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

K H Al-Gubory, M Arianmanesh¹, C Garrel², S Bhattacharya³, P Cash³ and P A Fowler⁴

INRA, UMR 1198 Biologie du Développement et Reproduction, Département de Physiologie Animale et Système d'Élevage, 78350 Jouy-en-Josas, France, ¹Department of Anatomical Sciences, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran, ²Unité de Biochimie Hormonale et Nutritionnelle, Département de Biologie – Toxicologie – Pharmacologie, Centre Hospitalier Universitaire de Grenoble, 38043 Grenoble Cedex 9, France, ³Division of Applied Health Sciences, Section of Immunology and Infection and ⁴Division of Applied Medicine, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK

Correspondence should be addressed to K H Al-Gubory; Email: kais.algubory@jouy.inra.fr

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 P16 and C16. 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.

Reproduction (2014) **147** 599–614

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, Domínguez *et al.* 2009, Parmar *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 epithelio-chorial 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 trophoblast 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

Figure 1 (A) Experimental design and days of collection of endometrium tissues from cyclic and pregnant ewes. (B) Macroscopic view of sheep endometrium and haematoxylin and eosin (H&E) staining of caruncular and intercaruncular tissues (magnification, $\times 250$). Aglandular caruncles are sites of conceptus implantation, and glandular intercaruncles secrete histotroph required for conceptus development. e, epithelium; s, stroma; g, glands.

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% (w/v), pH 3–10, resolyte (Merck Eurolab Ltd, Poole, Dorset, UK) and protease inhibitor cocktail (Roche Diagnostics). The tissues were disrupted using a Tissue Lyser (Qiagen Ltd) for 4 min at 30 Hz. Insoluble materials were removed from the lysates by centrifugation (50 000 g at 4°C) for 30 min. The protein content of the final supernatant had been determined by RC-DC assay (Bio-Rad Laboratories Ltd). The endometrial extracts were stored at -80°C until required for further analysis.

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, H00000307-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-α-tubulin (Abcam Ltd, ab7291) and 1–10 000 or rabbit anti-α-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-maX (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–Wilk 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.

Group comparison				
Group	Compared with	Up-regulated	Down-regulated	Percentage of total spots
P12	C12	14	41	6
P16	C16	54	31	10
P16	P12	31	14	5
P20	P16	34	28	7
C16	C12	30	30	7

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 ($P < 0.05$) 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 ($P < 0.05$) 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 ($P < 0.05$) 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 ($P < 0.05$) 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 ($P < 0.05$) 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 ($P < 0.05$) 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 ($P < 0.05$) changed between C12 and C16 (Table 1). Among these, 30 normalised spot volumes

Figure 3 Expression changes of (A) tryptophanyl tRNA synthetase (WARS), (B) endoplasmic reticulum resident protein 57 (ERP57), (C) annexin 4 (ANXA4), (D) mitochondrial manganese superoxide dismutase 2 (SOD2), (E) transferrin (TF) and (F) proteasome (prosome, macropain) subunit, alpha type 1 (PSMA1) in sheep caruncular 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 $P < 0.05$.

were up-regulated and 30 down-regulated at day 16 when compared with day 12 (Table 1). TF (Fig. 3E) significantly increased ($P < 0.05$) at C16 compared with C12 (Table 2). Conversely, WARS (Fig. 3A), ANXA4 (Fig. 3C), SOD2 (Fig. 3D) and PSMA1 (Fig. 3F) significantly decreased ($P < 0.05$) 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.

