MHR Basic science of reproductive medicine   **ORIGINAL RESEARCH**

Endometrial cysteine-rich secretory protein 3 is inhibited by human chorionic gonadotrophin, and is increased in the decidua of tubal ectopic pregnancy

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ABSTRACT: Ectopic pregnancy (EP) remains a considerable cause of morbidity and occasional mortality. Currently, there is no reliable test to differentiate ectopic from intrauterine gestation. We have previously used array technology to demonstrate that differences in gene expression in decidualized endometrium from women with ectopic and intrauterine gestations could be used to identify candidate diagnostic biomarkers for EP. The aim of this study was to further investigate the decidual gene with the highest fold increase in EP, cysteine-rich secretory protein-3 (*CRISP-3*). Decidualized endometrium from gestation-matched women undergoing surgical termination of pregnancy ($n = 8$), evacuation of uterus for miscarriage ($n = 6$) and surgery for EP ($n = 11$) was subjected to quantitative RT–PCR, morphological assessment, immunohistochemistry and western blot analysis. Sera were analysed for progesterone and human chorionic gonadotrophin (hCG) levels. Immortalized endometrial epithelial cells were cultured with physiological concentrations of hCG. *CRISP-3* mRNA and protein expression were greater in endometrium from ectopic when compared with intrauterine pregnancies ($P < 0.05$). *CRISP-3* protein was localized to epithelium and granulocytes of endometrium. *CRISP-3* serum concentrations were not different in women with ectopic compared with intrauterine pregnancies. *CRISP-3* expression in endometrium was not related to the degree of decidualization or to serum progesterone levels. Endometrial *CRISP-3* expression was inversely proportional to serum hCG concentrations ($P < 0.001$). Stimulation of endometrial epithelial cells with hCG *in vitro* caused a reduction in *CRISP-3* expression ($P < 0.01$). The measurement of CRISP-3 in endometrium could provide an additional tool in the diagnosis of failing early pregnancy of unknown location. The absence of a local reduction in expression of *CRISP-3* in decidualized endometrium of women with EP may be due to reduced exposure to hCG due to the ectopic location of the trophoblast.

Key words: ectopic pregnancy / decidualized endometrium / *CRISP-3* / hCG

Introduction

Tubal ectopic pregnancy remains a common cause of morbidity and mortality (Farquhar, 2005). In the UK, between 2003 and 2005, early pregnancy bleeding was the third most common cause of maternal death, and more than 60% of these cases were due to ruptured tubal ectopic pregnancies (Saving Mother's Lives, 2008). In the

USA, ruptured tubal ectopic pregnancy remains the most common cause of pregnancy-related death in the first trimester (Mukul and Teal, 2007). Unfortunately, our knowledge of the complex molecular and cellular interactions that contribute to tubal implantation is limited. Furthermore, despite the use of serial serum beta-human chorionic gonadotrophin (hCG) concentrations and transvaginal

ultrasonography, diagnosis remains difficult (ASRM, 2006). Indeed, tubal ectopic pregnancy is frequently not diagnosed, or misdiagnosed, at the initial visit (Duncan *et al.*, 1995; Robson and O'shea, 1996).

The development of a biomarker that can differentiate a tubal ectopic implantation from an intrauterine implantation is therefore important. In the pre-genomic era, a one-by-one scientific approach has been unsuccessful in revealing markers that could be used as a test for tubal ectopic pregnancy (Birkhahn *et al.*, 2001; Wegner and Mershon, 2001; Develioglu *et al.*, 2002; Florio *et al.*, 2007). However, we have recently shown that high-density oligonucleotide microarray analysis is helpful in identifying novel genes that are altered during tubal implantation (Horne *et al.*, 2008). Using this technology, we demonstrated that a secreted product, Activin B, from the decidualized endometrium was a candidate biomarker for tubal ectopic pregnancy (Horne *et al.*, 2008).

During that study, the gene with the highest fold increase in the decidualized endometrium of ectopic pregnancy, when compared with that of intrauterine pregnancy (FC = 13.10), was cysteine-rich secretory protein 3 (*CRISP-3*). *CRISP-3*, which is also known as specific granule protein of 28 kDa (SGP28), belongs to a family of CRISPs characterized by their size (220−230 amino acids), their secretory properties and a content of 16 highly conserved cysteine residues, which form intra-molecular disulphide bond (Kjeldsen *et al.*, 1996; Gibbs and O'Bryan, 2007). In humans, *CRISP-3* mRNA has been detected at high concentrations in the salivary glands, pancreas and prostate, and at lower levels in the epididymis, ovary, thymus and colon (Kratzschmar *et al.*, 1996). Furthermore, *CRISP-3* is secreted and can be detected in human tissue fluids such as saliva, sweat, blood and seminal plasma making it an ideal candidate biomarker for pathophysiological conditions, such as ectopic pregnancy (Udby *et al.*, 2002a; Bjartell *et al.*, 2006).

