Glucocorticoid Regulation of SLIT/ROBO Tumour Suppressor Genes in the Ovarian Surface Epithelium and Ovarian Cancer Cells

Rachel E. Dickinson, K. Scott Fegan, Xia Ren, Stephen G. Hillier, W. Colin Duncan*

Medical Research Council Centre for Reproductive Health, The University of Edinburgh, Edinburgh, United Kingdom

Abstract

The three SLIT ligands and their four ROBO receptors have fundamental roles in mammalian development by promoting apoptosis and repulsing aberrant cell migration. SLITs and ROBOs have emerged as candidate tumour suppressor genes whose expression is inhibited in a variety of epithelial tumours. We demonstrated that their expression could be negatively regulated by cortisol in normal ovarian luteal cells. We hypothesised that after ovulation the locally produced cortisol would inhibit SLIT/ROBO expression in the ovarian surface epithelium (OSE) to facilitate its repair and that this regulatory pathway was still present, and could be manipulated, in ovarian epithelial cancer cells. Here we examined the expression and regulation of the SLIT/ROBO pathway in OSE, ovarian cancer epithelial cells and ovarian tumour cell lines. Basal SLIT2, SLIT3, ROBO1, ROBO2 and ROBO4 expression was lower in primary cultures of ovarian cancer epithelial cells when compared to normal OSE (P<0.05) and in poorly differentiated SKOV-3 cells compared to the more differentiated PEO-14 cells (P<0.05). Cortisol reduced the expression of certain SLITs and ROBOs in normal OSE and PEO-14 cells (P<0.05). Furthermore blocking SLIT/ROBO activity reduced apoptosis in both PEO-14 and SKOV-3 tumour cells (P<0.05). Interestingly SLIT/ROBO expression could be increased by reducing the expression of the glucocorticoid receptor using siRNA (P<0.05). Overall our findings indicate that in the post-ovulatory phase one role of cortisol may be to temporarily inhibit SLIT/ROBO expression to facilitate regeneration of the OSE. Therefore this pathway may be a target to develop strategies to manipulate the SLIT/ROBO system in ovarian cancer.

Introduction

The secreted Slit glycoprotein and its Robo receptor were originally identified as important axon guidance molecules in the developing Drosophila nervous system [1,2]. Their role is evolutionarily conserved as vertebrate SLIT (SLIT1, SLIT2, SLIT3) and ROBO (ROBO1, ROBO2, ROBO3, ROBO4) also inhibit aberrant neuron migration [3]. However most members of the vertebrate SLIT and ROBO families are also expressed outside of the nervous system and have been linked with the development of a variety of organs including the mammary gland and ovary [4,5]. During organogenesis the SLIT/ROBO interaction is thought to regulate numerous processes including cell proliferation, apoptosis, adhesion and migration of non-neuronal cells [6,7].

Molecules that have important roles in development are often temporally regulated during the normal menstrual cycle in the endometrium and are expressed in the fallopian tube [21]. Furthermore there is increased expression of the SLITs and ROBOs in the adult corpus luteum during the late-luteal phase of the ovarian cycle. At this time the SLIT/ROBO interaction may act to promote its disintegration by stimulating apoptosis and inhibiting migration of luteal cells [22]. In the corpus luteum and endometrium expression of SLITs and ROBOs is hormonally regulated. There was reduced SLIT/ROBO expression in the decidualised endometrium of early pregnancy [21]. In addition the luteotrophic molecules, human chorionic gonadotrophin [23] and cortisol [24], that are increased in early pregnancy, reduce the expression of SLITs and ROBOs in luteal cells [22].

Reduced proliferation of fibrosarcoma, oesophageal, hepatocellular, colorectal, prostate and breast carcinoma cells [14–17]. SLIT2 also inhibited the invasion of numerous different types of tumour cells including those from the prostate, breast, endometrium and ovary [13,18,19].

The SLIT/ROBO pathway has now also been shown to have physiological roles in normal reproductive tissues [6]. SLIT/ROBO signalling seems to regulate placental angiogenesis and trophoblast function in an autocrine and/or paracrine manner [20]. In addition, most of the SLITs and ROBOs are also temporally regulated during the normal menstrual cycle in the endometrium and are expressed in the fallopian tube [21]. Furthermore there is increased expression of the SLITs and ROBOs in the adult corpus luteum during the late-luteal phase of the ovarian cycle. At this time the SLIT/ROBO interaction may act to promote its disintegration by stimulating apoptosis and inhibiting migration of luteal cells [22].
Around 90% of ovarian malignancies are classified as epithelial tumours that are thought to derive from the ovarian surface epithelium (OSE) [25]. The risk of ovarian cancer is positively correlated with the number of ovulations [26]. Thus recurrent injury and subsequent repair of the OSE during ovulation may predispose this tissue to neoplasia [27]. Ovulation is an inflammatory event disrupting the OSE, but requiring resolution. This repair is facilitated by an increased local production the anti-inflammatory steroid cortisol via up-regulation of 11b-hydroxysteroid dehydrogenase type 1 [28].

