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Proteomic analysis of uterine fluid during the pre-implantation period of pregnancy in cattle

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Abstract

The aims of this study were i) to characterize the global changes in the composition of the uterine luminal fluid (ULF) from pregnant heifers during pregnancy recognition (day 16) using nano-LC MS/MS; ii) to describe quantitative changes in selected proteins in the ULF from days 10, 13, 16 and 19 by Isobaric tags for Relative and Absolute Quantification (iTRAQ) analysis; and iii) to determine whether these proteins are of endometrial or conceptus origin, by examining the expression profiles of the associated transcripts by RNA sequencing. On day 16, 1652 peptides were identified in the ULF by nano-LC MS/MS. Of the most abundant proteins present, iTRAQ analysis revealed that RPB4, TIMP2 and GC had the same expression pattern as IFNT, while the abundance of IDH1, CST6 and GDI2 decreased on either day 16 or 19. ALDOA, CO3, GSN, HSP90A1, SERPINA31 and VCN proteins decreased on day 13 compared with day 10 but subsequently increased on day 16 ($P<0.05$). Purine nucleoside phosphorylase (PNP) and HSPA8 decreased on day 13, increased on day 16 and decreased and increased on day 19 ($P<0.05$). The abundance of *CATD*, *CO3*, *CST6*, *GDA*, *GELS*, *IDHC*, *PNPH* and *TIMP2* mRNAs was greater ($P<0.001$) in the endometrium than in the conceptus. By contrast, the abundance of *ACTB*, *ALDOA*, *ALDR*, *CAP1*, *CATB*, *CATG*, *GD1B*, *HSP7C*, *HSP90A*, *RET4* and *TERA* was greater ($P<0.05$) in the conceptus than in the endometrium. In conclusion, significant changes in the protein content of the ULF occur during the pre-implantation period of pregnancy reflecting the morphological changes that occur in the conceptus.

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Introduction

Available evidence supports an unequivocal role for endometrial secretions as primary regulators of conceptus survival, growth and development during pregnancy (Gray *et al.* 2002, Spencer & Gray 2006). In cattle, following successful fertilization, the embryo enters the uterus at approximately day 4 (el-Banna & Hafez 1970, Hackett *et al.* 1993), where it undergoes a number of cell divisions to form a blastocyst by day 7. On days 9–10 following fertilization, the embryo hatches from the zona pellucida and undergoes significant morphological changes that result in the formation of an ovoid conceptus (embryo proper and associated extra-embryonic membranes) on day 13. Prior to initiation of implantation on days 19–20 (Bazer *et al.* 2009), the conceptus trophectoderm elongates to form a filamentous conceptus that eventually occupies both uterine horns. While the conceptus undergoes these morphological changes, the endometrium undergoes changes to establish uterine receptivity to implantation, defined as a physiological state of the uterus when conceptus growth and implantation for establishment of pregnancy is possible.

During this period of elongation, the conceptus is bathed in, and supported by, uterine secretions from the epithelial cells of the endometrium, which exhibit high secretory activity and expression of nutrient transporters during the luteal phase of the oestrous cycle and pre-implantation period of pregnancy (Guillomot *et al.* 1981). Both the onset and rate of conceptus elongation in ruminants are dependent on uterine secretions. For example, while elongation *in vitro* can be physically provoked by culture of blastocysts in agar tunnels (Brandao *et al.* 2004, Vajta *et al.* 2004), the embryonic disk characterizing the pre-streak stage 1 is never established (Vejlsted *et al.* 2006). Furthermore, uterine gland knockout ewes (Gray *et al.* 2002) exhibit recurrent early pregnancy loss due to inadequate conceptus elongation; the absence of specific components of uterine luminal fluid (ULF) derived from the endometrial glands and likely luminal epithelia is proposed to be the primary cause of this recurrent pregnancy loss (Gray *et al.* 2002).

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Changes in the rate of conceptus elongation have been clearly demonstrated by modulating circulating concentrations of progesterone (P_4) in both sheep and cows (Garrett *et al*. 1988, Carter *et al*. 2008, Satterfield *et al*. 2009). While supplementation of culture media with P_4 *in vitro* has no effect on the rate of elongation after transfer of blastocysts to recipient heifers (Clemente *et al*. 2009), supplementation of animals with P_4 advances conceptus elongation in both cattle (Carter *et al*. 2008, Clemente *et al*. 2009) and sheep (Satterfield *et al*. 2006). Conversely, reduction of P_4 output from the corpus luteum (CL) results in delayed elongation (Forde *et al*. 2011*a*, 2011*b*), with these alterations to the rate of conceptus elongation mediated through changes to the endometrial transcriptome (Forde *et al*. 2009, 2010, 2012*a*, 2012*b*, Satterfield *et al*. 2009).