We hypothesized that, since *CRISP-3* is secreted, if decidual *CRISP-3* expression is higher in ectopic pregnancy, this could form the basis of a novel test for tubal implantation. This study aimed to further investigate *CRISP-3* expression, localization and regulation in the decidualized endometrium of women with tubal ectopic pregnancy. In addition we aimed to use such analyses to inform us about the molecular regulation of endometrial function during normal early pregnancy. Herein we report the identification, expression, localization, underlying biological regulation and serum concentrations of *CRISP-3* in gestation-matched women with tubal and intrauterine implantation.

Materials and Methods

Tissue collection

Ethical approval for this study was obtained from Lothian Research Ethics Committee and informed written consent was obtained from all patients before sample collection. First trimester decidualized endometrium was obtained from gestation-matched women (age 18−45 years) undergoing surgical termination of pregnancy (TOP, $n = 8$, Group 1, mean gestation 58.7 days), surgical management of embryonic missed miscarriage ($n = 6$, Group 2, mean gestation 57.7 days) and surgical management of tubal pregnancy ($n = 11$, Group 3, mean gestation 58.1 days). None of the women undergoing surgical management of tubal ectopic pregnancy presented acutely with hemodynamic shock, and all required serial serum hCG and ultrasound monitoring prior to diagnosis. The decidualized endometrium and trophoblast were obtained by suction curettage from Groups

1 and 2. The decidualized endometrium was obtained by suction endometrial biopsy (Pipelle™, Eurosurgical Ltd, Cranleigh, UK) from Group 3. The decidualized endometrium was isolated from the trophoblast macroscopically and was (i) immersed in RNAlater™ (Ambion, TX, USA) at 4°C overnight then flash frozen at −70°C; and (ii) fixed in 10% neutral buffered formalin overnight at 4°C, stored in 70% ethanol and wax embedded for staining with haematoxylin and eosin and immunohistochemistry. The presence of trophoblast was ruled out morphologically and by using immunohistochemical staining for cytokeratin as described previously (Ball *et al.*, 2006).

RNA extraction

Total RNA was extracted from the decidualized endometrium and cell culture experiments as detailed in the manufacturers' protocol (Qiagen, West Sussex, UK). The concentration and quality of the extracted RNA was assessed using an Agilent bioanalyser and quantified using an ND-1000 spectrophotometer (Nanodrop technologies, DE, USA). All samples were standardized for quality control and assigned an RNA integrity number (RIN). RNA samples were considered to be of good quality when a mean RIN value of 7.5 was obtained (Schroeder *et al.*, 2006).

Microarray analysis

The conduct and robust standardization and assessment of the microarray study that informed this study have been reported previously (Horne *et al.*, 2008). The microarray analysis involved 25 samples. Gene lists were created using a fold change threshold of >2 and a corrected *P*-value of <0.05, and *CRISP-3* had the highest fold change in ectopic pregnancy (13.10).

Quantitative RT−PCR

After DNAse treatment, using RQ1 DNase (Promega, Southampton, UK) the RNA was reverse transcribed into cDNA using random hexamers (Applied Biosystems, Foster City, CA, USA). Taqman Quantitative RT−PCR was then used to measure gene levels in all 25 samples. Applied Biosystems pre-validated 'assay-on-demand' specific primers and probes were used for analysis of tissue samples. cDNA from cell culture experiments was assayed for *CRISP-3* using validated primers and the Universal Probe Library (www.universalprobelibrary.com, Roche Diagnostics, Germany). All sample reactions were performed in triplicate. Using the 2-ΔΔCt method, *CRISP-3* mRNA expression results were normalized against ribosomal 18S internal control (Applied Biosystems) and expressed as the fold-change when compared with controls (TOP sample group or untreated control).