We hypothesised that the OSE express SLITs and ROBOs and that cortisol could temporarily reduce the expression of these tumour suppressor genes to facilitate survival, proliferation and migration of these cells during the repair process. If this was the case this pathway might have a role in ovarian cancer progression and if it remains active in malignant OSE cells it may offer therapeutic strategies to manipulate these genes. We therefore investigated the expression, localisation and regulation of the SLIT/ROBO pathway in the OSE. We also examined whether the SLITs and ROBOs were aberrantly expressed and hormonally regulated in ovarian cancer cells. Furthermore we analysed the functional significance of a perturbed SLIT/ROBO pathway in ovarian cancer cells.

Results

The SLIT/ROBO pathway is differentially expressed in human OSE, ovarian cancer cells and ovarian tumour cell lines

SLIT2 and ROBO1 could be immunolocalised to the normal human ovarian surface epithelium (Fig. 1A–C). RT-PCR analysis confirmed the expression of SLIT2 and ROBO1 in primary cultures of OSE and demonstrated that there was some expression of SLIT3, ROBO2 and ROBO4 in these cells (Fig. 1D). We then investigated the expression of these genes in primary cultures of malignant cells derived from the ascitic fluid of women with epithelial ovarian cancer. SLIT2, SLIT3, ROBO1, ROBO2 and ROBO4 were also expressed in these cells but quantitative analysis showed that they were reduced by 25–82% when compared to primary cultures of normal OSE. (Fig. 2A) (P<0.05).

In order to confirm that malignant OSE cells had a reduced expression of SLITs and ROBOs, we examined their expression in two different ovarian tumour cell lines. The PEO-14 cell line is derived from a well-differentiated ovarian adenocarcinoma and has similarities with more benign ovarian epithelial cells and early stage ovarian cancer. In contrast, the SKOV-3 cell line is derived from a poorly differentiated ovarian adenocarcinoma and is more characteristic of an advanced tumour [29]. Both these cell lines expressed SLIT2, SLIT3, ROBO1 and ROBO2 (Fig. 2B). However, paralleling our results in the primary cell culture, SLIT2, SLIT3 and ROBO2 expression was decreased by between 58% and 97% in the poorly differentiated SKOV-3 cells when compared to the well-differentiated PEO-14 cells (Fig. 2C) (P<0.05). Although PEO-14 and SKOV-3 cells may differ in other aspects as well as their differentiation status these data suggest that expression of the SLIT/ROBO tumour suppressor gene pathway may be reduced during tumour development or progression.

Blocking the SLIT/ROBO interaction decreases apoptosis in ovarian tumour cells

The effect of the SLIT/ROBO pathway on cell survival was investigated using a recombinant ROBO1/Fc chimera, which acts as a ligand trap to inhibit the SLIT/ROBO interaction, and direct inhibition of SLIT2 using siRNA. Treatment of PEO-14 and SKOV-3 cells with the ROBO1/Fc chimera did not affect cell proliferation (P>0.05, data not shown). However in both the PEO-14 and SKOV-3 cells blocking SLIT action with the ROBO1/Fc chimera reduced apoptosis by 20–21% as measured by an activated caspase-3/7 assay (P<0.05) (Fig. 3A). Transient transfection of SLIT2 siRNA reduced solely SLIT2 expression in both PEO-14 and SKOV-3 cells. PEO-14 and SKOV-3 cells with reduced SLIT2 expression had a significant 17–26% decrease in cleaved Caspase-3 and -7 activities (P<0.05, paired t-test) (Fig. 3B). This suggests that a reduction in the SLIT/ROBO gene pathway is associated with increased cell survival.

Cortisol negatively regulates the expression of SLITs and ROBOs in OSE and a well-differentiated ovarian cancer cell line

We then investigated whether the expression of the SLIT/ROBO pathway in ovarian epithelial cells was regulated. In human ovarian luteal cells the SLIT/ROBO pathway could be physiologically inhibited by cortisol [22]. As cortisol is produced locally in the OSE, and has an anti-inflammatory role after ovulation [30], we examined whether cortisol could regulate SLIT/ROBO expression in the OSE. SLIT2, SLIT3, ROBO1, ROBO2 and ROBO4 expression was reduced by 25–30% in cortisol (1000 nM) treated primary cultures of normal OSE. (P<0.05) (Fig. 4A). However, in primary cultures of malignant epithelial...
cells cortisol did not result in any further reduction in the expression of these genes (P>0.05) (Fig. 4B).

