Expression of the repulsive SLIT/ROBO pathway in the human endometrium and Fallopian tube

W.C. Duncan¹,*,†, S.E. McDonald¹,†, R.E. Dickinson¹, J.L.V. Shaw¹, P.C. Lourenco¹, N. Wheelhouse², K-F. Lee³, H.O.D. Critchley¹, and A.W. Horne¹

¹Centre for Reproductive Biology, Queen’s Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4SB, UK. ²Moredun Research Institute, Pentlands Science Park, Penicuik EH26 0PZ, UK. ³Department of Obstetrics and Gynaecology, University of Hong Kong, Pokfulam, Hong Kong, People’s Republic of China

*Correspondence address. Obstetrics and Gynaecology, Simpson Centre for Reproductive Health, Royal Infirmary of Edinburgh, 51 Little France Crescent, Edinburgh EH16 4SA, UK. Tel: +44-131-242-2687; Fax: +44-131-242-2686; E-mail: w.c.duncan@ed.ac.uk

Submitted on February 1, 2010; resubmitted on June 10, 2010; accepted on June 22, 2010

ABSTRACT: We investigated whether the repulsive SLIT/ROBO pathway is expressed in the endometrium and is negatively regulated during implantation. We also examined whether deficient expression in the Fallopian tube (FT) may predispose to ectopic pregnancy (EP). Endometrium (n = 21) and FT (n = 17) were collected across the menstrual cycle from fertile women with regular cycles. Decidualized endometrium (n = 6) was obtained from women undergoing termination, and FT (n = 6) was obtained from women with EP. SLIT/ROBO expression was quantified by reverse transcription–PCR and protein localized by immunohistochemistry. The regulation of SLIT/ROBO expression in vitro, by sex steroids and hCG, was assessed in endometrial (hTERT-EEpC) epithelial cells, and the effects of Chlamydia trachomatis infection and smoking were studied in oviductal (OE-E6/E7) epithelial cells. Endometrial SLIT3 was highest in the mid-secretory phase (P = 0.0003) and SLIT1,2 and ROBO1 showed a similar trend. ROBO2 was highest in proliferative phase (P = 0.027) and ROBO3,4 showed a similar trend. SLIT2,3 and ROBO1,4 were lower in decidua compared with mid-secretory endometrium (P < 0.05). SLITs and ROBOs, excepting ROBO2, were expressed in FT but there were no differences across the cycle or in EP. SLIT/ROBO proteins were localized to endometrial and FT epithelium. Treatment of hTERT-EEpC with a combination of estradiol and medroxyprogesterone acetate inhibited ROBO1 expression (P < 0.01) but hCG had no effect. Acute treatment of OE-E6/E7 with smoking metabolite, cotinine, and C. trachomatis had no effect. These findings imply a regulated role for the endometrial SLIT/ROBO interaction during normal development and pregnancy but that it may not be important in the aetiology of EP.

Key words: endometrium / decidua / ectopic pregnancy / Chlamydia trachomatis / smoking

Introduction

The Roundabout (ROBO) genes encode the transmembrane receptors for their ligands, the SLIT proteins. There are three SLIT (1–3) proteins that bind to and activate each of the four ROBO (1–4) receptors (Legg et al., 2008). The SLIT/ROBO pathway is widely expressed during development and one of its roles is to function as a repulsive cue in the regulation of cell migration (Dickinson and Duncan, 2010). It has an evolutionary conserved role in axon guidance during the development of the nervous system (Hinck, 2004). As well as being involved in the regulation of cell migration and angiogenesis (Jones et al., 2008), the SLIT/ROBO interaction can promote cell death through apoptosis (Dallol et al., 2005; Dickinson et al., 2008).

It therefore has particular roles in the development of major organs (Piper et al., 2000; Hinck, 2004), including the ovary (Dickinson et al., 2010). In addition, the various mutant mice suffer from multiple developmental abnormalities and the majority die in utero or shortly after birth (Xian et al., 2001; Grieshammer et al., 2004).