The composition of ULF is only partially defined and consists of a rather complex mixture of proteins, amino acids, sugars, lipids and ions that are derived from genes expressed in the endometrium as well as molecules that are transported selectively from maternal blood, primarily via the uterine epithelia. The protein content of ULF in the bovine uterus is not well defined but includes enzymes, growth factors and cytokines (Spencer *et al*. 2008). Recent studies have defined transcriptomic changes that occur in the endometrium of cattle during the oestrous cycle (Bauersachs *et al*. 2005, Forde *et al*. 2011*a*, 2011*b*) and peri-implantation period of pregnancy (Mansouri-Attia *et al*. 2009, Forde *et al*. 2011*a*, 2011*b*, Bauersachs *et al*. 2012) as well as in response to P_4 (Forde *et al*. 2009, 2010, 2011*a*, 2011*b*). In particular, the transcriptomic changes induced by manipulation of P_4 concentrations and timing of the post-ovulatory increase in P_4 have demonstrable effects on conceptus elongation following transfer of blastocysts into synchronized recipients on day 7 (Clemente *et al*. 2009, Forde *et al*. 2011*a*, 2011*b*). In cattle, changes in concentrations of circulating P_4 affect the abundance of candidate proteins (Costello *et al*. 2010, Mullen *et al*. 2012*a*, 2012*b*) as well as amino acids, glucose and ion content of the ULF (Hugentobler *et al*. 2010).

Despite extensive knowledge of the endometrial transcriptome, relatively little information has been published on the physical content of bovine ULF, particularly during early pregnancy. Some recent studies have described the protein content of ULF in cyclic heifers (Mullen *et al*. 2012*a*, 2012*b*), at a single time-point either considerably before (day 7: Faulkner *et al*. 2012) or after (day 18: Ledgard *et al*. 2009) pregnancy recognition, or in non-physiological models involving the transfer of up to 50 embryos per recipient (day 7: Munoz *et al*. 2012). This study provides new knowledge regarding the composition and quantitative changes in ULF during key morphological events in early pregnancy in cattle. The specific aims were to i) characterize the global changes in the composition of the ULF from pregnant heifers during pregnancy recognition (day 16) to identify the proteins that are present and potentially implicated in driving conceptus elongation; ii) describe quantitative changes in ULF protein content from hatching of the embryo from the zona pellucida (days 9–10) through the initiation of conceptus elongation (day 13) to pregnancy recognition (day 19) and beginning of implantation (day 19); and iii) determine whether these proteins are of endometrial or conceptus origin, by examining the expression profiles of the associated transcripts in both tissue types on day 16 of pregnancy using RNA sequencing technology.

Materials and methods

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act 1876 (Ireland) and the European Community Directive 86/609/EC and were sanctioned by the Animal Research Ethics Committee of University College Dublin. Unless otherwise stated, all chemicals and reagents were sourced from Sigma.

Animal model

The oestrous cycles of cross-bred beef heifers were synchronized using a controlled internal drug release (CIDR) device (1.38 g P_4; InterAg, Hamilton, New Zealand) placed intravaginally for 8 days. A 2 ml i.m. injection of a prostaglandin $F_{2\alpha}$ (PG) analogue (Estrumate, Shering-Plough Animal Health, Welwyn Garden City, Herts, UK: equivalent to 0.5 mg cloprostenol) was administered 1 day prior to CIDR removal. Following CIDR removal, animals were observed for oestrous behaviour and only those observed in standing oestrus (day 0; $n=45$) were inseminated with semen from a bull of proven fertility. Heifers were then assigned randomly for killing on day 10, 13, 16 or 19 following oestrus representing the beginning of conceptus elongation, pregnancy recognition and initiation of implantation respectively. At killing, the reproductive tract of each heifer was recovered and both uterine horns were flushed with 20 ml of a 10 mM Tris solution (pH 7.2). Only those heifers from which an appropriately developed conceptus was recovered were further processed, i.e. day 10 hatched blastocyst, day 13 ovoid conceptus and day 16 elongated conceptus (10 ± 0.7 cm). Following recovery of the ULF from the uterine horn ipsilateral to the corpus luteum, samples were centrifuged at 1000 ***g*** for 15 min at 4 °C. The supernatant was removed from the pelleted debris and snap frozen in 1 ml aliquots and stored at −80 °C prior to analysis.

Global proteomic analysis of ULF collected during pregnancy recognition in cattle

Identification of proteins in ULF was performed by Applied Biomics, Inc. (Hayward, CA, USA). In order to identify what proteins are present in the ULF of pregnant heifers on day 16, nano-LC MS/MS was carried out on $n=4$ individual samples of ULF from confirmed pregnant heifers on day 16 of pregnancy.