The PEO-14 and SKOV-3 ovarian tumour cell lines also have the potential to respond to cortisol treatment as they express the glucocorticoid receptor (GR). In addition they express the mineralocorticoid receptor (MR) but do not express the progesterone receptor (PR) (Fig. 5A). In the more differentiated PEO-14 cells, cortisol reduced the expression of \textit{SLIT2}, \textit{SLIT3}, \textit{ROBO1} and \textit{ROBO2} by 13–31\% (P<0.05) (Fig. 4C). Furthermore cortisol treatment reduced secreted \textit{SLIT2} protein concentration by 53\% (P<0.05) (Fig. 4E). Like the primary cell cultures, the regulation of these \textit{SLITs} and \textit{ROBOs}, as well as the secreted \textit{SLIT2} protein, was lost in the more malignant, and less differentiated, SKOV-3 cells (P<0.05) (Fig. 4D,F). This suggests that cortisol may have a physiological role in reducing the \textit{SLIT/ROBO} interaction during repair of the OSE and that this pathway may still be active in some early stage ovarian cancers.

**Manipulation of \textit{SLITs} and \textit{ROBOs} in ovarian tumour cells by targeting the glucocorticoid receptor**

Thus, although glucocorticoids have a theoretical detrimental effect on early ovarian cancer cells, it means that manipulation of the GR, with the aim of increasing \textit{SLIT/ROBO} gene expression, is a potential therapeutic target. We therefore “knocked down” GR using GR siRNA in cortisol-responsive PEO-14 cells. Transfection of the GR siRNA for 48 hours caused a significant 63\% reduction in GR expression (P<0.001) (Fig. 5C). This increased the expression of the \textit{SLIT2}, \textit{ROBO1} and \textit{ROBO2} tumour suppressor genes (P<0.05) (Fig. 5D). In addition, transfection of the GR siRNA resulted in a similar reduction in MR expression in the cortisol-unresponsive SKOV-3 cells (P<0.01) (Fig. 5B). Importantly the expression of the \textit{SLIT2} and \textit{ROBO1} tumour suppressor genes was also enhanced by GR siRNA transfection (P<0.05) (Fig. 5E).

PEO-14 and SKOV-3 cells were also treated with mifepristone (RU486), which functions as a GR antagonist. RU486 treatment...
alone did not influence the expression of SLIT2, SLIT3, ROBO1 or ROBO2 in either cell line (P>0.05, data not shown). However RU486 treatment did abolish the cortisol mediated negative regulation of SLIT/ROBO expression in PEO-14 cells (data not shown). This suggests that there may be ligand independent effects of GR on SLIT/ROBO expression and confirms that even in poorly differentiated cancer cells manipulation of GR can regulate the expression of tumour suppressor genes.

Discussion

In this study we established that the normal human adult OSE expresses, at the RNA level, SLIT2, SLIT3, ROBO1, ROBO2 and ROBO4. Using immunohistochemistry we also showed that SLIT2 and ROBO1 are also expressed at the protein level. As each of the SLITs is able to interact with each of the ROBOs, with the possible exception of ROBO4, it is likely that the SLIT/ROBO interaction is occurring in the OSE. This is not surprising as these molecules are expressed in other ovarian cells including the granulosa lutein, theca lutein and luteal fibroblasts cells of the adult corpus luteum [22] and the pre-granulosa cells and oocytes of primordial follicles within the developing fetal ovary [5]. The normal ovary is therefore a site of the physiological autocrine or paracrine actions of the SLIT/ROBO system.

We found that in normal OSE the SLIT/ROBO system can be regulated by cortisol. Cortisol reduced secreted levels of SLIT2 protein in the PEO-14 cells (control secretion is 0.5 ng/ml). Cortisol did not affect secreted levels of SLIT2 protein in the SKOV-3 cells (control secretion is 0.5 ng/ml). *

Figure 4. The effect of cortisol on the SLIT/ROBO pathway. A) Real-time quantitative PCR showing that Cortisol (1000 nM), compared to Ethanol carrier control, reduced SLIT2, SLIT3, ROBO1, ROBO2 and ROBO4 expression in primary cultures of hOSE. B) Cortisol did not alter the expression of SLITs and ROBOs in primary cultures of ovarian epithelial cancer cells. C) SLIT2, SLIT3, ROBO1 and ROBO2 expression was significantly reduced by Cortisol in more differentiated PEO-14 cells. D) However expression of these SLITs and ROBOs was not regulated by Cortisol in the poorly differentiated SKOV-3 cells. E) Cortisol reduced secreted levels of SLIT2 protein in the PEO-14 cells (control secretion is 0.5 ng/ml). F) Cortisol did not affect secreted levels of SLIT2 protein in the SKOV-3 cells (control secretion is 0.5 ng/ml). * = P<0.05; ** = P<0.01.

doi:10.1371/journal.pone.0027792.g004
Cortisol can inhibit the expression of \textit{SLIT2} and \textit{SLIT3} in primary cultures of luteinised granulosa cells and luteal fibroblast-like cells [22]. After ovulation there is an increase in the local production of cortisol in the OSE that may act to encourage tissue repair and renewal [28]. Over the range of physiologically relevant concentrations in OSE cells cortisol has been shown to have an anti-inflammatory action and can block interleukin-1 stimulated MMP-9 expression [30,31]. In addition we have previously shown that cortisol, by negatively regulating the expression of \textit{SLITs} and \textit{ROBOs}, inhibits apoptosis and facilitates cell migration [22]. This implies that after ovulation one of the effects of locally produced cortisol may be to temporarily reduce the expression of the \textit{SLIT}/\textit{ROBO} tumour suppressor genes to facilitate repair of the damaged OSE.

