The pro-social neurohormone oxytocin reverses the actions of the stress hormone cortisol in human ovarian carcinoma cells *in vitro*

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Abstract. The journey patients with ovarian cancer travel from non-specific symptoms causing delayed diagnosis through surgery and chemotherapy, culminating in a 5-year survival rate of 43%, must have a profound and detrimental psychological impact on patients. Emerging studies link higher levels of oxytocin (OT) and increased social support, an independent prognostic factor in cancer, with a moderating effect on stress. In contrast, there is a known association of tumour cell proliferation with elevated cortisol (stress hormone) levels. We hypothesise therefore that there is cross-talk between cortisol and oxytocin at a molecular level. Three ovarian cancer cell lines, used as *in vitro* models, were treated with cortisol at concentrations mimicking physiological stress *in vivo* in the presence or absence of OT. OT reduced cell proliferation and migration, induced apoptosis and autophagy for all three cell lines, partially reversing the effects of cortisol. Quantitative RT-PCR of tissue taken from ovarian cancer patients revealed that the glucocorticoid receptor (splice variant GR-P) and OT receptor (OTR) were significantly upregulated compared to controls. Tissue microarray revealed that the expression of GRα was lower in the ovarian cancer samples compared to normal tissue. OT is also shown to drive alternative splicing of the GR gene and cortisol-induced OTR expression. OT was able to transactivate GR in the presence of cortisol, thus providing further evidence of cross-talk *in vitro*. These data provide explanations for why social support might help distressed ovarian cancer patients and help define novel hypotheses regarding potential therapeutic interventions in socially isolated patients.

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Introduction

Stress responses and related psychosocial factors (e.g., hopelessness and social support) can be of significant prognostic value for cancer progression (1). Stress is defined as 'the state of threatened or perceived as threatened homeostasis, associated with activation of the stress system, mainly comprised by the hypothalamic-pituitary-adrenal axis and the arousal/sympathetic nervous systems' (2). Widely accepted as being a complex construct, stress, is divided into several stages: i) the stressors which are the event potentially leading to the psychophysiological features associated with stress, ii) the mediators which can involve the appraisal of, as well as coping with, the stressor, iii) the moderators where this can include the social support being available as well as the personality and resource of the individual, and finally, iv) the stress response itself which includes activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (3-6). The physiological response to stress usually involves activation of the HPA axis above the basal level, resulting in increased synthesis and secretion of glucocorticoids (GC) (such as cortisol) as well as activation of the sympathetic nervous system resulting in the secretion of catecholamines (such as adrenalin) (7). Concerning cancer prognosis, certain psychosocial factors such as little social support and hopelessness were found in many prospective studies and reviews to predict prognosis in cancer, independent of confounders such as cancer stage and treatments (1,8,9). Such responses are thought to partly affect cancer progression via the effects of glucocorticoids on cancer cells. Several studies have found that cortisol administration leads to greater cell invasiveness in some cancer cell lines (10).

Cortisol exerts its actions by binding to and activating glucocorticoid receptors (11,12). The glucocorticoid receptor (GR) is a nuclear receptor that acts as a phosphoprotein and a transcription factor modulating transcriptional events. To date, the cloning of four splice variants of the GR gene have been reported: GRα, GRβ, GRγ and GR-P (13). Moreover, growth arrest-specific transcript 5 (GAS5) encodes a single strand non-coding RNA (ncRNA). GAS5 ncRNA can be a repressor for the GR by acting as a decoy glucocorticoid response element (GRE), competing with DNA GREs for binding to

the GR. GAS5 ncRNA thus acts as a negative regulator by preventing GRs from binding to their DNA GRE and compromising the normal functions of the GR-cortisol complex (14).

Abnormal regulation of the immune system by stressors, whether suppression of anticancer cellular immunity or enhancement of pro-inflammatory cytokines (15,16) may result in significant adverse health consequences for tumour proliferation and metastatic events (17). In addition, patients diagnosed with cancer face psychosocial stressors that can lead to abnormal levels of cortisol (18). Indeed, nocturnal cortisol and cortisol variability show significant dysregulation in ovarian cancer patients, and this dysregulation is associated with greater functional disability, fatigue, and vegetative depression (19). In some other cancers, little cortisol variability was also found to predict poor prognosis (20).

The neurohormone OT is involved in various aspects of social cognition and prosocial behaviour such as trust (21,22). Central OT exerts anxiolytic and anti-depressive effects by activating its cognate receptor OTR which belongs to the GPCR superfamily (23). Early studies in a rodent model have shown that oxytocin decreases blood pressure and lowers circulating cortisol levels (24). Intranasal OT benefits some aspects of socio-emotional functioning (21). Furthermore, one study found that OT also interacts synergistically with social support in relation to cortisol secretion: during stress, people who received both OT and social support had lower cortisol levels than those receiving either treatment alone or no treatment (25). A number of studies proposed that OT can act as a negative regulator of cell proliferation in human breast carcinomas (26), human central nervous system tumours (27), and human osteosarcoma cell lines (28). Human endometrial carcinomas also express OTR, and OT inhibits the proliferation of endometrial cancer cells (29). Intraperitoneal administration of OT resulted in the reduction of intraperitoneal dissemination of ovarian carcinoma cells in a mouse model (30). Thus, there is regulatory cross-talk between OT and cortisol both at the socio-behavioural and at the systemic levels, which may also have consequences at the tumour level.

However, to this date, little is known about potential cross-talk between cortisol and oxytocin in the context of cancer in general and in ovarian cancer specifically. Because of the often grave prognosis of ovarian cancer due to it being diagnosed frequently at advanced stages (31), and since it is easy to administer OT, it seemed important to examine the effects of OT and cortisol on ovarian cancer cells first *in vitro*. Based on their opposing effects, we hypothesised that the activity of cortisol in ovarian cancer cells might be compromised if OTR signalling is activated.

Materials and methods

Patients. Clinical samples were of ovarian origin (n=12) and were taken from patients admitted to the First Department of Obstetrics and Gynecology, Papageorgiou General Hospital, Medical School, Aristotle University, Thessaloniki, Greece. Ethical approval was obtained by the local authority and Brunel University. The majority of ovarian cancers were deemed to be grade 3 stage III (poorly differentiated and involving the whole peritoneal cavity, not just confined to ovaries/tubes or pelvis) (10/12). Control samples (n=10) were also used in this study, obtained from patients undergoing total hysterectomy and bilateral salpingo-oopherectomy for benign reasons. None of the two groups (ovarian cancer and control) received hormone replacement therapy, and ovarian cancer patients were all post-menopausal. Table I provides further information on the stage, grade, age and CA125 status of ovarian cancer patients.

