Proteomic analysis of the sheep caruncular and intercaruncular endometrium reveals changes in functional proteins crucial for the establishment of pregnancy

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Abstract

The expression and regulation of endometrial proteins are crucial for conceptus implantation and development. However, little is known about site-specific proteome profiles of the mammalian endometrium during the peri-implantation period. We utilised a two-dimensional gel electrophoresis/mass spectrometry-based proteomics approach to compare and identify differentially expressed proteins in sheep endometrium. Caruncular and intercaruncular endometrium were collected on days 12 (C12) and 16 (C16) of the oestrous cycle and at three stages of pregnancy corresponding to conceptus pre-attachment (P12), implantation (P16) and post-implantation (P20). Abundance and localisation changes in differentially expressed proteins were determined by western blot and immunohistochemistry. In caruncular endometrium, 45 protein spots (5% of total spots) altered between day 12 of pregnancy (P12) and P16 while 85 protein spots (10% of total spots) were differentially expressed between C16 and C12. In intercaruncular endometrium, 31 protein spots (2% of total spots) were different between P12 and P16 while 44 protein spots (4% of total spots) showed differential expression between C12 and C16. The pattern of protein changes between caruncle and intercaruncle sites was markedly different. Among the protein spots with implantation-related changes in volume, 11 proteins in the caruncular endometrium and six proteins in the intercaruncular endometrium, with different functions such as protein synthesis and degradation, antioxidant defence, cell structural integrity, adhesion and signal transduction, were identified. Our findings highlight the different but important roles of the caruncular and intercaruncular proteins during early pregnancy.


Introduction

In humans and farm animals, early pregnancy loss occurs most frequently during the peri-implantation period mainly due to uterine dysfunction and/or abnormal conceptus (embryo and extraembryonic membranes) development (Tuckerman et al. 2004, Dixon et al. 2007, Patel & Lessey 2011, Koot et al. 2012). The incidence of early pregnancy loss in humans is estimated to be 30% prior to conceptus implantation and 30% before 6 weeks of pregnancy (Teklenburg et al. 2010). During early pregnancy, a complex cascade of events takes place in the endometrium to ensure the optimal development of the conceptus. Extending our knowledge of endometrium–conceptus interactions is a key step for the future development of effective and reliable therapeutic strategies that are aimed to improve conceptus implantation rates both following natural conception and/or assisted reproductive technologies.

Prior to the initial development of the fertilised ovum to the blastocyst stage, the preparation of a receptive endometrium through the actions of progesterone (Rider 2002) and oestradiol (Ma et al. 2003) is a prerequisite for the extraembryonic membranes–endometrial dialogue that supports conceptus attachment, implantation, development and survival (Imakawa et al. 2004, Spencer et al. 2004). In addition to the endometrial changes before implantation, other implantation and post-implantation changes in the endometrium may result from in utero presence of a conceptus. During the peri-implantation period, the stage-specific expression and regulation of proteins within the endometrium are critical determinants for successful establishment and progression of
pregnancy. It is not necessarily possible to accurately predict protein expression patterns and functions from quantitative mRNA determination. This is due to post-transcriptional regulatory mechanisms (mRNA export, surveillance, silencing and turnover) and post-translational modifications, all of which can determine protein activity, localisation, turnover and interactions with other proteins (Mann & Jensen 2003). Several studies have previously sought to identify proteins differentially expressed in the human endometrium between the proliferative and secretory phases of normal menstrual cycles (DeSouza et al. 2005, Chen et al. 2009, Dominguez et al. 2009, Pammar et al. 2009, Rai et al. 2010). There are also a number of studies describing endometrial phase-specific transcriptomic profiles (Kao et al. 2002, Ace & Okulicz 2004, Evans et al. 2012, Garrido-Gómez et al. 2013). To date, however, little is known about proteomic profiles of the mammalian endometrium during the peri-implantation period. Furthermore, the role of conceptus in the regulation of key endometrium proteins is unknown.

We hypothesised that endometrial proteome would display specific expression profile changes during the peri-implantation period and that the developing conceptus modulates the expression of the endometrial proteins prior to implantation and during early pregnancy. Testing this hypothesis requires the resolution of a number of challenges, including the ethical impossibility of obtaining human endometrium tissue samples during early pregnancy. The sheep, a species with epitheliochorial implantation, was considered to be a useful model to explore the physiological, molecular and biochemical events at the endometrial–extraembryonic membrane interface during early pregnancy (Spencer et al. 2007, Satterfield et al. 2009). In sheep, embryonic trophectoderm cells begin contact with the luminal endometrium epithelium between day 13 of pregnancy (P13) and P15 and then attach to endometrial cells on day 16, a process completed by day 22 post mating (Spencer et al. 2004). To test our hypothesis, endometrium was collected from cycling ewes on days 12 (C12) and 16 (C16) of the oestrous cycle and from pregnant ewes at conceptus pre-attachment (P12), implantation (P16) and early post-implantation (P20). Two-dimensional gel electrophoresis (2DE)-based proteomics (Fowler et al. 2007, Stephens et al. 2010, Arianmanesh et al. 2011) was employed to characterise peri-implantation-specific alterations in the endometrial proteome in order to enhance our understanding of the molecular endometrium environment supporting early conceptus development and survival.

### Materials and methods

#### Animals

All procedures relating to care and use of animals were approved by the French Ministry of Agriculture according to the French regulation for animal experimentation (authorisation no. 78-34). The study involved pregnant ewes and cyclic ewes of the Préalpes-du-Sud breed (18 months of age). All the ewes were treated for 14 days with intravaginal sponges containing 40 mg fluorogestone acetate (Intervet, Angers, France) to synchronise oestrus. Each ewe received 400 IU equine chorionic gonadotrophin (Intervet) immediately after removal of the sponges. Ewes assigned to the pregnant groups were mated at the time of the synchronised oestrus with fertile rams of the same breed, twice at an interval of 12 h. Ewes assigned to the pregnant group were mated and killed on P12 (n=4), P16 (n=4) and P20 (n=4) (Fig. 1A). As reproductive cycle of the ewe is about 17 days, control ewes were killed on C12 (n=4) and C16 (n=4) (Fig. 1A).