In many epithelial cancers there is an associated loss of the expression of \textit{SLIT2} and \textit{SLIT3} in primary cultures of luteinised granulosa cells and luteal fibroblast-like cells [22]. After ovulation there is an increase in the local production of cortisol in the OSE that may act to encourage tissue repair and renewal [28]. Over the range of physiologically relevant concentrations in OSE cells cortisol has been shown to have an anti-inflammatory action and can block interleukin-1 stimulated MMP-9 expression [30,31]. In addition we have previously shown that cortisol, by negatively regulating the expression of \textit{SLITs} and \textit{ROBOs}, inhibits apoptosis and facilitates cell migration [22]. This implies that after ovulation one of the effects of locally produced cortisol may be to temporarily reduce the expression of the \textit{SLIT}/\textit{ROBO} tumour suppressor genes to facilitate repair of the damaged OSE.

In many epithelial cancers there is an associated loss of the expression of members of the \textit{SLIT}/\textit{ROBO} family [9]. For example decreased expression of \textit{SLIT} and \textit{ROBO} transcripts has been observed in oesophageal squamous cell, hepatocellular, lung, prostate and breast carcinoma [10,15,16,32,33]. This reduction in expression however is not universal and some cancers, such as gliomas [34] and recurrent endometrial cancer [35] maintain or increase the \textit{SLIT}/\textit{ROBO} pathway. However alterations in the expression of this pathway in malignant epithelial cells from ovarian cancer has not previously been studied.

We cultured malignant epithelial cells from the ascitic fluid of patients with advanced epithelial ovarian cancer and compared \textit{SLIT}/\textit{ROBO} expression to that in normal OSE. We found reduced expression of \textit{SLIT2}, \textit{SLIT3}, \textit{ROBO1}, \textit{ROBO2} and \textit{ROBO4} in malignant cells. Although we believe these cultures are pure cultures of malignant ovarian cells [36], it is possible that there may be non-malignant contaminating cells in some cultures. We therefore also compared the well-differentiated PEO-14 cells with the poorly differentiated SKOV-3 cells. In both cases the more malignant cells had lower expression of the \textit{SLITs} and some of the \textit{ROBOs}. It is therefore likely that ovarian cancer can be added to the list of tumours with reduced \textit{SLIT}/\textit{ROBO} expression.

We went on to investigate what effects a less active \textit{SLIT}/\textit{ROBO} pathway may have on cell function. Recent studies have shown that cortisol can inhibit the expression of \textit{SLIT2} and \textit{SLIT3} in primary cultures of luteinised granulosa cells and luteal fibroblast-like cells [22]. After ovulation there is an increase in the local production of cortisol in the OSE that may act to encourage tissue repair and renewal [28]. Over the range of physiologically relevant concentrations in OSE cells cortisol has been shown to have an anti-inflammatory action and can block interleukin-1 stimulated MMP-9 expression [30,31]. In addition we have previously shown that cortisol, by negatively regulating the expression of \textit{SLITs} and \textit{ROBOs}, inhibits apoptosis and facilitates cell migration [22]. This implies that after ovulation one of the effects of locally produced cortisol may be to temporarily reduce the expression of the \textit{SLIT}/\textit{ROBO} tumour suppressor genes to facilitate repair of the damaged OSE.

Figure 5. The effect of manipulation of GR on SLITs and ROBOs in ovarian cancer cells. A) RT-PCR showing that PEO-14 and SKOV-3 cells expressed GR and MR but not PR. The breast tumour cell line HTB-19 was used as a positive control and –RT was used as a negative control. B) Real-time quantitative PCR demonstrating that transfection with the GR siRNA reduced GR expression in both cell lines when compared to the scrambled (sc) siRNA control. C) Confirmation that GRsiRNA did not affect MR expression in PEO-14 and SKOV-3 cells. D) Quantitative Real-time PCR showing an increase in \textit{SLIT2}, \textit{ROBO1} and \textit{ROBO2} expression after GR knock down by GRsiRNA in PEO-14 cells. E) Demonstration that GRsiRNA transfected SKOV-3 cells also had increased \textit{SLIT2} and \textit{ROBO1} expression. * = P<0.05. doi:10.1371/journal.pone.0027792.g005
implied that SLIT2 can inhibit the invasion of endometrial and ovarian tumour cell lines [19]. We have also shown that inhibition of SLIT/ROBO signalling in primary cultures luteal fibroblasts promoted cell migration [22]. In addition blocking SLIT action in luteal cells from the normal ovary inhibited apoptosis and reduced in Caspase-3/7 activity [22]. There was a reduction in Caspase-3/7 mediated apoptosis in PEO-14 and SKOV-3 cells when SLIT/ROBO signalling was inhibited by blocking SLIT activity using a ROBO1/Fc chimera and SLIT2 synthesis using siRNA technology. Increased apoptosis, associated with reduced expression of the Bcl-2 and Bcl-xl anti-apoptotic molecules, was seen in SLIT2 transfected fibrosarcomas and oesophageal squamous cell carcinomas [16]. SLIT2 could also induce apoptosis associated with activation of Caspase 3 in breast and lung tumour cell lines [37]. Overall a reduction in SLIT/ROBO activity is associated with increased cell survival and migration and this is likely to be relevant in ovarian cancer and its progression.