Function/protein	Spot no.	MW (kDa)	PI	MASCOT score	Accession no. (Swiss-Prot)	Fold change, <i>P</i> value				
						C16 vs C12	P16 vs P12	P20 vs P16	P12 vs C12	P16 vs C16
Protein synthesis										
Tryptophanyl tRNA synthetase	721	54.17	5.49	753	P17248	–1.33, <i>P</i> =0.043	+1.92, <i>P</i> =0.021	+1.45	–1.31, <i>P</i> =0.043	+1.96, <i>P</i> =0.021
Cytoplasmic (WARS/TrpRS)	867	52.44	5.74	275						
Iron transport and homeostasis										
Transferrin	15	79.80	6.75	659	Q29443	+2.53, <i>P</i> =0.011	+1.82, <i>P</i> =0.031	–1.69	–1.21	–1.68, <i>P</i> =0.029
Structure										
Lamin A/C (LMNA)	3	79.80	7.70	707	Q3SZ12	+2.75, <i>P</i> =0.021	–1.18	–1.08	–1.07	–3.49, <i>P</i> =0.021
	86	74.33	6.57	1209						
	700	79.80	7.70	279						
Lamin B2	686	68.21	5.35	229	516326 (NCBI) ^a	–1.91, <i>P</i> =0.004	+1.58, <i>P</i> =0.004	+1.16	–1.45, <i>P</i> =0.001	+2.07, <i>P</i> =0.004
Chaperones										
Endoplasmic reticulum resident protein 57 (ERP57/PDIA3/GRP58)	57	57.29	6.38	1584	P38657	–1.37	+1.47, <i>P</i> =0.002	+1.42, <i>P</i> =0.006	–1.372, <i>P</i> =0.006	+1.48, <i>P</i> =0.003
Protein degradation										
Proteasome (prosome, macropain) subunit, alpha type 1 (PSMA1)	111	29.81	6.00	470	Q3TS44	–1.65, <i>P</i> =0.011	+1.25, <i>P</i> =0.0008	+1.10	+1.47, <i>P</i> =0.002	+1.41, <i>P</i> =0.039
Signal transduction										
Annexin IV (ANXA4)	55	36.07	5.94	750	P13214	–1.77, <i>P</i> =0.001	+1.17	+1.35	–1.35, <i>P</i> =0.017	+1.54, <i>P</i> =0.0006
Calmodulin (CaM)	70	16.69	4.12	254	P62157	–1.49, <i>P</i> =0.042	+1.05	+1.54, <i>P</i> =0.038	–1.21	–1.30
Antioxidant cell defense										
Mitochondrial manganese superoxide dismutase 2 (SOD2)	13	25.00	8.09	486	P41976 (C8BKD6)	–1.83, <i>P</i> =0.002	+1.28	+1.66, <i>P</i> =0.01	–1.27	+1.85, <i>P</i> =0.004
Cytosolic NADP ⁺ -dependent isocitrate dehydrogenase (IDH1)	35	47.09	6.13	164	Q9XSG3	–1.63, <i>P</i> =0.001	–1.23, <i>P</i> =0.0001	–1.96, <i>P</i> =0.001	–1.09	+1.23
Cell adhesion										
Galectin 15 (LGALS15/OVGL11)	738	47.09	6.13	635						
	12	15.50	5.24	150	Q19MU7 ^b	+1.62, <i>P</i> =0.01	+2.73, <i>P</i> =0.048	–1.39	+1.27	+1.89

^aAccession from PubMed (NCBI) no Uniprot entry identified. ^bThe accession number is for ovine species as the accession number for bovine was not found.

Table 3 Numbers of protein spots of the intercaruncular 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 C16 and from pregnant ewes on days 12 (P12), 16 (P16) and 20 (P20) of pregnancy.

Group Comparison		Up-regulated	Down-regulated	Percentage of total spots
Group	Compared with			
P12	C12	36	8	3
P16	C16	17	17	3
P16	P12	6	25	2
P20	P16	13	26	3
C16	C12	37	15	4

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 spots, 17 were up-regulated and 17 down-regulated (Table 1). LGALS15 (Fig. 4B), VIM (Fig. 4C) and PSME1 (Fig. 4D) significantly increased ($P < 0.05$), whereas GSN (Fig. 4E) significantly decreased ($P < 0.05$) at the time of implantation (P16) when compared with the matching stage of the oestrous cycle (C16) (Table 4).

Comparison between pregnant ewes at implantation (P16) and pre-attachment (P12) periods revealed that 31 (2%) of protein spots were significantly ($P < 0.05$) changed (Table 3). Among these, six normalised spot volumes were up-regulated and 25 down-regulated at the implantation period (P16) compared with the pre-attachment period (P12) (Table 3). LGALS15 (Fig. 4B) and VIM (Fig. 4C) significantly increased ($P < 0.05$) at the time of implantation (P16) when compared with the pre-attachment period (P12) (Table 4). Comparison between pregnant ewes at implantation (P16) and post-implantation (P20) periods revealed that 39 (3%) of protein spots were significantly ($P < 0.05$) changed (Table 3). Among these spots, 13 normalised spot volumes were up-regulated and 26 down-regulated (Table 3). SM22 alpha (Fig. 4A) and SULT1A1 (Fig. 4F) significantly increased ($P < 0.05$) at the time of post-implantation (P20) when compared with the implantation day (P16) (Table 4).

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).

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). 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

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-preferring, 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 to bovine species. The accession number for ovine is given in parentheses if applicable.