Immunohistochemistry

Briefly, immunolocalization of *CRISP-3* was carried out using an anti-human *CRISP-3* monoclonal antibody in all 25 samples (MAB23971, R&D Systems, Minneapolis, USA). In order to perform the experiment in a controlled and easily repeatable manner, the Bond-X automated immunostaining machine (Vision Biosystems, Newcastle, UK) was utilized. Paraffin sections of uterine decidua (5 μm) were cut, dewaxed, rehydrated and subjected to antigen retrieval in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA, USA) for 15 min in the microwave before being placed on the Bond-X machine. This method on the Bond-X automated machine utilizes a specific polymer high-contrast program (Bond Refine Polymer detection kit). Slides were peroxidase blocked for 5 min, incubated for 3 h with the primary antibody at 2.5 μg/ml in the Bond diluent supplied and then incubated with the post-primary reagent for 8 min. To confirm antibody specificity, control sections were incubated with diluent alone or with non-specific mouse immunoglobulins, diluted

to the same concentration as primary antibody in supplied diluent. Prostate was used as a positive control (Udby *et al.*, 2005). Sections were then incubated with the polymer reagent for 8 min to increase sensitivity of detection prior to DAB detection for 10 min. Sections were counterstained in haematoxylin for 5 min. Slides were then removed from the machine and dehydrated and mounted using Pertex. The localization of *CRISP-3* was assessed by two independent observers blinded to tissue identity, and it was consistent throughout all samples.

Western blot analysis

Total protein from all 25 decidualized endometrial biopsies was prepared by homogenization (TissueLyser, Qiagen, UK) of RNALater™-treated frozen tissue in buffer (1% Triton X, 0.1% SDS, 0.5% deoxycholactic acid, 0.15 M NaCl, 0.02 M Tris) containing a protease inhibitor cocktail (Complete, Roche Diagnostics, Mannheim, Germany). The proteins in the supernatants were measured by the method of Bradford (Schleicher and Wieland, 1978) adapted for use on a Cobas Fara Centrifugal Analyser (Roche Diagnostics). Protein samples (20 μg) were run on 4–12% Novex bis-tris gels in NuPAGE MOPS SDS buffer (Invitrogen, CA, USA). Proteins were transferred onto a PVDF membrane (Immobilon-P, Millipore, MA, USA) in Tris/glycine/methanol. After blocking in 5% skimmed milk/PBS for 1 h, the membrane was washed with 0.5% BSA/PBS. The membrane was incubated overnight at 4°C with polyclonal rabbit anti-*CRISP-3* IgG (1 μg/ml) in 0.5% BSA/PBS (or *CRISP-3* monoclonal antibody, R&D Systems, Minneapolis, USA). Immunoblotting for β-actin (Abcam, Cambridge, UK) was used as a comparative control between the samples. After washes in 0.5% BSA/PBS, incubation with anti-rabbit HRP antibody (A1949, Sigma, Dorset, UK) followed for 1 h at room temperature. The membrane was washed as before, incubated with HRP substrate solution (Immobilon Western, Millipore, MA, USA) for 5 min and exposed to Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK). Images were also captured digitally using the VersaDoc Imaging System (BioRad, CA, USA) and band density was assessed and corrected for β-actin density on a Photoshop CS2 platform (Adobe, CA, USA).

Serum assays

Serum progesterone and hCG concentrations were measured using a standard radioimmunoassay in all 25 women (Corker and Davidson, 1978). The concentration of *CRISP-3* in serum samples was measured by a specific in-house research ELISA similar to the method reported previously (Udby *et al.*, 2002a), except that recently developed rabbit anti-CRISP antibodies raised against native human *CRISP-3* from granulocytes were used for capture and detection, and denaturation of samples was omitted. Native *CRISP-3* purified from human granulocytes was used as standard. The average intra-assay coefficient of variation was 2.7%, and the average inter-assay coefficients of variation were 6.6% (assays from same day) and 9.7% (assays from different days) (Udby *et al.*, 2002a).

Morphological analysis of sections

The samples of decidualized endometrium were classified blindly by a sub-specialist gynaecological pathologist according to the degree of endometrial change, as described previously (Horne *et al.*, 2008). In short, secretory transformation of the glandular epithelium (absent/early, high and exhausted) and stromal differentiation (non-decidualized, pre-decidual, confluent decidual change) were scored as follows: samples with exhausted glandular transformation with confluent stromal decidualization were classed overall as decidualized (+), those with absent/early secretory transformation and non-decidual stroma were classified as decidualized (−) and other samples were classified as decidualized (+/−).

