TGF β signaling regulates epithelial–mesenchymal plasticity in ovarian cancer ascites-derived spheroids

Samah Rafehi^{1,2}, Yudith Ramos Valdes¹, Monique Bertrand^{1,4,5}, Jacob McGee^{1,4}, Michel Préfontaine^{1,4}, Akira Sugimoto^{1,4,5}, Gabriel E DiMattia^{1,3,4,5} and Trevor G Shepherd^{1,2,4,5}

¹Translational Ovarian Cancer Research Program, London Regional Cancer Program, 790 Commissioners Road East, Room A4-836, London, Ontario, Canada N6A 4L6

²Department of Anatomy and Cell Biology, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, Ontario, Canada

³Department of Biochemistry, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, Ontario, Canada

⁴Department of Obstetrics and Gynaecology, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, Ontario, Canada

⁵Department of Oncology, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, Ontario, Canada

Correspondence should be addressed to T G Shepherd
Email
tshephe6@uwo.ca

Abstract

Epithelial–mesenchymal transition (EMT) serves as a key mechanism driving tumor cell migration, invasion, and metastasis in many carcinomas. Transforming growth factor-beta (TGF β) 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 TGF β 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 TGF β 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 TGF β 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
- ▶ TGF β
- ▶ spheroid
- ▶ EMT
- ▶ metastasis

Endocrine-Related Cancer
(2016) 23, 147–159

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 (TGF β) has been the most studied. The TGF β superfamily consists of a large number of structurally related polypeptide growth factors, including TGF β , 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). TGF β signaling has been implicated in the pathogenesis of many different cancers, including EOC (Polyak & Weinberg 2009, Vergara *et al.* 2010). Indeed, TGF β 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 TGF β is a key regulator of EMT in carcinomas (Zavadi & Bottinger 2005), we investigated its role in spheroid-induced EMT and found that treatment with the T β RI 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 μ g/ml penicillin–streptomycin). Cells were grown in a 37 °C humidified atmosphere of 95% air and 5% CO $_2$. 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, Mississauga, ON, Canada) and a Stratagene Mx3000P machine with data exported to Microsoft Excel for analysis. Human-specific primers for *CDH1* (E-cadherin), *SNAIL* (Snail), *TWIST1*, *TWIST2*, *ZEB2* and *VIM* (Vimentin) were used (Sigma). *GAPDH* served as an internal control for RNA input and quantification was performed using the $\Delta\Delta C_t$ method (Livak & Schmittgen 2001).

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 $MgCl_2$, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium

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).

Antibodies and other reagents

Antibodies against E-cadherin (#3195), Snail (#3879), β -Catenin (#9582P), 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 TGF β 1 was purchased from Millipore and prepared in distilled water, and used at a concentration of 10 ng/ml (Vergara *et al.* 2010). The TGF β receptor I (T β RI) inhibitor, SB-431542, was purchased from Sigma and prepared in DMSO according to manufacturer's instructions, and used at a concentration of 5 μ M (Inman *et al.* 2002).

TGF β signaling modulation

To test TGF β responsivity of primary EOC cells, adherent cells were treated with recombinant human TGF β 1 at 2, 24

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 5×10^4 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 μ l of reduced serum media (0.5% FBS) and added to the top chamber of a Transwell (6.5 mm in diameter; 8 μ m pore size) (Costar), with 600 μ l 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 1×10^4 per well to form adherent cultures, or ULA plates at a density of 5×10^4 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 μ l), 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 μ l 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 μ M 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 $\pm$ 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 *SNAIL1*, *TWIST1*, *TWIST2*, *ZEB2* and a coordinate down-regulation of *CDH1* (Fig. 1a). Of these mesenchymal markers, *SNAIL1* 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 *SNAIL1*,

Figure 1

EMT is induced during EOC ascites cell spheroid formation and reversed upon re-attachment. (a) Quantitative RT-PCR analysis of *CDH1*, *SNAIL1*, *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) 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.

