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Granulocyte colony-stimulating factor receptor signalling via Janus kinase 2/signal transducer and activator of transcription 3 in ovarian cancer

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Background: Ovarian cancer remains a major cause of cancer mortality in women, with only limited understanding of disease aetiology at the molecular level. Granulocyte colony-stimulating factor (G-CSF) is a key regulator of both normal and emergency haematopoiesis, and is used clinically to aid haematopoietic recovery following ablative therapies for a variety of solid tumours including ovarian cancer.

Methods: The expression of G-CSF and its receptor, G-CSFR, was examined in primary ovarian cancer samples and a panel of ovarian cancer cell lines, and the effects of G-CSF treatment on proliferation, migration and survival were determined.

Results: G-CSFR was predominantly expressed in high-grade serous ovarian epithelial tumour samples and a subset of ovarian cancer cell lines. Stimulation of G-CSFR-expressing ovarian epithelial cancer cells with G-CSF led to increased migration and survival, including against chemotherapy-induced apoptosis. The effects of G-CSF were mediated by signalling via the downstream JAK2/STAT3 pathway.

Conclusion: This study suggests that G-CSF has the potential to impact on ovarian cancer pathogenesis, and that G-CSFR expression status should be considered in determining appropriate therapy.

Ovarian cancer is the most lethal gynaecological malignancy and the fourth leading cause of cancer mortality in women (Gonzalez-Martin *et al*, 2010). This is largely due to the asymptomatic nature of the disease, such that women with ovarian cancer typically present with advanced-stage disease, when the cancer has already spread throughout the peritoneum and, in some cases, to distant sites (Lengyel, 2010). With aggressive surgical manipulation and subsequent chemotherapy, most patients can return to a state of microscopic disease with minimal residual tumours. However, such remission is commonly short-lived, with <40% survival at 5 years, due to recurrent disease (Gonzalez-Martin *et al*, 2010). In

addition, much remains to be understood about the aetiology of ovarian cancer at the molecular level, which would form the basis of more effective therapeutic approaches.

The vast majority of ovarian cancers arise from malignant transformation of either the ovarian surface epithelium (OSE) (Hudson *et al*, 2008) or the secretory cells of Fallopian tube epithelium (FTE) (Colombo *et al*, 2010; Karst and Drapkin, 2010). The process by which it occurs remains poorly understood; however, it has been postulated that the repetitive inflammatory stress to OSE or FTE leads to the clonal expansion of OSE and/or FTE secretory cells forming a neoplastic precursor lesion, which

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ultimately gives rise to ovarian tumours (Colombo *et al*, 2010). One key event in the progression of the disease is the spread of tumour cells into the peritoneum where they survive as cellular aggregates or spheroids prior to attachment to a suitable secondary site for further growth, invasion and metastasis (Burleson *et al*, 2006; Shield *et al*, 2009). Tumour cells are known to secrete cytokines and growth factors (Punnonen *et al*, 1998; Zeimet *et al*, 1998; Zebrowski *et al*, 1999), resulting in autocrine and paracrine loops that are able to support the anchorage-independent growth of ovarian cellular aggregates and stimulate invasion (Ahmed *et al*, 2005; Shield *et al*, 2007). Indeed, stimulation of the epidermal growth factor receptor (EGFR) has already been strongly implicated in the progression of this disease (Colomiere *et al*, 2009; Xu *et al*, 2010; Zeineldin *et al*, 2010).

Cytokine receptors are divided into a number of groups based on structural characteristics, which often translate into functional similarities. This includes the so-called 'interleukin-6 receptor (IL-6R) family', which employ glycoprotein 130 (GP130) or related receptor chains for signal transduction via the Janus kinase/Signal transducer and activator of transcription (JAK2/STAT3) pathway. A number of studies have described roles for altered signalling from IL-6R family members as a contributing factor to a range of malignancies. For example, leukaemia inhibitor factor (LIF) has been shown to stimulate growth of pancreatic carcinomas (Kamohara *et al*, 2007); both IL-6 and oncostatin M (OSM) are able to stimulate proliferation of prostate cancer cells (Godoy-Tundidor *et al*, 2005), while granulocyte colony-stimulating factor (G-CSF) has similar roles in leukaemia (Katayama *et al*, 1998) and squamous cell carcinoma (Hirai *et al*, 2001). Autocrine activation appears to have a key role, as described for IL-6/IL-6R in a variety of tumours (Grivennikov and Karin, 2008) and G-CSF/G-CSFR during malignant transformation in bladder cancer (Chakraborty *et al*, 2004). Meanwhile, activation of the downstream JAK2 and STAT3 has been shown to contribute to renal cell carcinoma (Wu *et al*, 2007), lung cancer (Gao *et al*, 2007) and breast cancer (Sansone *et al*, 2007), as well as ovarian cancer (Rose-John *et al*, 2007; Colomiere *et al*, 2009).

We and others have also demonstrated a role for aberrant IL-6/IL-6R signalling in ovarian cancer (Wang *et al*, 2008; Colomiere *et al*, 2009). However, studies on the G-CSF/G-CSFR pathway have been more limited. This is significant because G-CSF has been employed to aid haematopoietic recovery following chemotherapy for a range of malignancies including ovarian cancer (Bohlius *et al*, 2003; Ray-Coquard *et al*, 2007). Therefore, the potential exists for deleterious effects from use of G-CSF as part of ovarian cancer therapy. This current study demonstrates that both G-CSF and G-CSFR are expressed on high-grade ovarian tumours and ovarian cancer cells, and that G-CSF/G-CSFR signalling via JAK2/STAT3 participates in ovarian cancer cell migration and resistance against apoptosis.

MATERIALS AND METHODS

Clinical samples. Ovarian epithelial tumours of different pathological grades were collected from patients diagnosed with high-grade ovarian carcinoma, after obtaining written informed consent under protocols approved by the Human Research and Ethics Committee (HREC #09/09) of The Royal Women's Hospital, Melbourne, Australia, or via the Victorian Cancer Biobank and approved by the Deakin University Human Research Ethics Committee (DUHREC#2010-104). The histopathological diagnosis and tumour grades were determined by staff pathologists as part of the clinical diagnosis. Normal ovaries needed for control comparisons were obtained from patients undergoing surgery as a result of suspicious ultrasound images or a family history of

ovarian cancer. Specimens were snap-frozen under liquid nitrogen and subsequently stored at -80°C for expression analysis or preserved in Tissue-Tek (Torrance, CA, USA) embedding medium and frozen for immunohistochemical studies.

Cell lines. The human epithelial ovarian cancer lines TOV21G (Provencher *et al*, 2000), ES2, CAOV3, PA1 and SKOV3 (Fogh *et al*, 1977) were obtained from American Type Culture Collection, while OVCA433, OVCA429 and HEY (Ahmed *et al*, 2003) were obtained from Royal Women's Hospital Melbourne. Cell lines were grown as a monolayer in 25 or 75 cm^2 flasks (BD Biosciences, Sydney, NSW, Australia) in a 1:1 mixture of medium 199 (Life Technologies, Melbourne, VIC, Australia) and MCDB105 (Sigma Aldrich, Sydney, NSW, Australia), supplemented with 10% (*v/v*) heat-inactivated fetal bovine serum and 2 mM glutamine (Life Technologies, Rockville, MD, USA) at 37°C with 5% CO_2 . These cells were treated with recombinant human IL-6 (10 ng ml^{-1}) or G-CSF (10 ng ml^{-1}) (Cell Signaling, Beverly, MA, USA), cisplatin ($5\text{ }\mu\text{g ml}^{-1}$, from saline solution) (Pfizer, Perth, WA, Australia) or paclitaxel ($1\text{ }\mu\text{g ml}^{-1}$, from saline solution) (Hospira, Melbourne, VIC, Australia), with or without specific inhibitors for JAK2 (WP1066, $2.5\text{ }\mu\text{M}$) and STAT3 (LLL12, $2.5\text{ }\mu\text{M}$) (BioVision, Mountain View, CA, USA).