Cell culture

The immortalized endometrial epithelial cell line (hTERT-EEpC) (Hombach-Klonisch *et al.*, 2005) was maintained in HAM's F-10 media with L-glutamine supplemented with insulin, transferrin, selenium (ITS) (Lonza, MD, USA) and 10% foetal bovine serum (Sigma, MO, USA). Cells were starved of serum for 24 h prior to treatments and for the duration of the experiments. The experiments were performed in triplicate. The cells were treated with concentrations of hCG (Pregnyl™, Organon Laboratories, Cambridge, UK) mimicking concentrations that the maternal epithelia adjacent to an implanting embryo is likely to be exposed. These concentrations were modelled on those used in a previously published study (Nakayama *et al.*, 2003). In this study, the authors based their assumptions on the fact that, in pregnant women, 6–12 days after ovulation, the first sign of pregnancy is a urinary hCG concentration of greater than 0.325 mIU/ml on the day of blastocyst implantation. As the concentration of hCG in the urine and plasma is similar, and the plasma volume of a 50 kg woman is 1900 ml, the total amount of hCG is calculated to be 617.5 mIU ($=0.325$ mIU/ml $\times$ 1900 ml). Estimating that the local volume surrounding the blastocyst in the fallopian tube ranges from 1 to 10 μl, the intraluminal concentration of hCG around the blastocyst is estimated to be approximately 61.8 IU/ml. Therefore, in our study, we used concentrations of 10 and 100 IU/ml.

Statistical analysis

All data were assessed for normality of distribution using a computer programme (Prism 4; Graphpad Software, CA, USA). Where the data were normally distributed, three groups were analysed by ANOVA with Bonferroni pairwise comparisons. Two groups were analysed using a *t*-test. Where the data were not normally distributed, Kruskal–Wallis testing with Dunn's pairwise comparisons was used to compare three groups. Chi-squared test was used to compare proportions, and Spearman (non-parametric) or Pearson (parametric) coefficients were used to assess linear correlations. The statistical test used is given in the text and figure legend and significance was at the $P < 0.05$ level.

Results

CRISP-3 mRNA is increased in decidualized endometrium from tubal ectopic compared with intrauterine pregnancies

A previously published comparison of gene expression in the decidualized endometrium from women with intrauterine ($n = 14$) and tubal pregnancies ($n = 11$) revealed that of the 669 genes that were differentially expressed (FC $> \pm 2$, $P < 0.05$) (Horne *et al.*, 2008), *CRISP-3* was up-regulated in the women with tubal ectopic pregnancies with the highest fold increase (FC $= 13.10$). Quantitive RT–PCR confirmed its increased expression in the decidualized endometrium from tubal pregnancies when compared with both miscarriage ($P < 0.01$) and TOP ($P < 0.01$) groups (Fig. 1).

CRISP-3 protein is immunolocalized to the epithelium and granulocytes in decidualized endometrium

In order to further investigate *CRISP-3* in the decidualized endometrium, it was localized using immunohistochemistry. Specific immuno-staining for *CRISP-3* could be detected in the glandular epithelium, secreted within the glands (Fig. 2A–C) and in the
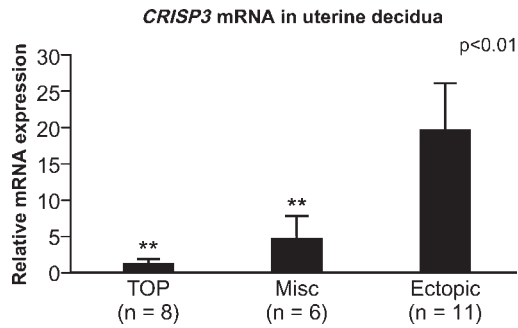
Figure 1 Expression of *CRISP-3* mRNA in uterine decidua; *CRISP-3* is differentially expressed.

