Ovine corpus luteum proteins, with functions including oxidative stress and lipid metabolism, show complex alterations during implantation

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Abstract

Progesterone (P₄) secreted by the corpus luteum (CL) is critical for *in utero* embryo survival and development, although CL proteins are key regulatory factors during the luteal phase. We, therefore, characterised protein expression patterns in ovine CL of pregnancy (days 12, 16 and 20) compared with those of controls, CL of oestrous cycle (days 12 and 16), using two-dimensional gel electrophoresis (2DE) gel-based proteomics. Proteins in 24 significantly altered spots were identified by tandem mass spectroscopy. At the time of embryo implantation (day 16), 77 spots were up-regulated and 101 spots were down-regulated in CL of pregnancy compared with regressed CL. Vimentin, lamin A/C (LMNA), [Mn] superoxide dismutase (SOD2), isocitrate dehydrogenase 1, annexin A1 and elongation factor Tu, mitochondrial (TUFM) altered during CL regression, whereas glutathione S-transferase A1, apolipoprotein A-1, myxovirus resistance protein 1, ornithine aminotransferase and enoyl-CoA hydratase, mitochondrial (ECHS1) tended to be altered during CL maintenance. biliverdin reductase B

(BLVRB), FDXR, guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 (GNB2) and cytochrome b-c1 complex subunit 1, mitochondrial (UQCRC1) showed divergent expression during CL regression and maintenance. The expression of two representative proteins, SOD2 and BLVRB, by western blot increased in CL of non-pregnant ewes on day 16 compared with that on day 12. SOD2 and BLVRB were localised in the large and small luteal cells and endothelial cells of CL over peri-implantation periods. 2DE gel and mass spectrometry have been used, for the first time, to study ovine CL function. We have identified proteins involved in key pathways, including oxidative stress, steroidogenesis, signal transduction and apoptosis, which have not previously been associated with changes occurring in the CL during the peri-implantation period. These proteins are most likely involved with mechanisms allowing the CL to produce P₄ during early pregnancy.

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Introduction

In mammals, the corpus luteum (CL) secretes progesterone (P₄) which is required for the establishment and maintenance of pregnancy. Rescue of the CL from periodic cyclic regression and the maintenance of P₄ production are crucial for uterine receptivity and are required for the implantation, survival and development of pre-implantation embryos, eventually leading to successful pregnancy outcomes (Schlafke & Enders 1975, Niswender *et al.* 2000). Many locally produced proteins are involved in CL function. Therefore, abnormal expression of luteal components may have adverse effects on the end target, the endometrium, leading to embryo–endometrial asynchrony, disruption of embryo implantation and failure to maintain

pregnancy (Tavaniotou *et al.* 2002, Mitwally *et al.* 2005). Luteal dysfunction, short luteal phases and subsequent endocrine abnormalities following the induction of ovulation are presumptive causes of early embryonic loss and pregnancy failure in livestock (Wathes 1992, Murdoch & Van Kirk 1998) and humans (Tavaniotou *et al.* 2002, Arredondo & Noble 2006). Defective P₄ production and/or inadequate luteal rescue by pituitary luteotrophic factors and trophoblast anti-luteolytic signals, produced by the conceptus during the peri-implantation period, are major causes of embryonic mortality and early recurrent abortions (Arredondo & Noble 2006) in humans (Porter & Scott 2005) and ruminants (Diskin & Morris 2008).

The CL is essential throughout pregnancy in the mouse, rat, hamster, rabbit, dog, sow and goat, but less than full term

in cat, guinea pig, sheep, cow, horse, macaque and human (Ryan 1969). In the latter species, the placenta subsequently becomes a predominant source of P_4 . The timing of this change varies between species: relatively early in pregnancy in the human (Csapo *et al.* 1972) and ovine (Al-Gubory *et al.* 1999) or during later gestation in bovine (Estergreen *et al.* 1967). The CL of the oestrous/menstrual cycle exhibits marked structural and functional alterations, depending on a balance between principal luteotrophic and luteolytic signals, LH and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) respectively (Vonnahme *et al.* 2006). In the absence of fertilization, the CL exhibits short periodic growth and differentiation followed by luteal regression, characterised by the loss of P_4 secretion and apoptosis (Al-Gubory *et al.* 2005, Vonnahme *et al.* 2006). Although several factors are known to be involved in the regulation of luteal growth and regression, these processes are not fully understood (Webb *et al.* 2002).