As the SLIT/ROBO interaction inhibits cell migration and promotes cell death, the SLITs and ROBOs also function as tumour suppressor genes (Chetodal et al., 2005; Dallol et al., 2005). There is increasing evidence for a loss of SLIT/ROBO expression, mainly through deletions and promoter region hypermethylation, in multiple cancers including those of the reproductive system (Dickinson et al., 2004; Narayan et al., 2006). We have recently shown however that SLITs and ROBOs also have a role in normal adult tissue physiology. We
have reported the expression of the SLIT/ROBO pathway in the human corpus luteum across the luteal phase of the menstrual cycle (Dickinson et al., 2008). The expression of both the ligands (notably SLIT2 and SLIT3) and receptors (notably ROBO2) peaked during luteolysis. In addition, using in vitro human luteal cell models, we showed that the luteotrophic molecules hCG (Myers et al., 2007a) and cortisol (Myers et al., 2007b) inhibited their expression. This suggests a regulated role in cyclical tissue remodelling in the ovary.

The uterus also undergoes tightly regulated cyclical remodelling under the control of systemic sex steroids and a number of other local factors (Jabbour et al., 2006). During the menstrual cycle, the endometrium undergoes morphological and functional changes to prepare for implantation and pregnancy. However, the paracrine interaction between the endometrial cells and the trophoblast to facilitate implantation is not yet fully understood. In 1.3–2% of all pregnancies, aberrant implantation occurs in the Fallopian tube (FT). Tubal ectopic pregnancy (EP) remains a major burden on health service resources (Wedderburn et al., 2010) and continues to be associated with notable maternal morbidity and mortality (Farquhar, 2005). Why the blastocyst implants in the FT is not clear as tubal EP does not occur in other species (Corpa, 2006; Shaw et al., 2010). There are however a number of known risk factors such as previous Chlamydia trachomatis infection and cigarette smoking (Faro, 1991; Bouyer et al., 2003).

As the SLIT/ROBO interaction is a regulated pathway known to be involved in remodelling in the human ovary, we believe that it may also have a functional role in the endometrium and FT. As implantation involves paracrine signalling associated with cell survival and migration, we hypothesized that the repulsive SLIT/ROBO interaction would be negatively regulated during implantation and that deficient expression in the FT may predispose to EP. This study therefore aimed to: (i) examine the expression and localization of the SLITs and ROBOs in human endometrium; (ii) determine if endometrial expression is inhibited at the time of implantation and during early pregnancy in vivo and study the effects of candidate regulatory molecules in vitro; (iii) examine the expression and localization of the SLITs and ROBOs in the FT; (iv) determine if their expression is inhibited in tubal EP in vivo and study the acute effects of chlamydial infection and smoking in vitro.

Materials and Methods

Subjects and tissue collection

Ethical approval was obtained from the Lothian Research Ethics Committee and written informed consent was obtained from all patients. Endometrial biopsies (n = 21) were collected from fertile women with regular menstrual cycles (25–35 days) who had not undergone hormonal treatment in the previous 3 months as described previously (King et al., 2009). These samples underwent histological dating (Noyes et al., 1950) and were classified as menstrual (n = 3), proliferative (n = 5), early-secretory (n = 4), mid-secretory (n = 5) and late-secretory (n = 4). This corresponded to circulating serum estradiol (E2) and progesterone concentrations as described previously (King et al., 2009).

Biopsies from the ampullary region of the FT were collected from a further 17 women undergoing hysterectomy for benign gynaecological conditions. All had regular menstrual cycles, no previous history of EP and had not taken any exogenous hormone preparations in the 3 months prior to surgery. These biopsies were also dated with reference to endometrial histology and serum hormone concentrations. They were classified as menstrual (n = 4), follicular (n = 5) and mid-luteal (n = 8). In addition, FT biopsies were obtained from six women undergoing surgical management of tubal EP [mean gestation 58.09 days ± SD 8.28; mean serum progesterone (P4) 58.53 nmol/L ± SD 47.22]. None of these women presented acutely with haemodynamic shock and all required serial serum hCG and ultrasound monitoring prior to diagnosis. Trophoblast-free decidual biopsies (n = 6) were obtained from women undergoing therapeutic surgical termination of pregnancy [mean gestation 69.75 days ± SD 7.07, mean serum P4 61.71 nmol/L ± SD 10.95] as described previously (Horne et al., 2008).

A representative part of each biopsy was immersed in RNAlater storage solution (Ambion, TX, USA) at 4°C overnight then stored frozen at −80°C. A further equal part of the biopsy was fixed in 4% neutral buffered formalin overnight at 4°C, transferred to 70% ethanol and subsequently embedded into paraffin wax.

