementary Figure S1, see section on supplementary data given at the end of this article). As such, we hypothesize that inhibiting TGFβ signaling in EOC ascites-derived cells in suspension will abrogate spheroid formation and induction of EMT. Inhibiting TGFβ signaling during spheroid formation using a TβRII small molecule inhibitor, SB-431542, efficiently blocked the EMT phenotype in spheroids as evidenced by a significant increase in CDH1 mRNA expression and a significant decrease in SNAI1, TWIST2 and ZEB2 mRNA expression (Fig. 3a). Increased E-cadherin protein and decreased Snail protein expression were consistently seen in SB-431542-treated spheroids compared with DMSO controls, indicating EMT is dramatically reduced in spheroids as a result of inhibiting TGFβ signaling (Fig. 3b). In fact, SB-431542 treatment resulted in spheroids that were much smaller in size and less cohesive than the compact control spheroids (Fig. 3c), which resulted in decreased viability as compared with adherent cells (Fig. 3d). We confirmed that SB-431542 treatment of adherent cells and spheroids resulted in blockade of TGFβ1-induced signaling by phospho-Smad2 western blotting (Supplementary Figure S2). In addition, SB-431542 treatment of adherent EOC ascites cells increases E-cadherin protein expression and promotes an exaggerated epithelial phenotype (data not shown).

**TGFβ signaling inhibition during EOC spheroid re-attachment enhances the epithelial phenotype of dispersing cells and reduces cell motility**

A common feature of EMT in cancer is the enhanced capacity for malignant cells to migrate; and the capacity of EOC ascites cell spheroids to re-attach, grow, and disperse defines their ability to form secondary metastases (Shield et al. 2009). Since inhibition of TGFβ signaling consistently blocks EMT and reduces cell–cell cohesion within spheroids, we sought to determine whether TGFβ signaling inhibition affects the ability of spheroids to re-attach and disperse. Thus, our first assessment was to quantify smaller and less cohesive spheroids compared with DMSO controls. Representative image from EOC75 patient sample at 72 h post treatment (scale bar = 500 μm). (d) Cell viability was determined using CellTiter-Glo assay following 3 days of SB-431542 treatment, or DMSO control across EOC patient ascites samples (n=8) cultured as adherent cells and spheroids. Data is represented as mean ± S.E.M. and Student’s t-test for statistical significance (**P<0.001).
SB-431542 treatment of spheroids potentiates carboplatin-induced cell death

Platinum-based chemotherapy is the standard for first-line treatment of metastatic EOC, yet the majority of patients will eventually recur with platinum-resistant disease (Cannistra 2004). Ovarian cancer spheroids are largely resistant to platinum treatment, likely due to their reduced proliferative state. To assess whether blocking TGFβ signaling can affect ovarian cancer spheroid sensitivity to platinum agents, we treated EOC spheroids with SB-431542 or vehicle control first, followed by treatment...
with carboplatin. We observed a dramatic reduction in viability in EOC ascites-derived cell spheroids that received a combined treatment of SB-431542 at day 0 followed by 72 h carboplatin treatment at day 3, when compared with either SB-431542 treatment alone or a combined treatment of vehicle plus carboplatin (Fig. 6a and b). These final results point to a critical role for TGFβ signaling in maintaining cell viability and achieving chemo resistance in ascites-derived cell spheroids.

Discussion

Spheroids have been postulated to act as important reservoirs of malignant EOC cells during spread throughout the peritoneal cavity in patients with late-stage disease (Shield et al. 2009, Lengyel et al. 2014) We had previously observed that EOC cells induce an endogenous EMT response upon spheroid formation (Peart et al. 2012). Herein, we have confirmed this phenomenon among a larger array of ascites-derived specimens and implicated the role of TGFβ signaling in its regulation. We also observed the plasticity of this process, since the cells undergo mesenchymal-to-epithelial transition (MET) to revert back to an epithelial phenotype upon spheroid reattachment to a substratum. Importantly, we identified that intact TGFβ signaling is required to elicit this EMT reattachment to a substratum. This also indicates that perhaps residual maintenance of TGFβ signaling acts to promote invasion and motility upon spheroid
reattachment. Lastly, SB-431542 disrupts efficient EOC ascites cell spheroid formation and renders suspended cells more susceptible to carboplatin-mediated cell death. EMT has been observed in tumor cell spheroids and implicated in EOC metastasis, and we provide some essential insight into its regulation, as well as the therapeutic potential of targeting this important pathological process in late-stage EOC metastasis.

Epithelial to mesenchymal transition is a universally accepted phenomenon that occurs in the malignant progression of most if not all human carcinomas (Kalluri & Weinberg 2009). Dynamics of EMT during EOC metastasis, particularly with its reversal, or MET, upon secondary tumor formation, has been discussed in the literature (Ahmed et al. 2007, Vergara et al. 2010). For example, E-cadherin down-regulation in ovarian cancer cells facilitates intraperitoneal spread (Sawada et al. 2008), yet E-cadherin expression in metastases can be similar to the primary tumor (Koensgen et al. 2010). We observed, however, that the level of E-cadherin expression upon spheroid re-attachment did not increase to initial levels completely, which may explain why secondary tumors oftentimes harbor low yet detectable E-cadherin in patients (Fujioka et al. 2001, Marques et al. 2004, Yuecheng et al. 2006).