Much of the molecular data on CL formation, function and regression originate from genomic and transcriptomic studies, partly due to limited availability of protein tools, such as suitable antibodies (Gonzalez-Fernandez *et al.* 2008). However, one of the main problems with genomic studies is that gene expression and biological effects are associated with complex protein synthesis. It is not necessarily possible to accurately predict protein expression patterns from mRNA due to post-transcriptional mechanisms (mRNA export, surveillance, silencing and turnover) and post-translational modifications, which can determine protein activity, localisation, turnover and interactions with other proteins (Mann & Jensen 2003). Therefore, the application of proteomics is important for the understanding of physiological and biological processes (Fowler *et al.* 2007). We aimed to identify CL proteins altering during the peri-implantation periods of pregnant ewes compared with matching stages of the luteal phase of cyclic ewes in order to facilitate better understanding of the molecular mechanisms underlying CL regression and maintenance.

Materials and Methods

Animals and sample collection

The French Ministry of Agriculture approved all procedures relating to the care and use of animals according to regulations for animal experimentation (authorization no. 78–34). All ewes (Préalpes-du-Sud breed, 18 months of age) were treated for 14 days with intra-vaginal sponges containing 40 mg fluorogestone (Intervet, Angers, France) to synchronise oestrous. An i.m. injection of 400 IU equine chorionic gonadotropin (Intervet) was administered to each ewe immediately after the removal of the sponges. Ewes assigned to the pregnant group were mated twice at oestrous with fertile rams, at an interval of 12 h. Pregnancy was confirmed by recovery of a visibly normal conceptus (embryo and associated extra-embryonic membranes) in uterine flashings using 20 ml sterile PBS, pH 7.4. Pregnant ewes ($n=12$) were

killed according to the protocols approved by the local INRA animal use committee on days 12 (pre-implantation, P12), 16 (implantation, P16) and 20 (post-implantation, P20) of pregnancy ($n=4$ ewes per group). A total of eight non-mated ewes used as controls were killed on days 12 (C12, $n=4$ ewes) and 16 (C16, $n=4$ ewes) of the oestrous cycle. After killing, the reproductive tracts were collected and immediately transported to the laboratory where the CLs were dissected from the surrounding ovarian tissue. For immunohistochemistry, a piece of each CL tissue was fixed in freshly prepared 4% paraformaldehyde in PBS, pH 7.4, for 24 h and embedded in wax. The rest of the CL was snap-frozen in liquid nitrogen and stored at -80°C to be processed for two-dimensional gel electrophoresis (2DE) and western blot.

P_4 assay

Blood samples were obtained from the jugular veins of the cyclic (days 12 and 16) and pregnant ewes (days 12, 16 and 20) into evacuated heparinised tubes. After centrifugation (3000 g, 4°C) for 30 min, plasma was stored at -20°C until assayed. The concentrations of P_4 were determined by RIA in unextracted plasma as described (Schanbacher 1979) and validated for sheep jugular venous plasma (Al-Gubory *et al.* 2005) with slight modifications (Al-Gubory *et al.* 2006). Tritiated P_4 (1,2,6, 7- ^3H - P_4 , sp act 88 Ci/mmol) was obtained from Amersham, and a specific anti- P_4 antibody was obtained from the Institut Pasteur (Paris, France). All plasma samples were analysed in a single RIA. The limit of assay sensitivity was 0.1 ng/ml and the intra-assay coefficient of variation was $<10\%$.

Preparation of tissue samples for electrophoretic analyses

Luteal tissues were combined with 5 μl lysis buffer per 1 mg wet weight of tissue (Fowler *et al.* 2007) and disrupted using a tissue lyser (Qiagen Ltd) for 4 min. Insoluble materials were removed from the lysates by centrifugation for 30 min (50 000 g at 4°C). The protein concentration of the supernatants was determined using the Bio-Rad RC-DC protein assay (Bio-Rad) and proteins were stored at -80°C for further analysis.

2DE analysis

We selected 2DE for the proteomic analysis because it is a powerful and flexible technique for investigation of protein expression in multiple sample groups (Stephens *et al.* 2010). Equal amounts of protein from each ewe, in each group, were combined to make five protein pools as follows: cyclic ewes on days 12 (C12) and 16 (C16) and pregnant ewes on days 12 (P12), 16 (P16), and 20 (P20). 2DE was performed as described previously (Cash & Kroll 2003, Uwins *et al.* 2006). For first dimension separation, 200 μg total protein from CL lysate was loaded on 11 cm, immobilised, non-linear pH gradient 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 12 bit images. Protein spot profiles were analysed using Progenesis SameSpots version 3 Software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK). This software allows very robust analysis of 2DE gels (Silva *et al.* 2010) using automated spot detection algorithms. Spot numbers assigned by the software at the start of image analysis were retained throughout the study to reduce potential errors. A reference gel was selected and the other gels were aligned to be closely matched to this reference gel using semi-automated procedures. Every spot included in the study was visually checked for reproducibility and robust selection. Background (based on all non-spot areas of the gel) was individually subtracted from each gel and spot volumes were normalised relative to gel 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. In order to be considered suitable for inclusion in the experiment, the 1409 spots included in this study had to conform to criteria of robust reproducibility and accuracy of the semi-automated spot outlining routines among all four replicate gels for each group. To be considered significantly different, log-normalised spot volumes had to differ between two groups at the level of $P < 0.05$ by ANOVA and *post hoc* testing. In addition, the 24 protein spots selected for identification by liquid chromatography–tandem mass spectrometry (LC–MS/MS) had to include a difference of a ≥ 1.25 -fold increase or decrease between two groups and be sufficiently intensely stained to allow a high probability of a positive identification.