TGF β 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 TGF β signaling pathway in mediating the pathobiological characteristics of EOC ascites spheroids. Previous microarray data from our lab

(Peart et al. 2012) revealed increased *TGF β 1* 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 TGF β 1 treatment. In doing so, patient ascites samples ($n = 4$) were treated with 10 ng/ml recombinant TGF β 1 ligand for 2, 24 and 72 h (Fig. 2a) and quantitative RT-PCR analysis was performed. Treatment of adherent cells with TGF β 1 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 TGF β 1 treatment (Fig. 2b), indicating induction of EMT and responsiveness of EOC ascites-derived cells to TGF β .

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 TGF β 1 ligand would potentiate EMT phenotype induced in spheroids, and compare endogenous EMT induction in EOC spheroids to TGF β 1-induced EMT in adherent EOC cells. Quantitative RT-PCR analysis revealed no significant changes in *CDH1*, *SNAI1*, *VIM*, *TWIST2* or *ZEB2* mRNA levels in TGF β 1-treated EOC ascites spheroids relative to untreated spheroids

Figure 2

TGF β 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 TGF β 1-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) TGF β 1 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 μ m). (c) Western blot analysis of E-cadherin, Claudin1 and Snail protein in adherent (A) and spheroid (S) samples treated with TGF β 1 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.

(data not shown). Western blot analysis confirmed that treatment of spheroids with TGF β 1 does not further increase Snail protein levels (Fig. 2c). Furthermore, there were higher levels of Snail protein in untreated spheroids compared with TGF β 1-treated EOC ascites cells indicating that spheroid formation alone may act as a potent inducer of EMT. We also observed that TGF β 1 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 TGF β 1 expression in EOC ascites-derived spheroids, we propose that endogenous TGF β 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 TGF β signaling in EOC ascites-derived cells in suspension will abrogate spheroid formation and induction of EMT. Inhibiting TGF β signaling during spheroid formation using a T β RI 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 TGF β 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 TGF β 1-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).

TGF β 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 TGF β signaling consistently blocks EMT and reduces cell-cell cohesion within spheroids, we sought to determine whether TGF β signaling inhibition affects the ability of spheroids to re-attach and disperse. Thus, our first assessment was to quantify

Figure 3

Inhibition of TGF β signaling in spheroids blocks EMT and compromises EOC spheroid morphology. (a) Quantitative RT-PCR analysis of *CDH1*, *SNAI1*, *TWIST2* and *ZEB2* mRNA in control and SB-431542-treated spheroids ($n=7$; $*P<0.05$ as determined by Student's t -test). (b) Western blot analysis of E-cadherin and Snail protein in spheroids formed from EOC ascites-derived patient samples ($n=6$). Actin was used as a loading control. (c) SB-431542 treatment of EOC ascites-derived cells at the time of seeding to ULA forms

smaller and less cohesive spheroids compared with DMSO controls. Representative image from EOC75 patient sample at 72 h post treatment (scale bar = 500 μ m). (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 $\pm$ s.e.m. and Student's t -test for statistical significance (**** $P<0.001$).

Figure 4

Inhibition of TGF β signaling in spheroids decreases migration of cells across a Transwell. (a) SB-431542 treatment of EOC ascites-derived cells at the time of seeding to ULA and transfer of day 3 spheroids to Transwell inserts decreases cell migration as compared with DMSO-treated controls. Representative EOC154 patient sample showing fewer migrated cells in SB-431542 treated spheroids compared with DMSO controls (scale bar = 100 μ m). (b) Transwell cell migration of treated spheroids from patient-derived samples ($n=9$) quantified using ImageJ software and averaged among five different fields per image (* $P<0.05$ as determined by Student's t -test).