RNA extraction and reverse transcription

RNA was extracted from frozen tissue biopsies using the QIAGEN RNaseasy Mini Kit and QIAGEN Tissue Lyser (QIAGEN, Crawley, UK) according to the manufacturer’s instructions and quantified using a ND-1000 spectrophotometer (NanoDrop technologies, DE, USA). All samples were treated with DNase I (QIAGEN) to remove genomic DNA contamination. Reverse transcription (RT) of RNA was performed in 30 μl reaction volumes containing 1 μ RT buffer, 25 mM magnesium chloride, dNTPs, random hexamers, RNase inhibitor and Multiscribe reverse transcriptase (PE Applied Biosystems, Warrington, UK). RT negative (containing template RNA but no reverse transcriptase enzyme) and RT water (containing reverse transcriptase but no template RNA) were included in every cDNA reaction as negative controls.

Quantitative real-time PCR

Tagman quantitative real-time PCR (Q-RT-PCR) was used to measure the gene expression levels. Specific primers were designed using the Universal Probe Library Assay Design Center (www.roche-applied-science.com) and used in conjunction with Universal Probe Library (UPL) probes (Roche Applied Science). Details of primer sequences and probes used are given in Table I. All reactions were performed in triplicate using an ABI 7900 sequence detection system. Using the 2-ΔΔCt method, mRNA expression results were normalized against ribosomal 18S internal control (PE Applied Biosystems, Oxford, UK) and expressed as relative expression compared with cellular or positive tissue controls.

Immunohistochemistry

Pilot studies showed we could reliably immunolocalize SLIT2, ROBO1, ROBO2 and ROBO4 using polyclonal antibodies (SantaCruz Biotechnology, Santa Cruz, CA, USA; Abcam, Cambridge, UK) and peptide-block controls. These proteins (excepting ROBO2 in the FT) were therefore immunolocalized by the standard methods using biotinylated secondary antibodies and ABC-Elite avidin–biotin–peroxidase complex (Vector Laboratories, Peterborough, UK; Dickinson et al., 2010). Briefly, 3 μm paraffin sections of endometrium and FT were cut, dewaxed in xylene, rehydrated and subjected to antigen retrieval by microwaving in Antigen Unmasking Solution (Vector Laboratories) before blocking endogenous peroxidase with 3% hydrogen peroxidase (Sigma, Dorset, UK). An avidin–biotin block (Vector Laboratories) and protein block (Dako, Ely, UK) were performed prior to overnight incubation with primary antibodies. Negative controls were included in every run where the primary antibody was pre-incubated with a blocking peptide. Sections were then incubated with biotinylated secondary antibody (Vector Laboratories) and ABC-Elite (Vector...
Laboratories). Positive immunostaining was visualized using 3,3-diaminobenzidine (Vector Laboratories). The stained sections were then counterstained in haematoxylin, mounted in Pertex (Cellpath PLC, Hemel Hempstead, UK) and compared with negative control sections.

**Cell culture**

The immortalized endometrial epithelial cell line (hTERT-EEpC; Hombach-Klonisch et al., 2005) was used as an in vitro model to investigate the effects of steroid hormones and hCG. Cells were maintained in Ham’s F-12 media (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum and transferred to a serum-free medium overnight prior to treatments. The cells were treated with concentrations of hCG (10 and 100 IU/ml; Organon Laboratories, Cambridge, UK) mimicking those at the maternal epithelia adjacent to an implanting embryo (Nakayama et al., 2003; Horne et al., 2009a). All experiments were conducted in triplicate and cells were collected after 6 and 24 h. These time points were established by our previous studies (Horne et al., 2009a). In addition, the cells were treated with E2 (10 nM), medroxyprogesterone acetate (MPA) (1 μM), vehicle control or a combination of E2 (10 nM) and MPA (1 μM) for 24 h. This treatment regimen has previously been validated as an in vitro model of steroid action and utilized the standard, and relevant, concentrations of steroids (Horne et al., 2006). Each experiment was conducted three times and cells were collected after 24 h.