Samples were exchanged into 50 mM ammonium bicarbonate buffer. Dithiothreitol was then added to a final concentration of 10 mM, and samples were incubated at 60 °C for 30 min followed by cooling to room temperature (RT). Iodoacetamide was added to a final concentration of 10 mM and incubated in the dark for 30 min at RT. A tryptic digestion was performed at 37 °C overnight. Nano-LC was carried out using a Dionex Ultimate 3000 (Thermos Scientific, Milford, MA, USA). Tryptic peptides were loaded into an α-Precolumn Cartridge and separated using an acetonitrile gradient (ranging from 5 to 60%) on the nano-LC column. Fractions were collected at 20-s intervals followed by mass spectrometry analysis on AB SCIEX TOF/TOF 5800 System (AB SCIEX, Framingham, MA, USA). Mass spectra were acquired in reflectron-positive ion mode. TOF/TOF tandem MS fragmentation spectra were acquired for each ion, averaging 4000 laser shots per fragmentation spectrum (excluding trypsin autolytic peptides and other known background ions). Identification of the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix Science, London, UK) to search the non-redundant database of National Center for Biotechnology Information. Searches were performed without constraining protein molecular weight or pI, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and with one missed cleavage also being allowed in the search parameters. In addition, a false discovery rate of 3.11% for day 16 was identified by submitting the list of identified peptides to a decoy database and significant hits were determined when $P<0.05$.

Overrepresented ontology groups and pathway analysis

To further interrogate the data generated, the list of proteins identified in at least 3/4 samples generated by nano-LC MS/MS was analysed using the functional annotation tool in DAVID (http://david.abcc.ncifcrf.gov/). The lists of detected proteins were converted into their corresponding gene identifiers and the overrepresented gene ontologies and KEGG pathways associated with the lists of proteins were determined, i.e. more proteins present in a given ontology or pathway than would be expected by chance.

Quantitative changes in protein abundance during the pre-implantation period of pregnancy

Quantitative changes in the protein content of the ULF were carried out by the Proteome Factory (Berlin, Germany) via Isobaric tags for Relative and Absolute Quantification (iTRAQ) eight-plex analysis. For each individual ULF sample ($n=4$ confirmed pregnant heifers from days 10, 13, 16 and 19 of pregnancy), 2 ml was precipitated in 10 ml 100% EtOH overnight. The resulting pellet was washed twice and resuspended in 40 µl of lysis buffer (20 mM TEAB, 5 mM TCEP, 0.1% SDS, 2 mM pefablock, 2.1 µM leupeptin and 2 mM benzamidine) and centrifuged at 13 000 ***g***. The supernatant was transferred to a clean tube, to which 10 mM iodoacetamide was added and this was incubated for 30 min at RT in the dark. Protein concentration was determined by Bradford assay and 100 µg total protein was subjected to trypsin digestion (Promega) at 37 °C overnight. Additional trypsin was added and the reaction continued for a further 3 h. The resulting peptides were acidified with formic acid (pH 2.0), desalted with Macro spin tips containing Vydac C18 material (Nest Group, Southborough, MA, USA) and lyophilized.

All samples were dissolved in 45 µl iTRAQ buffer (AB SCIEX) and 30 µl of each sample were reacted with appropriate iTRAQ reagent (Supplementary Tables 10 and 11, see section on supplementary data given at the end of this article) for 2 h at RT as per the manufacturer's protocol. The reaction was stopped with 50 µl of 20% formic acid (pH 2.0) and dried by lyophilization. Strong cation exchange (SCX) was performed on a PolySULFOETHYL A column (200 mm×2.1 mm, 5 µm, 200 Å, PolyLC, Columbia, MD, USA) using an Agilent 1100 HPLC system (Agilent, Karlsruhe, Germany) with 18 fractions collected per sample. Protein identification and quantification of iTRAQ reporter ions for each of the 18 SCX fractions were performed once using nano-LC-ESIMS/MS. The MS system consisted of an Agilent 1100 nano-LC system (Agilent), PicoTip emitter (New Objective, Langhorne, PA, USA) and a QExactive quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific, Bremen, Germany). The dried SCX peptide fractions were resuspended in 80 µl of MilliQ water containing 0.1% formic acid and 1% acetonitrile. After trapping 40 µl of each sample, the peptides were desalted for 5 min on an enrichment column (Zorbax SB C18, 0.3 mm×5 mm, Agilent) using a solution of 1% acetonitrile and 0.1% formic acid solution. All peptides were separated on a Zorbax 300 SB C18, 75 µm×150 mm column (Agilent) for 110 min, using an acetonitrile gradient containing 5–25% acetonitrile in 0.1% formic acid. The mass spectrometer was operated in a data-dependent mode by subjecting the ten most abundant ions of each survey spectrum (nominal resolution 35 000 at m/z 200) to HCD fragmentation (normalized collision energy at 40%, resolution 17 500 at m/z 200). MS/MS peak lists were extracted to mascot generic format files and searched by the Mascot search algorithm against the bovine IPI database (version 3.66) that had been curated from duplicate entries. The mass tolerance was set to 5 ppm for peptide masses and 0.02 Da for fragment ions. Protein identification and quantification were performed using Mascot version 2.2 (Matrix Science). For protein quantification, a significance threshold of $P<0.05$ (false discovery rate (FDR) 1%) and at least two peptides were required with the additional settings of protein ratio type=weighted, normalization=summed intensities and automatic outlier removal used.