cell migration from SB-431542-treated spheroids across a Transwell membrane. We observed that blockade of TGF β signaling significantly reduced the ability of cells ($n=9$) to migrate out of spheroids across a Transwell membrane (Fig. 4). Since we observed that SB-431542 treatment of spheroids reduces cell viability (Fig. 3d), we could not attribute these Transwell assay results to migration only. Therefore, we also used spheroid re-attachment assays to quantify cell motility, where only viable spheroid cells will re-attach and disperse. Spheroids formed over 3 days from patient ascites samples ($n=7$) were plated individually for re-attachment using standard tissue culture-treated plates. Treatment with SB-431542, or DMSO control, was started at the time of seeding for re-attachment. Remarkably, we observed less-cohesive spheroid cores within 24 h of SB-431542 treatment (Fig. 5a). We also observed a significant decrease in cell dispersion area in SB-431542-treated spheroids compared with DMSO controls (Fig. 5b). Since we demonstrated that SB-431542 treatment did not affect ascites cell proliferation over 72 h (Fig. 3d), this indicates that decreased dispersion from reattached spheroids is due to reduced cell motility via TGF β signaling inhibition.

Since inhibiting TGF β signaling in re-attached spheroids decreased cell motility of dispersing cells, we postulated that this was due to an enhanced epithelial

phenotype of dispersing cells. Indeed, EOC ascites-derived cell spheroids treated with SB-431542 during re-attachment yielded dispersing cells exhibiting an enhanced epithelial morphology compared with DMSO controls (Fig. 5c). To assess the epithelial morphology of cells dispersing from re-attached spheroids further, we performed immunofluorescence staining for several cytological markers. Since it was difficult to obtain membrane-specific immunofluorescence staining of E-cadherin, we chose to assess β -catenin. β -catenin is an integral component of the protein complex in adherens junctions and binds to the E-cadherin receptor intracellularly anchoring it to the actin cytoskeleton (Gumbiner 2005, Giannakouros *et al.* 2015). Furthermore, β -catenin protein expression was up-regulated in re-attached spheroids treated with SB-431542 by western blot analysis (data not shown). Immunofluorescence images showed enhanced plasma membrane localization of β -catenin in cells dispersing out of SB-431542 treated re-attached spheroids compared with DMSO controls (Fig. 5d). Moreover, phalloidin staining for F-actin showed reduced stress fiber formation in SB-431542 treated re-attached spheroids (Fig. 5e), also indicative of an enhanced epithelial phenotype of dispersing cells. To further validate this at the molecular level, we performed quantitative RT-PCR analysis of RNA isolated from re-attached spheroids from several independent EOC patient ascites samples. Re-attached spheroids treated with SB-431542 showed a significant up-regulation of *CDH1* mRNA and this was associated with a down-regulation of *SNAIL* (Supplementary Figure S3a, see section on supplementary data given at the end of this article). This was further validated by western blot analysis which showed enhanced E-cadherin protein expression associated with reduced Snail protein expression (in five out of six re-attached EOCs) in SB-431542 treated re-attached spheroids compared with DMSO controls (Supplementary Figure S3b).

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 TGF β signaling can affect ovarian cancer spheroid sensitivity to platinum agents, we treated EOC spheroids with SB-431542 or vehicle control first, followed by treatment

Figure 5

Inhibition of TGF β signaling in re-attached spheroids decreases motility of dispersing cells and enhances their epithelial phenotype. (a) SB-431542 treatment of EOC spheroids started at the time of re-attachment to standard tissue culture plates disrupts the spheroid core and decreases cell dispersion area. Representative image from EOC154 patient sample at 24 h post treatment (scale bar = 500 μ m). (b) Dispersion area was quantified using ImageJ software and averaged amongst 12 replicates per treatment condition (SB-431542 or DMSO control) for each EOC patient sample ($n = 7$). Dispersion area was calculated 24 h after spheroids had been re-plated to standard tissue culture plastic (* $P < 0.05$ as determined by Student's t -test).

(c) SB-431542 treatment of spheroids started at the time of re-attachment to standard tissue culture plates disrupts EOC spheroid core and changes morphology of dispersing cells to a more cuboidal epithelial phenotype. Representative image from EOC154 patient sample that was Hema-3-stained at 72 h post treatment (scale bar = 500 μ m). (d and e) Immunofluorescence images of cells dispersing out of SB-431542 treated re-attached spheroids shows enhanced β -catenin staining and reduced stress fiber formation (by rhodamine-phalloidin staining) compared with DMSO controls (scale bar = 100 μ m).