The OE-E6/E7 oviductal epithelial cell line (Lee et al., 2001) was used to assess the effects of smoking and chlamydial infection. Cells were maintained in Dulbecco’s modified Eagle’s medium F12 (Invitrogen Ltd, Paisley, UK) containing 10% fetal calf serum and plated in 48-well culture plates at a density of 2 × 10^5 cells/well. Prior to treatment, cells were transferred to a serum-free medium overnight prior to treatments. The cells were treated with cotinine, a metabolite of nicotine (Conklin et al., 2002). Cells were plated in 12-well culture plates at a density of 2 × 10^5 cells/well and transferred to the serum-free medium overnight prior to treatment. Cells were treated with cotinine (Sigma) at a concentration of either 40 ng/ml (representing the concentrations achieved in passive smoking) or 400 ng/ml (representing the concentrations achieved in heavy regular smoking; Haddow et al., 1987) or carrier control. Experiments were carried out three times and cells were collected at 4, 8, 12 and 24 h. In all culture experiments, the cells were collected for subsequent RNA extraction and analysis of mRNA expression by Q-RT-PCR as described previously. In these experiments, we focused on assessing the effects on the expression of the primary ROBO in these cells, ROBO1, and the representative SLIT, SLIT2.

**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. Analysis was performed using a one-way analysis of variance and Bonferroni’s post hoc test where three or more variables were being compared. A t-test was used when two variables were being compared. Where there were significant differences in variances the data were logarithmically transformed to ensure normal distribution in all cases prior to statistical analysis.

**Results**

**SLIT/ROBO genes are expressed in human endometrium**

Messenger RNA for all known genes in the SLIT/ROBO family was expressed in human endometrium (Fig. 1). The pattern of expression for each of the SLIT genes was similar across the menstrual cycle with maximal expression in the mid-secretory phase (Fig. 1a, c and e). This reached statistical significance for SLIT3 (P = 0.0003). The pattern of expression across the menstrual cycle for ROBO2, ROBO3 and ROBO4 was similar, although it was different to that of the SLITs (Fig. 1i, k and m). The peak ROBO2, ROBO3 and ROBO4 expression was seen in the proliferative phase and this reached statistical significance for ROBO2 (P = 0.027). Interestingly, the pattern of ROBO1 expression was more similar to the SLITs than the other ROBOs (Fig. 1g). Overall, this suggests that endometrial expression of the SLIT/ROBO system is hormonally regulated and may have a role in the endometrial function.

**SLIT/ROBO gene expression changes after decidualization**

The expression of SLIT2 (P < 0.05), SLIT3 (P < 0.005), ROBO1 (P < 0.05) and ROBO4 (P < 0.0005) was reduced in the decidualized endometrium of pregnancy when compared with the mid-secretory phase (Fig. 1). Unlike other members of the SLIT/ROBO family, SLIT1 (P < 0.05) expression was very low in the non-pregnant endometrium and increased in the decidualized endometrium of early pregnancy (Fig. 1b). With this exception, the pattern is consistent with an
inhibition of the endometrial SLIT/ROBO repulsive system during the establishment of pregnancy.

**SLIT and ROBO proteins are localized to the endometrial epithelium**

Immunohistochemistry was used to examine the endometrial localization of candidate SLIT and ROBO proteins (Fig. 2). SLIT2 and ROBO1 were predominantly expressed in the surface and glandular epithelium in the human endometrium. ROBO1 (Fig. 2b) and to a lesser extent SLIT2 (Fig. 2a) could also be detected in individual stromal cells near the surface epithelium. ROBO2 and ROBO4, the ROBO proteins that demonstrated a slightly different pattern of mRNA expression across the cycle, were also primarily expressed by epithelial cells (Fig. 2e and f). ROBO4 could also be localized to stromal cells that appeared to be endothelial in nature (Fig. 2f). The SLIT/ROBO system is therefore expressed in endometrial epithelial cells at the stage of the cycle when implantation occurs.

**SLIT/ROBO genes are expressed in human FT**

Expression of the SLIT and ROBO genes, with the exception of ROBO2, could also be detected in the FT across the menstrual cycle (Fig. 3). Although again there was a similar pattern of SLIT2 (Fig. 3c) and ROBO1 (Fig. 3b) expression, there were no statistical differences in expression of any of the SLIT or ROBO genes across the menstrual cycle (Fig. 3). In addition, there was no difference in the levels of expression of the SLIT/ROBO genes in the FTs of patients with tubal EP (Fig. 3). This suggests that there may be no reduction in the expression of the SLIT/ROBO system in tubal implantation.