Cell culture. SKOV3, and MDAH-2774 ovarian cancer cell lines (from American Type Culture Collection, USA), PEO1 (gift from Dr Helen Coley, University of Surrey) were cultured in RPMI phenol red-free complete media (Gibco) containing 10% fetal bovine serum (FBS), 5% penicillin/streptomycin (P/S) solution (5,000 μg/ml) and 5% Gibco 100X non-essential amino acids (NEAA) at 37˚C and 5% CO_2. For cell treatments, cells were seeded overnight into 6-well plates with 2 ml complete media before media were aspirated and wells washed with PBS solution. The cells were incubated for 3 h in serum free media (phenol red-free media with 5% NEAA and 5% P/S, without FBS). The cells were then treated with vehicle (NS), oxytocin (OT) and/or cortisol (C) to make a final concentration of 100 nM. Cell concentration and viability values were determined using a Countess™ automated cell counter based upon the method of trypan blue exclusion (Invitrogen, Paisley, Renfrewshire, UK), as previously described (32).

Wound healing assay. A solid line spanning the diameter of each well on a 6-well plate was drawn on the reverse side before cells were seeded at equal density and treated as stated above. The 'wound' was created using a 200-μl yellow pipette tip (Fisher) and scratching a line through the cells which was perpendicular to the line drawn along the well. Images of each wound at 0 hours (h), 6, 12 and 18 h after treatment were inspected by the Olympus IX71 Microscope and the images captured using the Photometrics Cool Snap™ CF camera. Percentage migration of cells into the wound after 18 h was calculated using the following formula: 1 - average width of wound at 18 h / average width of wound at 0 h*100.

RNA isolation, cDNA synthesis and quantitative RT-PCR. Total RNA was isolated using an RNA extraction kit (Sigma-Aldrich, UK), according to the manufacturer's instructions. RNA concentration was determined by spectrophotometric analysis (NanoDrop; Thermo Scientific, UK) and agarose gel electrophoresis. RNA (500 ng) was reverse-transcribed into cDNA using 5 IU/μl RNase H reverse transcriptase (Invitrogen). Relative expression of the genes of interest was assessed by quantitative PCR (Q-PCR) on an ABI7400 instrument (Applied Biosystems) and xxpress® (BJS Biotechnologies) using SYBR® Green-PCR reaction mixture (Sigma-Aldrich) and the primers for GR, GAS5 as previously described (33). For OTR the primers used were: (sense) 5'-TTACAATCACTA GGATGGCTACAA-3'; (antisense) 5'-CATTTACATTCCCAC CAACAATTTAA-3'. As a negative control for all the reactions, distilled water was used in place of the cDNA. RNAs were assayed from two to three independent biological replicates. The RNA levels were expressed as a 'relative quantification' using the housekeeping gene 18S RNA (RQ) value. 'ΔCt method' was employed for comparing relative expression results between treatments in Q-PCR (34).

Protein extraction from SKOV3, PEO1 and MDAH2774 cells. Ovarian cancer cells were cultured to 80% confluence,

Table I. Patient details (age, stage, grade, CA125) recruited for this study.

Histology	Grade	Stage	Age (years)	CA125
Serous	3	IIIC	64	474
Serous	3	IIIC	48	4,350
Serous	3	IIIC	61	858
Serous	2	IIIC	54	537
Serous	3	IIIC	69	534
Serous	3	IV	65	UKN
Serous	3	IIIC	75	242
Serous	3	IIIC	65	478
Serous	3	IIIC	56	UKN
Serous	3	IIIC	64	2,657
Serous	3	IIIC	64	339
Serous	2	IIIC	56	542

UKN, unknown.

and in the presence or absence of OT, cortisol, cortisol and oxytocin (100 nM for 48 h). Cells were then lysed using 200 μl 1X Laemmli buffer (Sigma-Aldrich) and denatured for 5 min at 100˚C before they were cooled on ice.

Western immunoblotting. Samples were separated on an SDS-10% polyacrylamide gel and the proteins were transferred to a nitrocellulose membrane. The membrane was blocked in TBS containing 5% dried milk powder (w/v) and 0.1% Tween-20, for 1 h at room temperature. After three washes with TBS-0.1% Tween-20, the nitrocellulose membranes were incubated with primary antibodies against caspase-3, Beclin-2 and GAPDH (Cell Signaling Technology). All primary antisera were used at a 1:1,000 dilution overnight at 4˚C. The membranes were washed thoroughly for 30 min with TBS-0.1% Tween, before incubation with the secondary HRP-conjugated immunoglobulin (1:2,000) for 1 h at room temperature and further washing for 30 min with TBS-0.1% Tween-20. Antibody complexes were visualised as previously described (33).

Ovarian tissue microarray. Unstained paraffin tissue microarray slides containing multiple ovarian carcinoma and normal tissue micro-array (70 cases of ovarian carcinoma, 5 cases of tumour adjacent normal ovary and 5 normal ovarian tissue from different biopsies; Biomax USA) were used for this study.

The paraffin-embedded slides were deparaffinised and rehydrated by a series of washes in reducing concentrations of ethanol (100, 95, 70 and 50%) followed by rinsing in tap water for 10 min. Antigen retrieval was accomplished by incubating the slide in sodium citrate (pH 6.0) for 20 min in a microwave. Slides were washed in 0.4% of PBS-T for 5 min and then incubated for 15 min in the PBS containing 0.3% H_2O_2 to stop the interference of the endogenous peroxidase activity. Blocking was carried out with 5% goat serum, followed by overnight incubation with primary GRα antibody. The following day, after several washes with PBS, slides were incubated with HRP conjugate-secondary antibody for 60 min. Further washing in PBS-T was carried out for 20 min before performing staining. Slides were then subjected to DAB staining, counterstained with haematoxylin and washed with 0.1% sodium bicarbonate. The extent of staining was scored based on the proportion of cells stained positive for GRα, as follows: 0, <5% of cells; 1, 5-25% of cells; 2, 26-50% of cells; 3, 51-75%; and 4, >75% of cells. Scoring was calculated from the mean of the two independently conducted assessments.