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 TGF β signaling in maintaining cell viability and achieving chemoresistance 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 TGF β 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 TGF β signaling is required to elicit this EMT response in spheroids since the potent small molecule type I receptor inhibitor SB-431542 blocks spheroid-induced EMT. SB-431542 treatment forces a more potent epithelial phenotype of dispersing cells from reattached spheroids, thereby reducing the spread of motile cells emanating from reattached spheroids. This also indicates that perhaps residual maintenance of TGF β signaling acts to promote invasion and motility upon spheroid

Figure 6

TGF β signaling in EOC ascites cells protects spheroids from platinum-induced cell death. (a) EOC ascites-derived cells ($n=4$) were treated with either DMSO or SB-431542 at the time of seeding to 24-well ULA cluster plate to form spheroids. Three days later, spheroids were treated with carboplatin (100 μ M) for 72 h. Representative EOC209 patient sample

showing reduced spheroid formation potential (fewer spheroids, more single cells) with combined carboplatin and SB-431542 treatment (scale bar = 1 mm). (b) Cell viability was determined using CyQUANT NF assay. Data is represented as mean $\pm$ S.E.M. and one-way ANOVA with Tukey's Multiple Comparison test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

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 pathobiological 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 TGF β 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 TGF β signaling pathway has been investigated for over 30 years in many human malignancies, including ovarian cancer (Massague 2008). Biologically-relevant concentrations of TGF β ligands have been detected in malignant ascites from patients (Abendstein *et al.* 2000, Dunfield *et al.* 2002). Primary EOC cells possess an intact TGF β 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 TGF β signaling was present in spheroids (Peart *et al.* 2012). This is consistent with TGF β 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 TGF β signaling in promoting the EMT phenotype of spheroids and their potential to reattach and spread. As such, TGF β 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 TGF β 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 TGF β 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 TGF β type I receptor inhibitor SB-431542. Most importantly, we also demonstrate that TGF β signaling blockade reduces EOC spheroid cell viability in concert with carboplatin treatment. It would be intriguing to test the numerous other TGF β 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 TGF β 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.

Funding

This research was supported by funding from an Ovarian Cancer Canada – Canadian Institutes for Health Research partnership grant (Grant number OVA-94083), a grant from the Canadian Cancer Society (Grant number 20109) and a London Regional Cancer Program Catalyst Grant with funds from the London Run for Ovarian Cancer. This work was also supported by the London Health Sciences Foundation with funds from the 100 Women Who Care and the Plunkett Foundation. S Rafehi was supported by a graduate scholarship from the Department of Obstetrics and Gynaecology at Western University.

Author contribution statement

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

reagents and materials: M Bertrand, J McGee, M Préfontaine and A Sugimoto. Wrote the paper: S Rafehi and T G Shepherd. Manuscript editing: S Rafehi, G E DiMattia and T G Shepherd.

Acknowledgements

We acknowledge the essential contribution of all members of our Translational Ovarian Cancer Research Program. In particular, we thank Christine Gawlik, Carrie Thornton and Kay Faroni for assistance with retrieval of clinical data, and Rachel Dales, Nicole Lesmeister, Meera Shah and Vinny Aggarwal for assisting with experiments. We are also extremely grateful to the women with ovarian cancer who generously donated their ascites samples to support our research.