If the glucocorticoid-mediated inhibition of the SLIT/ROBO pathway is still manifested in malignant epithelial cells in ovarian cancer then cortisol may have effects on cell survival and migration that would be detrimental to the patient. In our primary cultures of advanced ovarian cancer, as well as the poorly differentiated SKOV-3 ovarian cancer cell line, the regulation of SLITs and ROBOs by cortisol was lost. However it was maintained in the more differentiated PEO-14 tumour cell line. This implies that the pathway may still be active in the early stages of ovarian cancer or in less malignant phenotypes. Interestingly glucocorticoids could inhibit apoptosis during fibrosarcoma development [38] and in ovarian tumour cell lines and cells from the ascitic fluid of ovarian cancer patients [39]. Dexamethasone can also curtail apoptosis induced by chemotherapy in a variety of different tumour types including breast, prostate, cervical and ovarian carcinoma cells [40–42]. Therefore glucocorticoid inhibition of SLITs and ROBOs might still be possible in some malignant cells.

As the up-regulation of SLITs has been shown to have inhibitory effects on tumour growth and invasion it is possible that manipulation of the glucocorticoid pathway has therapeutic utility. RU486, a GR and PR antagonist, can induce apoptosis in prostate and ovarian cancer cells [43,44]. Blockade of cortisol activity in PEO-14 and SKOV-3 cells, which lack PR, using RU486 did not influence the expression of SLIT1 and ROBO2 in either cell line. However RU486 did abolish the negative regulation of SLIT/ROBO expression in PEO-14 cells.

More importantly when we inhibited endogenous GR expression, using siRNA, there was an increase in the expression of certain SLITs and ROBOs in both PEO-14 and SKOV-3 cells. This implies that the SLITs and ROBOs could be regulated, at the transcriptional level, by GR and that a major role for GR in controlling SLIT/ROBO expression may be ligand independent. Our bioinformatic analysis revealed several GR-responsive and related elements (GREs) in the promoter regions of SLIT2, SLIT3, ROBO1, ROBO2 and ROBO4. Intriguingly, in neuroblastoma cells, the activated GR directly interacts with p53 and inhibits p53 dependent cell cycle arrest and apoptosis [45]. However GR can act as a tumour suppressor in other types of tumours including skin cancer [46]. Therefore the exact function of GR in cancer could be dependent on the tumour type.

In summary, this study has provided further evidence that the SLIT/ROBO pathway has a role in normal ovarian physiology and is regulated by hormones including glucocorticoids. We have also shown that the SLITs and ROBOs seem to be aberrantly expressed in ovarian cancer. Furthermore reduction of this pathway could augment tumourigenesis and progression through a dysregulation of cellular processes including apoptosis and repulsion of cell migration. Overall these data support the concept that reduction of the SLIT/ROBO pathway is important in malignant development and progression. This research also suggests that targeting their physiological regulation by steroids may have utility in ovarian cancer.

**Materials and Methods**

**Cell and tissue collection**

All cells and tissues were obtained with informed written consent after approval from the Lothian Medical Research Ethics Committee. Human OSE cells were obtained from the ovaries of premenopausal women (n = 5) undergoing elective surgery for non-malignant gynaecological conditions as described previously [29]. Malignant epithelial cells were obtained from the ascitic fluid of women (n = 8) having surgery for advanced epithelial ovarian cancer as described previously [36]. Normal human ovarian tissue had been collected for a complimentary study [47]. The SKOV-3 and PEO-14 cell lines were kindly provided by P. Pujol, INSERM, Montpellier, France and S. Langdon, University of Edinburgh, Edinburgh, UK.