Immunohistochemistry. The expression of G-CSFR and relative activation status of STAT3 were assessed on paraffin-embedded primary tumour samples by immunohistochemistry as described previously (Colomiere *et al*, 2009). Briefly, tissue sections were blocked (1:50 human serum) and incubated with primary antibodies, washed and developed using appropriate secondary antibodies and the Peroxidase Universal Kit (Dako LSAB, Cambellfield, VIC, Australia) after elimination of non-specific peroxidase activity. Slides were counterstained with 1% (*w/v*) haematoxylin to highlight cell nuclei. Optical image capture and analysis was performed using AxioVision (Zeiss, North Ryde, NSW, Australia) and K5400 Zeiss software image analysis on a Zeiss Axioskop2 Microscope. Ten fields from each section were analysed in a 'blind' fashion and the percent tissue staining determined, which was scored as follows: 0 ($\leq 10\%$), 1 ($\geq 11-25\%$), 2 ($\geq 26-50\%$), 3 ($\geq 51-75\%$), 4 ($\geq 76-90\%$) and 5 ($\geq 91-100\%$). Control slides were processed in the absence of primary antibody, secondary antibody and chromogenic substrate, or with isotype control IgG to ensure specificity.

Flow cytometry. Cell lines were grown to confluence prior to harvest for fluorescence-activated cell sorter (FACS) analysis. Cell monolayers were washed twice with phosphate buffered saline (PBS), detached with 0.25% (*w/v*) trypsin-EDTA solution, collected by centrifugation and washed a further two times with PBS. Approximately 1×10^6 cells were fixed using 4% (*w/v*) paraformaldehyde (PFA) and subsequently blocked for 30 min in PBS containing 10% (*v/v*) goat serum. After incubation, the cells were washed twice with PBS, incubated with anti-G-CSFR-PE antibody (AbCam, Cambridge, MA, USA) at a concentration of $1\text{ }\mu\text{g }\mu\text{l}^{-1}$ for 30 min, followed by three washes in PBS. Samples were analysed on FACS Canto (BD Bioscience) using FACS DIVA software, in comparison to appropriate controls.

Protein extraction and western blot analysis. Proteins were extracted using RIPA buffer containing complete protease inhibitors (Sigma Aldrich) according to the manufacturer's instructions. The concentration of the protein was estimated using a Pierce BCA Protein Determination Kit (Thermo Scientific, Asheville, NC, USA) following the manufacturer's instructions, with bovine serum albumin used as standards, and the absorbance at 562 nm determined using a Perkin Elmer VICTOR X Multilabel Plate Reader (Perkin Elmer, Glen Waverly, VIC, Australia). Western blot was performed as previously described (Gits *et al*, 2006), using the following monoclonal antibodies: anti-

phospho-STAT3 (Tyr 705), anti-total-STAT3, anti-BCL2, anti-phospho-ERK (Tyr 204), anti-total-ERK, anti-phospho-AKT (Ser 473), anti-total-AKT, anti-phospho-NF κ B p65 (Ser 536), anti-total-NF κ B p65, and anti-ERCC1 (Cell Signaling), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Millipore Technologies, Sydney, NSW, Australia) and anti-CRP, anti-MDR1 and anti-GST-pi (AbCam). The secondary antibodies were anti-rabbit IRDye 680 CW (red) and anti-mouse IRDye 800 CW (green) (LI-COR Biosciences, Lincoln, NE, USA).

EMSA. Nuclear extracts were prepared and analysed by electrophoretic mobility shift assay (EMSA) using the m67 high-affinity STAT-binding site probe, as described previously (Ward *et al*, 1999a).

ELISA. ELISA was performed using Human G-CSF Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) as per the manufacturer's instructions.

Cell migration assay. The migratory potential of ovarian cancer cells was assessed as previously described (Lim *et al*, 2007). Briefly, cells were grown as a confluent monolayer in a six-well plate and then wounded using a sterile 200 μ l pipette tip. Three representative fields were marked and imaged immediately after wounding and 15 h later following incubation with cytokines and inhibitors as appropriate. Photos were taken using Olympus SC20 camera (Olympus, Tokyo, Japan) and the width of the scratches was measured at each time point and quantified using Cell Profiler (Lamprecht *et al*, 2007).

Growth assay. Cell growth rates in the presence and absence of cytokines were assessed as previously described (Ouellet *et al*, 2008). Briefly, 1×10^5 cells per well were seeded onto a six-well plate containing media with or without cytokines. Cells were trypsinised and resuspended in medium for counting using a hemocytometer daily over a 5-day period. Each experiment was performed in triplicate for each harvest and repeated twice.

Chemotaxis assay. Chemotaxis assays were performed using a two-chamber Transwell (Corning Inc, Tewksbury, MA, USA), as described previously (Zhao *et al*, 2011), using a polycarbonate filter with 3 μ m pores. Cells were trypsinised and suspended in medium containing 0.1% (*v/v*) FBS at a concentration of 1×10^5 cells per well. The cells were placed in the upper chamber and the medium containing 0.1% (*v/v*) was placed in the lower chamber. After 4 h at 37 °C, the cells in the upper chamber were wiped off with a cotton swab. The cells on the lower surface of the filter were fixed with 4% (*w/v*) PFA for 1 min at room temperature, permeabilised with 100% (*v/v*) methanol, washed with PBS and stained with Giemsa solution (Sigma Aldrich). Migration was quantitated by selecting 10 different views and the number of migrated cells was calculated.

Apoptosis assay. Apoptosis assays were performed using flow cytometry analysis of cells doubly stained with anti-Annexin V-FITC and propidium iodide (PI) using an Apoptosis Detection Kit (BD Bioscience).

RNA extraction and semi-quantitative RT-PCR. Total RNA was extracted from cells using Trizol (Life Technologies) according to the manufacturer's instruction. The concentration and purity of RNA were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). A 0.5 μ g per mg sample of RNA was used for cDNA synthesis using an iScript cDNA synthesis Kit (Bio-Rad, Gladesville, NSW, Australia) according to the manufacturer's instructions. PCR reactions were performed on a My-Cycler thermal cycler (Bio-Rad) using 25 μ l reaction volumes containing 500 ng cDNA template, 5.5 μ l sterile nuclease-free water (Life Technologies), 12.5 μ l $2 \times$ GoTaq Green Master Mix containing TaqDNA polymerase, dNTPs, MgCl $_2$ and reaction buffers at optimal concentrations (Promega, Alexandria, NSW, Australia)

and 0.4 μ M of each gene-specific forward and reverse primers. The following primer pairs were used:

G-CSFR (CSF3R) 5'-CCTGGAGCTGAGAAGTACCG-3'/5'-CTTCTGAAGGCAGGTGGAG-3';

G-CSF (CSF3) 5'-CAGAGCTTCCTGGAGGTGTC-3'/5'-ATGGGAGGACAGGAGCTTTT-3';

IL-6R 5'-CTCCTGCCAGTTAGCAGTCC-3'/5'-TCTTGCCAGGTGACACTGAG-3';

IL-6 5'-TACCCCCAGGAGAAGATTCC-3'/5'-TTTTCTGCCAGTGCCCTCTTT-3';

BCL2 5'-GGATGCCTTTGTGGAAGTGT-3'/5'-AGCCTGCAGCTTTGTTTCAT-3';

CRP 5'-ATACCCAGGCCACAAGAGTG-3'/5'-ACGTCCTCTCAGCTTGAAA-3';

β -ACTIN 5'-GGACTTCGAGCAAGAGATGG-3'/5'-AGCACTGTGTTGGCGTACAG-3';

GAPDH (GAPD) 5'-ATGTTTCGTCATGGGTGTGAA-3'/5'-GTTCTTGGGTGGCAGTGAT-3'.

Amplification conditions were typically 94 °C for 2 min, followed by 94 °C for 1 min, 58 °C for 1.5 min and 72 °C for 1 min for a total of 35 cycles, with a final condition of 72 °C for 10 min, unless otherwise specified. Control reactions were performed using no RT-control to confirm the absence of contaminating genomic DNA, and without cDNA template to ensure that amplicon products were not the result of contamination or primer-dimer effects on RT samples. PCR products were visualised on 1–3% (*w/v*) agarose gels containing SYBR Safe (Life Technologies) and imaged using a Chemidoc XRS Molecular Imager System (Bio-Rad).