Gene expression analysis in the endometrium and conceptus

Previously generated RNA sequencing data were interrogated in an attempt to determine the origin (endometrium or conceptus) of the proteins detected in the ULF. RNA was extracted from intercaruncular endometrial or conceptus tissues from pregnant heifers on days 13 or 16 ($n=5$ per day) as described previously (Mamo *et al.* 2011, Forde *et al.* 2012*a*, 2012*b*). Library preparation and cluster generation were performed as per manufacturer's instructions (www.illumina.com) and gene expression analysis was carried out on the

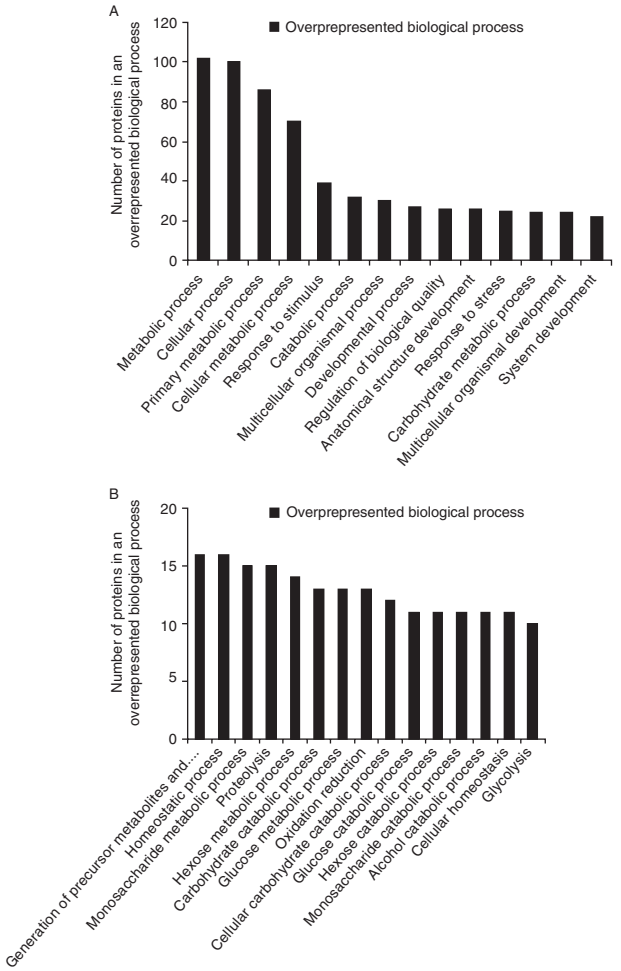

Figure 1 Gene ontology (GO) analysis depicting the proteins in uterine luminal fluid on day 16 of pregnancy that are overrepresented in (A) all biological processes and (B) GO fat terms for biological processes. The numbers represent the number of proteins associated with a given biological process (or GO FAT Term) identified on day 16 of pregnancy. All biological processes are significantly overrepresented in each sample set, i.e. more proteins detected in a specific biological process than would be expected by chance.

Illumina GA2 sequencer using the standard Illumina protocol for sequencing cDNA samples. The resulting 32 bp reads were processed through the standard software pipeline for the Genome Analyzer and aligned against the BosTau4 genome. A pseudochromosome containing potential splice junction sequences was generated. The ensGene table from the UCSC genome browser (http://hgdownload.cse.ucsc.edu/goldenPath/bosTau4/database/ensGene.txt.gz: Oct 2007 BosTau4) was used to provide exon location information to the CASAVA module. The moderated negative binomial test from the edgeR Bioconductor library (Robinson *et al.* 2010) was used to generate the lists of differentially expressed RNAseq transcripts, which were displayed as transcripts per million. An FDR adjusted P value of <0.05 was used as the cut-off for determining significance. The comparative analysis was restricted to the 26 957 protein coding transcripts in version 52 of Ensembl (www.ensembl.org) (Figs 1, 2 and 3).

Results

Changes in the ULF proteome during pregnancy recognition in cattle

Analysis of the ULF revealed 1652 detectable peptide sequences on day 16 of pregnancy with a range of 610–728 per sample (Supplementary Tables 1, 2, 3, 4, 5, 6, 7, 8 and 9, see section on supplementary data given at the end of this article). Three hundred and five proteins were detected in three of the four samples analysed while 600 were identified in two of the four samples. On day 16 of pregnancy, the most abundant proteins detected were three isoforms of albumin, haemoglobin subunit β, three