References

- Abendstein B, Stadlmann S, Knabbe C, Buck M, Muller-Holzner E, Zeimet AG, Marth C, Obrist P, Krugmann J & Offner FA 2000 Regulation of transforming growth factor- β secretion by human peritoneal mesothelial and ovarian carcinoma cells. *Cytokine* **12** 1115–1119. (doi:10.1006/cyto.1999.0632)
- Ahmed N, Thompson EW & Quinn MA 2007 Epithelial–mesenchymal interconversions in normal ovarian surface epithelium and ovarian carcinomas: an exception to the norm. *Journal of Cellular Physiology* **213** 581–588. (doi:10.1002/jcp.21240)
- Akhurst RJ 2006 Large- and small-molecule inhibitors of transforming growth factor- β signaling. *Current Opinion in Investigational Drugs* **7** 513–521.
- Akhurst RJ & Hata A 2012 Targeting the TGF β signalling pathway in disease. *Nature Reviews. Drug Discovery* **11** 790–811. (doi:10.1038/nrd3810)
- Bast RC Jr, Hennessy B & Mills GB 2009 The biology of ovarian cancer: new opportunities for translation. *Nature Reviews. Cancer* **9** 415–428. (doi:10.1038/nrc2644)
- Cannistra SA 2004 Cancer of the ovary. *New England Journal of Medicine* **351** 2519–2529. (doi:10.1056/NEJMra041842)
- Cao L, Shao M, Schilder J, Guise T, Mohammad KS & Matei D 2012 Tissue transglutaminase links TGF- β , epithelial to mesenchymal transition and a stem cell phenotype in ovarian cancer. *Oncogene* **31** 2521–2534. (doi:10.1038/ncr.2011.429)
- Correa RJ, Peart T, Valdes YR, DiMattia GE & Shepherd TG 2012 Modulation of AKT activity is associated with reversible dormancy in ascites-derived epithelial ovarian cancer spheroids. *Carcinogenesis* **33** 49–58. (doi:10.1093/carcin/bgr241)
- Davidson B, Trope CG & Reich R 2014 Epithelial–mesenchymal transition in ovarian carcinoma. *Frontiers in Oncology* **2** 33. (doi:10.3389/fonc.2012.00033)
- Do TV, Kubba LA, Du H, Sturgis CD & Woodruff TK 2008 Transforming growth factor- β 1, transforming growth factor- β 2, and transforming growth factor- β 3 enhance ovarian cancer metastatic potential by inducing a Smad3-dependent epithelial-to-mesenchymal transition. *Molecular Cancer Research* **6** 695–705. (doi:10.1158/1541-7786.MCR-07-0294)
- Dunfield LD & Nachtigal MW 2003 Inhibition of the antiproliferative effect of TGF β by EGF in primary human ovarian cancer cells. *Oncogene* **22** 4745–4751. (doi:10.1038/sj.onc.1206617)
- Dunfield LD, Dwyer EJ & Nachtigal MW 2002 TGF β -induced Smad signaling remains intact in primary human ovarian cancer cells. *Endocrinology* **143** 1174–1181. (doi:10.1210/endo.143.4.8733)
- Elloul S, Vaksman O, Stavnes HT, Trope CG, Davidson B & Reich R 2010 Mesenchymal-to-epithelial transition determinants as characteristics of ovarian carcinoma effusions. *Clinical & Experimental Metastasis* **27** 161–172. (doi:10.1007/s10585-010-9315-2)
- Fujioka T, Takebayashi Y, Kihana T, Kusanagi Y, Hamada K, Ochi H, Uchida T, Fukumoto M & Ito M 2001 Expression of E-cadherin and β -catenin in primary and peritoneal metastatic ovarian carcinoma. *Oncology Reports* **8** 249–255.
- Giannakouros P, Comamala M, Matte I, Rancourt C & Piche A 2015 MUC16 mucin (CA125) regulates the formation of multicellular aggregates by altering β -catenin signaling. *American Journal of Cancer Research* **5** 219–230.