GR luciferase reporter assay. SKOV-3 cells were incubated in 12-well plates with McCoy's 5A medium supplemented with 1.5 mM L-glutamine and 2.2 g/l sodium bicarbonate (Hyclone, Logan UT, USA (1 ml/well). The cells were co-transfected with 1 μg of pGRE-Luc vector (a gift from Dr John A. Cidlowski, NIEHS) and 0.5 μg of the pRL-TK vector (Renilla luciferase, Promega, Madison, WI, USA) to correct for transfection efficiency in transfection media containing OptiMed and Lipofectamine 2000 (Invitrogen) as described previously (35). The transfection medium were replaced after 6 h with fresh culture medium containing 100 nM cortisol, 100 nM oxytocin, alone or in combination. The cells were grown overnight until 90% confluent. Cell extracts were assayed using a dual-luciferase reporter assay system (Promega) following the manufacturer's instructions. Firefly and Renilla luciferase activities were measured for 10 sec each, respectively, using a CLARIOstar luminometer and the data were analyzed with Mars software (BMG Labtechnologies Inc., Durham, NC, USA). The relative luciferase activity level of each treatment (n=3) was expressed as the ratio of firefly/Renilla luciferase activity values.

In silico analysis of gene expression from microarray data. Oncomine (www.onocomine.org) is an online database consisting of previously published patient microarray data available to the public. We used this *in silico* dataset to compare the expression of OTR in normal and ovarian cancer tissues.

Statistical analysis. Statistical analysis was performed by the Student's t-test. A value of $P<0.05$ was regarded as statistically significant. For the immunohistochemistry studies, a Student's t-test where the assumptions of equal variances were not met, we used Levine's test, which uses often non-integer degrees of freedom. Q-PCR and western blot analysis data are reported as the mean ± SEM.

Results

Cortisol inhibits the anti-proliferative effects of OT in vitro. SKOV3, PEO1 and MDAH-2774 ovarian cancer cells were treated for 48 h with oxytocin (OT), cortisol (C), and cortisol plus oxytocin (C+OT) at 100 nM. This concentration was chosen in accordance with previous studies that demonstrated that 100 nM cortisol doses simulate stress conditions *in vitro* and this resembles physiological levels of circulating steroid *in vivo* (36). OT concentration was also chosen at 100 nM as it is the concentration at which the OTR was maximally activated in a number of *in vitro* studies (30). Staurosporine (ST) at 100 nM was also used as an extra control agent for reduction of cell proliferation (37). In SKOV3 and MDAH-2774 cells, OT partially, but significantly, reversed the proliferative

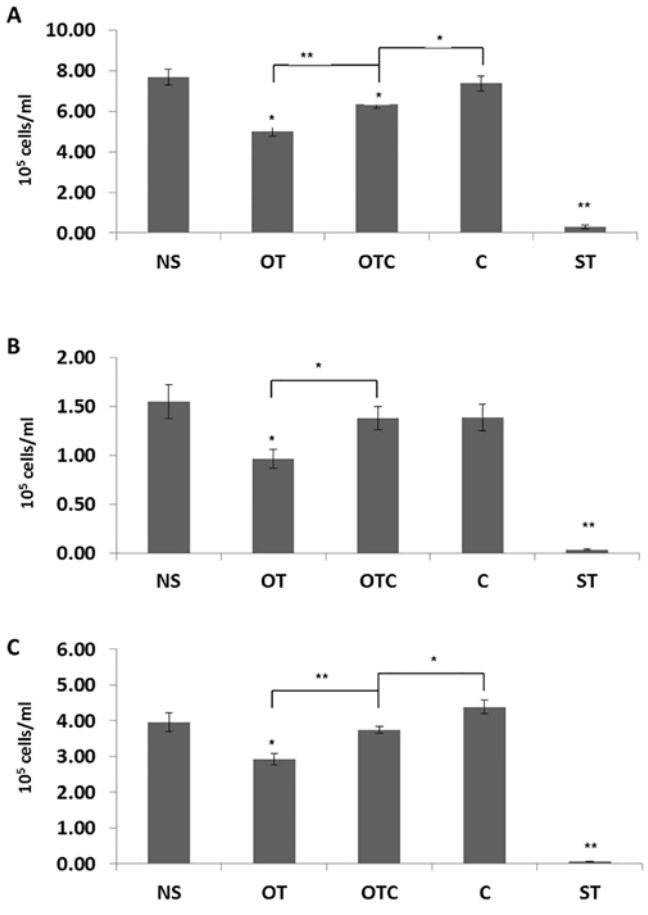

Figure 1. Cell viability assay for control cells (NS), cells treated with 100 nM oxytocin (OT), 100 nM oxytocin and 100 nM cortisol (OTC), 100 nM cortisol (C) and 1 μM staurosporine (ST). (A) Viable cell count for SKOV3 cell lines treated for 48 h. (B) Viable cell count for MDAH-2774 cell lines treated for 48 h. (C) Viable cell count for PEO1 cell lines treated for 48 h. Data are expressed as the mean ± SEM, *P<0.05, **P<0.01.