Quantitative reverse transcriptase PCR (qRT-PCR). Gene expression was quantified by qRT-PCR on an Agilent Stratagene MX3000P. Reactions (25 μ l) contained 3.125 μ l nuclease-free water, 3.125 μ l cDNA template (1:10 dilution), 12.5 μ l iQ 2X SYBR Green Supermix (Bio-Rad) and 3.125 μ l each of forward and reverse primer (2.4 μ M). Typical PCR conditions consisted of 95 °C for 30 s, followed by 45 cycles of 95 °C for 10 s, 60 °C for 30 s, 72 °C for 20 s, then 95 °C for 1 min, 58 °C for 1 min, and finally incremental increases (0.5 °C) from 58 °C to 95 °C to establish the melting curve for each sample. Appropriate control reactions were performed to ensure products were not the result of DNA contamination or due to primer-dimer formation. Data retrieved from these assays were analysed using the Livak method (Livak and Schmittgen, 2001).

Statistical analysis. All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software Ltd., La Jolla, CA, USA). Data were expressed as mean \pm s.e.m. The statistical correlation of data between groups was analysed by one-way analysis of variance (ANOVA) and a two-tailed Student's *t*-test, where *P* < 0.05 was considered significant.

RESULTS

Expression of G-CSFR and its ligand in ovarian cancer. To investigate the role of G-CSFR in ovarian cancer, semi-quantitative RT-PCR was used to determine the expression of the genes encoding G-CSFR and its ligand G-CSF in a series of primary ovarian cancers of different grades in comparison to the genes encoding IL-6R and its ligand IL-6 (Figure 1A). This revealed strong G-CSFR expression in three out of four Grade 3 serous samples, with broad expression of G-CSF across all samples. IL-6R and IL-6 were expressed in a range of samples, irrespective of grade. The involvement of G-CSFR was further investigated on a panel of Grade 3 ovarian tumours by qRT-PCR (Figure 1B), which confirmed a majority of samples showed elevated levels of G-CSFR compared with normal controls. Immunohistochemistry

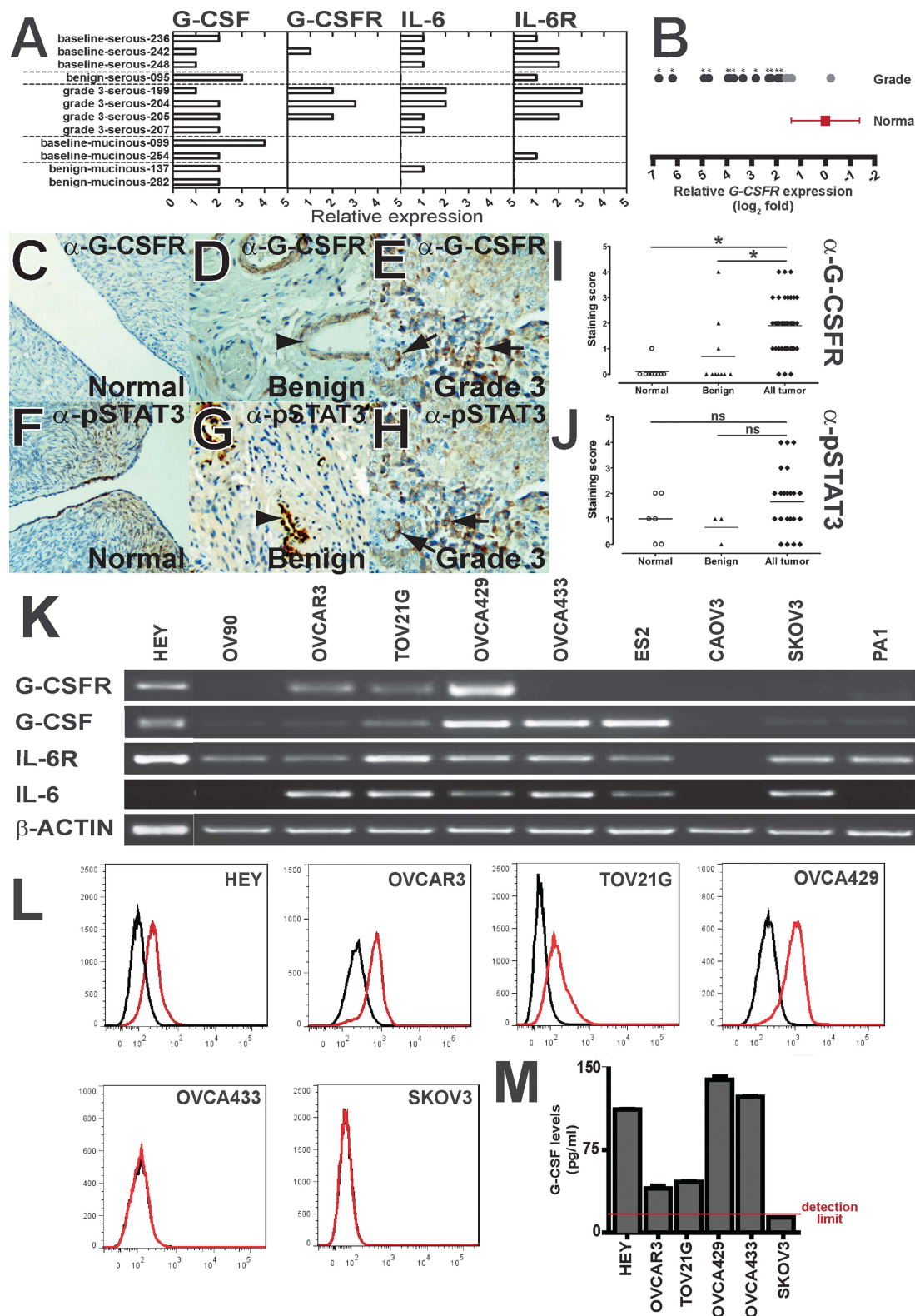


Figure 1. Expression of G-CSF/G-CSFR in ovarian cancer. (A–B) Expression of G-CSF/G-CSFR in primary ovarian cancers. RNA derived from the indicated patient samples were analysed with primers specific for *G-CSF*, *G-CSFR*, *IL-6*, *IL-6R* and β -*ACTIN* as a control. This analysis used either semi-quantitative RT-PCR with expression of *G-CSF*, *G-CSFR*, *IL-6* and *IL-6R* scored on a five-point scale on the indicated tumour samples (A), or by qRT-PCR for *G-CSFR* relative to β -*ACTIN* on Grades 3 tumour samples (B). (C–J) Detection of G-CSFR and phospho-STAT3 in ovarian cancer. Immunohistochemical staining of normal ovary (C, F), benign tumour (D, G) or Grade 3 (E, H) tumour samples with anti-G-CSFR (C–E) or anti-pSTAT3 (F–H), as indicated. Arrows indicate scattered epithelial staining with both antibodies in Grade 3 tumours, and arrowheads indicate vessel-associated staining. Immunohistochemical staining with anti-G-CSFR or anti-pSTAT3 was scored on a scale of 0–5, and represented as a scatter-plot for normal ovary, benign and pooled tumour groups, with the level of statistical significance indicated (I–J, * $P < 0.05$, ns: not significant). (K–M) Expression of G-CSF/G-CSFR in a panel of ovarian cancer cell lines. Cells were analysed by RT-PCR for expression of the indicated genes (K), subjected to FACS analysis using anti-G-CSFR-PE (red line) or an isotype control (black line) (L), or conditioned media obtained and analysed for G-CSF by ELISA (M).

on primary tissue sections confirmed an absence of G-CSFR expression on normal ovaries (Figure 1C), occasional weak staining on benign tumours (Figure 1D), but significant scattered epithelial staining within a cohort of tumours (Figure 1E), along with some vessel-associated staining. In total, ~60% (22 out of 36) of tumour samples tested showed significant G-CSFR staining (score ≥ 2), compared with 0% (0 out of 11) in normal controls and 20% (2 out of 10) in benign tumours. Staining with anti-pSTAT3 revealed an overall low level epithelial staining on normal ovary and benign tumours (Figures 1F and G), but significant staining of vessels (Figure 1G). In contrast, a cohort of Grade 1–3 tumours again exhibited strong scattered pSTAT3 epithelial staining (Figure 1H). Importantly, the majority of samples with a significant G-CSFR staining score were also positive for pSTAT3 staining (Figures 1I and J, and data not shown), consistent with active G-CSFR signalling.