It is increased in women with tubal ectopic pregnancy (Ectopic) when compared with that of women undergoing surgical management of miscarriage (Misc) ($P < 0.01$; Kruskal–Wallis) and surgical termination of pregnancy (TOP) ($P < 0.01$; Kruskal–Wallis).
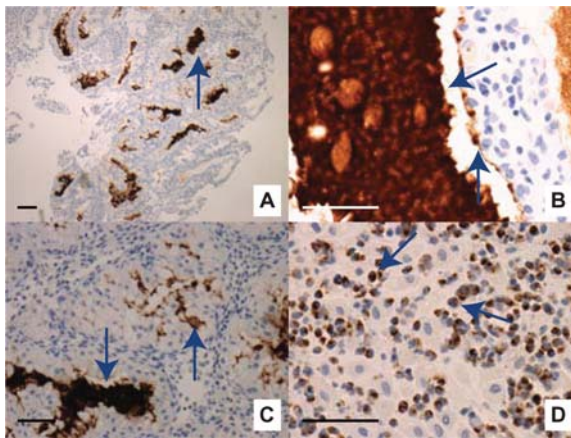

Figure 2 Immunohistochemistry for *CRISP-3* in a representative biopsy of decidualized endometrium from a woman with a tubal ectopic pregnancy.

Specific immuno-staining for *CRISP-3* in all 25 samples was similar and could be detected in the glandular epithelium (**A–C**), secreted within the glands (**A–C**) and in the granulocytes of the decidualized endometrium (**D**). Scale bar = 50 μm.

granulocytes of the decidualized endometrium (Fig. 2D). This localization pattern was consistent in all the decidual samples.

CRISP-3 protein is increased in decidualized endometrium from tubal ectopic compared with intrauterine pregnancies

Western blot analysis of decidual biopsies from patients with tubal ectopic ($n = 11$), miscarriages ($n = 6$) and viable pregnancies ($n = 8$) revealed bands of 29 and 27 kDa corresponding to *N*-glycosylated and non-glycoslyated *CRISP-3* (Udby *et al.*, 2005) (Fig. 3). Glycosylated *CRISP-3* was increased in the decidualized endometrium from tubal
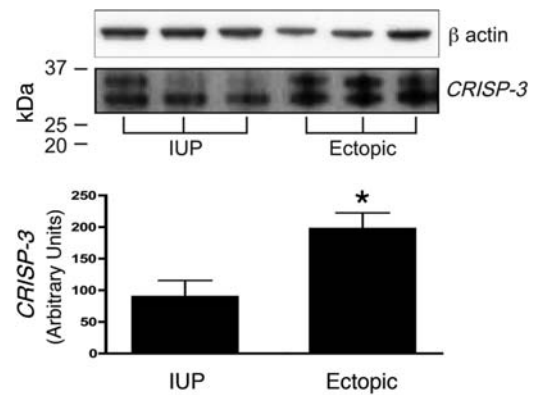

Figure 3 Representative western blot analysis of *CRISP-3* protein in uterine decidua. Western blot analysis revealed bands of 29 and 27 kDa corresponding to *N*-glycosylated and non-glycoslyated *CRISP-3*. Miscarriage and TOP groups were combined as they showed a similar pattern. Glycosylated *CRISP-3* was increased in the decidualized endometrium from tubal ectopic pregnancy when compared with that from intrauterine pregnancies ($P < 0.05$; *t*-test).

ectopic pregnancy when compared with that from miscarriages and viable pregnancies (Fig. 3).

CRISP-3 serum concentrations are similar in women with tubal ectopic compared with intrauterine pregnancies

As *CRISP-3* is a secreted protein that is increased in the decidualized endometrium of ectopic pregnancy, serum concentrations were analysed to determine if these mirrored endometrial expression. Serum samples from patients with tubal ectopic pregnancies ($n = 11$), miscarriages ($n = 6$) and viable pregnancies ($n = 8$) were assayed for *CRISP-3* protein. There were no significant differences in measured *CRISP-3* concentrations (Fig. 4).
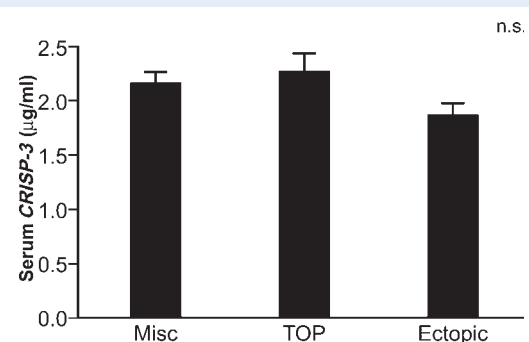

Figure 4 Serum *CRISP-3* concentrations.

Serum samples from patients with tubal ectopic pregnancies ($n = 11$), miscarriages ($n = 6$) and viable pregnancies ($n = 8$) were assayed for *CRISP-3* protein. There were no significant differences in measured *CRISP-3* concentrations ($P > 0.05$; ANOVA).