#### Tissue collection

The sheep endometrium consists of well-delimited aglandular caruncular and glandular intercaruncular areas (Fig. 1B). The caruncular areas are dense stroma protuberances covered by a simple luminal epithelium, which are the sites of implantation and placentation, whereas intercaruncular areas contain large numbers of branched glands that synthesise a variety of molecules, collectively referred to as histotroph, that are required for conceptus survival and development (Gray et al. 2001). Furthermore, the rate of protein synthesis in caruncular...
and intercaruncular endometrium is greater in pregnant than in cyclic ewes and these tissues respond differently to ovarian steroids early in pregnancy (Findlay et al. 1981). Given the distinct morphology and function of sheep caruncular and intercaruncular endometrium, these areas were used in this study to identify protein variation during the peri-implantation period. Pregnant ewes were randomly allocated for killing on P12, P16 and P20. The stages of pregnancy were confirmed by the recovery of normal concepti with viable embryos in uterine flashings. Cyclic ewes were randomly allocated for killing on C12 and C16. The ewes were slaughtered at a local abattoir (INRA, Jouy-en-Josas, France) in accordance with protocols approved by the local institutional animal use committee. After killing, the reproductive tracts were collected and immediately transported to the laboratory. Immediately after dissection, caruncular and intercaruncular tissues were snap-frozen in liquid nitrogen and stored at −80°C until processed. For immunohistochemistry, small pieces of caruncular and intercaruncular tissues were fixed overnight in freshly prepared 4% paraformaldehyde in PBS (pH 7.4) and then embedded in wax. Although sheep caruncular and intercaruncular zones are visible to the naked eye, we used routine histology (Al-Gubory et al. 2008) to check the accuracy of the dissection of the two morphologically different endometrial tissues (Fig. 1B).

Preparation of tissue samples for electrophoretic analyses

The caruncular and intercaruncular tissues were processed separately for 1DE and 2DE gel electrophoresis as described previously (Fowler et al. 2007). Briefly, tissues were combined with 5 μl lysis buffer/1 mg wet weight of tissue. The lysis buffer (0.01 M Tris–HCl, pH 7.4) contained 1 mM EDTA, 8 M urea, 0.05 M dithiothreitol, 10% (v/v) glycerol 5% (v/v), NP40, 6% (0.01 M Tris–HCl, pH 7.4) contained 1 mM EDTA, 8 M urea,

2DE gel electrophoresis analysis

2DE was performed as described by Cash & Kroll (2003). Equal amounts of protein from caruncular or intercaruncular tissues of each ewe were combined to make five protein pools: cyclic ewes on days 12 and 16 and pregnant ewes on days 12, 16 and 20. For first-dimension separation, 200 μg total protein from endometrium lysate was loaded on 11 cm, immobilised non-linear pH gradient (IPG) strips of pH 3–10 (Bio-Rad). The second dimension was carried out using 16 cm 10% polyacrylamide gels. Quadruplicate 2DE gels were prepared for each of the five groups. Proteins were visualised using Colloidal CBB G-250 and scanned using a Molecular Dynamics Personal Densitometer SI (Molecular Dynamics Ltd, Bucks, England) at 50 μm resolution to produce 16-bit images. Protein spot profiles were analysed using Progenesis SameSpots Software, version 3 (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK). Briefly, a reference gel was selected and the other gels were aligned to be closely matched to this reference gel. Background was subtracted individually from each gel and spot volumes were normalised relative to total spot volume individually for each gel. Virtual ‘average gels’ were generated from the four sets of the gels in each group and then used to compare the log-normalised protein spot volumes (ANOVA) between groups. Spots demonstrating ≥1.25-fold changes (P<0.05) between two or more of the groups were ranked in terms of size, reproducibility across the quadruplicate gels within each group and magnitude of difference. The spots having all the above characteristics were chosen for spot cutting and protein identification. Representative 2D gels and zoom boxes of identified sheep endometrium proteins are shown in (Fig. 2).

Mass spectrometry

In order to identify proteins, spots were excised from stained gels and subjected to in-gel digestion with trypsin (sequencing grade, modified; Promega) as described previously (Fowler et al. 2008). Peptide solutions were analysed using an HCTultra PTM Discovery System (Bruker Daltonics Ltd, Coventry, UK) coupled to an UltiMate 3000 LC System (Dionex (UK) Ltd,

Figure 2 Representative 2D gels and zoom boxes of identified sheep endometrium proteins. In (A and B) a caruncular 2D gel is shown with enlarged views of protein spot 57 while in (C and D) an intercaruncular 2D gel is shown with enlarged views of protein spot 94. The arrows highlight the relevant spot in each gel image.
Camberley, Surrey, UK). Protein ID’s are based on at least two peptides. Peptides were separated on a Monolithic Capillary Column (200 μm i.d. × 5 cm; Dionex part no. 161409). Peptide fragment mass spectra were acquired in data-dependent AutoMS (2) mode with a scan range of 300–1500 m/z; three averages and up to three precursor ions selected from the MS scan 100–2200 m/z. Precursors were actively excluded within a 1.0 min window and all singly charged ions were excluded. Peptide peaks were detected and deconvoluted automatically using Data Analysis Software (Bruker). Mass lists in the form of Mascot Generic Files were created automatically and used as the input for Mascot MS/MS ions searches of the NCBI nr database using the Matrix Science web server (www.matrixscience.com). The default search parameters used were: Enzyme=Trypsin; Max. Missed cleavages=1; Fixed modifications=Carbamidomethyl (C); Variable modifications=Oxidation (M); Peptide tolerance±1.5 kDa; MS/MS tolerance±0.5 Da; Peptide charge=2 + and 3 +; Instrument=ESI–TRAP. Statistically significant MOWSE scores and good sequence coverage were considered to be positive identifications.