Function/protein	Spot no.	MW (kDa)	PI	MASCOT score	Accession no. (Swiss-Prot)	Fold change (<i>P</i> value)				
						C16 vs C12	P16 vs P12	P20 vs P16	P12 vs C12	P16 vs C16
Cell adhesion	7	15.17	5.30	483	Q19MU7 ^a	–2.3	+2.38, <i>P</i> =0.021	+1.56	–1.25	+2.30, <i>P</i> =0.008
Galectin 15 (LGALS15/OVGL11)										
Structure	95	53.72	5.02	1858	P48616 (Q9MZA9)	–1.09	+1.73, <i>P</i> =0.028	+1.02	–1.58	+1.52, <i>P</i> =0.026
Vimentin (VIM)										
Actin binding proteins	890	80.96	5.54	931	Q3SX14	+1.37, <i>P</i> =0.039	–1.13	+1.22	–1.03	–1.60, <i>P</i> =0.034
Gelsolin isoform b (GSN)	121	22.60	8.87	641	Q9T587	+1.03	–1.40	+1.25, <i>P</i> =0.039	–1.57, <i>P</i> =0.044	–1.03
Transgelin (TAGLN/SM22 alpha)										
Antigen presentation	119	28.84	5.78	562	Q4U5R3	–1.34, <i>P</i> =0.013	+1.15	+1.25	–1.23	+1.25, <i>P</i> =0.035
Proteasome (prosome, macropain) activator subunit 1 (PA28 alpha (PSME1))										
Lipid metabolism										
Sulfotransferase family, cytosolic, 1A, phenol-prefering number 1 (SULT1A1)	77	34.32	6.52	213	P50227	–1.20	+1.09	+1.61, <i>P</i> =0.006	–1.00	+1.20

^aThe accession number is for ovine species as the accession number for bovine was not found.

12 to 16 (Fig. 3A) in contrast to a tendency for elevation in western blot (WB) of the same groups (Fig. 5A). This is probably because non-isoform-specific WARS antibody was used to quantify WARS bands, both cytoplasmic form (WARS) and mitochondrial form (WARS2), while in the 2DE gel, cytoplasmic form was identified.

SOD2 was detected at 25 kDa (Fig. 5B). There was a significant (*P*<0.05) elevation of SOD2 band volume at the time of implantation (P16) when compared with matching day of the oestrous cycle (C16) (Fig. 5C) that it is in agreement with 2DE gel findings for SOD2 alterations quantified by 2DE analysis in caruncular tissue of pregnant ewes (Fig. 3D). In addition, SOD2 expression increased significantly in pregnant ewes from day 12 (P12) to day 16 (P16).

ANXA4 expression decreased significantly (*P*<0.05) from C12 to C16 (Fig. 5C). Although no significant changes of ANXA4 expression could be observed between any stage of pregnancy examined, its expression was significantly higher at the time of implantation (P16) when compared with matching day of the oestrous cycle (C16) (Fig. 5C), in agreement with 2DE gel findings for ANXA4 alterations quantified by 2DE analysis in caruncular tissue of pregnant ewes (Fig. 3C).

Quantification of intercaruncular proteins

To quantify protein changes, WB were performed on proteins whose known functions suggested important roles in the endometrium. WARS was detected at 54 kDa (Fig. 6A). WARS band volume increased at the time of implantation (P16) when compared with pre-attachment period (P12) (Fig. 6A). In addition, its band volume was markedly higher at the time of implantation (P16) when compared with the matching day of the oestrous cycle (C16) (Fig. 6A).

GSN was detected at 82 kDa (Fig. 6B). No significant changes could be detected between groups by WB (Fig. 6B). However, the 2DE gel spot volume of GSN was significantly down-regulated at the time of implantation (P16) when compared with matching day of the oestrous cycle (C16) (Fig. 4E). It may be because non-isoform-specific gelsolin antibody was used to quantify gelsolin bands while in the 2DE gel, gelsolin isoform b was identified.

SM22 alpha was detected with 23 kDa (Fig. 6C). Changes in SM22 expression between groups by WB corresponded to its expression on 2DE gel (Fig. 4A), although it did not achieve at the significance level.

Immunohistochemistry of differentially expressed proteins

WARS immunoreactivity was present in the epithelium and stroma cells of both caruncular and intercaruncular tissues in all groups (Fig. 7). The stromal cells of both

Figure 5 Western blot analysis of (A) tryptophanyl tRNA synthetase (WARS), (B) mitochondrial superoxide dismutase 2 (SOD2) and (C) annexin 4 (ANXA4) expression in sheep caruncular 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. In all WBs, there were no significant changes in α -tubulin band volumes between the samples and all groups, indicating its validity as a load control. Normalised band volumes are shown as means $\pm$ S.E.M. for four ewes per group. The acceptable level of significance was set at $P < 0.05$.