CRISP-3 expression is not related to the morphology of the decidualized endometrium

As the decidualization of endometrium is different in the tubal ectopic, miscarriage and viable pregnancy groups (Horne *et al*., 2008), the relationship of *CRISP-3* expression to decidual morphology was investigated. As previously demonstrated (Horne *et al*., 2008), in decidualized endometrium from women with tubal ectopic pregnancies, the glandular compartment ($P < 0.05$) showed a lesser degree of secretory transformation and the stromal compartment ($P < 0.05$) showed less decidualization when compared with intrauterine pregnancies (data not shown). When the samples were stratified based on the overall degree of morphological decidualization, there was however no clear correlation with *CRISP-3* mRNA expression (Fig. 5A) or serum concentrations (data not shown).

CRISP-3 mRNA expression is not related to serum progesterone, but is inversely proportional to serum hCG concentrations

As the degree of decidualization did not explain the observed differences in *CRISP-3*, its expression was investigated in relation to serum progesterone and hCG concentrations (Fig. 5B and C, respectively). There was no clear correlation between *CRISP-3* mRNA expression and serum progesterone ($P > 0.05$; Fig. 5B). However, when the samples were analysed according to serum hCG, there was a marked correlation with *CRISP-3*. Expression of *CRISP-3* mRNA in decidualized endometrium was inversely proportional to serum hCG ($P = 0.0006$; $r = -0.64$; Fig. 5C).

hCG regulates *CRISP-3* mRNA expression in endometrial epithelial cells *in vitro*

As endometrial epithelial *CRISP-3* mRNA expression was inversely related to serum hCG concentrations *in vivo*, we hypothesized that hCG could inhibit *CRISP-3* epithelial expression. We therefore assessed the effect of hCG treatment on *CRISP-3* expression in endometrial epithelial cells *in vitro*. At both 6 and 24 h, hCG treatment of the cell line was associated with a dose-dependent decrease in endometrial epithelial *CRISP-3* mRNA expression ($P < 0.01$; Fig. 6).

Discussion

This study demonstrates a clear difference between the decidualized endometrium of tubal ectopic and intrauterine pregnancies of similar gestational ages, that may be reflected by an alteration in endometrial secretory function. We have shown that decidual expression of *CRISP-3* is increased in tubal ectopic pregnancies when compared with intrauterine pregnancies. In addition, the expression of *CRISP-3* mRNA in the decidualized endometrium appears to be negatively regulated by hCG. These data suggest that during early intrauterine pregnancy, endometrial *CRISP-3* expression may be inhibited by hCG and that this does not occur to the same degree in tubal ectopic pregnancy.

Disappointingly, although we demonstrated increased *CRISP-3* mRNA and protein expression in the decidualized endometrium of women with tubal ectopic pregnancies, we were unable to replicate
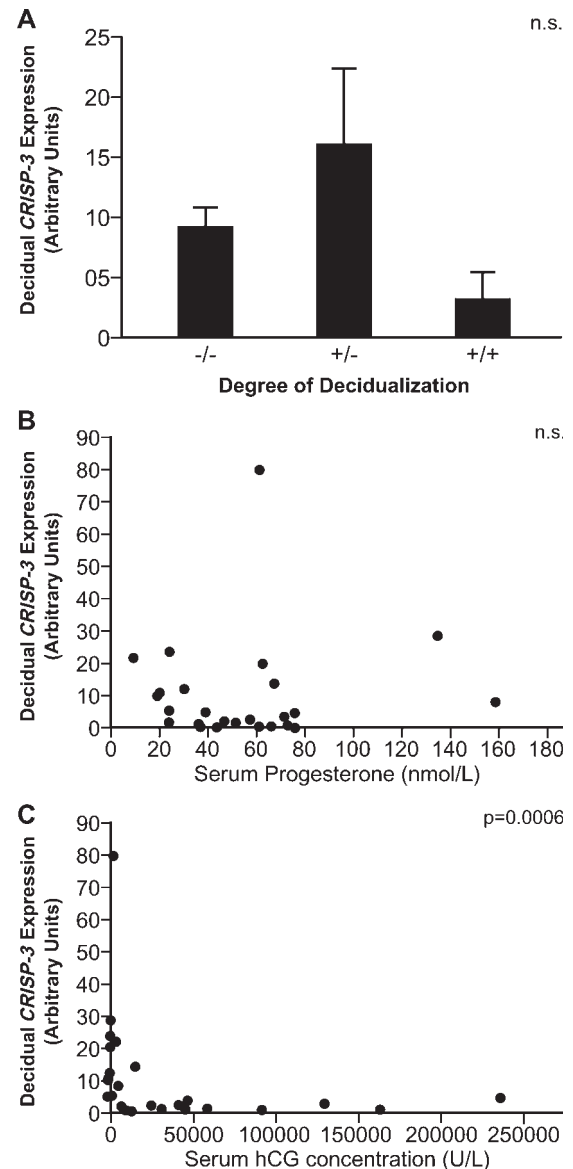