The analysis of G-CSFR and its ligand was extended to a panel of ovarian cancer cell lines, with expression of β -ACTIN used as a control (Figure 1K). This revealed four cell lines (HEY, OVCAR3, TOV21G and OVCA429) that were positive for G-CSFR expression and all but two positive for G-CSF, confirming the two genes are not co-ordinately regulated. All cell lines except CAOV3 were positive for IL-6R and IL-6 was expressed in the majority, consistent with previous reports (Watson *et al*, 1990). FACS analysis was used to confirm cell-surface expression of G-CSFR (Figure 1L), with strong staining observed in the four cell lines that were positive for G-CSFR expression by RT-PCR, but not the two RT-PCR negative lines tested (OVCA433 and SKOV3). As it has been previously reported that stimulation with IL-6 and EGF can affect the expression of IL-6 family cytokine receptors and their ligands (Colomiere *et al*, 2009), we also examined the relative expression of G-CSF/G-CSFR and IL-6/IL-6R following treatment with IL-6 and G-CSF. However, only modest changes were observed (Supplementary Figure 1). Finally, conditioned media were analysed using a G-CSF-specific ELISA, which confirmed robust production of G-CSF, particularly in HEY, OVCA429 and OVCA433 cells (Figure 1M), despite the latter not expressing G-CSFR.

G-CSF stimulates ovarian cancer cell migration. Having established the expression of G-CSFR in primary ovarian cancer samples and ovarian cancer cell lines, we sought to investigate the functional significance of this with regard to key cancer phenotypes. We first examined migration using a wound-healing assay (Jones *et al*, 2008), which was performed either without cytokines or in the presence of G-CSF or IL-6 (Figure 2A). Migration of cells after 15 h was found to be increased upon G-CSF stimulation in the G-CSFR-positive cell lines (OVCA429, HEY and TOV21G) but not in the G-CSFR-negative cell lines. In contrast, IL-6 enhanced migration in each of the cell lines tested, all of which were IL-6R-positive.

We next explored whether the migration observed was part of a chemotactic response using a Transwell assay to measure directional cell migration. Addition of G-CSF again increased migration in all G-CSFR-positive cell lines (Figure 2B). However, no significant difference in migration was observed whether the G-CSF was placed in the upper or lower chamber (data not shown), suggesting G-CSF-stimulated increased cell migration was not chemotactic in nature.

G-CSF does not affect proliferation but protects against apoptosis. Cytokines are known to stimulate proliferation and survival in responsive cells. Therefore, we examined those parameters in ovarian cancer cell lines in the presence or absence of cytokines. IL-6 elicited a significant increase in proliferation in all cell lines, but no enhancement in proliferation was observed with G-CSF (Figure 2C). We next sought to investigate the possible effects of G-CSF on survival. However, the cell lines tested showed

very low levels of apoptosis, even in low serum, and so apoptosis was induced by the addition of sodium azide (0.5%) in order to evaluate anti-apoptotic responses. G-CSF was able to provide variable but statistically significant protection against apoptosis in all G-CSFR-positive cell lines (Figure 2D). IL-6 also generally enhanced survival in this setting.

Effects of G-CSF are mediated by the JAK2/STAT3 pathway. Granulocyte colony-stimulating factor is known to activate many different signalling pathways, but principal among these is the JAK2/STAT3 pathway (Ward, 2007). To investigate whether this was functionally important in ovarian cancer cells, lysates were prepared from cell lines stimulated with G-CSF or IL-6 as a control, and analysed for STAT3 activation using phospho-STAT3-specific antibodies (Figure 3A). Stimulation of the G-CSFR-positive cells OVCA429, HEY and TOV21G with G-CSF led to robust phosphorylation of STAT3, whereas the G-CSFR-negative OVCA433 and SKOV3 did not show STAT3 phosphorylation upon G-CSF stimulation. In contrast, all cell lines showed IL-6-induced STAT3 phosphorylation. Antibodies to detect total levels of STAT3 and GAPDH confirmed equivalent loading in each case. To verify the integrity of the JAK2/STAT3 pathway in these cells, specific inhibitors for JAK2 and STAT3 were used in combination with cytokine stimulation. Both inhibitors effectively blocked STAT3 phosphorylation by both cytokines (Figure 3B). The potential activation of ERK and AKT was also examined using phospho-specific antibodies (Figure 3C). G-CSF induced strong activation of ERK, but not AKT, in these cells, despite robust activation of AKT by IL-6. The addition of a JAK2 inhibitor was able to block the G-CSF-mediated ERK activation (Figure 3D), indicating it lay downstream of JAK2. To confirm that signalling via the JAK2-STAT3 pathway also contributed to the G-CSF-induced phenotypes, responsive cell lines were re-analysed in combination with the specific inhibitors. Both inhibitors were able to suppress G-CSF-mediated migration (Figure 3E and Supplementary Figure 2) and survival responses (data not shown).

We next examined whether the effects of G-CSF on STAT3 phosphorylation translated into active STAT3-mediated transcription. Nuclear extracts were analysed by EMSA using a STAT3-binding site. G-CSF induced strong binding to this probe, which was also blocked by both JAK2 and STAT3 inhibitors (Figure 4A). The expression of two STAT3-responsive genes, *CRP* and *BCL2*, was then examined by RT-PCR. This revealed strong induction of both genes by G-CSF in the G-CSFR-positive cell lines, OVCA429, HEY and TOV21G, but not in the G-CSFR-negative cell lines OVCA433 and SKOV3 (Figure 4B). This was confirmed by both qRT-PCR (Figure 4C) and western blot analysis (Figure 4D). The addition of JAK2 or STAT3 inhibitors effectively blocked the induction of *BCL2* (Figure 4E), confirming it as a STAT3 target.

Interaction between chemotherapy agents and the G-CSF/G-CSFR pathway. Both cisplatin and paclitaxel are common and effective chemotherapy treatments for ovarian cancer patients (Neijt *et al*, 2000; Bookman, 2012). Hence, it was relevant to investigate the status of G-CSF and G-CSFR expression in response to these chemotherapeutic agents in ovarian cancer cells. Addition of cisplatin resulted in increased expression of G-CSFR (nearly two- to fourfold) in the four cell lines tested and of G-CSF (nearly two- to fivefold) in three of these lines, as determined by real-time PCR (Figure 5A). This was further confirmed by ELISA on cell supernatants, which identified increased levels of secreted G-CSF in two of the cell lines, albeit to a modest extent (Figure 5B), and by FACS analysis, which showed increased levels of cell-surface G-CSFR on the four cell lines identified by qRT-PCR, but not SKOV3, CAOV3, ES2 or PA1 (Figure 5C, and data not shown). Addition of paclitaxel led to a similar induction of G-CSF and G-CSFR (Figures 5D and E). The transcription factor NF κ B is