Western blot
Caruncular or intercaruncular tissue lysates were loaded (30 μg protein/lane) onto 26-lane 1DE gels (NUPAGE Novex Midi gels, 4–12%, Invitrogen) under reducing conditions and then electroblotted onto immobilon-FL membrane (Millipore Ltd, Watford, UK) as described previously (Fowler et al. 2008). After blotting, membranes were incubated in blocking buffer, 1:1 Odyssey blocking buffer (LI-COR Biosciences UK Ltd, Cambridge, UK) and PBS at 4 °C overnight. Primary antibodies were diluted in Odyssey blocking buffer 1:1 with 0.2 μm filtered PBST as follows: for caruncular tissues, mouse anti-manganese superoxide dismutase 2 (SOD2, Abcam Ltd, Cambridge, UK, ab16956), 1–1500, rabbit anti-tryptophanyl tRNA synthetase (WARS, Abcam Ltd, ab31536), 1–4500, rabbit anti-endoplasmic reticulum resident protein 57 (ERP57, Abcam Ltd, ab13507), 1–1000 and mouse anti-annexin 4 (ANXA4, Abnova Corp., Taipei, Taiwan, H0000307-M13), 3 μg/ml; for intercaruncular tissues, rabbit anti-transgelin (SM22, Abcam Ltd, ab14106), 1–600, mouse anti-gelsolin (GSN, Abcam Ltd, ab55070), 3 μg/ml and rabbit anti-tryptophanyl tRNA synthetase (WARS, Abcam Ltd, ab31536), 1–4500 all combined with mouse anti-z-tubulin (Abcam Ltd, ab7291) and 1–10 000 or rabbit anti-z-tubulin (Abcam Ltd, ab4074), 1 μg/ml. The membranes were incubated with primary antibodies at 4 °C overnight and then incubated with secondary antibodies for 60 min at room temperature. Secondary antibodies including anti-mouse IgG IRDye800 (all secondary antibodies were provided from LI-COR, 610-732-124), 1–10 000 and anti-mouse IRDye700DX (610-730-124) 1–5000 were diluted in Odyssey blocking buffer 1:1 with 0.2 μm filtered PBST+0.01% SDS. After washing the membranes, digital images were captured using Odyssey LI-COR Infrared Imager (LI-COR). The band volumes and molecular weights (kDa) were then obtained following a background subtraction using Phoretix-1D Advanced Software (Nonlinear Dynamics Ltd).

Immunohistochemistry
Caruncular and intercaruncular tissue sections (5 μm) of all groups were either stained with haematoxylin and eosin or mounted onto ChemMate slides (DakoCytomation Ltd, Ely, Bucks, UK) and stained using the Bond-maxX (Leica Microsystems) automated immunostaining machine. EDTA-based buffer (pH 8.8) was used for epitope retrieval. The sections were incubated with primary antibodies for 30 min and the Bond DAB Enhancer was used to maximise the level of staining intensity and to create a counterstaining between chromogen-specific staining and the haematoxylin. All immunohistochemistry analyses were performed with antigen retrieval. Antibodies were used as follows: for caruncular sections, mouse anti-manganese SOD2 (Abcam Ltd, ab16956), 1–600, rabbit anti-tryptophanyl tRNA synthetase (WARS, Abcam Ltd, ab31536), 1–500, rabbit anti-ERP57 (Abcam Ltd, ab13507), 1–600, rabbit anti-transgelin (SM22, Abcam Ltd, ab14106), 1–700, mouse anti-vimentin (VIM, Abcam Ltd, Ab7752), 1–500; for intercaruncular sections, rabbit anti-transgelin (SM22, Abcam Ltd, ab14106), 1–700, mouse anti-VIM (Abcam Ltd, Ab7752), 1–500, mouse anti-manganese SOD2 (Abcam Ltd, ab16956), 1–600, rabbit anti-ERP57 (Abcam Ltd, ab13507) and 1–600, rabbit anti-tryptophanyl tRNA synthetase (WARS, Abcam Ltd, ab31536), 1–500. Sections were visualised with Bond ‘Refine’ DAB, washed in water and counter-stained with haematoxylin. Negative control sections incubated in the absence of the primary antibody showed no positive immunostaining. Slides were assessed using an Olympus BX41 microscope (Olympus, USA) and Progres CapturePro 2.6 Image Software with a Progress C5 (Jenoptik, Jena, Germany).

Statistical analysis
Normality of data was tested with the Shapiro–Wilks test. Normally distributed data were subjected to one-way ANOVA and Bonferroni–Dun post hoc test using SPSS 17.0 Software to assess significance of differences. Differences were considered to be significant if $P<0.05$ and statistical comparisons between specific groups were carried out by Student’s t-test. Mann–Whitney unpaired two-sample tests were used to analyse non-normally distributed data.

Table 1 Numbers of protein spots of the caruncular endometrium tissue significantly ($P<0.05$) changed between groups. Tissues were collected from cycling ewes on days 12 (C12) and 16 (C16) of the oestrous cycle and from pregnant ewes on days 12 (P12), 16 (P16) and 20 (P20) of pregnancy.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of spots</th>
</tr>
</thead>
<tbody>
<tr>
<td>P12</td>
<td>14</td>
</tr>
<tr>
<td>P16</td>
<td>54</td>
</tr>
<tr>
<td>P16</td>
<td>31</td>
</tr>
<tr>
<td>P20</td>
<td>34</td>
</tr>
<tr>
<td>C16</td>
<td>30</td>
</tr>
</tbody>
</table>

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Results

Proteome profile of caruncular endometrial tissue

Overall, 500 protein spots were included (on the basis of clear, reproducible, expression and absence of noise in all four gels for each group) for analysis from a total of 867 distinct protein spots detected by automatic detection with Progenesis SameSpots Software. The number of spots showing statistically significant differences in normalised spot volumes between groups are summarised in Table 1. The greatest number of changes occurred in pregnant ewes at the time of implantation (P16) when compared with the matching stage of the oestrous cycle (C16) and the fewest spots altered between pre-implantation (P12) and implantation (P16) periods.

Comparison between pregnant ewes at the pre-implantation period (P12) and cyclic ewes at the matching stage of the oestrous cycle (C12) revealed that 55 (6%) of protein spots were significantly ($P \leq 0.05$) changed (Table 1). Among these, 14 normalised spot volumes were up-regulated and 41 down-regulated (Table 1). WARS (Fig. 3A), ERP57 (Fig. 3B) and ANXA4 (Fig. 3C) significantly decreased ($P \leq 0.05$) at P12 when compared with the matching stage of the oestrous cycle (C12) (Table 2). Comparison between pregnant ewes at the time of implantation (P16) and cyclic ewes at the matching stage of the oestrous cycle (C16) revealed that 85 (10%) of protein spots were significantly ($P \leq 0.05$) changed (Table 1). Among these, 54 normalised spot volumes were up-regulated and 31 down-regulated (Table 1). WARS (Fig. 3A), ERP57 (Fig. 3B), ANXA4 (Fig. 3C) and SOD2 (Fig. 3D) significantly increased ($P \leq 0.05$) at implantation (P16) when compared with C16 (Table 2).