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

Figure 6 Western blot analysis of (A) tryptophanyl tRNA synthetase (WARS), (B) gelsolin (GSN) and (C) transgelin (SM22) expression 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. In all WBs, there were no significant changes in α -tubulin band volumes between the samples and all groups, indicating its validity as a load control in this experiment. Normalised band volumes are shown as means $\pm$ S.E.M. for four ewes per group. The acceptable level of significance was set at $P < 0.05$.

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 trophoblast cells during the period of blastocyst elongation and attachment, i.e. between P12 and P21 (Godkin *et al.* 1982), identified as an interferon τ (IFN τ) 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 IFN γ (type II IFN) and lightly by IFN α (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 IFN τ . As IFN τ (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 IFN τ 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

Figure 7 Representative immunohistochemical localisation of tryptophanyl tRNA synthetase (WARS) 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 WARS 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 arrows, lymphocytes. Scale bars, 100 μ m (C12-car, C16-car and C16-icar, P12-car and P12-icar, P16-car and P16-icar) and 50 μ m; (C12-icar).

that the endometrium protein synthesis and secretion increase during the peri-implantation period.

Antioxidant cell defence

Imbalance between generation and elimination of superoxide radical ($\cdot\text{O}_2^-$) 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 $\cdot\text{O}_2^-$ 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 $\cdot\text{O}_2^-$ 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 $\cdot\text{O}_2^-$ 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,

Figure 8 Representative immunohistochemical localisation of endoplasmic reticulum resident protein 57 (ERP57) 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 ERP57 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. Arrow heads, large granulated cells. Scale bars, 100 μ m.

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.

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 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, P16-car and P16-icar) and 50 µm (P12-car and P12-icar).

Figure 11 Representative immunohistochemical localisation of transgelin (SM22) 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 SM22 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 arrows, endothelial cells; black arrow heads, myoepithelial cells. Scale bars, 100 μ m.

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 anti-coagulation 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 stroma 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, thus preventing assembly of actin monomers to the filament (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 α /PSME1 is a regulatory complex for 20S proteasome, which is inducible by IFN γ (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 leukocytes at these times. Subsequent secretion of IFN γ 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.

Funding

This research project was funded by NHS Grampian R&D (project number RG05/019).

Acknowledgements

The authors are grateful to Margaret Fraser, Gillian Smith, Evelyn Argo, Ian Davidson and Elizabeth Stewart for their expert technical assistance. They also thank the staff of the sheep sheds of Brouessy and Jouy-en-Josas (INRA, France) for outstanding technical help and sheep management. Dr Mitra Arianmanesh would like to thank the Iranian Ministry of Health and Medical Education and Zanjan University of Medical Sciences for awarding her a 'pursue' (engage in an activity) scholarship to prepare her PhD. The authors would also like to thank the anonymous editorial board member and reviewers for their close examination of this review article and their useful comments.

References

- Ace CI & Okulicz WC 2004 Microarray profiling of progesterone-regulated endometrial genes during the rhesus monkey secretory phase. *Reproductive Biology and Endocrinology* **2** 54. (doi:10.1186/1477-7827-2-54)
- Al-Gubory KH & Garrel C 2012 Antioxidative signalling pathways regulate the level of reactive oxygen species at the endometrial–extraembryonic membranes interface during early pregnancy. *International Journal of Biochemistry & Cell Biology* **44** 1511–1518. (doi:10.1016/j.biocel.2012.06.017)
- Al-Gubory KH, Bolifraud P & Garrel C 2008 Regulation of key antioxidant enzymatic systems in the sheep endometrium by ovarian steroids. *Endocrinology* **149** 4428–4434. (doi:10.1210/en.2008-0187)
- Al-Gubory KH, Fowler PA & Garrel C 2010 The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. *International Journal of Biochemistry & Cell Biology* **42** 1634–1650. (doi:10.1016/j.biocel.2010.06.001)
- Arianmanesh M, McIntosh RH, Lea RG, Fowler PA & Al-Gubory KH 2011 Ovine corpus luteum proteins, with functions including oxidative stress and lipid metabolism, show complex alterations during implantation. *Journal of Endocrinology* **210** 47–58. (doi:10.1530/JOE-10-0336)
- Bartmann AK, Romo GS, Ramos Eda S & Ferriani RA 2004 Why do older women have poor implantation rates? A possible role of the mitochondria. *Journal of Assisted Reproduction and Genetics* **21** 79–83. (doi:10.1023/B:JARG.0000027018.02425.15)
- Can A, Tekeliolu M & Baltaci A 1995 Expression of desmin and vimentin intermediate filaments in human decidual cells during first trimester pregnancy. *Placenta* **16** 261–275. (doi:10.1016/0143-4004(95)90113-2)
- Cash P & Kroll JS 2003 Protein characterization by two-dimensional gel electrophoresis. *Methods in Molecular Medicine* **71** 101–118. (doi:10.1385/1-59259-321-6:101)
- Chen JI, Hannan NJ, Mak Y, Nicholls PK, Zhang J, Rainczuk A, Stanton PG, Robertson DM, Salamonsen LA & Stephens AN 2009 Proteomic characterization of midproliferative and midsecretory human endometrium. *Journal of Proteome Research* **8** 2032–2044. (doi:10.1021/pr801024g) (doi:10.1021/pr801024g)
- Demir R, Yaba A & Huppertz B 2009 Vasculogenesis and angiogenesis in the endometrium during menstrual cycle and implantation. *Acta Histochemica* **112** 203–214. (doi:10.1016/j.acthis.2009.04.004)
- DeSouza L, Diehl G, Yang EC, Guo J, Rodrigues MJ, Romaschin AD, Colgan TJ & Siu KW 2005 Proteomic analysis of the proliferative and secretory phases of the human endometrium: protein identification and differential protein expression. *Proteomics* **5** 270–281. (doi:10.1002/pmic.200400920)