**Cell culture and treatments**

Primary OSE cells and primary ovarian cancer cells were routinely maintained in culture media consisting of Medium 199 (Invitrogen, Paisley, UK) and MCDB105 (Sigma-Aldrich Corp., Gillingham, UK) (pH 7.3; 1:1 v/v) supplemented with 15% (v/v) Fetal Bovine Serum, 50 IU/ml Penicillin, 50 µg/ml Streptomycin and 2 mM L-Glutamine. PEO-14 and SKOV-3 cells were cultured in the same media however it was supplemented with 10% (v/v) FBS. For the cortisol treatment studies cells were seeded in six-well plates at a density of 2 × 10⁵ cells/well. After 24 hours fresh media containing either 1000 nM Cortisol [22,30,31] in ethanol with or the equivalent volume of ethanol (0.001% v/v) was added to the cells. In other experiments 50 µM RU486 [24] (Sigma) with and without cortisol was used. Each treatment was carried out in technical triplicate. After 24 hours 1 ml of media was removed from each well and stored at −20°C for the enzyme-linked immunosorbent assay (ELISA) experiment and RNA was extracted from the cells as described below.

For the ROBO1/Fc treatment studies cells were seeded at 2 × 10⁴ cells/well in 96-well plates. After 24 hours fresh media containing either recombinant rat ROBO1/Fc chimera (R&D Systems, Inc., Abingdon, UK; 1 µg/ml) or the equivalent volume of PBS/0.1% (w/v) BSA was added to the cells. Treatments were carried out in technical quadruplicate. Forty-eight hours later the cells were analysed for apoptosis using the Caspase-Glo 3/7 assay as described below.

**Short interfering RNA technology**

Twenty-four hours before transfection PEO-14 or SKOV-3 cells were seeded in antibiotic free media so that cells were 50% confluent at the time of transfection. Short interfering RNA oligonucleotides against GR and SLIT2 as well as a negative control, with no significant sequence similarity to human gene sequences, were obtained from Applied Biosystems (Warrington, UK). They were transiently transfected into the cells using Oligofectamine transfection reagent according to manufacturers’ instructions (Invitrogen). Briefly, the siRNA oligonucleotides were diluted to a final concentration of 200 nM in Opti-MEM 1 Reduced Serum Media (Invitrogen) and combined with diluted Oligofectamine to allow the siRNA/Oligofectamine complexes to form at room temperature. Then siRNA/Oligofectamine complex was then added dropwise to each well and the cells were then
incubated at 37°C, 5% CO₂ for 48–72 hours. For expression analysis experiments the cells were grown in 6-well plates and each transfection was performed in triplicate. For the caspase-3/7 activity assay experiments the cells were grown in 96-well plates and each transfection was performed in quadruplicate.

Expression analysis

RNA was extracted from each well of the cells using the RNeasy Mini kit (Qiagen Ltd., Crawley, UK) and treated with deoxyribonuclease I (Qiagen). RNA was used as a template for cDNA synthesis using Taqman reverse transcriptase reagents (Applied Biosystems). Primers specific for the SLITs and ROBOs have been previously described in detail [22]. PCR was performed on an Eppendorf Mastercycler gradient authorised thermocycler (PerkinElmer, Inc., Waltham, MA) using GoTaq Flexi DNA polymerase (Promega Ltd., Southampton, UK). The PCR thermocycle consisted of an initial denaturation of 5 min at 95°C followed by 35 cycles of 95°C for 30 sec, annealing temperature for 30 sec, 72°C for 30 sec, and a final extension of 10 min at 72°C. PCR products were visualised on a 2% (w/v) agarose gel with added ethidium bromide.

Real-time quantitative PCR

RNA was extracted and reverse transcribed as described above. A standard curve was generated with serial dilutions of cDNA synthesised from human fetal brain total RNA (Stratagene Europe, Amsterdam, The Netherlands). Real-time PCR amplification was then performed in duplicate 10 μl reactions using Power SYBR Green PCR master mix (Applied Biosystems) following the manufacturer’s instructions and using the ABI 7500HT fast real-time PCR system instrument (Applied Biosystems). Primers used were the same as for the expression analysis and have been described previously [22]. The ABI instrument’s default settings were used for the cycling program and the melting curve analysis.

The ABI analysis software calculated quantitative values for each sample by comparing the sample threshold cycle number, where the increase in the signal associated with exponential growth of PCR products begins to be detected, to the standard curve, according to the manufacturer’s manuals. In all cases the level of gene expression within the samples lay within the boundaries of the corresponding standard curve. Since the precise quality and amount of cDNA that was added to each reaction mix was difficult to assess, transcripts of glucose-6-phosphate dehydrogenase (G6PDH), a housekeeping gene, were also quantified for each sample as described above. This gene is not regulated in the samples under investigation and therefore acted as an endogenous control. Each sample was normalised on the basis of its G6PDH content by dividing the amount of target gene by the amount of housekeeping gene.

SLIT2 ELISA

Since SLIT2 is a secreted protein, the concentration of this protein from PEO-14 and SKOV-3 cells was assessed using culture media from these experiments. Human SLIT2 protein concentration was determined quantitatively using an immunoassay kit (USCN Life Science & Technology Co., Wuhan, China).

Statistical analysis

Statistical analysis was conducted using a Prism software package (GraphPad Software Inc., La Jolla, CA, USA) with significance defined as \( P<0.05 \). After confirmation of normal distribution of samples they were analysed using either a paired or unpaired t-test as appropriate.