Mass spectrometry

In order to identify proteins, spots were excised from stained gels and subjected to in-gel trypsin digestion as described previously (Uwins *et al.* 2006). The peptide fragment mass spectra were acquired on a PerSeptive Biosystems Voyager-DE STR MALDI-TOF mass spectrometer operated in the reflection delayed extraction mode. Tryptic peptides from the MS/MS spectra were applied to search the National Centre for Biotechnology Information (NCBI) database with the MASCOT program (<http://www.matrixscience.com>). Search parameters for the programme that included maximum allowed error of peptide mass 250 ppm, cysteine as *S*-carbamidomethyl derivative and oxidation of methionine were allowed.

Western blot

Individual CL 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 (UK) 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: mouse anti-superoxide dismutase [Mn] (SOD2: Abnova Corp, Taipei, Taiwan, H00006648), 1–2500, and mouse anti-biliverdin reductase B (BLVRB, Abnova Corp, H00000645), 1–1000, all combined with an anti- α tubulin load control (mouse 1–10 000 ab7291, AbCam Ltd, Cambridge, UK). 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. Following the washing of membranes, the 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). Initially, we tested anti- β -actin (mouse: 1–5000 and rabbit: 1–10 000, both AbCam Ltd, ab6227 and ab8227 respectively) and then anti-GAPDH (rabbit: 1–5000, AbCam Ltd, ab9485) as load controls. However, the band volumes of β -actin and GAPDH were significantly different between the groups, demonstrating the unsuitability of these as load controls. In contrast, α -tubulin showed no significant differences ($P > 0.05$) between groups and was, therefore, employed as our load control.

Immunohistochemistry

CL sections of all groups: control groups on days 12 and 16 and pregnant groups on days 12, 16 and 20 (5 µm, two sections per slide) were either stained with haematoxylin and eosin (H&E) or mounted onto ChemMate slides (Dako-Cytomation Ltd, Ely, UK) and stained using the Bond-maX (Leica Microsystems Newcastle Ltd., Newcastle Upon Tyne, UK) automated immunostaining machine. For immunostaining, two consecutive sections (5 µm thickness) from all groups were used: one section for main antibody (SOD2/BLVRB) and the other for 3 β -hydroxysteroid dehydrogenase (3 β HSD) to label steroidogenic cells. Antigen retrieval (72 °C in EDTA-based buffer, pH 8.8) was performed *in situ* in the autostainer. Bond Polymer 3,3'-diaminobenzidine (DAB) Detection and Bond Polymer alkaline phosphatase (AP) Red Detection were employed to visualise the sections with DAB and AP staining respectively. For DAB staining, endogenous peroxidase activity was blocked using HRP for 10 min. The sections were then incubated with primary antibodies for 40 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.

The two antibodies used for DAB staining were i) SOD2 (1:500, with antigen retrieval: mouse polyclonal, Abnova Corp, H00006648) and ii) BLVRB (1:200, with antigen retrieval: mouse polyclonal, Abnova Corp, H00000645). Sections were visualised with Bond 'Refine' DAB, washed in water and counterstained with haematoxylin. For AP staining, the sections were incubated with primary antibody for 30 min. Then, the slides were incubated in post-primary antibody solution for 20 min and in poly-AP anti-mouse/rabbit IgG for 30 min. To visualise the desired antigen via a red precipitate, a substrate, chromogen, Fast Red, was applied for overall 20 min and then counterstained by 0.02% haematoxylin for 5 min. For AP staining, 3 β HSD antibody (1:200, with antigen retrieval: rabbit polyclonal, provided by Prof. J Ian Mason) was used. Negative control sections were included, incubated in the absence of the primary antibody, using non-immune serum, showed no positive immunostaining. Slides were assessed using an Olympus BX41 microscope (Olympus Microscopy, Southend-on-Sea, Essex, UK) and Progres CapturePro 2.6 image Software with a Progress C5 attachment (Jenoptik, Jena, Germany). Different cell types of the CL were recognised according to their shapes: large luteal cells (LLCs) and small LCs (SLCs) are round with a central big nucleus and endothelial cells are small and flat (O'Shea *et al.* 1979).

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 (IBM Ltd., Portsmouth, UK) 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. The Mann–Whitney unpaired two-sample tests were employed to analyse non-normally distributed data.