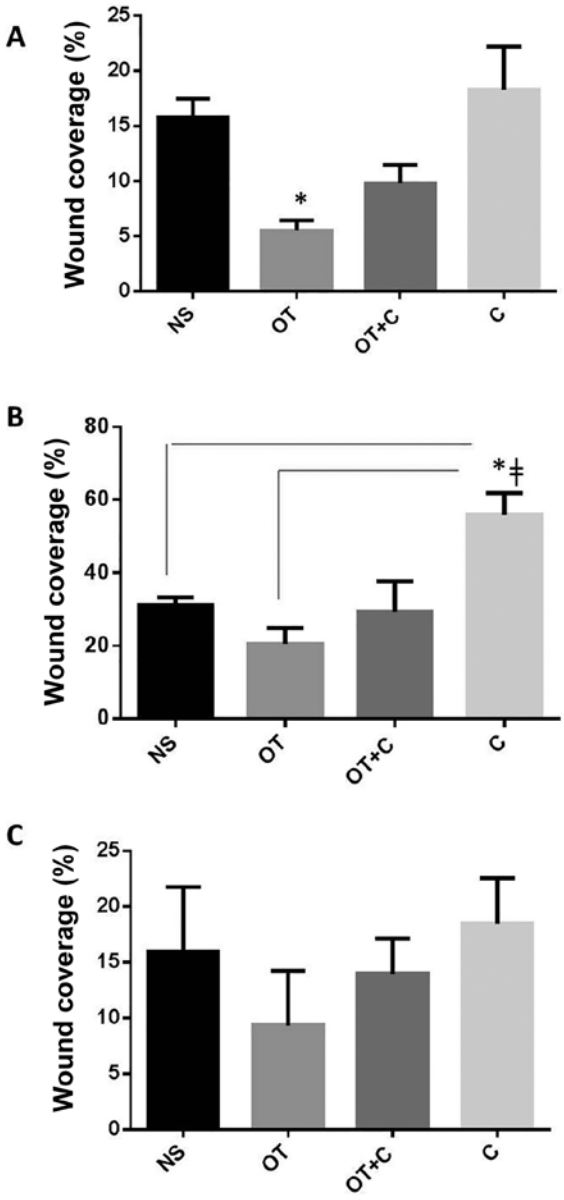

Figure 2. Wound healing assay for control cells (NS), cells treated for 48 h with oxytocin (OT) ± cortisol (OT+C, 100 nM) and cortisol (C, 100 nM) alone for SKOV3 (A), PEO1 (B) and MDAH-2774 (C). Data are expressed as the mean ± SEM, *P<0.05 to NS, ‡P<0.05 to OT.

effects of cortisol when compared to the effects of cortisol alone (Fig. 1A and C). In all three cell lines used, OT alone was able to significantly reduce the proliferation of ovarian cancer cells (Fig. 1). The extent of the inhibition varied, with OT having a more profound effect on PEO1 and SKOV3 cells.

Effects of cortisol and OT on cell migration. We then assessed the effects of C and OT on cell migration in scratch conditions. OT significantly reduced the migratory ability of SKOV3 cells when compared to controls (Fig. 2A), whereas in PEO1 cells, C alone induced a significant cell migration compared to controls and to OT treated cells (Fig. 2B). In MDAH2774, although the differences did not reach statistical significance, they followed a similar trend towards inhibition of cell migration by OT and induction by C (Fig. 2C).

Effects of cortisol and OT on apoptosis. To further understand the potential pro-apoptotic mechanisms of OT *in vitro*, we measured the levels of apoptosis-associated proteins in the presence or absence of cortisol.

After exposure to OT±C and C alone for 48 h, cleaved caspase-3 over total caspase-3 and Beclin-1 over GAPDH protein levels were examined by western blotting. Quantitative analysis of cleaved caspase-3 over total caspase-3 bands by scanning densitometry revealed a significant increase in this ratio in OT treated SKOV3 (56% P<0.01) and MDAH-2774 (76% P<0.01) cells compared to cortisol alone (Fig. 3A and C). Surprisingly, in PEO1 cells, cortisol treatment induced more cleavage of caspase-3 when compared to OT (Fig. 3B). The effects of cortisol remained unaltered in the presence of oxytocin in all three cell lines, thus suggesting that there is no involvement of caspase-3 as a cross-talk mechanism in these models between C and OT.

We next measured the expression of the autophagy-related protein Beclin-1. Autophagy is a highly conserved cellular process that is involved in several catabolic processes, including degradation of long-lived proteins and organelles, and cell death (38). Although autophagy is initiated as a protective response to stress, the constitutive activation of