**SLIT/ROBO proteins are expressed by the tubal epithelium**

Although there was some faint immunostaining in some smooth muscle and stromal cells, the primary site of SLIT2 and ROBO1,4 expression in the FT, in both the follicular and the luteal phases of the cycle, was the epithelium (Fig. 4). Again the SLIT/ROBO system seems to be expressed at the epithelial site of ectopic attachment of the conceptus in the oviduct. However, the localization of SLIT and ROBO in the FTs from women with a tubal EP was similar to that of the non-pregnant tubes (Fig. 4e and f).

**Regulation of SLIT/ROBO expression in the endometrial epithelial cells**

In order to investigate factors involved in the regulation of the SLIT/ROBO pathway in the decidua an immortalized endometrial epithelial cell line was studied in vitro. Neither E2 alone, MPA alone nor a combination of E2 and MPA had any effect on the expression of SLIT2 over 24 h (Fig. 5a). ROBO1 was not regulated by either E2 or MPA but a combination of E2 and MPA inhibited its expression over 24 h (P < 0.01; Fig. 5b). Treatment with either 10 or 100 IU/ml hCG showed no effect on either SLIT2 (Fig. 5c) or ROBO1 (Fig. 5d) expression after 6 and 24 h of culture.
Regulation of SLIT/ROBO expression in oviductal epithelial cells by known risk factors for EP

In order to determine if known risk factors for tubal EP could regulate the expression of the SLIT/ROBO pathway in tubal epithelial cells, an in vitro model was used to investigate the acute effects of smoking and chlamydial infection. The effect of smoking on the FT was modelled using an oviductal epithelial cell line exposed to physiological concentrations of cotinine. Treatment with a low dose (40 ng/ml; representative of passive smoking) or a high dose (400 ng/ml; representative of regular smoking) had no effect on the expression of either SLIT2 or ROBO1 at any of the time points over the 24-h assessment (Fig. 5e and f).

The acute effect of *C. trachomatis* infection on the SLIT/ROBO pathway in the FT was then investigated using this oviductal epithelial cell line exposed to physiological concentrations of cotinine. Treatment with a low dose (40 ng/ml; representative of passive smoking) or a high dose (400 ng/ml; representative of regular smoking) had no effect on the expression of either SLIT2 or ROBO1 at any of the time points over the 24-h assessment (Fig. 5e and f).

However, there seemed to be a bimodal effect on the expression of ROBO1. After 24 h, there was a trend for an increase in expression, but after 48 h, the expression of ROBO1 tended to be lower than the control cells. At the highest *Chlamydia* inoculation titres, there was a significant reduction in ROBO1 expression between 24 and 48 h exposure ($P < 0.05$; Fig. 5h).

**Discussion**

To our knowledge, this is the first comprehensive report of the patterns of expression of the SLIT/ROBO system in the human endometrium and FT at different phases of the menstrual cycle. We also report on SLIT/ROBO expression at normal endometrial and pathological tubal implantation sites. In addition, we have used an in vitro model system to investigate the impact of acute exposure to factors thought to influence implantation on SLIT/ROBO expression in these tissues.

We demonstrate that all of the known SLITs and ROBOs are expressed in the endometrium across the menstrual cycle. Only the expression of SLIT3 significantly changed across the menstrual cycle but each of the SLITs followed the same pattern with maximal
expression in the mid-secretory phase. As each of the SLITs seems to have the same action at each of the ROBO receptors, this suggests a peak of repulsive ligand expression at the time of expected implantation. This implies that their expression is hormonally regulated and that the SLITs may have a specific role in endometrial function. However, unlike the corpus luteum (Dickinson et al., 2008), in the endometrium cell death does not follow the pattern of SLIT expression. In the endometrium, cell death increases in the late-secretory phase and is highest during the menstrual phase (Otsuki, 2001; Jabbour et al., 2006). Endometrial tissue remodelling and angiogenesis however occur after menstruation, are primarily driven by E2 and vascular endothelial growth factor and are largely complete by the mid-secretory phase (Fraser et al., 2008). It is therefore possible that the peak in SLIT expression at the mid-secretory phase may reflect or influence this process (Legg et al., 2008). However, the nature of the role for SLIT in the endometrium remains unclear.