Results

CL function

Plasma concentrations of P_4 on day 16 of the oestrous cycle were lower ($P = 0.015$) than those on day 12 of the oestrous cycle and on days 12, 16 and 20 of pregnancy (Fig. 1). Concentrations of P_4 on day 12 of the oestrous cycle were similar to those on days 12, 16 and 20 of pregnancy (Fig. 1).

The CL proteome profile

Overall 1409 distinct protein spots were located in the 2DE gels, with representative spots shown in Fig. 2A. The numbers of ovine CL protein spots showing statistically significant differences in normalised spot volumes between groups are summarised in Table 1. Of the 24 protein spots excised for tandem mass spectroscopy, four spots (74, 145, 656 and 1036)

did not yield significant hits (MOWSE score < 150 , peptide coverage $< 5\%$). Some proteins (vimentin (VIM), glutathione S-transferase (GST), isocitrate dehydrogenase 1 (NADP⁺), soluble (IDH1), guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 (GNB2), apolipoprotein A-1 (APOA1) and interferon (IFN)-induced GTP-binding protein Mx1 (or myxovirus resistance protein1 (MX1))) were identified in more than a single 2DE protein spot. In order to maximise the accuracy of the analysis, normalised volumes of each spot containing the same protein were combined. This enabled statistical analysis to be performed, not just on individual spots but also on combined spot volumes where they shared a common protein, allowing a more accurate indication of overall abundance of those proteins.

Changes in CL proteins during CL regression

The greatest number of alterations in spot volumes ($P < 0.05$) was observed between the fully functional (day 12) and regressed CL (day 16): 139 protein spots were up-regulated and 69 were down-regulated (15% of total spots) in the regressed CL compared with functional CL (Table 1). The expression of VIM, IDH1, SOD2; Fig. 2B), BLVRB (Fig. 2C), NADPH, adrenodoxin oxidoreductase (mitochondrial FDXR), annexin A1 (ANXA1), cytochrome b-c1 complex subunit 1, mitochondrial (UQCRC1), elongation factor Tu, mitochondrial (TUFM) and GNB2 increased from day 12 to day 16 in CL of cyclic ewes (Table 2). However, only lamin A/C (LMNA) and APOA1 were significantly ($P < 0.05$) reduced in CL on day 16 compared with day 12 of the oestrous cycle (Table 2).

Figure 1 Concentrations of progesterone in plasma of jugular blood collected on days 12 (C12) and 16 (C16) of the oestrous cycle and on days 12 (P12), 16 (P16) and 20 (P20) of pregnancy. Progesterone concentrations declined in the plasma at the end of the oestrous cycle (C16) compared with mid luteal phase day (C12). Plasma progesterone concentrations were higher on day P16 than that at matching stage of the oestrous cycle (C16). Values are shown as mean $\pm$ s.e.m. (ng/ml, $n = 4$ per group).

Changes in CL proteins during early pregnancy

In total, 52 protein spots were significantly ($P < 0.05$) up-regulated and 14 down-regulated in CL of pregnant ewes at the time of implantation (day 16) compared with ewes on pre-implantation (day 12; Table 1). The smallest number of significant changes in protein spot volumes (13 up-regulated spots and 24 down-regulated in the CL of pregnant ewes on day 20 compared with day 16, $P < 0.05$) occurred between days 16 and 20 of pregnancy (Table 1). The expression of FDXR increased (6.07-fold) between pre-implantation and implantation; however, its expression fell at post-implantation (day 20; Table 2). The expression of GSTA1, ornithine aminotransferase, isoform 2 (OAT), GNB2 and MX1 significantly increased ($P < 0.05$) between days 12 and 16 and tended to increase on day 20 (Table 2). The expression of APOA1 secreted highly during implantation and early pregnancy. In addition, expression of enoyl-CoA hydratase and mitochondrial (ECHS1) declined 4.26-fold ($P < 0.05$) on day 20 compared with day 16 (Table 2).

Divergent protein profiles in CL of pregnancy compared with CL of the oestrous cycle

There were small but significant differences in the protein profiles between pregnant and cyclic groups on day 12 (P12 versus C12). At this stage, 21 normalised spot volumes were significantly ($P < 0.05$) up-regulated and 15 down-regulated (Table 1) in CL of pregnant ewes compared with the matching day of the luteal phase of cyclic ewes. Furthermore, reduced expression of FDXR and LMNA was observed in the CL of pregnant ewes compared with the CL of cyclic ewes