- Gumbiner BM 2005 Regulation of cadherin-mediated adhesion in morphogenesis. *Nature Reviews. Molecular Cell Biology* **6** 622–634. (doi:10.1038/nrm1699)
- Han XY, Wei B, Fang JF, Zhang S, Zhang FC, Zhang HB, Lan TY, Lu HQ & Wei HB 2013 Epithelial–mesenchymal transition associates with maintenance of stemness in spheroid-derived stem-like colon cancer cells. *PLoS One* **8** e73341. (doi:10.1371/journal.pone.0073341)
- Hanahan D & Weinberg RA 2011 Hallmarks of cancer: the next generation. *Cell* **144** 646–674. (doi:10.1016/j.cell.2011.02.013)
- Haslehurst AM, Koti M, Dharsee M, Nuin P, Evans K, Geraci J, Childs T, Chen J, Li J, Weberpals J et al. 2012 EMT transcription factors snail and slug directly contribute to cisplatin resistance in ovarian cancer. *BMC Cancer* **12** 91. (doi:10.1186/1471-2407-12-91)
- Huang RY, Chung VY & Thiery JP 2012 Targeting pathways contributing to epithelial–mesenchymal transition (EMT) in epithelial ovarian cancer. *Current Drug Targets* **13** 1649–1653. (doi:10.2174/138945012803530044)
- Inman GJ, Nicolas FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, Laping NJ & Hill CS 2002 SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Molecular Pharmacology* **62** 65–74. (doi:10.1124/mol.62.1.65)
- Iwanicki MP, Davidowitz RA, Ng MR, Besser A, Muranen T, Merritt M, Danuser G, Ince TA & Brugge JS 2011 Ovarian cancer spheroids use myosin-generated force to clear the mesothelium. *Cancer Discovery* **1** 144–157. (doi:10.1158/2159-8274.CD-11-0010)
- Jakowlew SB 2006 Transforming growth factor- β in cancer and metastasis. *Cancer Metastasis Reviews* **25** 435–457. (doi:10.1007/s10555-006-9006-2)
- Jemal A, Bray F, Center MM, Ferlay J, Ward E & Forman D 2011 Global cancer statistics. *CA: A Cancer Journal for Clinicians* **61** 69–90. (doi:10.3322/caac.20107)
- Kalluri R & Weinberg RA 2009 The basics of epithelial–mesenchymal transition. *Journal of Clinical Investigation* **119** 1420–1428. (doi:10.1172/JCI39104)
- Koensgen D, Freitag C, Klamann I, Dahl E, Mustea A, Chekerov R, Braicu I, Lichtenegger W & Sehouli J 2010 Expression and localization of E-cadherin in epithelial ovarian cancer. *Anticancer Research* **30** 2525–2530.
- Kurrey NK, Amit K & Bapat SA 2005 Snail and Slug are major determinants of ovarian cancer invasiveness at the transcription level. *Gynecologic Oncology* **97** 155–165. (doi:10.1016/j.ygyno.2004.12.043)
- Lengyel E 2010 Ovarian cancer development and metastasis. *American Journal of Pathology* **177** 1053–1064. (doi:10.2353/ajpath.2010.100105)
- Lengyel E, Burdette JE, Kenny HA, Matei D, Pilrose J, Haluska P, Nephew KP, Hales DB & Stack MS 2014 Epithelial ovarian cancer experimental models. *Oncogene* **33** 3619–3633. (doi:10.1038/ncr.2013.321)
- Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C(T))$ method. *Methods* **25** 402–408. (doi:10.1006/meth.2001.1262)
- Lu ZY, Dong R, Li D, Li WB, Xu FQ, Geng Y & Zhang YS 2012 SNAI1 overexpression induces stemness and promotes ovarian cancer cell invasion and metastasis. *Oncology Reports* **27** 1587–1591. (doi:10.3892/or.2012.1685)
- Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M et al. 2008 The epithelial–mesenchymal transition generates cells with properties of stem cells. *Cell* **133** 704–715. (doi:10.1016/j.cell.2008.03.027)