Comparison between pregnant ewes at implantation (P16) and pre-attachment (P12) periods revealed that 45 (5%) of protein spots were significantly ($P \leq 0.05$) changed (Table 1). Among these, 31 normalised spot volumes were up-regulated and 14 down-regulated at the implantation period (P16) compared with the pre-attachment period (P12) (Table 1). WARS (Fig. 3A), ERP57 (Fig. 3B), transferrin (TF) (Fig. 3E) and proteasome (prosome, macropain) subunit, alpha type 1 (PSMA1) (Fig. 3F) significantly increased at the time of implantation (P16) when compared with the pre-attachment (P12) period (Table 2). Comparison between pregnant ewes at implantation (P16) and post-implantation (P20) periods revealed that 62 (7%) of protein spots were significantly ($P \leq 0.05$) changed (Table 1). Among these spots, 34 were up-regulated and 28 down-regulated. ERP57 (Fig. 3B) and SOD2 (Fig. 3D) significantly increased at the time of post-implantation (P20) when compared with the implantation day (P16) (Table 2).

In cyclic ewes, 60 (7%) of protein spots were significantly ($P \leq 0.05$) changed between C12 and C16 (Table 1). Among these, 30 normalised spot volumes were up-regulated and 30 down-regulated at day 16 when compared with day 12 (Table 1). TF (Fig. 3E) significantly increased ($P \leq 0.05$) at C16 compared with C12 (Table 2). Conversely, WARS (Fig. 3A), ANXA4 (Fig. 3C), SOD2 (Fig. 3D) and PSMA1 (Fig. 3F) significantly decreased ($P \leq 0.05$) at C16 when compared with C12 (Table 2).

Proteome profile of intercaruncular endometrial tissue

Overall, 998 protein spots were included (on the basis of clear, reproducible, expression and absence of noise in all four gels for each group) for analysis from a total of 1324 distinct protein spots detected by automatic detection with Progenesis SameSpots Software. The number of spots...
Table 2: Sheep caruncular endometrium proteins identified from 2DE spots. Statistically significant fold changes between groups are shown in bold, together with their corresponding P values. Increases in spot volumes are denoted by a + and decreases are denoted by a − prefix to the fold change values. The comparisons between groups follow the rule that the fold changes are calculated on the basis that the first group is being compared with the second group. Accession number is given regarding to bovine species. The accession number for ovine is provided in parentheses if applicable.

<table>
<thead>
<tr>
<th>Function/protein</th>
<th>Spot no.</th>
<th>MW (kDa)</th>
<th>PI</th>
<th>MASCOT score</th>
<th>Accession no. (Swiss-Prot)</th>
<th>C16 vs C12</th>
<th>P16 vs P12</th>
<th>P20 vs P16</th>
<th>P12 vs C12</th>
<th>P16 vs C12</th>
<th>P16 vs C16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein synthesis</td>
<td>Tryptophanyl tRNA synthetase</td>
<td>721</td>
<td>54.17</td>
<td>5.49</td>
<td>753</td>
<td>P17248</td>
<td>−1.33, P = 0.043</td>
<td>+1.92, P = 0.021</td>
<td>+1.45</td>
<td>−1.31, P = 0.043</td>
<td>+1.96, P = 0.021</td>
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<td></td>
<td>Cytoplasmic (WARS/TrpRS)</td>
<td>867</td>
<td>52.44</td>
<td>5.74</td>
<td>275</td>
<td>Q3Z43</td>
<td>+2.53, P = 0.011</td>
<td>+1.82, P = 0.031</td>
<td>−1.69</td>
<td>−1.21</td>
<td>−1.68, P = 0.029</td>
</tr>
<tr>
<td></td>
<td>Iron transport and homeostasis</td>
<td>Transferrin</td>
<td>15</td>
<td>79.80</td>
<td>6.75</td>
<td>659</td>
<td>Q29443</td>
<td>+2.75, P = 0.021</td>
<td>−1.18</td>
<td>−1.08</td>
<td>−1.07</td>
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<td>Structure</td>
<td>Lamin A/C (LMNA)</td>
<td>3</td>
<td>79.80</td>
<td>7.00</td>
<td>707</td>
<td>Q3SZI2</td>
<td>+2.53, P = 0.011</td>
<td>+1.82, P = 0.031</td>
<td>−1.69</td>
<td>−1.21</td>
<td>−1.68, P = 0.029</td>
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<td>Lamin B2</td>
<td>866</td>
<td>68.21</td>
<td>5.35</td>
<td>229</td>
<td>516326 (NCBI)</td>
<td>+2.53, P = 0.011</td>
<td>+1.82, P = 0.031</td>
<td>−1.69</td>
<td>−1.21</td>
<td>−1.68, P = 0.029</td>
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<tr>
<td>Chaperones</td>
<td>Endoplasmic reticulum resident protein 57 (ERP57/PDIA3/GRP58)</td>
<td>57</td>
<td>57.29</td>
<td>6.38</td>
<td>1584</td>
<td>P38657</td>
<td>+2.53, P = 0.011</td>
<td>+1.82, P = 0.031</td>
<td>−1.69</td>
<td>−1.21</td>
<td>−1.68, P = 0.029</td>
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<td>Proteasome (prosome, macropain) subunit, alpha type 1 (PSMA1)</td>
<td>111</td>
<td>29.81</td>
<td>6.00</td>
<td>470</td>
<td>Q3S54</td>
<td>+2.53, P = 0.011</td>
<td>+1.82, P = 0.031</td>
<td>−1.69</td>
<td>−1.21</td>
<td>−1.68, P = 0.029</td>
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<td>Signal transduction</td>
<td>Annexin IV (ANXA4)</td>
<td>55</td>
<td>36.07</td>
<td>5.94</td>
<td>254</td>
<td>P13214</td>
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<td>+1.47, P = 0.002</td>
<td>+1.42, P = 0.006</td>
<td>−1.37, P = 0.006</td>
<td>+1.48, P = 0.003</td>
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<td>Calmodulin (CaM)</td>
<td>70</td>
<td>16.69</td>
<td>4.12</td>
<td>254</td>
<td>P62157</td>
<td>+1.37</td>
<td>+1.47, P = 0.002</td>
<td>+1.42, P = 0.006</td>
<td>−1.37, P = 0.006</td>
<td>+1.48, P = 0.003</td>
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<td></td>
<td>Mitochondrial manganese superoxide dismutase 2 (SOD2)</td>
<td>13</td>
<td>25.00</td>
<td>8.09</td>
<td>486</td>
<td>P41976 (C8BK6)</td>
<td>+1.37</td>
<td>+1.47, P = 0.002</td>
<td>+1.42, P = 0.006</td>
<td>−1.37, P = 0.006</td>
<td>+1.48, P = 0.003</td>
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<td>Cytosolic NADP⁺-dependent isocitrate dehydrogenase (IDH1)</td>
<td>738</td>
<td>47.09</td>
<td>6.31</td>
<td>635</td>
<td>Q3XSG3</td>
<td>+1.37</td>
<td>+1.47, P = 0.002</td>
<td>+1.42, P = 0.006</td>
<td>−1.37, P = 0.006</td>
<td>+1.48, P = 0.003</td>
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<tr>
<td>Cell adhesion</td>
<td>Galectin 15 (LGALS15/OVGL11)</td>
<td>12</td>
<td>15.50</td>
<td>5.24</td>
<td>150</td>
<td>Q19MU7</td>
<td>+1.37</td>
<td>+1.47, P = 0.002</td>
<td>+1.42, P = 0.006</td>
<td>−1.37, P = 0.006</td>
<td>+1.48, P = 0.003</td>
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aAccession from PubMed (NCBI) no Uniprot entry identified. bThe accession number is for ovine species as the accession number for bovine was not found.
protein spots were significantly \((P<0.05)\) changed between groups. Tissues were collected from cycling ewes on days 12 (C12) and 16 (C16) of the oestrous cycle and C16 and from pregnant ewes on days 12 (P12), 16 (P16) and 20 (P20) of pregnancy.