(Table 2). The normalised volumes of 178 spots were significantly ($P < 0.05$) altered in CL of pregnant ewes at the time of implantation compared with matching stage of the luteal phase of cyclic ewes: 77 up-regulated and 101 down-regulated in CL of pregnant ewes (Table 1). The expression of VIM and SOD2 (Fig. 2B) was higher (4.81- and 2.01-fold respectively, $P < 0.05$) in CL of cyclic ewes compared with CL of pregnant ewes at the time of implantation (Table 2). In contrast, the expression of MX1 and APOA1 was markedly elevated (4.52- and 1.29-fold respectively, $P < 0.05$) in CL of pregnant ewes. The expression of UQCRC1, FDXR, ANXA1 and BLVRB (Fig. 2C) was lower in the CL of pregnant ewes compared with cyclic ewes (Table 2). Comparing the P16 with C12 group enabled further analysis of differences between the pregnant and the non-pregnant but functional CL. Of the total protein spots, 51 were significantly

Table 1 Numbers of ovine corpus luteum protein spots significantly ($P < 0.05$) differing between both control (C) and pregnant (P) groups during the oestrous cycle and early implantation

Groups compared		Up-regulated	Down-regulated	Percentage of total spots
Group	Compared with			
C16	C12	139	69	15
P16	P12	52	14	5
P20	P16	13	24	3
P12	C12	21	15	3
P16	C16	77	101	13
P16	C12	51	21	5

Table 2 Ovine corpus luteum proteins identified from two-dimensional gel electrophoresis spots demonstrating significant differences in expression during pre-implantation, implantation and early pregnancy ($P<0.05$). Statistically significant ($P<0.05$) fold changes are shown in bold. Increases in spot volumes are denoted by '+' and decreases by '-' prefixes to the fold change values. The comparisons between groups (group 1 versus group 2) follow the rule that the fold changes are calculated on the basis that the first group is being compared with the second group. The accession numbers for bovine proteins are given. The accession numbers for ovine proteins are in brackets where available

Protein	Spot no.	MW (kDa)	PI	MOWSE score (MASCOT)	Swiss-prot	Fold change						
						C16 versus C12	P16 versus P12	P20 versus P16	P12 versus C12	P16 versus C16	P16 versus C12	
Structural proteins												
	VIM	1	53.8	5.06	828	P48616	+7.89	+2.75	-2.53	-1.67	-4.81	+1.63
		7	52.9	5.10	156							
LMNA	584	12.1	9.02	330	Q3SZI2	-1.87	+1.05	+1.41	-1.46	+1.35	-1.38	
Oxidative stress												
	GSTA1	27	25.5	8.66	516	Q28035	+1.38	+1.67	+1.01	-1.19	+1.01	+1.40
		1196	25.5	8.66	453							
SOD2	51	25.6	9.10	564	P41976	+1.86	+1.09	+1.17	-1.18	-2.01	-1.08	
BLVRB	589	22.2	6.58	197	P52556	+3.10	-1.00	+1.02	+1.49	-1.54	+1.82	
IDH1	6	47	6.13	1214	Q9XSG3	+2.01	+1.28	+1.06	+1.33	-1.17	+1.71	
	41	47.1	6.34	1231								
Steroidogenesis												
	APOA1	45	28.4	5.57	1308	P15497	-1.29	+1.53	+1.47	-1.53	+1.29	+1.00
		147	28.4	5.57	807							
FDXR	3	54.6	8.83	882	P08165	+3.21	+6.07	-1.76	-3.00	-1.59	+2.02	
ECHS1	1205	31.5	8.82	513	Q58DM8	-1.04	+1.56	-4.26	+1.09	+1.78	+1.71	
Signal transduction												
	ANXA1	26	39.1	6.44	568	P46193	+3.07	+1.68	+1.32	-1.51	-2.77	+1.10
	GNB2	158	36.3	6.06	391	P11017	+1.28	+1.46	+1.11	+1.01	+1.13	+1.45
	88	36.3	6.06	349								
Protein synthesis												
	TUFM	132	49.7	6.75	1235	P49410	+1.68	-1.34	+1.12	+1.11	-1.12	1.50
	OAT	547	48.8	6.77	179	Q3ZCF5	+1.65	+1.85	+1.05	+1.17	+1.32	+2.18
Electron transfer												
	UQCRC1	42	49.8	5.46	1238	P31800	+2.06	+1.57	+1.11	-1.12	-1.47	+1.39
Cytokine												
	MX1	4	75.9	5.74	563	P79135	-1.01	+5.87	+1.48	-1.31	+4.52	+4.46
		38	75.9	5.74	399							

VIM, vimentin; LMNA, lamin A/C; GSTA1, glutathione S-transferase A1; SOD2, mitochondrial super-oxide dismutase; BLVRB, biliverdin reductase B; IDH1, isocitrate dehydrogenase 1 (NADP+), soluble; APOA1, apolipoprotein A-1; FDXR, NADPH: adrenodoxin oxidoreductase, mitochondrial; ECHS1, enoyl coenzyme A hydratase, short chain, 1, mitochondrial; ANXA1, annexin A1; GNB2, guanine nucleotide-binding protein G(i)/G(s)/G(t) subunit beta-2; TUFM, elongation factor 1u, mitochondrial; OAT, ornithine aminotransferase, isoform 2; UQCRC1, cytochrome b-c1 complex subunit 1, mitochondrial; MX1, myxovirus resistance protein 1.