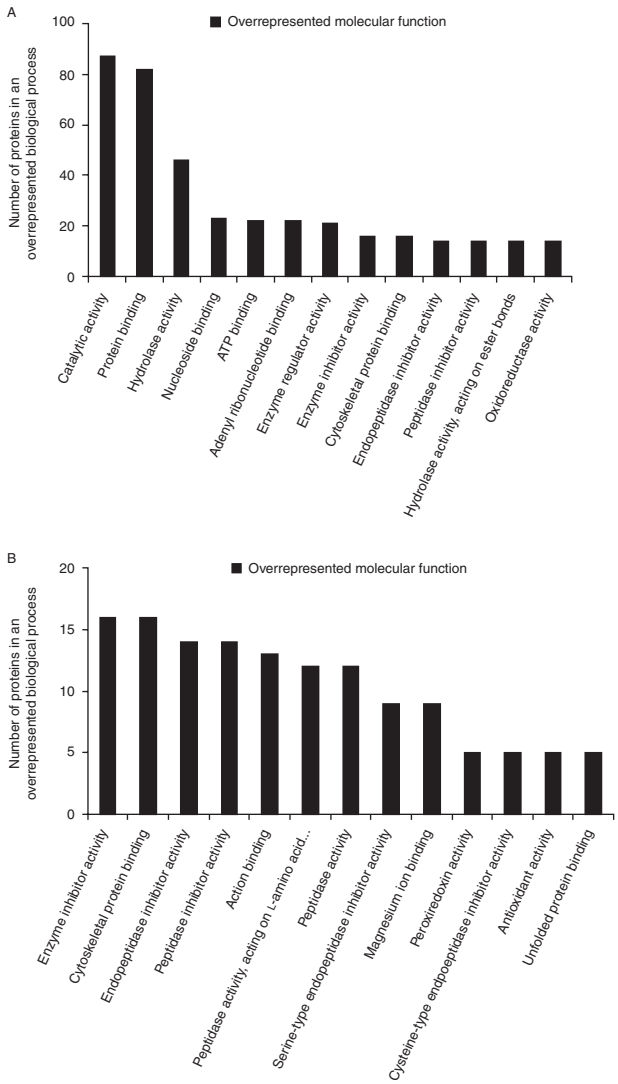

Figure 2 Gene ontology (GO) analysis depicting the proteins in uterine luminal fluid on day 16 of pregnancy that are overrepresented in (A) all molecular functions and (B) GO fat terms for molecular functions. The numbers represent the number of proteins associated with a given molecular function (or GO FAT Term) identified on day 16 of pregnancy. All molecular functions are significantly overrepresented in each sample set, i.e. more proteins detected in a specific biological process than would be expected by chance.

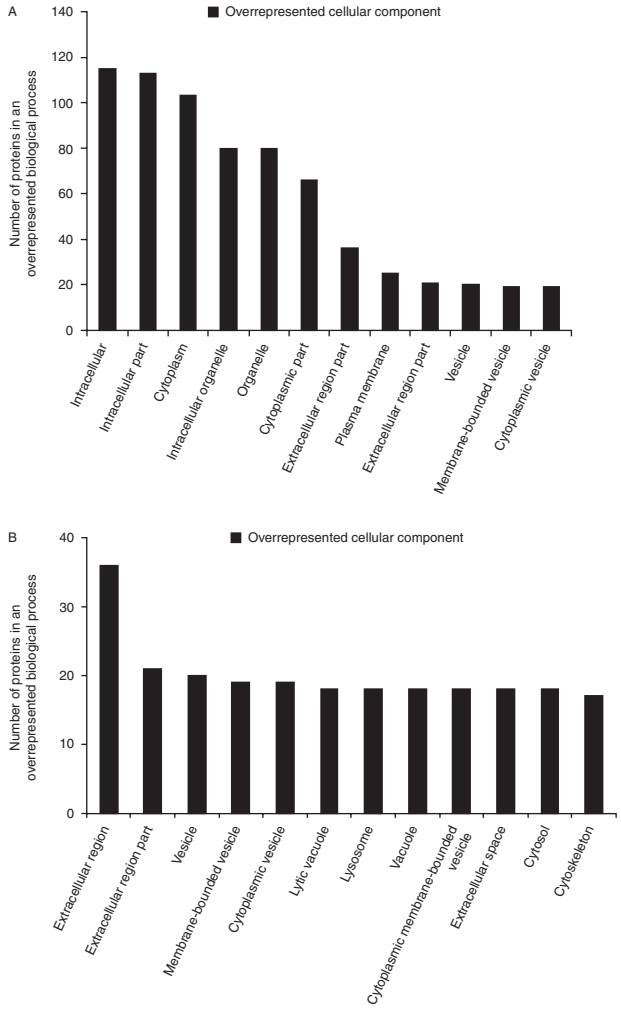

Figure 3 Gene ontology (GO) analysis depicting the proteins in uterine luminal fluid on day 16 of pregnancy that are overrepresented in (A) all cellular components and (B) GO fat terms for cellular component. The numbers represent the number of proteins associated with a given cellular component (or GO FAT Term) identified on day 16 of pregnancy. All cellular components are significantly overrepresented in each sample set, i.e. more proteins detected in a specific biological process than would be expected by chance.

isoforms of serotransferrin precursor (TF), two of ovalbumin as well as isocitrate dehydrogenase [NADP] cytoplasmic (IDHA), purine nucleoside phosphorylase (PNP), cystatin-M precursor (CST6), retinol-binding protein 4, aldose reductase (ALDR), cathepsin D (CATD), heat-shock cognate 71 kDa protein (HSP7C), actin, cytoplasmic 1 (ACTB), prepro complement component C3 (CO3), cathepsin B precursor (CATB), PREDICTED: transitional endoplasmic reticulum ATPase isoform 3 (*Canis lupus familiaris*), heat-shock protein HSP 90-alpha, fructose-bisphosphate aldolase A (ALDOA), guanine deaminase (GDA), rab GDP dissociation inhibitor beta (GD1B), legumain precursor, metalloproteinase inhibitor 2 precursor (TIMP2), CAP1 protein (CAP1), gelsolin isoform b (GELS), serpin A3-1 precursor (SERPINA31) and vitamin D-binding protein precursor (Table 1).