Differential E-cadherin expression supports the idea that switching between EMT and MET occurs during ovarian cancer metastasis and is important in disease progression. Our results provide further insight into the mechanism controlling this phenomenon of epithelial–mesenchymal plasticity within spheroids, which act as carriers of transiting metastatic cells in the peritoneal cavity. We demonstrate that the expression of classical EMT markers is rapidly induced during spheroid formation, yet the simple re-attachment to a substratum is sufficient to stimulate an MET cell phenotype. Our results imply that transcriptional repressors have immediate functions to induce EMT in spheroid formation and their reduction triggers the start of MET at reattachment since expression changes are rapid and sustained. It is peculiar that an EMT response would be induced in spheroids since these cells would not necessarily require a mesenchymal phenotype in these fairly static structures. However, one could envision that an EMT phenotype of spheroids primes the resident cells for invasive capabilities upon their subsequent reattachment (Iwanicki et al. 2011).

On that note, E-cadherin is less immediately responsive particularly upon reattachment, perhaps indicating a delay to complete MET thereby facilitating spheroid cell motility and invasion. Induction of an EMT phenotype has been observed in other spheroid systems, usually in concert with stem-like cells (Mani et al. 2008, Han et al. 2013); however, these reports have not described whether or not the EMT phenotype is reversible. It may be that this
plasticity is a particularly unique characteristic of metastatic ovarian cancer, not unlike its almost exclusive mode of intraperitoneal spread which is unique when compared among other carcinomas.

The induced expression of EMT markers in spheroids implies that their gene products may act to facilitate disease progression. Snail expression in ovarian cancer portends poor patient prognosis (Yoshida et al. 2009) and platinum resistance (Haslehurst et al. 2012). Ectopic Snail expression in ovarian cancer cell lines enhances oncogenic transformation properties and promotes tumor growth in mouse xenografts, yet similar manipulation of Slug expression failed to elicit the same effect (Lu et al. 2012). Snail and Slug may have different functions in controlling EMT in ovarian cancer cells (Kurrey et al. 2005, Xu et al. 2010), which supports our findings that SNAI2/Slug expression is not consistently altered during spheroid formation and reattachment (data not shown). This exemplifies the utility of using our experimentally-tractable in vitro system using patient-derived cells not only to look at the dynamics of gene expression, but use it to dissect out the impact of these markers on spheroid cell biology. To this end, we are currently focusing our efforts on investigating Snail function and its requirement on the EMT phenotype in spheroids, anoikis-resistance, and cell invasion upon spheroid reattachment. In addition, it will be important to determine whether TGFβ signaling directly regulates the genes controlling EMT, e.g. SNAI1 and TWIST1/2, in EOC spheroid cells, as well as other pathobiological processes that this metastasis-associated pathway may regulate during spheroid formation and transcoelomic metastasis.

The TGFβ signaling pathway has been investigated for over 30 years in many human malignancies, including ovarian cancer (Massague 2008). Biologically-relevant concentrations of TGFβ ligands have been detected in malignant ascites from patients (Abendstein et al. 2000, Dunfield et al. 2002). Primary EOC cells possess an intact TGFβ signaling pathway, which can induce cytostasis upon its activation as long as CDKN2B encoding p15 is intact (Dunfield et al. 2002). We showed previously that TGFβ signaling was present in spheroids (Pearl et al. 2012). This is consistent with TGFβ treatment regulating tissue transglutaminase 2 in ovarian cancer cells to promote EMT and spheroid formation (Cao et al. 2012). In the present report, we have now uncovered a new function for active maintenance of endogenous TGFβ signaling in promoting the EMT phenotype of spheroids and their potential to reattach and spread. As such, TGFβ signaling may be essential to promote late-stage ovarian cancer progression, thus serving as a potential therapeutic target to reduce intraperitoneal spread and disease burden upon recurrence.

Several attempts have been made in the past to test whether TGFβ signaling could be targeted with inhibitors in cancer treatment (Akhurst & Hata 2012). Perhaps the majority of these studies have looked at malignancies where metastasis had already occurred and thus the inhibitors may be less efficacious at this step of progression. Ovarian cancer represents an entirely different case, since after primary debulking and chemotherapy the majority of disease is reduced. The eventual recurrence of disease and the continual re-seeding of the peritoneal cavity with malignant cells imply that cyclic TGFβ signaling activity may be required to drive this process. Our results show that the underlying pathobiology of spheroid cells can be dramatically altered by treatment with the potent TGFβ type I receptor inhibitor SB-431542. Most importantly, we also demonstrate that TGFβ signaling blockade reduces EOC spheroid cell viability in concert with carboplatin treatment. It would be intriguing to test the numerous other TGFβ signaling inhibitors available (Akhurst 2006), and apply them in an in vivo intraperitoneal ovarian cancer metastasis model to offer further supportive evidence that the therapeutic potential of targeting TGFβ signaling can be re-evaluated in ovarian cancer.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-15-0383.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Conceived and designed the experiments: S Rafahi, G E DiMattia and T G Shepherd. Performed the experiments: S Rafahi and Y R Valdes. Analyzed the data: S Rafahi, G E DiMattia and T G Shepherd. Contributed...
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