Acknowledgments

The authors would like to thank the patients involved and the clinical research nurses for their help in tissue collection particularly Catherine Murray and Sharon McPherson. Dr Mick Rae, Dr Chris Harlow and Dr Simon Langdon were involved with parallel studies and gave helpful advice and support.

Author Contributions

Conceived and designed the experiments: RED KSF WCD. Performed the experiments: RED KSF XR. Analyzed the data: RED WCD. Contributed reagents/materials/analysis tools: KSF XR SGH. Wrote the paper: RED WCD XR KSF SGH.
SLITs and ROBOs in Ovarian Surface Epithelium

7. Hinck L (2004) The versatile roles of “axon guidance” cues in tissue morphogenesis. Dev Cell 7: 783–793.
8. Chedotal A, Kerjan G, Moreau-Fauvarque C (2005) The brain within the tumor: new roles for axon guidance molecules in cancers. Cell Death Differ 12: 1044–1056.
9. Dallol A, Dickinson RE, Latif F (2005) Epigenetic disruption of the SLIT-ROBO interactions in human cancer. In: Esteller M, ed. DNA Methylation, Epigenetics and Metastasis. Springer Netherlands. pp 191–214.
10. Latil A, Chene L, Cochant-Priollet B, Mansin P, Fournier G, et al. (2003) Quantification of expression of netrin, slits and their receptors in human prostate tumors. Int J Cancer 103: 306–315.
11. Naranay G, Goparajoo C, Arias-Pulido H, Kaufmann AM, Schneider A, et al. (2006) Promoter hypermethylation-mediated inactivation of multiple Slit-Robo pathway genes in cervical cancer progression. Mol Cancer 5: 16.
12. Singh RK, Indra D, Mitra S, Mondal RK, Baus PS, et al. (2007) Deletions in chromosome 4 differentially associated with the development of cervical cancer: evidence of slit2 as a candidate tumor suppressor gene. Hum Genet 122: 71–81.
13. Yu J, Cao Q, Wu L, Dallol A, Li J, et al. (2010) The neuronal repellent SLIT2 is a target for repression by EZH2 in prostate cancer. Oncogene 29: 3570–3580.
14. Dallol A, Morton D, Maher ER, Latif F (2003) SLIT2 axon guidance molecule is frequently inactivated in colorectal cancer and suppresses growth of colorectal carcinoma cells. Cancer Res 63: 1054–1058.
15. Jin J, You H, Yu B, Deng Y, Tang N, et al. (2009) Epigenetic inactivation of SLIT2 in human hepatocellular carcinomas. Biochem Biophys Res Commun 379: 86–91.
16. Kim HK, Zhang H, Li H, Wu TT, Siewer S, et al. (2008) Slit2 inhibits growth and metastasis of fibrosarcoma and squamous cell carcinoma. Neoplasia 10: 1411–1429.
17. Marrows R, Strickland P, Lee JS, Wu X, Pebenito M, et al. (2008) SLITs suppress tumor growth in vivo by silencing Sdf1/Cxcr4 within breast epithelium. Cancer Res 68: 7819–7827.
18. Prasad A, Fernandis CA, Chuang CS, Guo SW (2012) Slit2 protein–medicated inhibition of CXCR4-induced chemotactic and chemoinvasive signaling pathways in breast cancer cells. J Biol Chem 287: 9122–9131.
19. Stella MC, Trusolino L, Comoglio PM (2009) The Slit/Robo system suppresses hepatocellular growth-factor-dependent invasion and morphogenesis. Mol Biol Cell 20: 642–657.
20. Liao WX, Wing DA, Geng JG, Chen DB (2010) Perspectives of SLIT-ROBO signaling in placental angiogenesis. Histois Histopatol 25: 1181–1190.
21. Duncan WC, McDonald SE, Dickinson RE, Shaw JL, Lauwereins PC, et al. (2010) Expression of the repulsive SLIT-ROBO pathway in the human endometrium and Fallopian tube. Mol Hum Reprod 16: 950–959.
22. Dickinson RE, Myers M, Duncan WC (2008) Novel Regulated Expression of the SLIT/ROBO Pathway in the Ovary. Possible Role during Luteolysis in Women. Endocrinology 149: 5024–5034.
23. Duncan WC, Myers M, Dickinson RE, van den Driessche S, Fraser HM (2009) Luteal development and luteolysis in the primate corpus luteum. Animal Reprod 6: 34–46.
24. Myers M, Lamont MC, van den Driessche S, Mary N, Thong KJ, et al. (2007) Role of luteal glucocorticoid metabolism during maternal recognition of pregnancy in women. Endocrinology 148: 5769–5779.
25. Leung PC, Choi JH (2007) Endocrine signaling in ovarian surface epithelium and cancer. Hum Reprod Update 13: 143–162.