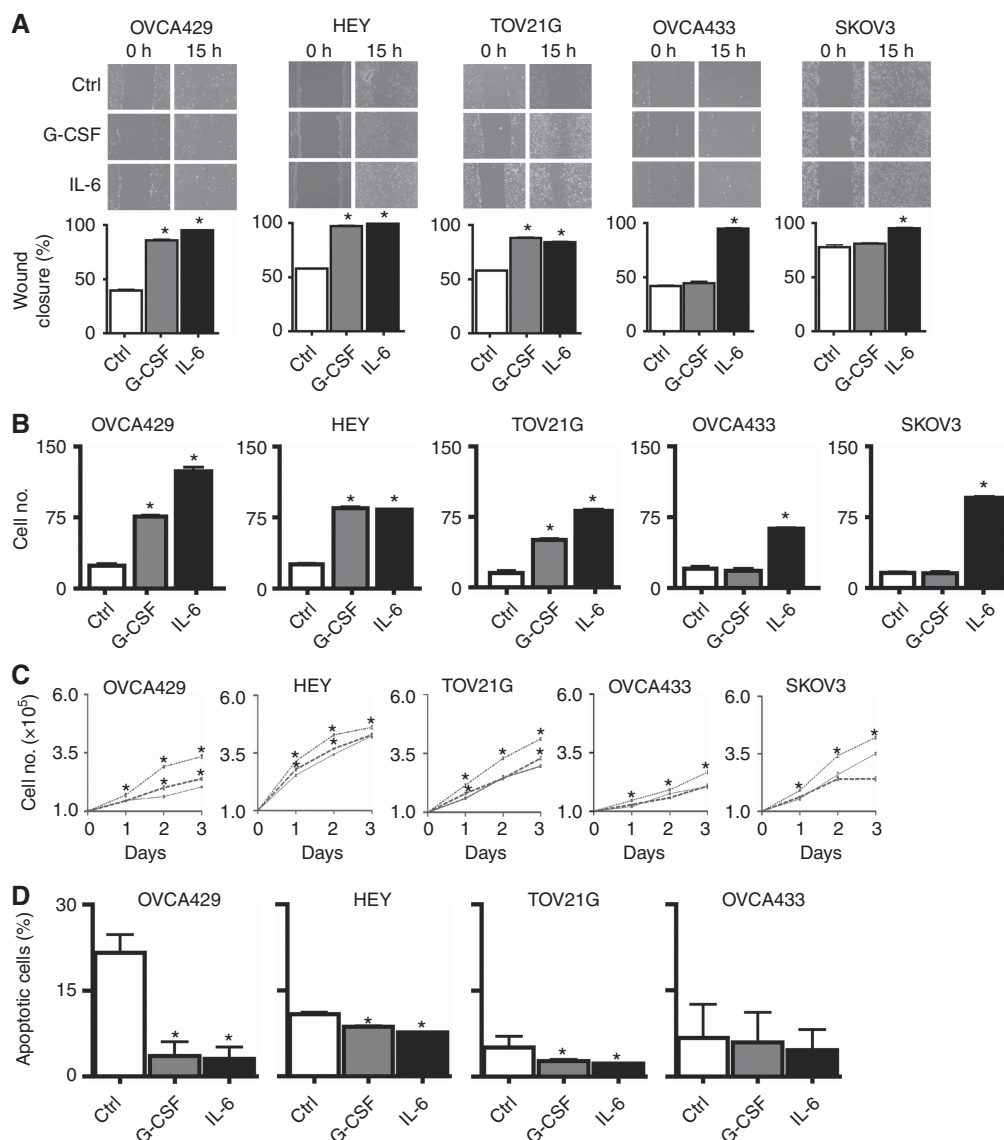


Figure 2. Phenotypic effects of G-CSF on G-CSFR-positive ovarian cancer cells. **(A)** Wound-healing assay. Indicated cells were incubated in a medium lacking cytokines (Ctrl) or containing G-CSF (10 ng ml^{-1}) or IL-6 (10 ng ml^{-1}). Images are representative of single independent experiments performed in triplicate. Graphs represent the mean percentage area of wound closure from three independent experiments ($*P < 0.05$). **(B)** Transwell migration assay. Indicated cells were analysed for migration in a transwell chamber in the presence and absence of G-CSF and IL-6. Mean number of cells migrated through the chamber was counted from five different areas from three independent experiments ($*P < 0.05$). **(C)** Proliferation assay. Indicated cells were incubated over a period of 5 days in the presence and absence of G-CSF and IL-6. The graph represents the mean cell count of three independent experiments ($*P < 0.05$). **(D)** Apoptosis assay. Indicated cells were treated with sodium azide alone (Ctrl) or in the presence of G-CSF and IL-6. Graph shows the mean percentage of apoptotic cells ($*P < 0.05$).

known to be activated by chemotherapeutic agents (Lagunas and Melendez-Zajgla, 2008) and to regulate genes encoding cytokines (Lawrence, 2009), including G-CSF (Dunn *et al*, 1994). Treatment with either cisplatin or paclitaxel induced NF κ B activation (Figure 5F), suggesting that it may be involved in the induction of G-CSF and potentially G-CSFR.

Cisplatin was found to enhance wound closure to a similar extent of that induced by G-CSF (Figure 6A), which was blocked by inhibitors of the JAK2/STAT3 pathway (Figure 6A and Supplementary Figure 3), shown to be effective in this setting (Figure 6B). Both cisplatin and paclitaxel were also able to induce significant apoptosis in these cell lines (Figures 6C and D, respectively). This could be ameliorated by treatment with G-CSF (or IL-6), but not in the presence of STAT3 or JAK2 inhibitors, consistent with robust activation of STAT3 by G-CSF in

combination with either agent (Figure 7A). In this context, ERK was also activated by G-CSF, but not AKT (Figure 7B). Finally, G-CSF was able to induce MDR1, GST-pi and ERCC1 to various extents in all three cell lines tested, including in the presence of cisplatin or paclitaxel (Figure 7C), suggesting these proteins likely contribute to the G-CSF-induced chemoresistance phenotype.

DISCUSSION

The G-CSFR is expressed on a range of haematopoietic cells including haematopoietic stem cells, myeloid progenitors, mature neutrophilic granulocytes, monocytes and lymphocytes, as well non-haematopoietic tissues including cardiomyocytes, neuronal