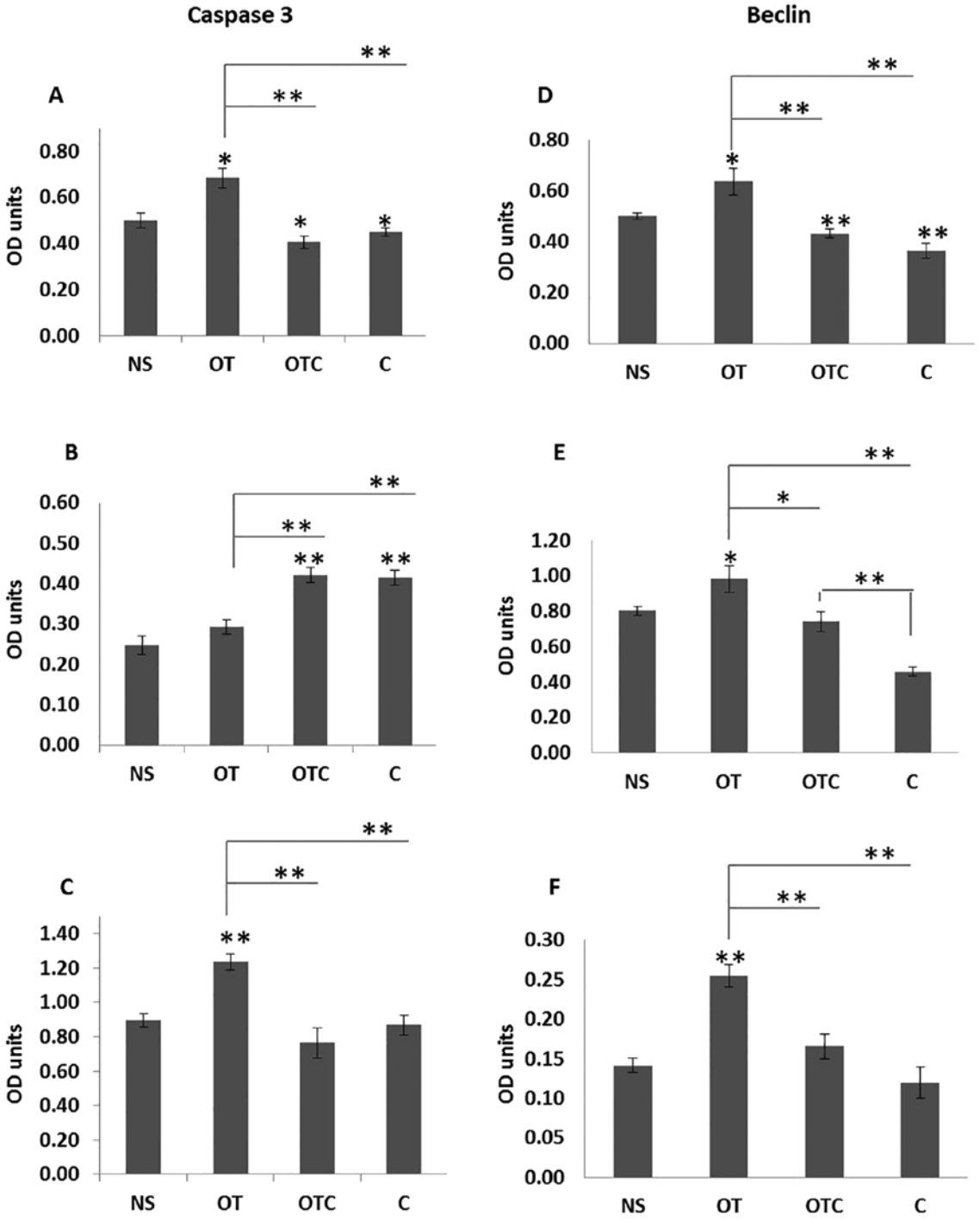

Figure 3. Expression of cleaved caspase-3 as a ratio of total caspase-3 and Beclin-1 in SKOV3 (A and D), PEO1 (B and E) and MDAH-2774 (C and F) cell lines treated for 48 h in NS, OT, OTC and C. Data are expressed as the mean ± SEM, $^{*}P<0.05$, $^{**}P<0.01$.

autophagy can lead to cell death by excessive self-degradation of essential cellular components (38,39). Beclin-1 protein levels were significantly increased in all three OT-treated cell lines when compared to cortisol alone or to cortisol and oxytocin (Fig. 3D-F). In all cell lines, OT appeared to reverse the reduction of Beclin-1 that was due to cortisol, since Beclin-1 levels were moderately elevated in SKOV3 and MDAH-2774 cells in cortisol and OT treated samples when compared to cortisol alone. Moreover, in PEO1, the induction of Beclin-1 was significant in the same preparations (Fig. 3E), thus suggesting that the effects of OT+C on cell proliferation described above may have been mediated by a potential cross-talk with mechanisms regulating autophagy and subsequently cell death.

Expression of OTR, GRs and GAS5 in ovarian cancer patients. Quantitative RT-PCR revealed that GAS5 and all GR variants were expressed at the ovarian level (Fig. 4). No apparent differences in the expression of GRα, GRβ, or GRγ between control (n=10) and ovarian cancer (n=12) patients were found. However, GR-P was significantly upregulated in

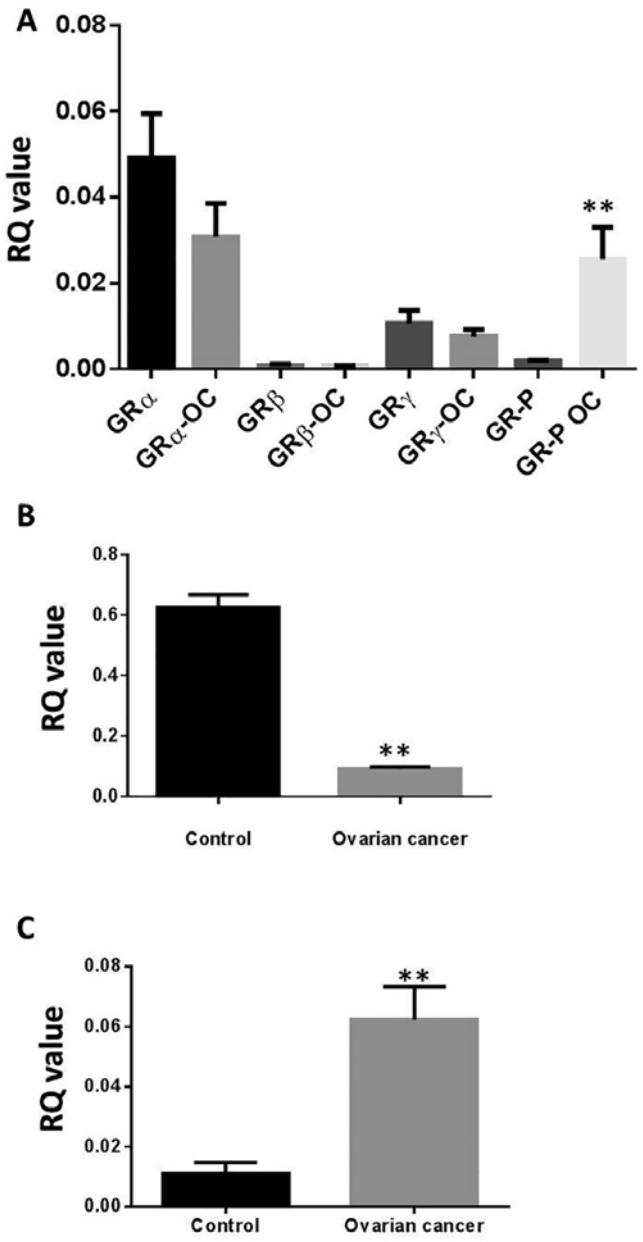

Figure 4. Gene expression of GR splice variants GRα, GRβ, GRγ, and GR-P (A), GAS5 (B) and oxytocin receptor (OTR) (C) in ovarian cancer (OC) patients (n=12) and control (n=10). Data are expressed as the mean ± SEM, **P<0.01 to controls.

ovarian cancer patients when compared to the control group (Fig. 4A). Moreover, the pseudo-GRE GAS5 was significantly downregulated in ovarian cancer (Fig. 4B), whereas OTR was significantly upregulated in the same cohort (Fig. 4C) when compared to controls. We also analysed the expression of gene copy number of OTR, using Oncomine datasets. OTR gene copy number showed an increased expression in the Bonome dataset in ovarian carcinoma patients (n=185) against normal ovarian surface epithelium (n=10; data not shown).

We then expanded on these observations at protein level using tissue microarray slides containing 70 samples of ovarian cancer and 10 samples of normal tissue. Results are presented as a percentage of total cells positive for GRα expression. The immunohistochemistry study shows 49% positive staining for GRα in the normal ovaries (n=10) and 31% in malignant tissues [n=70; χ^2 (1) = 1.28; P>0.5]. Of the malignant tissues, 31% of epithelial origin ovarian cancers were stained positive for GRα and 26% of germ cell cancers were positive for GRα.

In the sample 43.9% were at stage I, 21.1% stage II, 28.1 stage III and 7% stage IV. Because of the relatively small sample and to increase statistical power, we grouped the patients in early stages (I and II) versus late (III and IV; Fig. 5A). After log transforming the GRα expression, the late stage patients had significantly higher expression of GRα (1.2) than early stage patients [0.4; t(54.3)=2.5; P=0.015]. There was no further correlation of GRα expression with clinico-pathological features such as age and histological type of tumour.