($P < 0.05$) up-regulated and 21 were down-regulated in the CL of pregnant ewes on day 16 compared with CL of cyclic ewes on day 12 (P16 versus C12; Table 1). The expression of IDH1, FDXR, GNB2, TUFGM, OAT and MX1 was significantly ($P < 0.05$) higher in the CL of pregnant ewes compared with cyclic ewes, whereas the expression of LMNA was significantly ($P < 0.05$) lower in CL of pregnant ewes (Table 2).

Validation of selected proteins

It was not possible to identify all the protein spots of interest and representative examples were identified by LC-MS/MS. Tightly controlled reactive oxygen species (ROS) generation is an important constitutive process and is one of the central elements in cell signalling (Finkel 1998), gene expression (Allen & Tresini 2000) and maintenance of redox homeostasis and signal transduction pathways involved in cell function, growth, differentiation and death (Valko *et al.* 2007). Therefore, immunohistochemistry was performed on two representative antioxidant proteins, SOD2 and BLVRB. These proteins are fundamental for CL function, especially during pregnancy (Sugino *et al.* 2000, Fang *et al.* 2004). They were also selected based on the availability of specific antibodies.

The expression of both SOD2 (Fig. 3A) and BLVRB (Fig. 3B) significantly increased ($P < 0.05$) in the individual CL of cyclic ewes on day 16 compared with day 12, that is in agreement with increased expression of these proteins at the late luteal phase in 2DE gel (Fig. 2B and C). The expression of both proteins by WB was greater ($P < 0.05$) in CL of cyclic ewes compared with CL of pregnant ewes on day 16 (Fig. 3A and B). SOD2, BLVRB and β HSD were immunolocalised in CL over peri-implantation periods. Positive SOD2 immunostaining was observed in all five groups in LCs and endothelial cells (Fig. 4A, left panel). BLVRB was localised to the cytoplasm of LLCs and SLCs (Fig. 4B, left panel). LLCs and SLCs showed β HSD-positive immunostaining compared with stromal cells, which were negative for β HSD (Fig. 4A, right panel; Fig. 4B, right panel). The pattern of immunostaining in CL from day 16 non-pregnant and pregnant ewes was similar to that shown in Fig. 4.

Discussion

We have demonstrated that 58% of detected protein spots in the ovine CL showed divergent expression during the periods of CL regression and maintenance. We identified 15 proteins with involvement in diverse CL functions including enzymatic antioxidant pathways and steroidogenesis, which are discussed below grouped by biological function.

Oxidative stress

ROS and antioxidant enzyme systems have been implicated in the regulation of reproductive processes, including ovarian

Figure 3 Western blot quantitation of two proteins identified from proteomic gel analysis. Each band is a representative band for a single CL. Band volumes normalised against α -tubulin are shown as mean $\pm$ S.E.M. ($n = 4$ per group). (A) SOD2 band volumes significantly increased ($P < 0.05$) in the CL of cyclic ewes at the end of the oestrous cycle. SOD2 expression declined in the CL of pregnant ewes compared with the CL of cyclic ewes at the time of implantation ($P < 0.05$). (B) BLVRB band volumes were significantly elevated in CL of cyclic ewes from days 12 to 16 and significantly reduced in CL of pregnant ewes in comparison with CL of cyclic ewes across the implantation period ($P < 0.05$).

follicular development, luteal steroidogenesis, endometrial receptivity and shedding, embryonic development and implantation (Al-Gubory *et al.* 2010). Cytosolic copper/zinc-containing SOD (Cu, Zn-SOD or SOD1) and mitochondrial manganese-containing SOD2 contribute to the first line of antioxidant pathway defence against ROS. Our finding of a significant increase in SOD2 expression in ovine CL in the late luteal phase agrees with the previous findings that CL SOD2 activity increases from mid luteal to late luteal phase in women (Sugino *et al.* 2000) and sheep (Al-Gubory *et al.* 2005). Moreover, SOD2 expression in CLs

Figure 4 Representative immunohistochemical localisation of two proteins identified from proteomic gel analysis in the CL of cyclic and pregnant ewes. 3 β HSD immunostaining in sections consecutive to SOD2 and BLVRB-immunostained sections is shown to highlight steroidogenic cells. (A) Left panel, SOD2 immunostaining; right panel, 3 β HSD immunostaining. (B) Left panel, BLVRB immunostaining; right panel, 3 β HSD immunostaining. (C12) Cyclic ewe on day 12 (P12), pregnant ewe on day 12 (IgG negative), negative control (non-immune serum). Black arrows indicate large luteal cells (LLCs), white arrows show small luteal cells (SLCs) and blue arrows show stromal cells. Scale bars: (A; C12) and (B; C12, P12), 100 μ m; (A; P12), 50 μ m.

of pregnant ewes on days 12, 16 and 20 did not alter, in agreement with the previous findings for SOD2 activity (Al-Gubory *et al.* 2004). SOD2 mRNA is induced in the rat CL by inflammatory cytokines (Sugino *et al.* 1998), which are involved in CL regression (Niswender *et al.* 2000). Therefore, SOD2 expression increasing during the late luteal phase may be up-regulated by inflammatory cytokines.