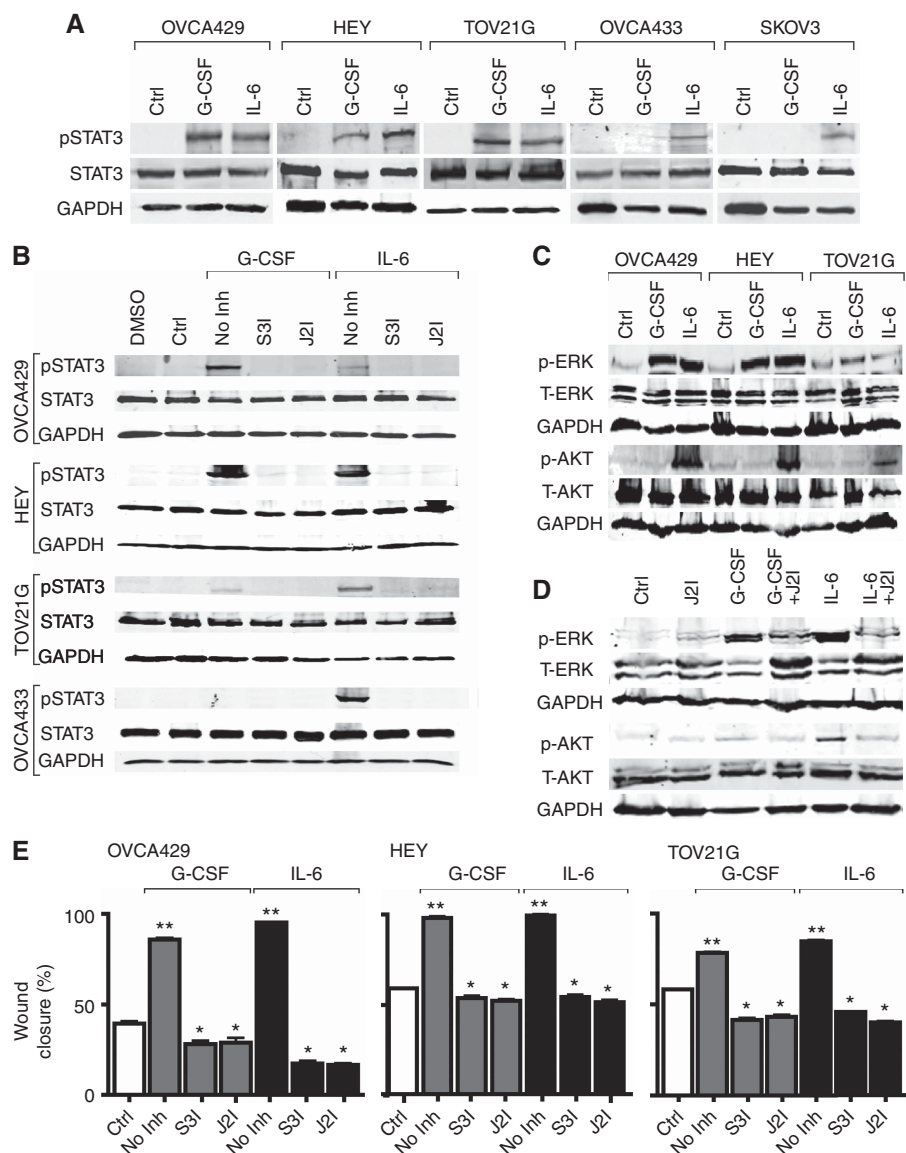


Figure 3. G-CSFR signals via JAK2/STAT3 to mediate its effects on ovarian cancer cells. **(A)** Activation of STAT3 by G-CSF. Lysates from either untreated (Ctrl) cells or those treated with G-CSF or IL-6 were immunoprecipitated with anti-STAT3 and analysed by western blot with anti-phospho-STAT3 and anti-STAT3 antibodies. The input lysate was analysed using anti-GAPDH to confirm equivalent amounts of protein was examined in each case. Experiments were performed thrice and blots are representative of one experiment. **(B)** G-CSF-induced STAT3 activation is blocked by specific inhibitors of the JAK2/STAT3 pathway. Cell lysates were prepared from cells either untreated or treated with G-CSF or IL-6 with or without specific inhibitors for STAT3 (S31) or JAK2 (J21), as indicated, and analysed for STAT3 phosphorylation as per panel **(A)**. **(C–D)** G-CSF-induced ERK activation and its inhibition by a JAK2 inhibitor. Cell lysates were prepared from untreated (Ctrl) cells or those treated with G-CSF or IL-6, and analysed by western blot with anti-phospho-ERK, anti-ERK, anti-phospho-AKT, anti-AKT and anti-GAPDH **(C)**. This analysis was repeated for OVCA429 cells in the presence of JAK2 inhibitor (J21) **(D)**. **(E)** Migration is blocked by specific inhibitors of the JAK2/STAT3 pathway. Wound closure in OVCA429 cells by G-CSF and IL-6, with or without specific inhibitors for STAT3 or JAK2, as indicated (* $P < 0.05$, ** $P < 0.005$, compared with control untreated cells).

precursors, endothelial cells and placental tissue (Touw and Van De Geijn, 2007). Signalling through the G-CSFR has several important functions including effective mobilisation of haematopoietic progenitor cells and neutrophilic granulocytes from the bone marrow (Christopher and Link, 2007), as well as stimulation of myelopoiesis (Touw and Van De Geijn, 2007; Liongue *et al*, 2009a). These properties have seen G-CSF used widely to restore neutrophil numbers following chemotherapy, including for ovarian cancer (Ray-Coquard *et al*, 2007). Therefore, it is of strong clinical interest whether G-CSF can exert additional effects on ovarian cancer cells. In this study, we examined the expression of G-CSFR

in primary ovarian cancer samples and a panel of ovarian cancer cell lines and determined the effects of G-CSF treatment on proliferation, migration and survival.

The G-CSFR has previously been implicated in a diverse range of malignancies. In the case of haematological cancers, its pathogenic effects are mediated via the expression of hyperactive truncated forms, generated through either somatic mutation or disrupted splicing (Liongue *et al*, 2009b). In solid tumours, mis-expression of both the G-CSFR and its ligand have been implicated. Thus, a significant proportion of invasive bladder carcinoma cells have been shown to express both G-CSF and

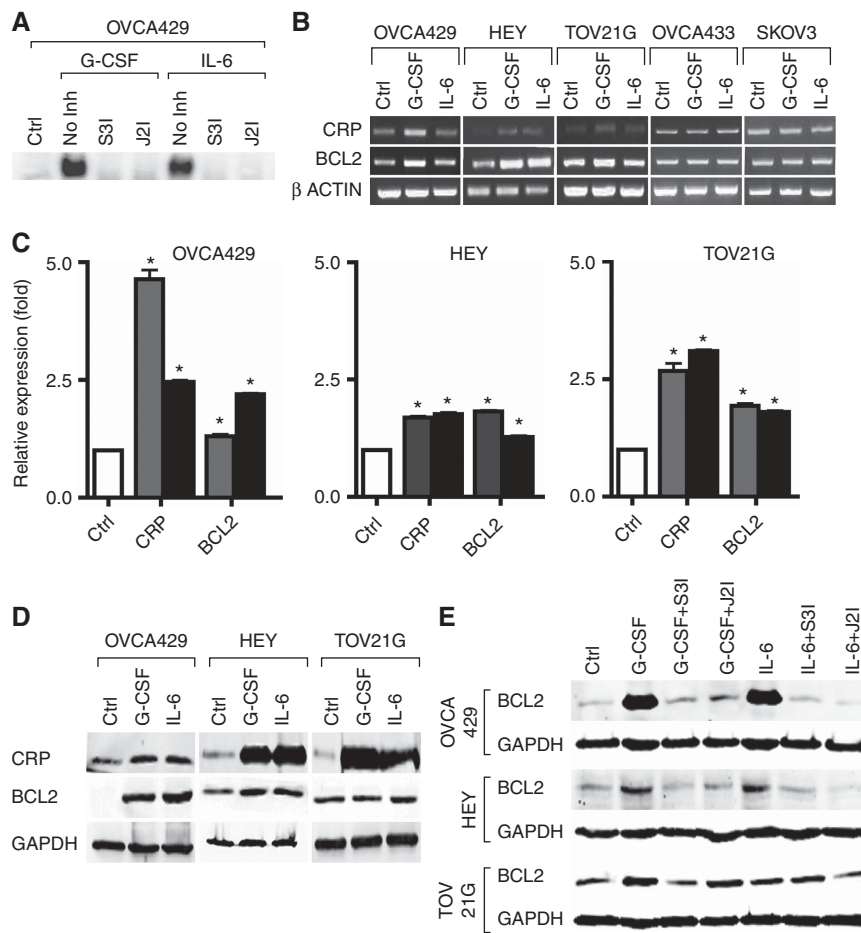


Figure 4. Induction of STAT3 transcriptional activity by G-CSF. **(A)** Induction of STAT3 DNA binding by G-CSF. Nuclear extracts were prepared from OVCA429 cells either untreated (Ctrl) or stimulated by G-CSF or IL-6 alone (No Inh) or in combination with inhibitors for STAT3 (S3I) or JAK2 (J2I), as indicated. **(B–E)** Expression of STAT3-responsive genes by G-CSF. Cells either untreated or treated with G-CSF or IL-6 were analysed by semi-quantitative RT-PCR **(B)**, with the fold change determined by subsequent real-time RT-PCR **(C)**, (* $P < 0.05$ compared with the control). Expression of the encoded proteins in these cells was determined by western blot analysis as indicated **(D)**. For BCL2, this analysis was repeated in the presence of inhibitors for JAK2 (J2I) and STAT3 (S3I) **(E)**.

G-CSFR, with subsequent autocrine signalling contributing to their proliferation and survival *in vitro* (possibly via STAT3), as well as the size of their induced tumours *in vivo* (Chakraborty *et al*, 2004). Both G-CSF and G-CSFR have also been found to be expressed in a series of Ewing's sarcoma patient samples and cell lines, with *in vivo* tumour growth significantly increased by G-CSF treatment (Morales-Arias *et al*, 2007). Similarly, dysplastic and squamous cell carcinomas (SCCs) have been shown to exhibit higher expression of G-CSF and G-CSFR than normal controls (Hirai *et al*, 2001). Granulocyte colony-stimulating factor has also been demonstrated to stimulate the migration of tumour cells derived from patients with head and neck squamous cell carcinoma, with G-CSFR-positive tumours showing increased invasion (Gutschalk *et al*, 2006).

The role of G-CSF/G-CSFR signalling in ovarian cancer has remained controversial. Previous reports have shown that G-CSFR is expressed on primary ovarian carcinomas (Brandstetter *et al*, 1998; Ninci *et al*, 2000; Brandstetter *et al*, 2001; Savarese *et al*, 2001). G-CSF is often co-expressed in the cancer cells or surrounding stroma, with the potential for both autocrine and paracrine activation (Savarese *et al*, 2001). However, the importance of G-CSF expression is ambiguous, with one study suggesting that it does not represent an adverse prognostic factor in ovarian cancer (Munstedt *et al*, 2010), but another showing that overall survival was worse if present as part of a paracrine loop (Savarese

et al, 2001). Granulocyte colony-stimulating factor has also been demonstrated to stimulate the proliferation of a subset of primary ovarian cells and cell lines (Connor *et al*, 1994; Spinner *et al*, 1995; Brandstetter *et al*, 1998), although in other ovarian cancer cell lines increased proliferation was only observed in synergy with EGF (Savarese *et al*, 2001), and in others there was either no effect (Brandstetter *et al*, 2001; Savarese *et al*, 2001), or indeed inhibition (Spinner *et al*, 1995). Consistent with these studies, our data failed to identify an effect of the G-CSF/G-CSFR pathway on ovarian cancer cell proliferation. In contrast, our work has identified a role for G-CSF/G-CSFR signalling in ovarian cancer cell migration and survival – including in response to chemotherapy agents. This has not been reported previously, but has a parallel in a recent study that autocrine IL-6R can confer chemoresistance, including to cisplatin, in ovarian cancer cells (Wang *et al*, 2010). Interestingly, G-CSF/G-CSFR signalling directly enhances the motility of human neutrophils (Nakamae-Akahori *et al*, 2006) and is essential for the directional migration of myeloid cells during embryonic development (Liongue *et al*, 2009b), and also has a key role in myeloid cell survival (Eyles *et al*, 2006; Ward, 2007). Our data suggest that these downstream functions are able to be 'hijacked' by ovarian cancer cells.