Transactivation of GR by OTR and effects of OT and C on GR splicing in vitro. The effects of C and OT alone or in combination on transactivation of human GR were assessed using a GRE-luciferase reporter system in SKOV3 cells. Treatment of SKOV3 cells for 16 h with 100 nM OT did not induce any changes in the luciferase activity, whereas C alone exerted a significant increase. Interestingly, when SKOV3 cells were treated with C+OT, the increase in GRE activity was significantly higher compared to the effects of C alone (Fig. 6). The effect was not additive.

OT drives differential splicing of GR isoforms in a cell-specific manner. In SKOV3 (Fig. 7A) and PEO1 (Fig. 7B) cells, OT significantly induced GRβ expression, whereas only in PEO1, the expression of GR-P was significantly augmented. With regards to GAS5, it was induced in PEO1 (Fig. 7B) and MDAH-2774 cells (Fig. 7C).

Given the increase of OTR seen in the clinical samples, and since this may result from the cancer-induced stress responses as well, we tested the hypothesis that cortisol, a stress hormone, might affect OTR expression directly. SKOV3, PEO1 and MDAH-774 were treated for 48 h with cortisol, in an attempt to resemble a sustained moderate stress environment *in vitro*. When SKOV3, PEO1 and MDAH-774 cells were treated with cortisol (100 nM), the expression of OTR was significantly upregulated by 5-fold, 2-fold and in 81%, respectively, when compared to basal levels (data not shown).

Discussion

In this study, we provide to the best of our knowledge, for the first time, evidence for cross-talk between a stress hormone (cortisol) and a 'social' hormone (OT) at the molecular level in ovarian cancer cells. These findings have wider implications especially for ovarian cancer patients who frequently exhibit stress, depression, anxiety, and poorer overall quality of life (QOL) (40,41). A major finding of this study is the inhibitory role that oxytocin can exert over the cortisol effects in tumour cells. We demonstrate that OT reversed the effects of cortisol by inducing autophagy, as evident from the upregulation of Beclin-1. Interestingly, there is a correlation of decreased expression of Beclin-1 with poorer outcomes in patients with ovarian carcinoma (42); and between decreased expression of Beclin-1 with the development of epithelial ovarian tumours (43). This is of particular importance since synthetic glucocorticoids can promote cell survival in epithelial tumours,

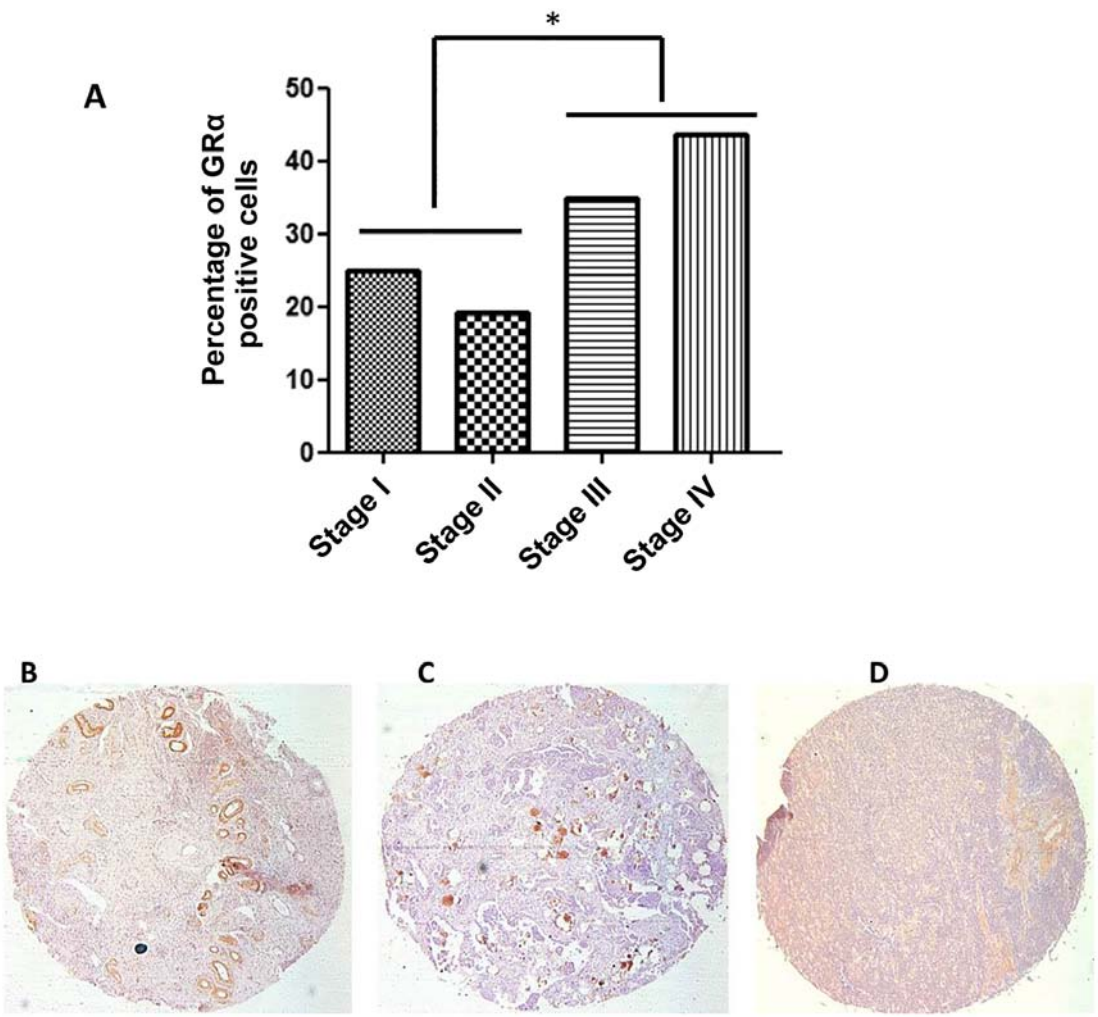

Figure 5. Protein expression of GRα using tissue microarray. Positive staining for GRα as detected by stage (A). Immunohistochemical analysis of ovarian cancers expressing GRα in normal controls (B) and in patients with serous papillary carcinoma, presented as either early stage (C) or late stage (D).