In the corpus luteum, the expression of ROBOs mimics the pattern of expression of the SLITs (Dickinson et al., 2008). Although this was true in the endometrium for ROBO1, it was not for the other ROBOs. In the fetal ovary, the SLITs and ROBOs also show a discrepancy in their pattern of expression (Dickinson et al., 2010). The nature of this differential expression is unclear but it does suggest that the regulation of ROBOs is different to that of the SLITs in both tissues. However, there are receptors to the SLIT ligands in the endometrium across the menstrual cycle.

Another interesting feature of the ROBOs was the endometrial stromal cell immunostaining. It has been reported that vascular endothelial cells express ROBO4 (Park et al., 2003; Legg et al., 2008), and our findings suggest that this is likely to be the case in the human endometrium but we also demonstrate specific epithelial staining. Although SLIT2 could be detected in some individual cells in the stroma, this was particularly obvious for ROBO1. The nature of those specific cells is not clear but they may represent immune cells. Certainly, the SLIT/ROBO pathway has previously been described in lymphocytes (Prasad et al., 2007). However, these undefined immunostained cells were particularly marked in the proliferative phase and it is the mid-secretory stage that the immune population peaks with the accumulation of uterine CD56+ve natural killer cells (King, 2000; Figure 3).
Although it is attractive to speculate that immune cells are involved in the SLIT/ROBO interaction in the endometrium, further work needs to be done to confirm this.

As steroids are the primary regulatory molecules controlling the human endometrium (Jabbour et al., 2006), we hypothesized that they would regulate SLIT/ROBO expression. Certainly, there are steroid response elements on the promoter regions of these genes (Dickinson RE, unpublished observations) and glucocorticoids inhibit both SLIT and ROBO expression in ovarian cells (Dickinson et al., 2008). In addition, there are steroid receptors on uterine immune cell populations (Henderson et al., 2003). As progesterone receptors are lost from endometrial glandular epithelium in the secretory phase (Koh et al., 1995), it is possible that progesterone is suppressive and that the absence of progesterone receptors is responsible for the peak in expression at this time. However, MPA alone did not suppress SLIT2 or ROBO1 and E2 in combination with MPA did not suppress SLIT2 in vitro. Nonetheless, we did discover that ROBO1 expression was inhibited by the combination of E2 and MPA. The biological significance of this, and whether the addition of E2 promotes progesterone receptor expression (Jabbour et al., 2006), is uncertain and requires further study. Recently, it has been suggested that SLIT/ROBO1 is increased in ovarian endometriomas (Shen et al., 2009). Sex steroid receptors however are maintained or increased in ectopic endometrium (Fujishita et al., 1997; Jones et al., 1998). Although it remains possible that steroids are involved in the physiological regulation of endometrial SLIT/ROBO, we have not been able to fully confirm this in vitro.

We speculated that the SLIT/ROBO system would have a role in the regulation of implantation. If the system functions as a repulsive cue, which can be regulated, we hypothesized that the endometrial expression of SLITs or ROBOs would be down-regulated during implantation. Although there was no down-regulation during the mid-secretory implantation window, we found some evidence for this in early gestation. The decidualized endometrium of pregnancy demonstrated a significant reduction in the expression of SLIT2, SLIT3, ROBO1 and ROBO4. The factors involved in regulating this expression are not clear but in vitro the decidua is exposed to a combination of estrogen and progesterone as well as increased local cortisol, as a consequence of HSD11B1 expression (Michael et al., 2003; McDonald et al., 2006). Increased local cortisol is associated with reduced SLIT/ROBO expression in the ovary (Dickinson et al., 2008). It is not yet known whether trophoblast cells express SLIT or ROBO or if trophoblast products can regulate endometrial SLIT or ROBO expression. We found no evidence for acute regulation by hCG using a treatment.