Figure 5 Association between the degree of morphological decidualization, serum progesterone and hCG concentrations and the decidual *CRISP-3* mRNA expression. There was no significant association between the degree of morphological decidualization and *CRISP-3* mRNA expression (**A**) ($P > 0.05$; Kruskal–Wallis). There was no clear association between *CRISP-3* mRNA expression and serum progesterone ($P > 0.05$; Spearman Rank Correlation) (**B**). Expression of *CRISP-3* mRNA in decidualized endometrium was inversely proportional to serum hCG ($P = 0.0006$; $r = -0.64$) (**C**).

this finding in matched serum samples. As other organs and tissue types, such as the salivary glands and pancreas, can contribute to the blood level of *CRISP-3*, the total endometrial impact may be low. The determination of *CRISP-3* concentration in serial samples across the menstrual cycle and after hysterectomy may address this issue in more detail. At present, this study was not able to demonstrate a role for *CRISP-3* as a serum biomarker. However, it may be that further assay refinements would increase the sensitivity and
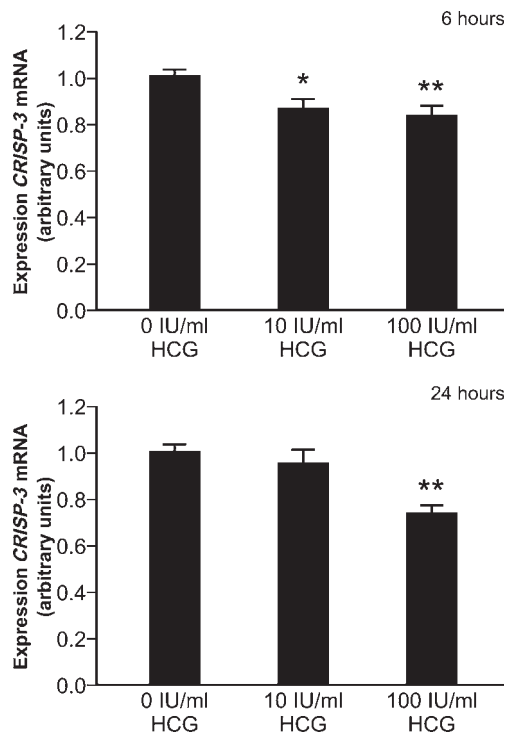
Figure 6 Regulation of endometrial epithelial *CRISP-3* expression *in vitro*.

At both 6 and 24 h, hCG treatment (100 IU/ml) was associated with a dose dependent decrease in endometrial *CRISP-3* mRNA expression (*n* = 3) (*P* < 0.01; ANOVA).

specificity of the assay and *CRISP-3* remains a possible candidate for a biomarker in the future.

If there are no useful differences in serum *CRISP-3* concentrations, it remains possible that *CRISP-3* could function as a biomarker in other instances. For example, the rapid measurement of *CRISP-3* in a small biopsy of the uterine decidua could provide the clinical pathologist with an additional means of diagnosing ectopic pregnancy in any woman with a failing early pregnancy of unknown location. The use of outpatient endometrial sampling in the rapid assessment of pregnancy location requires further study.

However, the endometrium in early pregnancy does express and secrete *CRISP-3* and the difference between intrauterine and tubal gestation suggests that this expression is regulated. However, its role within the human decidualized endometrium is not yet clear. It has been suggested that *CRISP-3* may have a role in the human innate immune defence system. It is likely that the poorly understood innate immune mechanisms and their involvement during implantation have roles in facilitating successful human fertility. The role for *CRISP-3* in human innate immune defence is supported by the expression of *CRISP-3* in neutrophils and in exocrine glands, and sequence similarities with 'pathogenesis-related proteins', involved in plant anti-microbial defence (Stintzi *et al.*, 1993; Udby *et al.*, 2002a). Certainly in normal early pregnancy, it appears that there is lower endometrial *CRISP-3* expression. This has clear parallels with the decidual expression of another key mediator of the innate immune system. Secretory leuco-cyte protease inhibitor is also relatively increased in the decidualized

endometrium of women with tubal ectopic pregnancies when compared with those with intrauterine implantation (Dalgetty *et al.*, 2008). Together, these findings suggest that the endometrium has an innate immunity that is locally regulated during early pregnancy.