GSTs are involved in the intracellular transport of compounds and their delivery to sites for subsequent transformation and/or excretion (Kaplowitz 1980) and are important in detoxifying reactive metabolites. GSTs are abundant in steroid-producing ovarian cells throughout the menstrual cycle (Rahilly *et al.* 1991), in bovine follicular and LC (Rabahi *et al.* 1999), and increase in activity during growth of porcine ovarian follicles and CL (Keira *et al.* 1994). GSTA1 is involved in detoxifying environmental toxins and products of oxidative stress (Ahn *et al.* 2006) and binds to steroid hormone metabolites, which it helps transport intracellularly (Listowsky *et al.* 1998). Increased expression of GSTA1 in CL from day 12 to day 16 of pregnancy (this study) is likely an important mechanism protecting the early developing CL against ROS-mediated cell damage. This agrees with the observations that ovine CL GST activity significantly increases from day 15 to day 40 of pregnancy (Al-Gubory *et al.* 2004).

BLVRB catalyses the conversion of biliverdin to bilirubin (McDonagh *et al.* 1981), which is a potent antioxidant (Stocker *et al.* 1987, Kwak *et al.* 1991). The depletion of cellular bilirubin increases ROS production and induces cell death by apoptosis (Baranano *et al.* 2002). The catalysing activity of BLVRB is an important mechanism in cellular signal transduction pathways, gene expression and oxidative response (Florczyk *et al.* 2008). The increased expression of

BLVRB in CL of cyclic ewes on day 16 compared with day 12 (present study) may be an adaptive response mechanism to overcome ROS-induced oxidative damage.

Maintaining cellular redox state is one of the primary functions of NADP⁺-dependent IDH1 (Lee *et al.* 2002). IDH1 abundance and activity in the immature rat ovary increased markedly following gonadotropin treatment (Jennings & Stevenson 1991) and thus IDH1 may support ovarian development by maintaining cellular redox state and activities of ROS-scavenging enzymes. In our study, the expression of IDH1 tended to be more abundant in CL of cyclic rather than pregnant ewes at the time of implantation. However, in the absence of IDH1 activity data, this finding requires further study.

Steroidogenesis

High-density lipoprotein (HDL) is a main substrate for luteal steroidogenesis in rodents (Plump *et al.* 1996), human (Azhar *et al.* 1998) and pig (Miranda-Jiménez & Murphy 2007). The bovine CL synthesises APOA1, the main component of HDL, across the oestrous cycle and pregnancy (Ndikum-Moffor *et al.* 1997). Thus, APOA1 stimulates P₄ production in the CL and promotes PGE₂ production (a luteotrophic PG) (Chandras *et al.* 2004). In our study, APOA1 decreased from early to late luteal phase during CL regression but was highly expressed in the early CL of pregnancy at the time of embryo implantation (day 16) and post-implantation (day 20). The elevated expression of APOA1 in sheep CL during early pregnancy may, therefore, be related to the high demand for HDL required for P₄ production.

The rate-limiting step in steroidogenesis is the conversion of cholesterol to pregnenolone by CYP11A, in which

electrons are driven from NADPH through an electron transfer chain, consisting of FDXR and adrenodoxin (Hanukoglu 1996). The concentration of FDXR in porcine granulosa cells markedly increased during follicle growth and luteinisation (Tuckey & Stevenson 1986). Similarly, FDXR increases in growing CL of cow (Hanukoglu & Hanukoglu 1986) and rat (McLean *et al.* 1992), indicating increased CL steroidogenic activity due to FDXR induction. In this study, however, FDXR was, unexpectedly, more abundant during the late luteal phase in CL of cyclic ewes relative to CL of early pregnancy. In addition, FDXR expression increased in CL of pregnant ewes at the time of implantation (day 16) compared with pre-implantation (day 12). Surprisingly, there was a reduction in FDXR expression in CL of early pregnancy (present study), at a time when P₄ secretion is essential. This might be due to the presence of an inactive form of FDXR, or masking of true abundance by other proteins in the particular protein spot.