**Figure 4** Representative immunolocalization of SLIT2 and ROBO1 proteins (brown) in human FT. (a) SLIT2 in follicular phase FT. (b) ROBO1 in follicular phase FT. (c) SLIT2 in luteal phase FT. (d) ROBO1 in luteal phase FT. (e) SLIT2 in the tube collected from an EP. (f) ROBO1 in the tube collected from an EP. Negative controls are seen in the insets and the tubal lumen is labelled L. Scale bar represents 100 μm.
Figure 5  Manipulation of SLIT2 and ROBO1 expression in vivo. (a and b) The effect of E2 (10 nM) and MPA (10 nM) in endometrial epithelial cells (hTERT EEpCs). (a) SLIT2 expression does not change after steroid exposure for 24 h. (b) ROBO1 expression is reduced (\( **P < 0.01 \)) by a combination of E2 and MPA for 24 h. (c and d) The effect of hCG (10 and 100 IU/ml) in hTERT EEpCs. (c) SLIT2 and (d) ROBO1 are not altered by hCG after 6 h (grey bars) or 24 h (black bars). (d) ROBO1 is also not altered by hCG after 6 h (grey bars) or after 24 h (black bars). (e and f) The effect of cotinine in oviductal epithelial cells. There were no significant differences (\( P > 0.05 \)) of treatment with cotinine at 40 ng/ml (grey bars) and 400 ng/ml (white bars) on the expression of SLIT2 (e) and ROBO1 (f) relative to control (black bars) at four different time points up to 24 h. (g and h) The effect of C. trachomatis exposure in oviductal epithelial cells at different MOI for 24 (grey bars) or 48 h (black bars) on the relative expression of SLIT2 (g) and ROBO1 (h). A significant difference (\( P < 0.05 \)) in ROBO1 expression between 24 and 48 h exposure to the highest infective concentration was noted.
regimen that has been shown to influence endometrial gene expression (Horne et al., 2009a). It is likely that there is some regulation of the SLIT/ROBO interaction during intrauterine implantation but the importance and cause of this regulation requires further study.

We have also shown that the SLITs and ROBOs, with the exception of ROBO2, are expressed in the FT and SLIT/ROBO protein is localized to the tubal epithelium. However, unlike the endometrium, there was no change in mRNA expression across the cycle. Differential regulation of specific proteins in the FT compared with endometrium has also been shown for sex steroid receptors. In contrast to the endometrium, epithelial steroid receptors are consistently expressed in the FT across the menstrual cycle (Amso et al., 1994; Horne et al., 2009b).

It is not clear if the expression of SLIT and ROBO in the tubal epithelium provides a repulsive cue to tubal implantation. We did not detect any changes in SLIT/ROBO expression in the FTs of EP compared with the non-pregnant FT. This might imply that there is no local down-regulation of SLITs or ROBOs in the FT by the implanted embryo in contrast to that seen in the endometrium. However, it is not possible, for ethical reasons, to study the expression of the SLITs and ROBOs in the FT of a woman with an intrauterine pregnancy. Thus, it is feasible that the reduced sex steroid receptor expression that has been recently observed in the FT of women with EP (Horne et al., 2009b) may also be seen in the FT in an intrauterine pregnancy.

The function of the SLIT/ROBO system in the FT is unclear. We hypothesized that if their function is to inhibit implantation/invasion of ectopic trophoblast or to affect embryo transport, their expression would be altered by components known to act as risk factors for EP such as smoking or chlamydial infection (Farquhar, 2005). We assessed whether an acute exposure to cotinine or Chlamydia infection alters the expression of SLITs and ROBOs in the FT. We did not detect any changes to SLIT/ROBO expression that could be accounted for by cotinine or Chlamydia infection.

We have also shown that the SLITs and ROBOs, with the exception of ROBO2, are expressed in the FT and SLIT/ROBO protein is localized to the tubal epithelium. However, unlike the endometrium, there was no change in mRNA expression across the cycle. Differential regulation of specific proteins in the FT compared with endometrium has also been shown for sex steroid receptors. In contrast to the endometrium, epithelial steroid receptors are consistently expressed in the FT across the menstrual cycle (Amso et al., 1994; Horne et al., 2009b).

However, unlike the endometrium, there was no change in mRNA expression across the cycle. Differential regulation of specific proteins in the FT compared with endometrium has also been shown for sex steroid receptors. In contrast to the endometrium, epithelial steroid receptors are consistently expressed in the FT across the menstrual cycle (Amso et al., 1994; Horne et al., 2009b).

In addition, salivary or serum concentrations of cotinine have been widely used to establish and quantify an individual’s exposure of smoking (Haddow et al., 1987). We were therefore able to assess the effects of biologically relevant concentrations of cotinine. We focused on endometrial gene expression after infection with increasing concentrations of Chlamydia at different stages of the menstrual cycle and the menopause. Hum Reprod 1994;9:1027–1037.