- Dixon AB, Knights M, Winkler JL, Marsh DJ, Pate JL, Wilson ME, Dailey RA, Seidel G & Inskeep EK 2007 Patterns of late embryonic and fetal mortality and association with several factors in sheep. *Journal of Animal Science* **85** 1274–1284. (doi:10.2527/jas.2006-129)
- Domínguez F, Garrido-Gómez T, López JA, Camafeite E, Quiñero A, Pellicer A & Simón C 2009 Proteomic analysis of the human receptive versus non-receptive endometrium using differential in-gel electrophoresis and MALDI-MS unveils stathmin 1 and annexin A2 as differentially regulated. *Human Reproduction* **24** 2607–2617. (doi:10.1093/humrep/dep230)
- Evans GE, Martínez-Conejero JA, Phillipson GT, Simón C, McNoe LA, Sykes PH, Horcajadas JA, Lam EY, Print CG, Sin IL *et al.* 2012 Gene and protein expression signature of endometrial glandular and stromal compartments during the window of implantation. *Fertility and Sterility* **97** 1365–1373. (doi:10.1016/j.fertnstert.2012.03.007)
- Findlay JK, Ackland N, Burton RD, Davis AJ, Walker FM, Walters DE & Heap RB 1981 Protein, prostaglandin and steroid synthesis in caruncular and intercaruncular endometrium of sheep before implantation. *Journal of Reproduction and Fertility* **62** 361–377. (doi:10.1530/jrf.0.0620361)
- Fleckner J, Rasmussen HH & Justesen J 1991 Human interferon γ potently induces the synthesis of a 55-kDa protein (γ 2) highly homologous to rabbit peptide chain release factor and bovine tryptophanyl-tRNA synthetase. *PNAS* **88** 11520–11524. (doi:10.1073/pnas.88.24.11520)
- Fowler PA, Tattum J, Bhattacharya S, Klonisch T, Hombach-Klonisch S, Gazvani R, Lea RG, Miller I, Simpson WG & Cash P 2007 An investigation of the effects of endometriosis on the proteome of human eutopic endometrium: a heterogeneous tissue with a complex disease. *Proteomics* **7** 130–142. (doi:10.1002/pmic.200600469)
- Fowler PA, Dorá NJ, McFerran H, Amezaa MR, Miller DW, Lea RG, Cash P, McNeilly AS, Evans NP, Cotinot C *et al.* 2008 *In utero* exposure to low doses of environmental pollutants disrupts fetal ovarian development in sheep. *Molecular Human Reproduction* **14** 269–280. (doi:10.1093/molehr/gan020)
- Frickel EM, Frei P, Bouvier M, Stafford WF, Helenius A, Glockshuber R & Ellgaard L 2004 ERp57 is a multifunctional thiol-disulfide oxidoreductase. *Journal of Biological Chemistry* **279** 18277–18287. (doi:10.1074/jbc.M314089200)
- Garret M, Pajot B, Trézéguet V, Labouesse J, Merle M, Gandar JC, Benedetto JP, Sallafranque ML, Alterio J, Gueguen M *et al.* 1991 A mammalian tryptophanyl-tRNA synthetase shows little homology to prokaryotic synthetases but near identity with mammalian peptide chain release factor. *Biochemistry* **30** 7809–7817. (doi:10.1021/bi00245a021)
- Garrido-Gómez T, Ruiz-Alonso M, Blesa D, Diaz-Gimeno P, Vilella F & Simón C 2013 Profiling the gene signature of endometrial receptivity: clinical results. *Fertility and Sterility* **99** 1078–1085. (doi:10.1016/j.fertnstert.2012.12.005)
- Godkin JK, Bazer FW, Moffatt J, Sessions F & Roberts RM 1982 Purification and properties of a major, low molecular weight protein released by the trophoblast of sheep blastocysts at day 13–21. *Journal of Reproduction and Fertility* **65** 141–150. (doi:10.1530/jrf.0.0650141)
- Gray CA, Taylor KM, Ramsey WS, Hill JR, Bazer FW, Bartol FF & Spencer TE 2001 Endometrial glands are required for pre-implantation conceptus elongation and survival. *Biology of Reproduction* **64** 1608–1613. (doi:10.1095/biolreprod64.6.1608)
- Imakawa K, Anthony RV, Kazemi M, Marotti KR, Polites HG & Roberts RM 1987 Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophoblast. *Nature* **330** 377–379. (doi:10.1038/330377a0)
- Imakawa K, Chang KT & Christenson RK 2004 Pre-implantation conceptus and maternal uterine communications: molecular events leading to successful implantation. *Journal of Reproduction and Development* **50** 155–169. (doi:10.1262/jrd.50.155)
- Ivaska J, Pallari HM, Nevo J & Eriksson JE 2007 Novel functions of vimentin in cell adhesion, migration, and signaling. *Experimental Cell Research* **313** 2050–2062. (doi:10.1016/j.yexcr.2007.03.040)
- Jessop CE, Tavender TJ, Watkins RH, Chambers JE & Bulleid NJ 2002 Substrate specificity of the oxidoreductase ERp57 is determined primarily by its interaction with calnexin and calreticulin. *Journal of Biological Chemistry* **274** 2194–2202. (doi:10.1074/jbc.M808054200)