Signal transduction and apoptosis

ANXA1 plays a role in phospholipase A2 activity (Solito *et al.* 2003), as well as mediating apoptosis (Petrella *et al.* 2005). Therefore, it is logical that ANXA1 was dramatically up-regulated in the regressed CL in comparison with the functional CL (this study). On day 12 of pregnancy, the rat ovary expresses lower ANXA5 mRNA levels than in pseudopregnancy. Therefore, P₄ may down-regulate ANXA5 expression because the CL on day 12 of pregnancy secretes large amounts of P₄, although the pseudopregnant CL is regressed (Kawaminami *et al.* 2003). The sheep CL exhibits higher apoptotic DNA fragmentation during the late luteal phase than in early and mid luteal phase, concomitantly with a diminution in the plasma P₄ concentrations from mid to late luteal phase (Al-Gubory *et al.* 2005). Thus, high expression of ANXA1 in the regressed CL (present study) is likely because P₄ titres at the late luteal stage are inadequate to maintain ANXA1, contributing to a subsequent increase in apoptosis.

GNB2 has GTPase activity, participating in G protein-coupled receptor activity (Hamm 1998). Elevation of GNB2 expression in regressed CL (this study) is likely due to increased PGF_α levels during the late luteal phase. PGF_α, a stimulator of apoptosis in the sheep CL (Garrel *et al.* 2007), exerts its function through a G protein-coupled receptor on LCs. Thus, it can activate the Gq/phospholipase C/protein kinase C pathway, leading to a reduction in steroidogenesis (Yadav *et al.* 2005). In addition, GNB2 was highly expressed in CL at the time of implantation and early pregnancy (this study), possibly due to binding of LH to its G protein-coupled receptor on the LCs. This may result in the activation of cAMP/PKA signalling pathway that plays a role in the regulation of steroidogenesis (Chin & Abayasekara 2004, Stocco *et al.* 2007).

VIM may be involved in apoptosis, as a candidate substrate of caspase-mediated proteolysis (Prasad *et al.* 1999). Although VIM expression in the rat CL did not change during the luteal

life span (Nilsson *et al.* 1995), it was markedly increased in the regressed ovine CL (this study). We suggest that VIM may participate in LCs apoptosis, a major factor in the control of CL formation, function and regression (Stocco *et al.* 2007).

Protein synthesis

OAT is involved in the synthesis of polyamines (Wu *et al.* 2005), which play key roles in protein synthesis and cell proliferation and differentiation (Igarashi & Kashiwagi 2000). OAT activity in the porcine placenta was elevated during early pregnancy, reflecting the synthesis of polyamines essential for conceptus development through ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis (Wu *et al.* 2004, 2005). Inhibition of the preovulatory rise of ovarian ODC leads to a decrease in CL P₄ synthesis during the murine oestrous cycle (Bastida *et al.* 2002). In our study, OAT was most abundant in CL during early pregnancy. We propose that OAT plays a role in the steroidogenic pathway of ovine CL. This is supported by the fact that the OAT protein expression did not change from day 12 to day 16 of the oestrous cycle (this study).

Miscellaneous functions

MX proteins are anti-viral GTPases induced by cytokines, in particular type I IFNs (Thomas *et al.* 2006). MX mRNA expression is stimulated in the sheep endometrium (Charleston & Stewart 1993) and CL (Spencer *et al.* 1999) by IFN- τ (IFN τ). This is the ovine conceptus trophoblast signal for maternal recognition pregnancy between days 10 and 20 of pregnancy, with maximum production at the time of implantation (Gray *et al.* 2006). MX is highly expressed in the endometrium between days 13 and 20 of sheep pregnancy (Charleston & Stewart 1993). In this study, MX1 increased at the time of implantation and remained at high abundance during early pregnancy. Logically, this is due to MX1 induction by IFN τ because IFN τ can exert its effects: 1) directly on the CL, 2) indirectly via some mediators such as chemokines and 3) systemically by circulating immune system. Although MX1's function in the CL is not yet understood, it may participate in modulating immune system activity.

It is very difficult to characterise the entire luteal proteome because of the very large number of proteins contained in tissue lysates. Furthermore, one of the main limitations of the current proteomic technology is that low-abundance proteins in biological samples cannot be easily detected during the analysis of the proteome. Proteomics identifies alterations in proteins, thus further studies are required to determine whether those changes are of functional significance or due to tissue remodelling, for instance. Furthermore, most antibodies are not isoform specific. Therefore, where the 2DE gels yielded specific isoforms separately, the WB reflected total immunoreactive quantities of the protein and this may cause a disconnect between the 2DE and the WB

quantitations of proteins. Nevertheless, although there are limitations to the 2DE gel-based proteomic analysis, we have identified novel proteins, with potentially relevant biological functions, in ovine CL.

In conclusion, 2DE gel and mass spectrometry have been used for the first time to study ovine CL function. We have identified proteins involved in key pathways, including oxidative stress, steroidogenesis, signal transduction, and apoptosis, which have not previously been associated with changes occurring in the CL during the peri-implantation period. These proteins are most likely involved with mechanisms allowing rescue of the CL and maintenance of P₄ production during 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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