Granulocyte colony-stimulating factor receptor signalling involves several distinct downstream intracellular signalling cascades, including the JAK2/STAT3 pathway, which ultimately

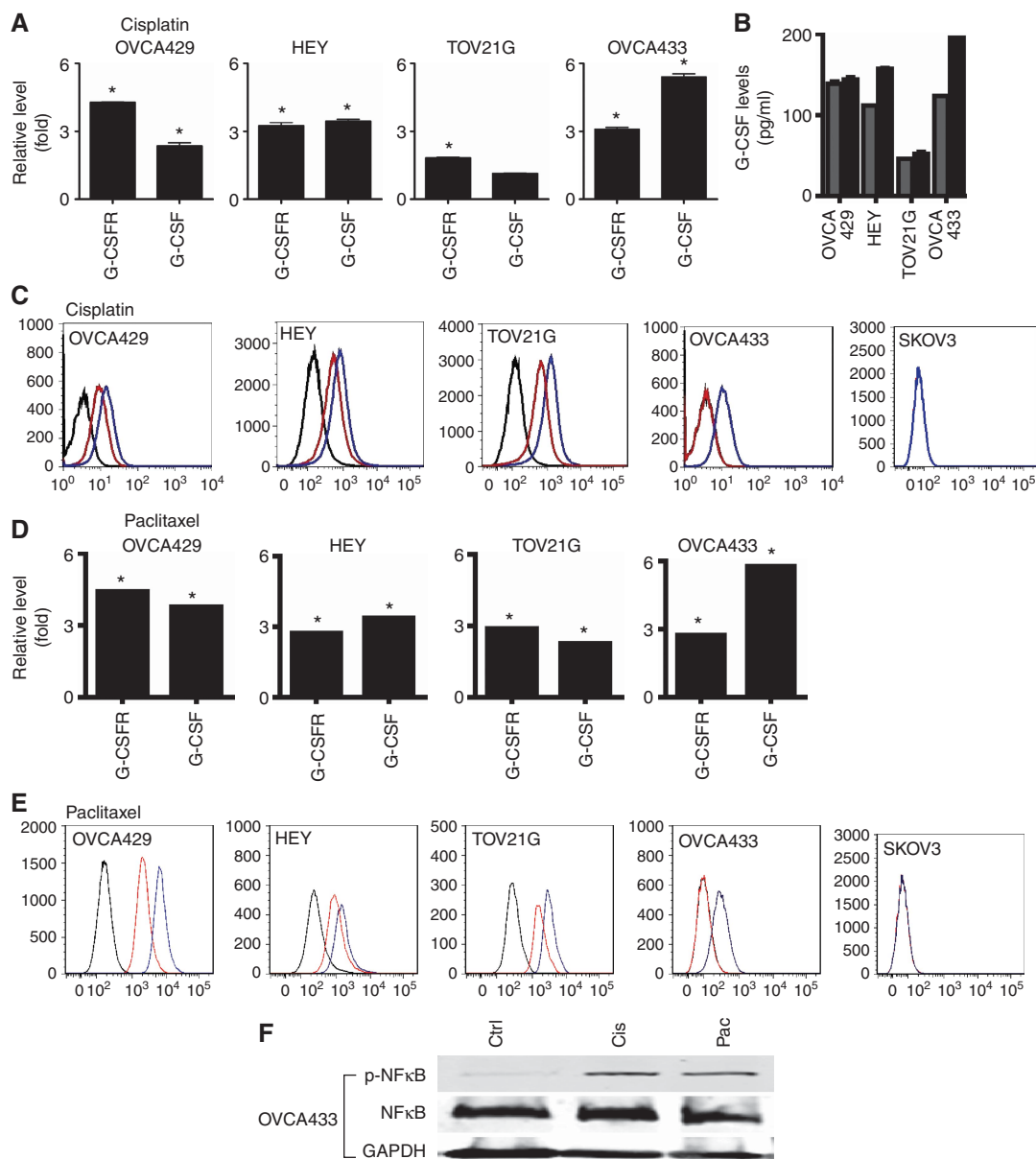


Figure 5. Interaction between chemotherapeutic agents and G-CSF/G-CSFR. (A–E) The effects of the chemotherapeutic agents cisplatin and paclitaxel on the expression of G-CSF/G-CSFR. The impact of 5-day chemotherapy treatment on the indicated ovarian cancer cell lines was determined by RT-PCR for G-CSFR and G-CSF in response to cisplatin (A) or paclitaxel (D), analysis of cell supernatants using ELISA for G-CSF in response to cisplatin (B), or FACS analysis for G-CSFR expression in response to cisplatin (C) or paclitaxel (E) (red line: untreated, blue line: chemotherapy agent) or isotype control (black line). (F) Chemotherapeutic agents activate NFκB. Cell lysates were prepared from OVCA433 cells treated with cisplatin (Cis) or paclitaxel (Pac) as indicated and subjected to western blot analysis with anti-phospho-NFκB, anti-NFκB or anti-GAPDH.

lead to transcriptional changes that impact on survival, proliferation, differentiation and migration (Liongue *et al.*, 2009b). Our data show that the effects of G-CSF/G-CSFR in augmenting both ovarian cancer cell migration and survival are mediated via the JAK2/STAT3 pathway. We have previously shown that G-CSF-mediated STAT3 activation contributes to myeloid cell survival (Ward *et al.*, 1999b), while STAT3 activation has additionally been shown to contribute to both migration (Yoon *et al.*, 2013) and chemoresistance (Zhou *et al.*, 2010) of solid tumours. We have also previously demonstrated that chemoresistant ovarian tumours express higher levels of STAT3 in their non-adherent population (Latifi *et al.*, 2012), while cisplatin treatment was able to generate cells with mesenchymal properties (Latifi *et al.*, 2011). In this study, we demonstrate that treatment with either cisplatin or paclitaxel can also enhance expression of both G-CSF and G-CSFR, potentially via NFκB activation, which may augment STAT3

activation and possibly contribute to chemoresistance. This is consistent with studies that have demonstrated that NFκB can synergise with STAT3 (Yoon *et al.*, 2013) and that NFκB inhibition can increase the efficacy of paclitaxel (Mabuchi *et al.*, 2004). Finally, three well-established contributors to chemoresistance, the P-glycoprotein pump MDRI (Goda *et al.*, 2009), the DNA repair component ERCC1 (Kirschner and Melton, 2010) and the detoxifying enzyme GST-pi (Vasieva, 2011) were all induced by G-CSF, providing several mechanisms for mediating the effects observed.

This study has shown that functional G-CSFR is expressed on high-grade ovarian tumours and ovarian cancer cell lines, with G-CSF stimulation able to induce migration and survival signalling in G-CSFR-positive ovarian cancer cell lines via the JAK2/STAT3 pathway. This was enhanced by both cisplatin and paclitaxel in a number of cell lines. G-CSF is commonly used in the context of