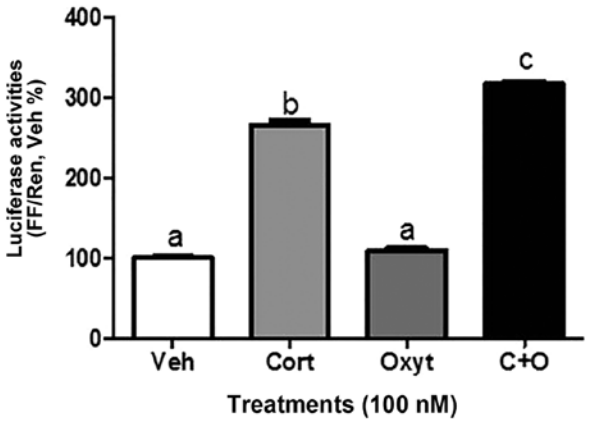

Figure 6. Effects of 16-h treatment with 100 nM cortisol (Cort), 100 nM oxytocin (Oxyt) alone and in combination (C+O) on transactivation of human GR using a GRE-luciferase reporter system in SCOV3 cells. Bars represent means ± SEM, n=3. Different letters denote values significantly different from each other (P<0.01) analyzed by one-way ANOVA and Tukey post hoc test.

including breast and ovarian cancers. For example, cortisol can enhance the invasive potential of SKOV3 ovarian cancer cells (10). Moreover, to prevent chemotherapy-related side effects, dexamethasone is routinely administered to patients with ovarian cancer. In a recent clinical study, ovarian cancer patients receiving dexamethasone showed a significant induction of pro-cancer cell survival genes, including serum and glucocorticoid-regulated kinase 1 (SGK1) and map kinase phosphatase 1 (MKP1)/dual specificity phosphatase 1 (DUSP1) at the tumour site. It appears therefore that glucocorticoids (GCs) may decrease chemotherapy effectiveness via induction of anti-apoptotic gene expression (44). In another study, dexamethasone not only induced therapy resistance of primary ovarian carcinomas *in vivo*, it also led to a faster basal growth of the xenografts (45). Collectively, these data suggest that while the anti-emetic effects by glucocorticoids may be of benefit to patients to tolerate the side effects of the treatment, their counteracting of cytotoxic treatments may minimize the treatment-induced growth retardation of ovarian cancer.

Furthermore, in the cell lines SKOV3 and MDAH-2774 OT induced caspase-3, indicative of initiating apoptotic events. A similar effect has been observed in rat neuronal cells (46). However, the effects of cortisol on caspase-3 cleavage remained unaltered in the presence of oxytocin in all three cell lines, implying that OT exerts a direct apoptotic effect,

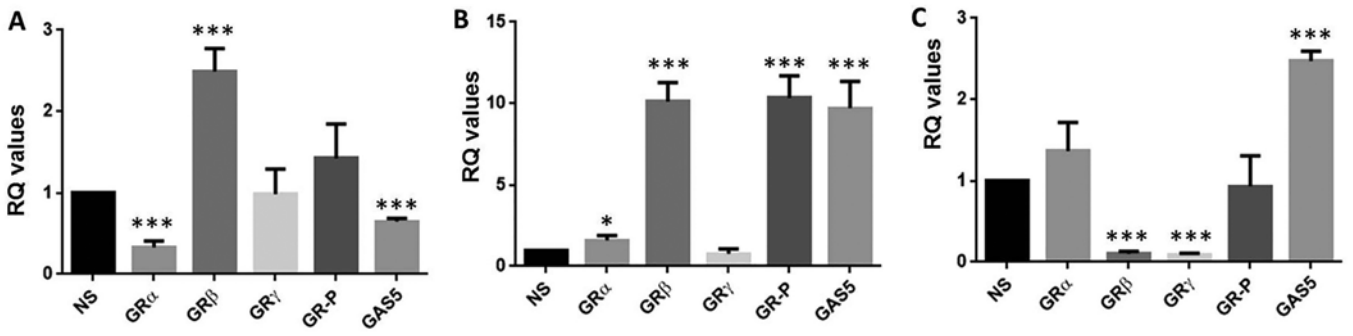

Figure 7. Expression of the GR splice variants GRα, GRβ, GRγ, GR-P, and GAS5 following OT treatment (100 nm) in SKOV3 (A), PEO1 (B) and MDAH-2774 (C) cells. Data are expressed as the mean ± SEM, *P<0.05, ***P<0.01 to no supplement (NS).