- Marques FR, Fonsechi-Carvasan GA, De Angelo Andrade LA & Bottcher-Luiz F 2004 Immunohistochemical patterns for α - and β -catenin, E- and N-cadherin expression in ovarian epithelial tumors. *Gynecologic Oncology* **94** 16–24. (doi:10.1016/j.ygyno.2004.03.037)
- Massague J 2008 TGF β in cancer. *Cell* **134** 215–230. (doi:10.1016/j.cell.2008.07.001)
- Micalizzi DS, Farabaugh SM & Ford HL 2010 Epithelial–mesenchymal transition in cancer: parallels between normal development and tumor progression. *Journal of Mammary Gland Biology and Neoplasia* **15** 117–134. (doi:10.1007/s10911-010-9178-9)
- Pearl TM, Correa RJ, Valdes YR, Dimattia GE & Shepherd TG 2012 BMP signalling controls the malignant potential of ascites-derived human epithelial ovarian cancer spheroids via AKT kinase activation. *Clinical & Experimental Metastasis* **29** 293–313. (doi:10.1007/s10585-011-9451-3)
- Polyak K & Weinberg RA 2009 Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nature Reviews. Cancer* **9** 265–273. (doi:10.1038/nrc2620)
- Pon YL, Zhou HY, Cheung AN, Ngan HY & Wong AS 2008 p70 S6 kinase promotes epithelial to mesenchymal transition through snail induction in ovarian cancer cells. *Cancer Research* **68** 6524–6532. (doi:10.1158/0008-5472.CAN-07-6302)
- Rustin G, van der Burg M, Griffin C, Qian W & Swart AM 2011 Early versus delayed treatment of relapsed ovarian cancer. *Lancet* **377** 380–381. (doi:10.1016/S0140-6736(11)60126-8)
- Sawada K, Mitra AK, Radjabi AR, Bhaskar V, Kistner EO, Tretiakova M, Jagadeeswaran S, Montag A, Becker A, Kenny HA et al. 2008 Loss of E-cadherin promotes ovarian cancer metastasis via $\alpha 5$ -integrin, which is a therapeutic target. *Cancer Research* **68** 2329–2339. (doi:10.1158/0008-5472.CAN-07-5167)
- Shepherd TG, Theriault BL, Campbell EJ & Nachtigal MW 2006 Primary culture of ovarian surface epithelial cells and ascites-derived ovarian cancer cells from patients. *Nature Protocols* **1** 2643–2649. (doi:10.1038/nprot.2006.328)
- Shield K, Ackland ML, Ahmed N & Rice GE 2009 Multicellular spheroids in ovarian cancer metastases: biology and pathology. *Gynecologic Oncology* **113** 143–148. (doi:10.1016/j.ygyno.2008.11.032)
- Turley EA, Veiseth M, Radisky DC & Bissell MJ 2008 Mechanisms of disease: epithelial–mesenchymal transition – does cellular plasticity fuel neoplastic progression? *Nature Clinical Practice. Oncology* **5** 280–290. (doi:10.1038/ncponc1089)
- Vergara D, Merlot B, Lucot JP, Collinet P, Vinatier D, Fournier I & Salzet M 2010 Epithelial–mesenchymal transition in ovarian cancer. *Cancer Letters* **291** 59–66. (doi:10.1016/j.canlet.2009.09.017)
- Xu Z, Jiang Y, Steed H, Davidge S & Fu Y 2010 TGF β and EGF synergistically induce a more invasive phenotype of epithelial ovarian cancer cells. *Biochemical and Biophysical Research Communications* **401** 376–381. (doi:10.1016/j.bbrc.2010.09.059)
- Yoshida J, Horiuchi A, Kikuchi N, Hayashi A, Osada R, Ohira S, Shiozawa T & Konishi I 2009 Changes in the expression of E-cadherin repressors, Snail, Slug, SIP1, and Twist, in the development and progression of ovarian carcinoma: the important role of Snail in ovarian tumorigenesis and progression. *Medical Molecular Morphology* **42** 82–91. (doi:10.1007/s00795-008-0436-5)
- Yuecheng Y, Hongmei L & Xiaoyan X 2006 Clinical evaluation of E-cadherin expression and its regulation mechanism in epithelial ovarian cancer. *Clinical & Experimental Metastasis* **23** 65–74. (doi:10.1007/s10585-006-9020-3)
- Zavadil J & Bottinger EP 2005 TGF- β and epithelial-to-mesenchymal transitions. *Oncogene* **24** 5764–5774. (doi:10.1038/sj.onc.1208927)

Received in final form 30 November 2015

Accepted 8 December 2015

Made available online as an Accepted Preprint

8 December 2015