- Johnson GA, Burghardt RC, Joyce MM, Spencer TE, Bazer FW, Pfarrer C & Gray CA 2003 Osteopontin expression in uterine stroma indicates a decidualization-like differentiation during ovine pregnancy. *Biology of Reproduction* **68** 1951–1958. (doi:10.1095/biolreprod.102.012948)
- Kaetzel MA, Chan HC, Dubinsky WP, Dedman JR & Nelson DJ 1994 A role for annexin IV in epithelial cell function. Inhibition of calcium-activated chloride conductance. *Journal of Biological Chemistry* **269** 5297–5302.
- Kao LC, Tulac S, Lobo S, Imani B, Yang JP, Germeyer A, Osteen K, Taylor RN, Lessey BA & Giudice LC 2002 Global gene profiling in human endometrium during the window of implantation. *Endocrinology* **143** 2119–2138. (doi:10.1210/endo.143.6.8885)
- Kisselev L, Frolova L & Haenni AL 1993 Interferon inducibility of mammalian tryptophanyl-tRNA synthetase: new perspectives. *Trends in Biochemical Sciences* **18** 263–267. (doi:10.1016/0968-0004(93)90178-P)
- Kloetzel PM & Ossendorp F 2004 Proteasome and peptidase function in MHC-class-I-mediated antigen presentation. *Current Opinion in Immunology* **16** 76–81. (doi:10.1016/j.coi.2003.11.004)
- Koot YE, Teklenburg G, Salker MS, Brosens JJ & Macklon NS 2012 Molecular aspects of implantation failure. *Biochimica et Biophysica Acta* **1822** 1943–1950. (doi:10.1016/j.bbdis.2012.05.017)
- Korgun ET, Cayli S, Asar M & Demir R 2007 Distribution of laminin, vimentin and desmin in the rat uterus during initial stages of implantation. *Journal of Molecular Histology* **38** 253–260. (doi:10.1007/s10735-007-9095-4)
- Kudo Y, Hara T, Katsuki T, Toyofuku AK, Takikawa O, Fujii T & Ohama K 2004 Mechanisms regulating the expression of indoleamine 2,3-dioxygenase during decidualization of human endometrium. *Human Reproduction* **19** 1222–1230. (doi:10.1093/humrep/deh218)
- Kwiatkowski DJ 1999 Functions of gelsolin: motility, signaling, apoptosis, cancer. *Current Opinion in Cell Biology* **11** 103–108. (doi:10.1016/S0955-0674(99)80012-X)
- Langereis JD, Prinsen BH, de Sain-van der Velden MG, Coppens CJ, Koenderman L & Ulfman LH 2009 A 2D-DIGE approach to identify proteins involved in inside-out control of integrins. *Journal of Proteome Research* **8** 3824–3833. (doi:10.1021/pr8010815)
- Lee RSF, Wheeler TT & Peterson J 1998 Large-format. Two-dimensional polyacrylamide gel electrophoresis of ovine periimplantation uterine luminal fluid proteins: identification of aldose reductase, cytoplasmic actin, and transferrin as conceptus-synthesized proteins. *Biology of Reproduction* **59** 743–752. (doi:10.1095/biolreprod59.4.743)
- Li B, Dedman JR & Kaetzel MA 2003 Intron disruption of the annexin IV gene reveals novel transcripts. *Journal of Biological Chemistry* **278** 43276–43283. (doi:10.1074/jbc.M306361200)
- Ma WG, Song H, Das SK, Paria BC & Dey SK 2003 Estradiol is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. *PNAS* **100** 2963–2968. (doi:10.1073/pnas.0530162100)
- Mann M & Jensen ON 2003 Proteomic analysis of post-translational modifications. *Nature Biotechnology* **21** 255–261. (doi:10.1038/nbt0303-255)
- Norwitz ER, Fernandez-Shaw S, Barlow DH & Starkey PM 1991 Expression of intermediate filament in endometrial glands changes with the onset of pregnancy and in endometriosis. *Human Reproduction* **6** 1470–1473.
- Oliveira SF, Greca CP, Abrahamsohn PA, Reis MG & Zorn TM 2000 Organization of desmin-containing intermediate filaments during differentiation of mouse decidual cells. *Histochemistry and Cell Biology* **113** 319–327. (doi:10.1007/s004180000141)
- Orrenius S, Gogvadze V & Zhivotovsky B 2007 Mitochondrial oxidative stress: implications for cell death. *Annual Review of Pharmacology and Toxicology* **47** 143–183. (doi:10.1146/annurev.pharmtox.47.120505.105122)
- Parmar T, Gadkar-Sable S, Savardekar L, Katkam R, Dharma S, Meherji P, Puri CP & Sachdeva G 2009 Protein profiling of human endometrial tissues in the midsecretory and proliferative phases of the menstrual cycle. *Fertility and Sterility* **92** 1091–1103. (doi:10.1016/j.fertnstert.2008.07.1734) (doi:10.1016/j.fertnstert.2008.07.1734)
- Patel BG & Lessey BA 2011 Clinical assessment and management of the endometrium in recurrent early pregnancy loss. *Seminars in Reproductive Medicine* **29** 491–506. (doi:10.1055/s-0031-1293203)