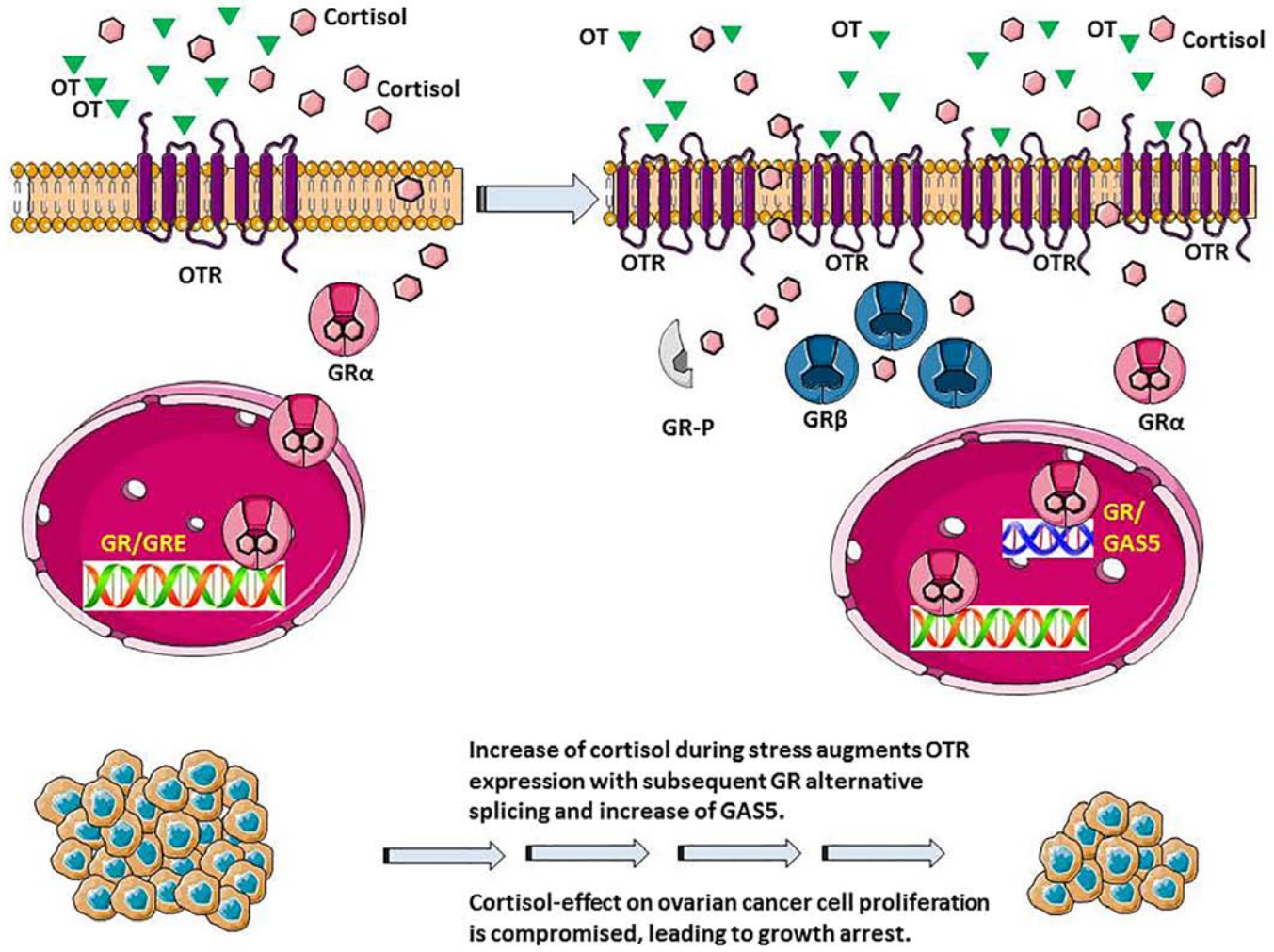

Figure 8. Proposed model of the potential interactions between oxytocin (triangles) and cortisol (polygons) at concentrations mimicking high stress *in vivo* during ovarian cancer.

involving a particular caspase, independent of cortisol. Over the past decade, a substantial amount of data implicates OT in proliferative and anti-proliferative effects *in vitro*, and here we have shown that OT can exert a cytostatic/cytotoxic effect in ovarian cancer cells. The OTR, is another 'promiscuous' GPCR in terms of its capacity to induce multiple signalling pathways involving PLC, cAMP, IP3, MAPK to name a few. Here we provide evidence for a unique cross-talk between OTR and GR, since OT can transactivate the GR gene when in the presence of cortisol. This can have implications in GR splicing events. For example, OT can compromise GR signalling by inducing multiple splicing isoforms (GRβ and GRγ) that can act in a dominant negative manner, including the induction of the pseudo-GRE GAS5.

It is possible therefore for OT to act in a cell- or tissue-specific manner and exert a dual role as a result. In the only *in vivo* study, intraperitoneal administration of OT resulted in the reduction of intraperitoneal dissemination of ovarian cancer cells followed by suppression of MMP2 and increases in expression of E-cadherin (30). Breastfeeding - a state where OT is markedly elevated for more than one year, reduces the risk of developing ovarian cancer compared with never breast-feeding (47), and may also reduce endometrioid ovarian cancer risk to a greater extent than other subtypes (48,49). Our results are also in line with other studies showing anti-proliferative effects of OT in several, but not all, cancer cell types (26-28).

These data have wider implications in stress management for cancer patients. The severe emotional distress accompanying a diagnosis of cancer generally and ovarian cancer specifically and its initial treatment has been extensively documented (40). In ovarian cancer, social support has been related to higher NK cytotoxicity in PBMC and tumour-infiltrating lymphocytes (TIL), whereas distress was related to lower NK cytotoxicity in TIL (50). Ovarian cancer patients suffering from chronic stress, depression and low social support have increased MMP-9 levels in tumour-associated macrophages (51), of importance for tumour invasion and metastasis. Moreover, there is a substantial body of longitudinal research relating initial social support to lower morbidity and mortality from a variety of cancers (8). The results observed in the present study provide important evidence for possible mechanisms linking social support to better cancer prognosis since social support is positively related to OT levels (52) and our results propose that OT may minimise the anti-apoptotic effects or stress-related cortisol in ovarian cancer.

The three ovarian cancer cell lines exhibit differences in GR expression levels but similarities in the response to OT (proliferation) and wound (mechanical scratch assay). SKOV3 human ovarian clear cell adenocarcinoma cells were derived from the ascites of a Caucasian 64-year-old female. PEO1 is an adherent ovarian cancer cell line derived from a malignant effusion from the peritoneal ascites of a patient with a poorly differentiated serous adenocarcinoma. MDAH-2774 cells are of human ovarian endometrioid adenocarcinoma origin, isolated from the ascites of an untreated female patient. MDAH-2774 cells show a trend towards triploidy, particularly trisomy of chromosomes 1, 2, 3, 6, 11, 12, 16 and X and monosomy of chromosomes 17 and 21. SKOV3 have mutations in TP53 and PIK3CA genes, whereas MDAH2774 have mutations in TP53, PIK3CA, KRAS, BRCA1 (silent) and BRCA2 (silent) (53). It should also be noted that there is heterogeneity in cultured cells and the SKOV3 cell line has been shown to contain cells with high and low invasive and migratory potential (54). This may provide an explanation of the variability seen in experiments involving SKOV3, PEO1 and MDAH-2774 cell lines.

We have provided conclusive evidence that cortisol can induce OTR expression at concentrations that mimic a stressful milieu *in vitro*. This could be a compensatory defensive and regulatory response. In our study, there was also an induction of OTR in ovarian cancer patients compared to controls and, a slight decrease in GRα. We propose that OT may exert a dual beneficial effect: at the CNS level allowing patients to cope better with stress by seeking social support and by possibly feeling less lonely on one hand, and at the ovarian level by partially-reversing the deleterious effects of glucocorticoids on tumour cell viability, on the other; whilst retaining the useful anti-emetic effects of glucocorticoids (Fig. 8). However, this study did not include direct evidence linking these two levels in patients; including assessment of their psychosocial profile. Future studies need to examine prospectively the relationship between social support, systemic and *in situ* levels of OT and cortisol, and prognosis in ovarian cancer patients. This will enable to extend the observations found in this study and then point at possible clinical implications.

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