Table 3 Numbers of protein spots of the intercaruncular endometrium tissue significantly \((P<0.05)\) changed between groups. Tissues were collected from cycling ewes 12 (C12) and 16 (C16) of the oestrous cycle and C16 and from pregnant ewes on days 12 (P12), 16 (P16) and 20 (P20) of pregnancy.

<table>
<thead>
<tr>
<th>Group Compared with</th>
<th>Up-regulated</th>
<th>Down-regulated</th>
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<tbody>
<tr>
<td>P12 vs C12</td>
<td>36</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>P16 vs C16</td>
<td>17</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>P16 vs P12</td>
<td>6</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>P20 vs P16</td>
<td>13</td>
<td>26</td>
<td>3</td>
</tr>
<tr>
<td>C16 vs C12</td>
<td>37</td>
<td>15</td>
<td>4</td>
</tr>
</tbody>
</table>

showing statistically significant differences in normalised spot volumes between groups is summarised in Table 3. The largest number of changes were detected between C12 and C16 (C12 vs C16) and the fewest changes observed between P12 and P16 (P12 vs P16).

Comparison between pregnant ewes at the pre-implantation period (P12) and cyclic ewes at the matching stage of the oestrous cycle (C12) revealed that 44 (3%) of protein spots were significantly \((P<0.05)\) changed (Table 3). Among these, 36 normalised spot volumes were up-regulated and eight down-regulated (Table 3). SM22 alpha (Fig. 4A) significantly increased \((P<0.05)\) at P12 when compared with C12. Comparison between pregnant ewes at the time of implantation (P16) and cyclic ewes at the matching stage of the oestrous cycle (C16) revealed that 34 (3%) of protein spots were significantly \((P<0.05)\) changed (Table 3). Among these, 17 were up-regulated and 17 down-regulated (Table 3).

LGALS15 (Fig. 4B), VIM (Fig. 4C) and PSME1 (Fig. 4D) significantly increased \((P<0.05)\) at the time of post-attachment (P12) (Table 3). PSME1 (Fig. 4D) significantly decreased \((P<0.05)\) at the time of post-attachment (P16) when compared with the matching day of the oestrous cycle (C16) (Fig. 5A). Therefore, the WB finding for WARS was in agreement with 2DE gel findings, although the latter showed a significant reduction in cyclic ewes from days C12 to C16 (Fig. 5A).

Quantification of caruncular proteins

To quantify protein changes, WBs were performed on proteins whose known functions suggested important roles in the endometrium. WARS was a prime candidate for WB in caruncular tissue as it appeared in two spots and significantly changed in both cyclic and pregnant groups. WARS was detected at 54 kDa (Fig. 5A). The expression of WARS in caruncular tissue of pregnant ewes showed a significant \((P<0.05)\) increase from P12 to P16. Comparison of WARS expression between cyclic and pregnant groups revealed a significant \((P<0.05)\) elevation in caruncular tissue at the time of implantation (P16) when compared with the matching day of the oestrous cycle (C16) (Fig. 5A).

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In cyclic ewes, 52 (4%) of protein spots were significantly \((P<0.05)\) changed between C12 and C16 (Table 3). Among these, 37 normalised spot volumes were up-regulated and 15 down-regulated at day 16 when compared with day 12 (Table 3). PSME1 (Fig. 4D) significantly decreased \((P<0.05)\) and GSN (Fig. 4E) significantly increased \((P<0.05)\) from C16 to C12 (Table 4).

Figure 4 Expression changes of (A) transgelin (SM22), (B) galectin 15 (LGALS15), (C) vimentin (VIM), (D) proteasome (prosome, macropain) activator subunit 1 (PSME1), (E) gelsolin (GSN) and (F) sulfotransferase family, cytosolic, 1A phenol-prefering, member 1 (SULT1A1) in sheep intercaruncular endometrial tissues collected on days 12 (C12) and 16 (C16) of the oestrous cycle and on days 12 (P12), 16 (P16) and 20 (P20) of pregnancy. The acceptable level of significance was set at \(P<0.05\).
Table 4 Sheep intercaruncular endometrium proteins identified from 2DE spots. Statistically significant fold changes between groups are shown in bold, together with their corresponding P values. Increases in spot volumes are denoted by a + and decreases are denoted by a − prefix to the fold change values. The comparisons between groups follow the rule that the fold changes are calculated on the basis that the first group is being compared with the second group. Accession number is given regarding bovine species. The accession number for ovine is given in parentheses if applicable.