- Ponnampalam AP & Rogers PA 2006 Cyclic changes and hormonal regulation of annexin IV mRNA and protein in human endometrium. *Molecular Human Reproduction* **12** 661–669. (doi:10.1093/molehr/gal075)
- Punyadeera C, Dassen H, Klompc J, Dunselman G, Kampha R, Dijkse F, Ederveene A, de Goeij A & Groothuis P 2005 Oestrogen-modulated gene expression in the human endometrium. *Cellular and Molecular Life Sciences* **62** 232–250. (doi:10.1007/s00018-004-4435-y)
- Rai P, Kota V, Sundaram CS, Deendayal M & Shivaji S 2010 Proteome of human endometrium: identification of differentially expressed proteins in proliferative and secretory phase endometrium. *Proteomics. Clinical Applications* **4** 48–59. (doi:10.1002/prca.200900094)
- Rider V 2002 Progesterone and the control of uterine cell proliferation and differentiation. *Frontiers in Bioscience* **7** d1545–d1555. (doi:10.2741/rider)
- Riesewijk A, Martin J, van Os R, Horcajadas JA, Polman J, Pellicer A, Mosselman S & Simon C 2003 Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. *Molecular Human Reproduction* **9** 253–264. (doi:10.1093/molehr/gag037)
- Rubin BY, Anderson SL, Xing L, Powell RJ & Tate WP 1991 Interferon induces tryptophanyl-tRNA synthetase expression in human fibroblasts. *Journal of Biological Chemistry* **266** 24245–24248.
- Russell SJ, Ruddock LW, Salo KE, Oliver JD, Roebuck QP, Llewellyn DH, Roderick HL, Koivunen P, Myllyharju J & High S 2004 The primary substrate binding site in the b' domain of ERp57 is adapted for endoplasmic reticulum lectin association. *Journal of Biological Chemistry* **279** 18861–18869. (doi:10.1074/jbc.M400575200)
- Satterfield MC, Song G, Kochan KJ, Riggs PK, Simmons RM, Elsik CG, Adelson DL, Bazer FW, Zhou H & Spencer TE 2009 Discovery of candidate genes and pathways in the endometrium regulating ovine blastocyst growth and conceptus elongation. *Physiological Genomics* **39** 85–99. (doi:10.1152/physiolgenomics.00001.2009)
- Schwarz K, Eggers M, Soza A, Koszinowski UH, Klotzel PM & Groettrup M 2000 Proteasome subunit composition. *European Journal of Immunology* **30** 3672–3679. (doi:10.1002/1521-4141(200012)30:12<3672::AID-IMMU3672>3.0.CO;2-B)
- Shaw TJ, Terry V, Shorey CD & Murphy CR 1998 Alterations in distribution of actin binding proteins in uterine stromal cells during decidualization in the rat. *Cell Biology International* **22** 237–243. (doi:10.1006/cbir.1998.0245)
- Silacci P, Mazzolaib L, Gaucia C, Stergiopulos N, Yinc HL & Hayoz D 2004 Gelsolin superfamily proteins: key regulators of cellular functions. *Cellular and Molecular Life Sciences* **61** 2614–2623. (doi:10.1007/s00018-004-4225-6)