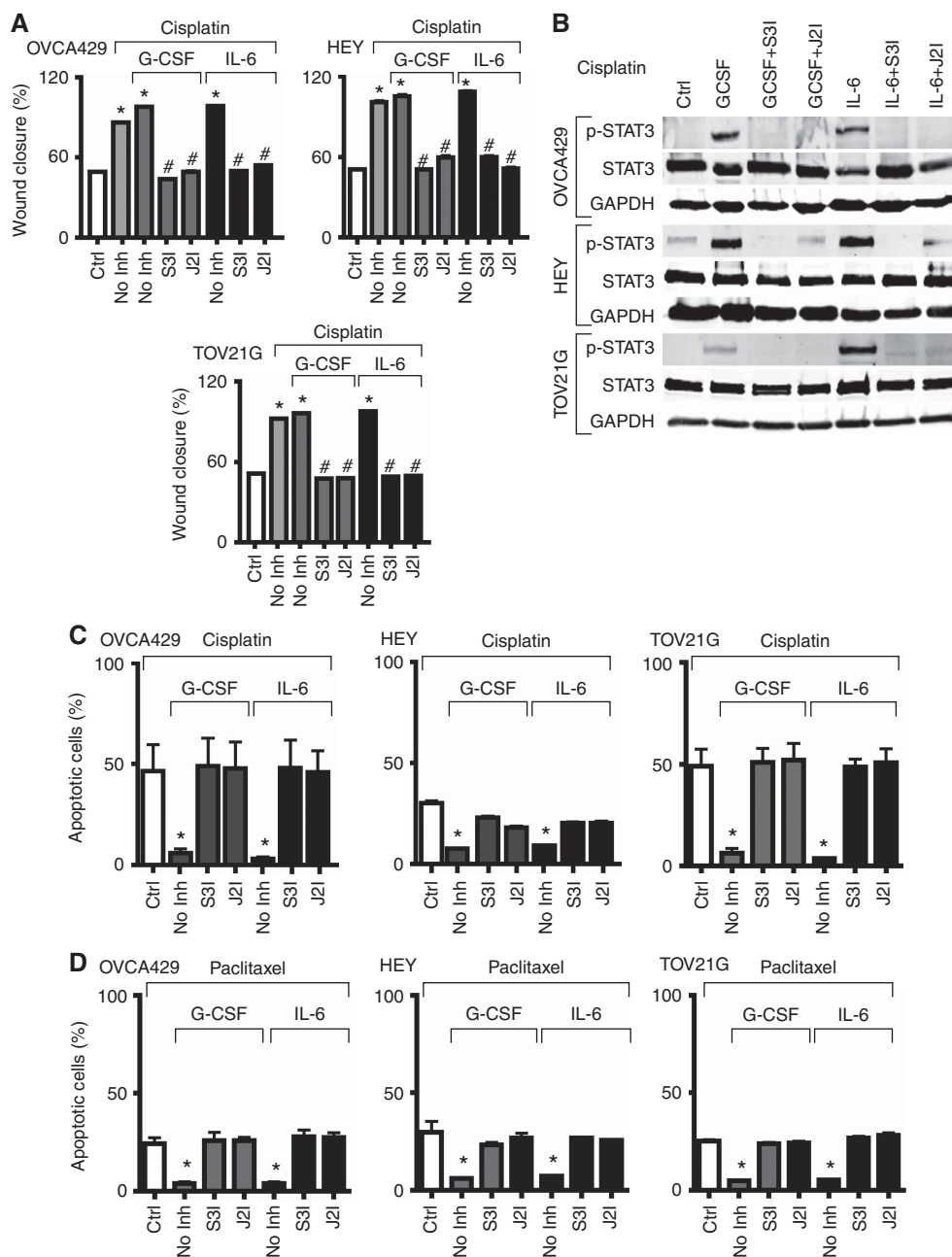


Figure 6. Effects of chemotherapeutic agents on cell survival and migration. **(A)** Wound-healing assay. Indicated cells were treated with cisplatin alone, or with G-CSF and IL-6 treatment, in the presence or absence of inhibitors for STAT3 (S31) and JAK2 (J21). Graph shows the percentage wound closure, expressed as mean + s.e.m. (* $P < 0.05$ compared with control, # $P < 0.005$ compared with cisplatin). **(B)** Induction of STAT3 activity. Cell lysates were prepared from OVCA429, HEY and TOV21G cells treated with cisplatin (Cis) as indicated, and subjected to western blot analysis with anti-phospho-STAT3, anti-STAT3 and anti-GAPDH. **(C–D)** Apoptosis assay. Indicated cells were treated with 12.5 mg ml^{-1} cisplatin **(C)** or paclitaxel **(D)**, or with G-CSF and IL-6 treatment, in the presence or absence of inhibitors for STAT3 and JAK2. Graph shows the percentage of apoptotic cells, expressed as mean + s.e.m. (* $P < 0.005$ compared with chemotherapeutic agent). For TOV21G cells, cisplatin was increased to $20 \mu\text{g ml}^{-1}$ in order to achieve significant levels of apoptosis.

ovarian cancer to restore neutrophils ablated by standard chemotherapeutic drugs used to treat patients (Yamada *et al.*, 2001). However, if the tumour expresses G-CSFR, either basally or in response to chemotherapy, these treatments could contribute to tumour development by increasing migration and/or survival, irrespective of G-CSF expression. Therefore, screening patient tumours for G-CSFR expression by histology or FACS-based approaches prior to administration of G-CSF could be beneficial, potentially indicating alternative therapies for patients with G-CSFR-positive tumours. Of relevance, co-administration of

granulocyte-macrophage CSF factor and recombinant interferon gamma 1b in concert with carboplatin treatment in women with recurrent ovarian cancer was shown to be efficacious and elicited a favourable haematological profile (Schmeler *et al.*, 2009), providing a potential option in this regard. Finally, inhibitors of JAK2 and STAT3 have now been developed and used in various clinical settings such as myeloproliferative disorders (Pardanani *et al.*, 2011). Consistent with our data, inhibition of JAK2/STAT3 has been shown to induce apoptosis in ovarian tumours (Kandala and Srivastava, 2012) and increase the chemo-sensitivity of ovarian

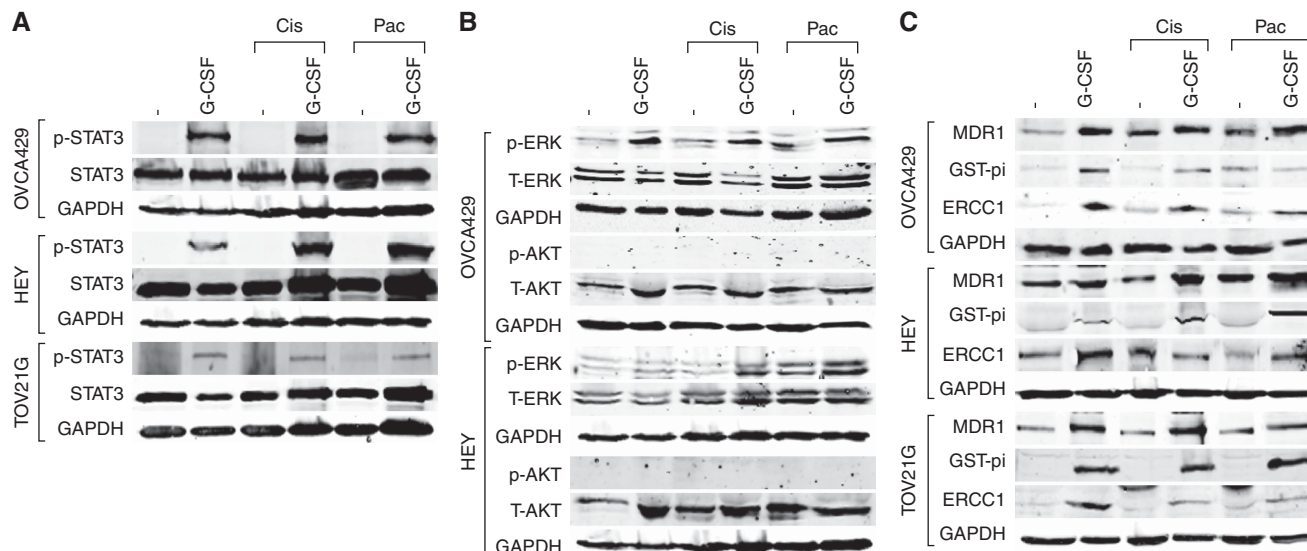


Figure 7. Effects of chemotherapeutic agents on downstream pathways. Cell lysates were prepared from OVCA429, HEY and TOV21G cells treated with cisplatin (Cis) or paclitaxel (Pac) as indicated, and subjected to western blot analysis with anti-phospho-STAT3, anti-STAT3 and anti-GAPDH (A), anti-phospho-ERK, anti-ERK, anti-phospho-AKT, anti-AKT and anti-GAPDH (B), or anti-MDR1, anti-GST-pi, anti-ERCC1 and anti-GAPDH (C).

cancers (Hedvat *et al*, 2009; Lee *et al*, 2011). This suggests that inhibitors of the JAK2/STAT3 pathway may also be efficacious in the treatment of chemoresistant ovarian cancer.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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