26. Purdie DM, Bain CJ, Siskind V, Webb PM, Green AC (2005) Ovulation and risk of epithelial ovarian cancer. Int J Cancer 109: 228–232.
27. Aragno M, Hoffmann F, Gauchez AS (2008) Ovarian cancer detection and treatment: current situation and future prospects. Anticancer Res 28: 3135–3138.
28. Hillier SG, Tetuoka M (1998) An anti-inflammatory role for glucocorticoids in the ovaries. J Reprod Immunol 39: 21–27.
29. Gubhaju G, Gao W, Rae MT, Niven D, Langdon SP, et al. (2005) Inflammation-associated gene expression is altered between normal human ovarian surface epithelial cells and cell lines derived from ovarian adenocarcinomas. Br J Cancer 92: 1927–1933.
30. Rae MT, Niven D, Critchley HO, Harlow CR, Hillier SG (2004) Anti-inflammatory steroid action in human ovarian surface epithelial cells. J Clin Endocrinol Metab 89: 4538–4544.
31. Rae MT, Price D, Harlow CR, Critchley HO, Hillier SG (2009) Glucocorticoid receptor-mediated regulation of MAPK gene expression in human ovarian surface epithelial cells. Fertil Steril 92: 705–713.
32. Avci ME, Kono O, Yagci T (2008) Quantification of SLIT-ROBO transcripts in hepatocellular carcinoma reveals two groups of genes with coordinate expression. BMC Cancer 8: 381.
33. Dallol A, Da Silva NF, Viacava P, Minna JD, Birche I, et al. (2002) SLIT2, a human homologue of the Drosophila Slit2 gene, has tumor suppressor activity and is frequently inactivated in lung and breast cancers. Cancer Res 62: 5074–5080.
34. Mertzch S, Schmitz J, Jehannin A, Geng JG, Paulus W, et al. (2008) Slit2 involvement in glial cell migration is mediated by Robo receptor. J Neurosci 28: 1–7.
35. Ma S, Lin X, Geng JG, Gao SW (2010) Increased SLIT immunoreactivity as a biomarker for recurrence in endometrial carcinoma. Am J Obstet Gynecol 202: 68.e1–68.e11.
36. Papacopoulos G, Hogg K, Fegan KS, Critchley HO, Hillier SG, et al. (2009) Regulation of Beta-hydroxysteroid dehydrogenase type 1 and type 2 gene expression and function in the human ovarian surface epithelium by cytokines. Mol Hum Reprod 15: 379–92.
37. Morrissey C, Dallol A, Latif F, Gaskar A, Minna JD (2004) The candidate tumor suppressor gene SLIT-2 suppresses growth and induces apoptosis in lung and breast cancer. Proc Amer Assoc Cancer Res 45: Abstract 3519.
38. Gascoyne DM, Kypta RM, Vivanco MM (2003) Glucocorticoids inhibit apoptosis during fibrosarcoma development by transcriptionally activating Bcl-xl. J Biol Chem 278: 10022–10029.
39. Rasmussen IB, Bruning A (2005) Glucocorticoids inhibit cell death in ovarian cancer and up-regulate caspase inhibitor cIAP2. Clin Cancer Res 11: 6325–6332.
40. Sui M, Chen F, Chen Z, Fan W (2006) Glucocorticoids interfere with therapeutic efficacy of paclitaxel against human breast and ovarian xenograft tumors. Int J Cancer 119: 712–717.
41. Zhang C, Beckerman B, Kallidrits G, Liu Z, Rittgen W, et al. (2006) Corticosteroids induce chemotherapy resistance in the majority of tumour cells from bone, brain, breast, cervix, melanoma and neuroblastoma. Int J Oncoal 29: 1295–1301.
42. Zhang C, Matern J, Haefkamp A, Pfitznermaier J, Hohenfellber M, et al. (2006) Corticosteroid-induced chemotherapy resistance in urological cancers. Cancer Biol Ther 5: 59–64.
43. El Etreby MF, Liang Y, Lewis RW (2000) Induction of apoptosis by mifepristone and tamoxifen in human LNCaP prostate cancer cells in culture. Prostate 43: 31–42.
44. Goyeneche A, Caron RW, Telleria CM (2007) Mifepristone inhibits ovarian cancer cell growth in vitro and in vivo. Clin Cancer Res 13: 3370–3379.
45. Sengupta S, Vonesch JL, Walzinger C, Zheng H, Wasylyk B (2000) Negative regulation of oestrogen receptors in the human corpus luteum. Reproduction 119: 6051–6064.
46. Chodova D, Yemelyanov A, Budunova I (2007) The mechanism of tumor suppressor effect of glucocorticoid receptor in skin. Mol Carcinog 46: 732–740.
47. van den Driessche S, van der Sande MA, Smit SM, Myllynsi M, Duncan WC (2008) Expression and regulation of oestrogen receptors in the human corpus luteum. Reproduction 135: 509–17.