It is possible that *CRISP-3* has a role in endometrial tissue remodelling as it is localized to specific and gelatinase-containing granules in human neutrophils. This suggests a matrix degradative role, as these granules are partially exocytosed during neutrophil migration and inflammation (Udby *et al.*, 2002b). In addition, *CRISP-3* appears to be up-regulated in chronic pancreatitis (Friess *et al.*, 2001), further supporting a role for *CRISP-3* in localized tissue inflammatory and immune response. Although there are clear morphological and functional changes in the endometrium across the menstrual cycle, the ontogeny of *CRISP-3* expression is unclear. Further work therefore needs to be done to investigate *CRISP-3* expression in the endometrium and its role across the menstrual cycle and at the time of implantation.

Whatever the role of *CRISP-3* at the time of implantation, it is clear that endometrial expression in early gestation is higher in tubal ectopic pregnancy. The most obvious difference in the decidualized endometrium from the tubal pregnancy compared with the intrauterine groups is the absence of a local trophoblast. The trophoblast has important effects on the endometrium. Indeed, the effect of pregnancy on the endometrium using the model of ectopic pregnancy has been studied in another microarray study, demonstrating effects on the complement and coagulation cascade, phospholid degradation, glycosphingolipid biosynthesis, retinol metabolism, antigen presentation pathway, glycosphingolipid biosynthesis and O-glycan biosynthesis (Savaris *et al.*, 2008). Recent *in-vitro* studies, also using a functional genomics approach, have shown that conditioned media from trophoblast alter the local immune environment of the uterine decidua to facilitate embryo implantation by causing a significant induction of pro-inflammatory cytokines and cells (Hess *et al.*, 2007). This suggests that differences between decidualized endometrium in ectopic and intrauterine gestations may be the result of local trophoblast products.

In order to investigate why *CRISP-3* was increased in the decidua of ectopic pregnancies, we analysed whether the variation in *CRISP-3* expression correlated with local and systemic variables in early pregnancy. The first strategy was to examine whether it changed during decidualization. The endometrium of ectopic pregnancies is less decidualized than that of intrauterine pregnancies (Horne *et al.*, 2008). Unlike Activin B, which appears to be a decidual-related marker of ectopic pregnancy (Horne *et al.*, 2008), *CRISP-3* expression is not related to the degree of decidualization. As progesterone concentrations tend to be lower in ectopic pregnancy, we also assessed whether progesterone concentrations were related to *CRISP-3* expression and found no relationship between serum progesterone and decidual *CRISP-3* expression. This negative finding is perhaps not surprising as progesterone receptors disappear from the endometrial glands during the secretory phase (Koh *et al.*, 1995) of the cycle.

We found a highly significant negative correlation between decidual *CRISP-3* expression and serum hCG concentrations. During the peri-implantation period of pregnancy in primates, hCG is the first clear signal of the trophoblast's presence and viability (Hearn *et al.*, 1988). Indeed, primate studies have shown that hCG induces alterations in the endometrial expression of genes that regulate the

modulation of the immune response around the implanting trophoblast (Sherwin *et al.*, 2007). We realize the limitations our *in-vitro* experimentation with hCG and short-term culture but, together with our *in-vivo* observations, our findings suggest that *CRISP-3* may be an endometrial gene that can be regulated by hCG during early pregnancy. It is therefore suggested that with tubal implantation, the absence of a local reduction in the expression of *CRISP-3* in the decidualized endometrium of women with tubal ectopic pregnancy may be due to the impaired production, or local effect, of hCG from the trophoblast.

In summary, *CRISP-3* is expressed in the decidualized endometrium of pregnancy and is higher in tubal ectopic pregnancy. Its inverse relationship with serum hCG concentrations and the inhibition of *CRISP-3* by hCG *in vitro* suggests that it may be directly inhibited by the conceptus. This may have a role in the control of innate immunity during implantation.

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