- Spencer TE, Johnson GA, Bazer FW & Burghardt RC 2004 Implantation mechanisms: insights from the sheep. *Reproduction* **128** 657–668. (doi:10.1530/rep.1.00398)
- Spencer TE, Johnson GA, Bazer FW & Burghardt RC 2007 Fetal–maternal interactions during the establishment of pregnancy in ruminants. *Society of Reproduction and Fertility Supplement* **64** 379–396.
- Stephens AN, Hannan NJ, Rainczuk A, Meehan KL, Chen J, Nicholls PK, Rombauts LJ, Stanton PG, Robertson DM & Salamonsen LA 2010 Post-translational modifications and protein-specific isoforms in endometriosis revealed by 2D DIGE. *Journal of Proteome Research* **9** 2438–2449. (doi:10.1021/pr901131p)
- Sugino N, Shimamura K, Takiguchi S, Tamura H, Ono M, Nakata M, Nakamura Y, Ogino K, Uda T & Kato H 1996 Changes in activity of superoxide dismutase in the human endometrium throughout the menstrual cycle and in early pregnancy. *Human Reproduction* **11** 1073–1078. (doi:10.1093/oxfordjournals.humrep.a0192REF7=10.1016/0143-4004(95)90113-2)
- Tavernier J & Fiers W 1984 The presence of homologous regions between interferon sequences. *Carlsberg Research Communications* **49** 359–364. (doi:10.1007/BF02913963)
- Teklenburg G, Salker M, Heijnen C, Macklon NS & Brosens JJ 2010 The molecular basis of recurrent pregnancy loss: impaired natural embryo selection. *Molecular Human Reproduction* **16** 886–895. (doi:10.1093/molehr/gaq079)
- Tolstrup AB, Beijder A, Fleckner J & Justesen J 1995 Transcriptional regulation of the interferon-inducible tryptophanyl-tRNA synthetase includes alternative splicing. *Journal of Biological Chemistry* **270** 397–403. (doi:10.1074/jbc.270.1.397)
- Tuckerman E, Laird SM, Stewart R, Wells M & Li TC 2004 Markers of endometrial function in women with unexplained recurrent pregnancy loss: a comparison between morphologically normal and retarded endometrium. *Human Reproduction* **19** 196–205.
- Walker CG, Meier S, Littlejohn MD, Lehnert K, Roche JR & Mitchell MD 2010 Modulation of the maternal immune system by the pre-implantation embryo. *BMC Genomics* **11** 474. (doi:10.1186/1471-2164-11-474)
- Zhang JC, Kim S, Helmke BP, Yu WW, Du KL, Lu MM, Strobeck M, Yu QC & Parmacek MS 2001 Analysis of SM22a-deficient mice reveals unanticipated insights into smooth muscle cell differentiation and function. *Molecular and Cellular Biology* **21** 1336–1344. (doi:10.1128/MCB.2001.21.4.1336-1344.2001)

Received 22 November 2013

First decision 19 November 2013

Revised manuscript received 9 January 2014

Accepted 20 January 2014