<table>
<thead>
<tr>
<th>Function/protein</th>
<th>Spot no.</th>
<th>MW (kDa)</th>
<th>PI</th>
<th>MASCOT score</th>
<th>Accession no. (Swiss-Prot)</th>
<th>Fold change (P value)</th>
<th>C16 vs C12</th>
<th>P16 vs P12</th>
<th>P20 vs P16</th>
<th>P12 vs C12</th>
<th>P16 vs C16</th>
</tr>
</thead>
<tbody>
<tr>
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<td>15.17</td>
<td>5.30</td>
<td>483</td>
<td>Q19MU7*</td>
<td>−2.3</td>
<td>+2.38, P=0.021</td>
<td>+1.56</td>
<td>−1.25</td>
<td>+2.30, P=0.008</td>
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<tr>
<td>Structure</td>
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<td>53.72</td>
<td>5.02</td>
<td>1858</td>
<td>P48616 (Q9MZA9)</td>
<td>−1.09</td>
<td>+1.73, P=0.028</td>
<td>+1.02</td>
<td>−1.58</td>
<td>+1.52, P=0.026</td>
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<tr>
<td>Vimentin (VIM)</td>
<td>890</td>
<td>8096</td>
<td>5.54</td>
<td>931</td>
<td>Q13S14</td>
<td>+1.37, P=0.039</td>
<td>−1.13</td>
<td>+1.22</td>
<td>−1.03</td>
<td>−1.60, P=0.034</td>
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<tr>
<td>Actin binding proteins</td>
<td>121</td>
<td>22.60</td>
<td>8.87</td>
<td>641</td>
<td>Q13T367</td>
<td>+1.03</td>
<td>−1.40</td>
<td>+1.25, P=0.039</td>
<td>−1.37, P=0.044</td>
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<tr>
<td>Transgelin (TAGLN)</td>
<td>119</td>
<td>28.84</td>
<td>5.78</td>
<td>562</td>
<td>Q13U513</td>
<td>−1.34, P=0.013</td>
<td>+1.15</td>
<td>+1.25</td>
<td>−1.23</td>
<td>+1.25, P=0.035</td>
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<tr>
<td>Proteasome (prosome, macropain) activator subunit 1 (PSME1)</td>
<td>77</td>
<td>34.32</td>
<td>6.52</td>
<td>213</td>
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<td>+1.09</td>
<td>+1.61, P=0.006</td>
<td>−1.00</td>
<td>+1.20</td>
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<td>Sulfotransferase family, cytosolic, IA, phenol-prefering number 1 (SULT1A1)</td>
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<td></td>
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</table>

*The accession number is for ovine species as the accession number for bovine was not found.

To quantify protein changes, WB were performed on tissues in all groups (Fig. 7). The stromal cells of both caruncular and intercaruncular tissues in all groups (Fig. 7). The stromal cells of both caruncular and intercaruncular tissues in all groups (Fig. 7). The stromal cells of both caruncular and intercaruncular tissues in all groups (Fig. 7). The stromal cells of both caruncular and intercaruncular tissues in all groups (Fig. 7). The stromal cells of both caruncular and intercaruncular tissues in all groups (Fig. 7).
caruncular and intercaruncular tissues on C12 were characterised by a weak immunostaining for WARS. The stromal cells of caruncular and intercaruncular tissues from pregnant ewes were characterised by increased immunoreactivity for WARS on P12 and P16 compared with matching days of the oestrous cycle. Overall, WARS immunostaining in the epithelial and stromal cells of both caruncular and intercaruncular tissues is markedly increased on P16. ERP57 immunoreactivity was present in epithelium and stromal cells of both caruncular and intercaruncular tissues in all groups (Fig. 8). ERP57 immunoreactivity tended to be stronger in the epithelium than in the stroma of both caruncular and intercaruncular tissues irrespective of days of oestrous cycle and pregnancy examined. SOD2 immunoreactivity was present in the epithelium and stromal cells of both caruncular and intercaruncular tissues in all groups (Fig. 9). VIM immunoreactivity was present in the epithelium and stromal cells of both caruncular and intercaruncular tissues in all groups (Fig. 10). It appeared that VIM immunostaining is markedly decreased in the epithelium and stromal cells of both caruncular and intercaruncular tissues from C12 to C16. There was no positive immunostaining for SM22 in epithelial cells of both caruncular and intercaruncular tissues in all groups (Fig. 11). SM22 immunoreactivity was stronger in stromal cells of caruncular tissue than that in stromal cells of intercaruncular tissue irrespective of days of oestrous cycle and pregnancy examined. The endothelial cells of blood vessels in both caruncular and intercaruncular tissues are characterised by strong immunoreactivity for SM22 (Fig. 11).

Discussion

In this study, we provide the profile of protein expression in sheep aglandular caruncular and glandular intercaruncular endometrium during the peri-implantation period. Proteins with different functions in protein synthesis and degradation, antioxidant cell defence, cell structural integrity, adhesion and signal transduction exhibited implantation-specific, tissue-specific expression characteristics. Our findings highlight the different but important roles of the caruncular and intercaruncular proteins during implantation and early pregnancy. The differentially expressed proteins in both endometrial tissues are discussed with regard to their important functions.

Protein synthesis and degradation

WARS is a member of aminoacyl-tRNA synthetase family that catalyses the aminoacylation of tRNA_{trp} with tryptophan, a critical step in cellular protein synthesis (Fleckner et al. 1991, Garret et al. 1991). The expression of WARS mRNA increases during decidualisation of human
endometrium early in pregnancy (Kudo et al. 2004). Similarly, WARS mRNA expression increases 2.4-fold in the endometrium of pregnant cows on day 17 compared with the matching stage of the oestrous cycle (Walker et al. 2010). Up-regulation of WARS protein expression observed in sheep endometrium at conceptus implantation had not been reported previously. What is particularly worth noting is that WARS expression increased dramatically in both the caruncular and intercaruncular tissues. In addition, we show that WARS is localised in endometrium luminal epithelium and stromal tissue over the peri-implantation periods (days 12–20). Importantly, WARS staining increased markedly in luminal epithelium and in stromal tissue of pregnant ewes at the time of implantation compared with the matching stage of the oestrous cycle. These results suggest that factor(s), probably produced by the implanting conceptus, enhance endometrium WARS protein expression.