Overrepresented gene ontology categories and pathways associated with proteins in the ULF during pregnancy recognition

On day 16 of pregnancy, there were 177 overrepresented biological processes associated with proteins in the ULF. The top ten terms identified, i.e. those with the largest numbers of proteins, were associated with metabolic process (47.6% of all genes associated with this gene ontology (GO) term: Fig. 4 left panel), cellular process (46.7%), primary metabolic process (40.2%), cellular metabolic process (32.7%) and response to stimulus (18.2); catabolic process (14.9%), multicellular organismal process (14.0%), developmental process (12.6%), regulation of biological quality (12.2%) and anatomical structure development (12.2%). The top ten GO FAT biological processes (Fig. 4 right panel) were generation of precursor metabolites and energy (16 proteins), homeostatic process (16), monosaccharide metabolic process (15), proteolysis (15), hexose metabolic process (14), carbohydrate catabolic process (13), glucose metabolic process (13), oxidation reduction (13), cellular carbohydrate catabolic process (12) and glucose catabolic process (11).

Proteins involved in the top ten molecular functions of catalytic activity (40.7%), protein binding (38.3%), hydrolase activity (21.5%), nucleoside binding (10.7%), ATP binding (10.3%), adenyl ribonucleotide binding (10.3%), enzyme regulator activity (9.8%), enzyme inhibitor activity (7.5%), cytoskeletal protein binding (7.5%) and endopeptidase inhibitor activity (6.5%) were detected in ULF on day 16 (Fig. 5 left panel), with similar GO FAT molecular function terms including enzyme inhibitor activity (16 proteins), cytoskeletal protein binding (16), endopeptidase inhibitor activity (14), peptidase inhibitor activity (14), actin binding (13), peptidase activity, acting on L-amino acid peptides (12), peptidase activity (12), serine-type endopeptidase inhibitor activity (9), magnesium ion binding (9) and peroxiredoxin activity (5: Fig. 5 right panel). Similar GO and GO FAT terms for cell component were observed as overrepresented on day 16 of pregnancy and included extracellular region (36 proteins), extracellular region part (21), vesicle (20), membrane-bounded vesicle (19), cytoplasmic vesicle (19), lytic vacuole (18), lysosome (18), vacuole (18), cytoplasmic membrane-bounded vesicle (18) and extracellular space (18: Fig. 5).

In total, the proteins in the ULF on day 16 were overrepresented in 20 distinct KEGG pathways (Table 2). The top five pathways included proteins involved in lysosome (18 proteins), glycolysis/gluconeogenesis (11), regulation of actin cytoskeleton (10), antigen processing

Table 1 Top 20 proteins, as determined by total peptide counts, identified in the uterine luminal fluid of cattle ($n=4$) on day 16 of pregnancy as determined by nano-LC MS/MS. A dashed line in a cell indicates that specific protein type was not identified in that animal.

		Heifer 1		Heifer 2		Heifer 3		Heifer 4	
Accession no.	**Protein name**	Rank	emPAI	Rank	emPAI	Rank	emPAI	Rank	emPAI
gi|30794280	Serum albumin precursor (*Bos taurus*)	1	104.15	1	59.78	1	59.01	1	154.08
gi|74267962	ALB protein (*Bos taurus*)	2	104.94	2	44.84	–	–	2	86.76
gi|229552	Albumin	3	52.50	3	33.83	2	31.42	3	85.24
gi|114326282	Serotransferrin precursor (*Bos taurus*)	4	20.47	4	9.79	3	8.06	4	20.88
gi|2501351	RecName: full=serotransferrin; short=transferrin; AltName: full=β-1 metal-binding globulin; AltName: full=siderophilin; flags: precursor	5	17.90	–	–	–	–	–	–
gi|75832090	Isocitrate dehydrogenase [NADP] cytoplasmic (*Bos taurus*)	6	9.26	5	13.32	6	7.95	14	5.80
gi|51247896	Chain A, calf spleen purine nucleoside phosphorylase (Pnp) binary complex with 9-(5,5-difluoro-5-phosphonopenthyl)guanine	7	12.60	6	9.14	8	8.69	7	9.14
gi|1042206	Purine nucleoside phosphorylase, PNP, purine nucleoside:orthophosphate ribosyltransferase (EC 2.4.2.1) (cattle, spleen, peptide, 284 aa)	8	11.06	7	9.47	9	7.58	8	9.47
gi|61097917	Cystatin-M precursor (*Bos taurus*)	9	47.06	8	48.09	4	60.38	23	18.99
gi|262073106	Cathepsin D precursor (*Bos taurus*)	10	5.61	26	3.27	14	5.43	22	4.34
gi|132403	RecName: full=retinol-binding protein 4; AltName: full=plasma retinol-binding protein; short=PRBP; short=RBP	11	15.16	16	12.00	5	37.89	24	5.46
gi|113594	RecName: full=aldose reductase; short=AR; AltName: full=20-α-hydroxysteroid dehydrogenase; short=20-α-HSD; AltName: full=aldehyde reductase	12	6.87	14	8.15	7	7.76	12	9.51
gi|12697815	Cathepsin D (*Bos taurus*)	13	5.58	33	3.14	19	4.71	34	3.66
gi|148887198	RecName: full=heat-shock cognate 71 kDa protein; AltName: full=heat-shock 70 kDa protein 8	14	2.05	–	–	11	1.80	6	4.02
gi|4501885	Actin, cytoplasmic 1 (*Homo sapiens*)	15	2.27	24	2.29	–	–	–	–
gi|83764016	Prepro complement component C3 (*Bos taurus*)	16	0.61	227	0.08	140	0.20	43	0.53
gi|27806671	Cathepsin B precursor (*Bos taurus*)	17	4.03	19	3.44	23	3.92	35	2.38
gi|73971210	PREDICTED: transitional endoplasmic reticulum ATPase isoform 3 (*Canis lupus familiaris*)	18	1.72	209	0.32	71	0.47	28	1.07
gi|297470328	PREDICTED: ovalbumin (*Bos taurus*)	19	3.51	20	3.55	12	4.55	60	1.26
gi|60592792	Heat-shock protein HSP 90-α (*Bos taurus*)	20	2.05	60	1.03	31	1.52	18	1.89
gi|156120479	Fructose-bisphosphate aldolase A (*Bos taurus*)	28	2.50	9	7.52	10	5.38	16	4.84
gi|56119114	Purine nucleoside phosphorylase (*Bos taurus*)	–	–	10	7.69	15	6.16	10	6.44
gi|78101017	Chain A, crystal structure of bovine Hsc70(Aa1–554)e213aD214A MUTANT	–	–	11	3.03	–	–	–	–
gi|296484769	TPA: guanine deaminase (*Bos taurus*)	–	–	12	3.32	27	2.46	31	3.76
gi|108750	Ig heavy chain precursor (B/MT.4A.17.H5.A5) – bovine	29	1.19	13	2.26	26	1.16	11	1.95
gi|157834116	Chain A, purine nucleoside phosphorylase	–	–	15	6.82	22	5.43	–	–
gi|91982959	Immunoglobulin γ1 heavy-chain constant region (*Bos taurus*)	22	2.01	17	4.26	29	1.96	9	3.58
gi|76253900	Rab GDP dissociation inhibitor β (*Bos taurus*)	66	0.99	18	1.96	34	1.89	37	1.68
gi|297470328	PREDICTED: ovalbumin (*Bos taurus*)	19	3.51	20	3.55	12	4.55	60	1.26
gi|27806555	Legumain precursor (*Bos taurus*)	38	2.34	42	1.75	13	4.38	145	0.66
gi|294459577	Hemoglobin β (*Bos taurus*)	–	–	53	20.24	16	26.13	41	20.24
gi|296476071	TPA: metalloproteinase inhibitor 2 precursor (*Bos taurus*)	21	3.98	38	3.11	17	4.95	75	1.24
gi|152001126	CAP1 protein (*Bos taurus*)	–	–	–	–	18	3.18	29	1.40
gi|313507212	Chain A, the structure of crystalline profilin-β-actin	–	–	25	2.30	20	1.87	20	3.19
gi|296490958	TPA: serotransferrin precursor (*Bos taurus*)	–	–	–	–	–	–	5	18.32
gi|113594	RecName: full=aldose reductase; short=AR; AltName: full=20-α-hydroxysteroid dehydrogenase; short=20-α-HSD; AltName: full=aldehyde reductase	12	6.87	14	8.15	7	7.76	12	9.51
gi|157832110	Chain A, structural basis of the 70 kDa heat-shock cognate protein Atp hydrolytic activity, Ii. Structure of the active site with Adp or Atp bound to wild-type and mutant Atpase fragment	–	–	–	–	32	2.97	13	7.25
gi|77736201	Gelsolin isoform b (*Bos taurus*)	23	1.09	88	0.75	65	0.62	15	1.24
gi|31340900	Serpin A3-1 precursor (*Bos taurus*)	73	1.12	45	1.64	–	–	17	2.64
gi|296486435	TPA: vitamin D-binding protein precursor (*Bos taurus*)	79	0.59	93	0.75	111	0.44	19	2.37