Between the period of blastocyst hatching on about day 8 post-mating and initial endometrial attachment of the conceptus on P16, the sheep endometrium responds to ovarian steroid hormones and conceptus signals to provide an essential microenvironment that permits early establishment of pregnancy. This involves the production of a trophoblast protein produced transiently by the mononuclear trophectoderm cells during the period of blastocyst elongation and attachment, i.e. between P12 and P21 (Godkin et al. 1982), identified as an interierson (IFN) that inhibits functional corpus luteum regression (Imakawa et al. 1987). WARS is an IFN-inducible gene (Fleckner et al. 1991, Rubin et al. 1991, Kisselev et al. 1993). In addition, WARS expression is stimulated by IFNγ (type II IFN) and lightly by IFNα (type I IFN) in different cell types (Tolstrup et al. 1995). The increase in WARS expression in luminal epithelium and in stromal tissue during early pregnancy reported may be due to stimulation by conceptus IFNγ. As IFNγ (type I IFN) is produced and secreted by sheep conceptus during peri-implantation period and both types of IFN have some sequence homology (Tavernier & Fiers 1984), the ability of IFNγ to induce WARS expression in ovine endometrium requires further studies.

The formation of disulphide bonds is a key step in the folding of many proteins. ERP57 is a member of the protein disulphide isomerase family (Frickel et al. 2004). This ER-soluble protein interacts with the two lectin chaperones, calreticulin and calnexin, to promote folding of newly synthesised proteins by catalysing the formation of disulphide bonds (Russell et al. 2004). ERP57 interacts with glycoproteins during their folding and corrects disulphide bond formation and hence it catalyses the re-folding of mis-folded proteins (Jessop et al. 2002). In this study, ERP57 expression increased in the caruncular tissue at the time of implantation and during early pregnancy. This increase may be required for facilitating an elevation in protein flux via the ER as one can expect...
that the endometrium protein synthesis and secretion increase during the peri-implantation period.

**Antioxidant cell defence**

Imbalance between generation and elimination of superoxide radical (\( \cdot O_2^- \)) and other reactive oxygen species (ROS) is considered to be a promoter of defective embryogenesis, embryonic mortality and abortion (Al-Gubory et al. 2010). The \( \cdot O_2^- \) antioxidant scavenging enzymes comprise mitochondrial SOD2 and cytoplasmic SOD1. In the human endometrium, SOD2 activity increased at the mid-secretory phase, decreased at the late secretory phase and increased early during pregnancy (Sugino et al. 1996). The effectiveness of \( \cdot O_2^- \) scavenging capacity in ovine caruncular and intercaruncular tissues varies markedly from implantation to post-implantation periods. Indeed, caruncular tissues demonstrated increased SOD2 activity from days 16 to 21 whereas intercaruncular tissues demonstrated increased SOD1 and SOD2 activities from P16 to P21 (Al-Gubory & Garrel 2012). In this study, SOD2 protein expression was increased in the caruncular tissue at the time of implantation. Moreover, SOD2 protein was abundant in caruncular tissue during the early post-implantation period, whereas it declined at the end of the oestrous cycle. Ubiquitous mitochondrial \( \cdot O_2^- \) production is the first step in the formation and propagation of ROS within and out of the cell and it could be a mediator of oxidative chain reactions that alter cell function and integrity (Orrenius et al. 2007). Abnormal mitochondrial activity alters ROS production and reduces conceptus implantation in women (Bartmann et al. 2004). Therefore,
increased endometrium SOD2 protein expression (this study) and activity (Al-Gubory & Garrel 2012) during the peri-implantation period in ewe may represent a protective mechanism to prevent mitochondrial damage and oxidative insult early in pregnancy.

**Cell structural framework and adhesion**

VIM belongs to the intermediate filament family of proteins that contributes to the structural integrity of cells. Surface and glandular epithelial cells of human endometrium have been shown to express VIM during the menstrual cycle (Norwitz et al. 1991). Decidualisation of endometrial stromal fibroblasts into epithelial-like cells is suggested to limit conceptus trophoblast invasion through the uterine wall during invasive implantation. A role for VIM for decidualisation of humans (Can et al. 1995), mice (Oliveira et al. 2000) and rats (Korgun et al. 2007) has been suggested. Furthermore, VIM affects the organisation and expression of surface molecules critical for adhesion (Ivaska et al. 2007), a process known to be essential for conceptus implantation. In our study, VIM increased in the intercaruncular tissue of pregnant sheep at the time of implantation, probably as there is a mild transformation of fibroblastic stromal cells to the large and polyhedral cells following attachment of the conceptus to the luminal epithelial cells (Johnson et al. 2003). Further, elevation of VIM during implantation may be associated with its involvement in cellular integrity and uterine receptivity. Given the involvement of structural protein in cell differentiation, proliferation, apoptosis and

![Figure 9](image1.png)  
**Figure 9** Representative immunohistochemical localisation of mitochondrial manganese superoxide dismutase 2 (SOD2) in the sheep aglandular caruncular (left panels) and glandular intercaruncular (right panels) collected from cyclic ewes on days 12, (C12) and 16 (C16) of the oestrous cycle and on days 12, (P12) and 16 (P16) of pregnancy. Presence of SOD2 is indicated by the amount of red staining. IgG-ve, IgG-negative control; e, luminal epithelium; ses, sub-epithelial stroma; ps, profound stroma; g, uterine gland; eg, epithelium of uterine gland. Scale bars, 100 μm.

![Figure 10](image2.png)  
**Figure 10** Representative immunohistochemical localisation of vimentin (VIM) in the sheep aglandular caruncular (left panels) and glandular intercaruncular (right panels) collected from cyclic ewes on days 12, (C12) and 16 (C16) of the oestrous cycle and on days 12, (P12) and 16 (P16) of pregnancy. Presence of VIM is indicated by the amount of red staining. IgG-ve, IgG-negative control; e, luminal epithelium; ses, sub-epithelial stroma; ps, profound stroma; g, uterine gland; eg, epithelium of uterine gland. Black arrow heads, large granulated cells. Scale bars, 100 μm (C12-car and C12-icar, C16-car and C16-icar, P12-car and P12-icar) and 50 μm (P12-car and P12-icar).
cellular integrity, further study is needed, especially in connection with VIM.

**Signal transduction**

ANXA4 is involved in absorption and secretion across the epithelial cells (Kaetzel et al. 1994). It participates in a variety of physiological activities such as coagulant inhibition, inhibition of phospholipase A2, vesicular transport and Ca\(^{2+}\) ion channel activity (Ponnampalam & Rogers 2006). ANXA4 gene was up-regulated in human endometrium during the mid-secretory phase of the menstrual cycle (Kao et al. 2002, Riesewijk et al. 2003). ANXA4 mRNA increased in the human endometrium during the mid and late luteal phase of the menstrual cycle in association with high circulating progesterone concentrations. Further, ANXA4 mRNA and its protein were elevated in explants of human endometrium of the proliferative phase after treatment with progesterone (Ponnampalam & Rogers 2006). In our study, ANXA4 expression declined in the caruncular tissue at the end of oestrous cycle in both 2DE and 1DE, most probably due to the rapid decline in circulating progesterone concentrations from days 12 to 16 of the sheep oestrous cycle. By contrast, ANXA4 increased at the time of implantation in caruncular tissue because the sheep corpus luteum was secreting high levels of progesterone to maintain an environment for anticoagulation during implantation and its involvement in intracellular Ca\(^{2+}\) signalling and Ca\(^{2+}\) homeostasis, emphasising importance of Ca\(^{2+}\) at the time of implantation (Li et al. 2003).

**Actin binding proteins**

SM22 alpha is a smooth muscle actin-binding protein that is expressed in vascular smooth muscle cells and co-localised with actin filament bundles. Little is known about its function at present, but it may be involved in the organisation of the cytoskeleton to regulate smooth muscle cell morphology (Zhang et al. 2001). In this study, SM22 alpha was highly expressed in the intercaruncular tissue of pregnant ewes during the pre- and post-implantation periods but not at the time of implantation. WB of SM22 alpha demonstrated the same pattern as a 2DE gel, although not at the level of statistical significance. Immunohistochemical images showed that the number of positively stained endometrial stromal cells for SM22 alpha increased at the time of implantation compared with the pre-implantation period. This is probably because sub-epithelial stromal cells underwent transformation to other cell types or subjected to an increase in proliferation rate when implantation occurs. It could therefore be suggested that reduction in expression of SM22 alpha in sheep intercaruncular tissue at the time of implantation is probably due to endometrial cell differentiation while it is widely expressed in endothelial cells contributing to new formation of microvasculature at that time (Demir et al. 2009).

GSN plays a role in dynamic changes in the actin cytoskeleton. It severs assembled actin filaments in two and caps the growing plus end of the actin filaments (Kwiatkowski 1999). In addition, it is involved in apoptosis (Kwiatkowski 1999), integrin affinity regulation and cell adhesion (Langereis et al. 2009) and regulation of gene transcription (Silacci et al. 2004). GSN was highly expressed in decidual cells of rat endometrium (Shaw et al. 1998). GSN is an oestrogen-responsive gene in the human endometrium (Punyadeera et al. 2005). In our study, GSN was up-regulated in the intercaruncular tissue at the end of...
the oestrous cycle. As circulating oestrogen levels are high at the end of the sheep oestrous cycle, it may stimulate endometrium GSN expression.

Miscellaneous proteins

PA28 alpha/PSME1 is a regulatory complex for 20s proteasome, which is inducible by IFNγ (Schwarz et al. 2000) and resulting in the generation of antigenic peptides for presentation by class I molecules of the major histocompatibility complex (Kloetzel & Ossendorp 2004). Therefore, high expression of PSME1 in the intercaruncular tissue at implantation and during the early post-implantation period (this study) may be due to increased endometrium infiltration by leucocytes at these times. Subsequent secretion of IFNγ would induce PMSE1 and the formation of immunoproteasome to modulate the immune system during early pregnancy. TF is an iron-binding and transport protein with involvement in cell differentiation and proliferation. TF is present in uterine luminal fluid of pregnant ewes, originating from both conceptus and maternal serum (Lee et al. 1998). Further, TF is synthesised by the yolk sac of the sheep conceptus from day 15 prior to vasculogenesis and haematopoiesis, when the iron demand is extensively increased (Lee et al. 1998). Therefore, high expression of PSME1 in the intercaruncular tissue at implantation and during the early post-implantation period (this study) may be due to increased endometrium infiltration by leucocytes at these times. Subsequent secretion of IFNγ would induce PMSE1 and the formation of immunoproteasome to modulate the immune system during early pregnancy. TF is an iron-binding and transport protein with involvement in cell differentiation and proliferation. TF is present in uterine luminal fluid of pregnant ewes, originating from both conceptus and maternal serum (Lee et al. 1998). Further, TF is synthesised by the yolk sac of the sheep conceptus from day 15 prior to vasculogenesis and haematopoiesis, when the iron demand is extensively increased (Lee et al. 1998). TF up-regulation in the caruncular tissue at the time of implantation (present study) is possibly because of high demand of iron for endometrium angiogenesis at this time. However, no evidence was found in the literature to support our findings that TF was increased in caruncular tissue at the end of the oestrous cycle and then reduced during the early pregnancy. The fact that there were two different positive-identified proteins (TF and keratin 9) in this spot and that TF plays roles in cell differentiation and proliferation may be reasons for these unexpected changes in TF expression.

In conclusion, we have identified complex patterns of changes in a number of proteins involved in various biological pathways potentially crucial for the establishment of early pregnancy. The identified proteins have roles in signal transduction, protein synthesis and degradation, cell adhesion and antioxidant defence. Further, this study suggests that the conceptus is capable of modulating endometrial function, biasing the endometrial proteome in favour of proteins crucial for conceptus implantation and the establishment of the early pregnancy in sheep. Our findings highlight the different but important roles of the caruncular and intercaruncular endometrial proteins during implantation and early pregnancy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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