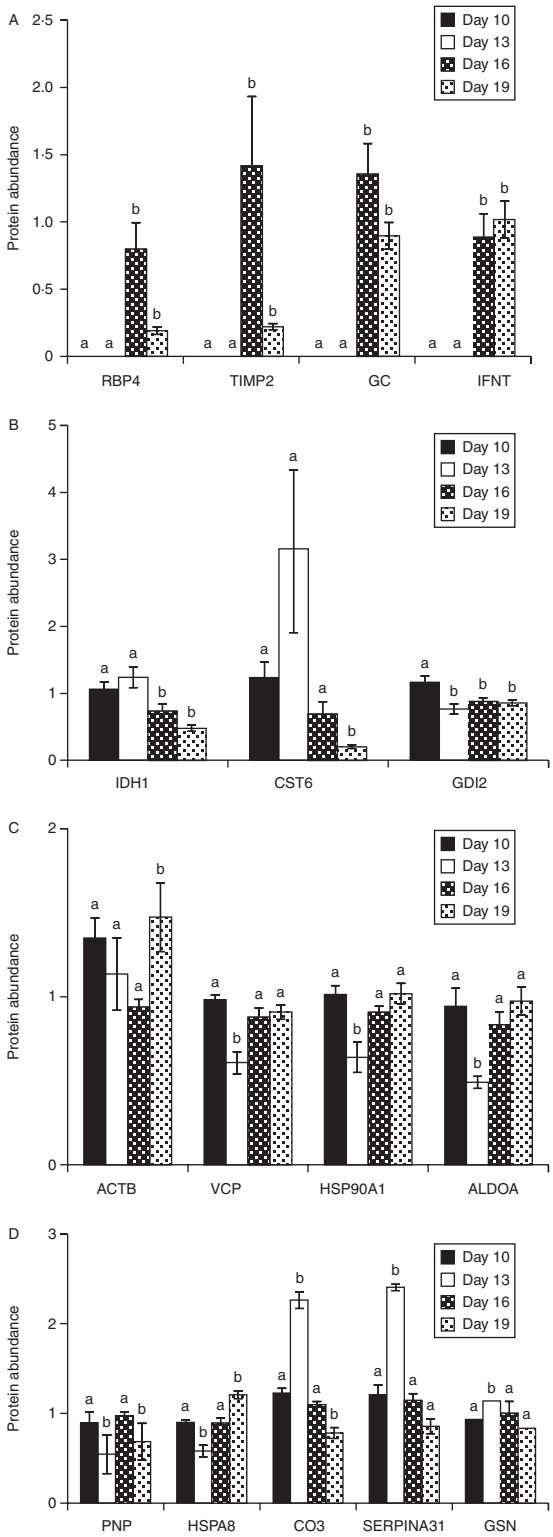

Figure 4 (A, B, C, and D) Quantitative differences in the abundance of proteins in the uterine luminal fluid on days 10, 13, 16 and 19 of pregnancy as determined by iTRAQ analysis. Significant differences in protein abundance between sequential days of pregnancy, i.e. day 10 vs 13 and day 16 vs 19) are indicated by different superscript letters (a,b) when $P<0.0$.

and presentation (9) and leukocyte transendothelial migration (7).

Quantitative changes in protein abundance in ULF during the pre-implantation period of pregnancy

The temporal changes in proteins not derived from albumin that were most abundant on day 16 were investigated using iTRAQ analysis (Fig. 4A, B, C and D), which detected 17 of these proteins (for a full list of proteins detected by iTRAQ analysis, see Supplementary Tables 10 and 11). Four distinct categories of proteins were identified. RPB4, TIMP2 and GC had the same expression pattern as IFNT, with no protein detected on day 10 or 13 but detected on both days 16 and 19 (Fig. 4A: $P<0.05$). The abundance of IDH1, CST6 and GDI2 decreased on either day 16 or day 19 of pregnancy (Fig. 4B). Three proteins decreased significantly on day 13 compared with day 10 but subsequently increased on day 16, which was maintained to day 19 of pregnancy (Fig. 4C). CO3, SERPINA31 and GSN increased on day 13 but decreased on subsequent days of pregnancy while PNP and HSPA8 decreased on day 13, increased on day 16 and decreased and increased on day 19 respectively (Fig. 4D).

Putative source of protein based on RNA sequencing analysis of the endometrium and conceptus

In order to gain some insight into whether the proteins detected in the ULF were predominantly of endometrial or conceptus origin, RNA sequencing data for both the endometrium and conceptus on days 13 and 16 was screened to assess transcript abundance for these proteins. The abundance of *CATD*, *CO3*, *CST6*, *GDA*, *GELS*, *IDHC PNPH* and *TIMP2* mRNAs was greater ($P<0.001$) in the endometrium than in the conceptus on both days examined (Fig. 5A and B). By contrast, the abundance of *ACTB*, *ALDOA*, *ALDR CAP1*, *CATB*, *CATG*, *GD1B*, *HSP7C*, *HSP90A*, *RET4* and *TERA* was greater ($P<0.05$) in the conceptus than in the endometrium on one or both days examined (Fig. 5B and C: $P<0.05$).

Discussion

This study characterized the protein content of ULF during key stages of peri-implantation conceptus development in cattle and demonstrates that significant changes in the abundance of a number of proteins occur as pregnancy progresses towards implantation. These novel proteomic data have been expanded by placing them in the context of RNA sequencing data to try to identify the putative source of the most abundant proteins identified in the ULF during the pregnancy recognition period. The abundance of *CATD*, *CO3*,

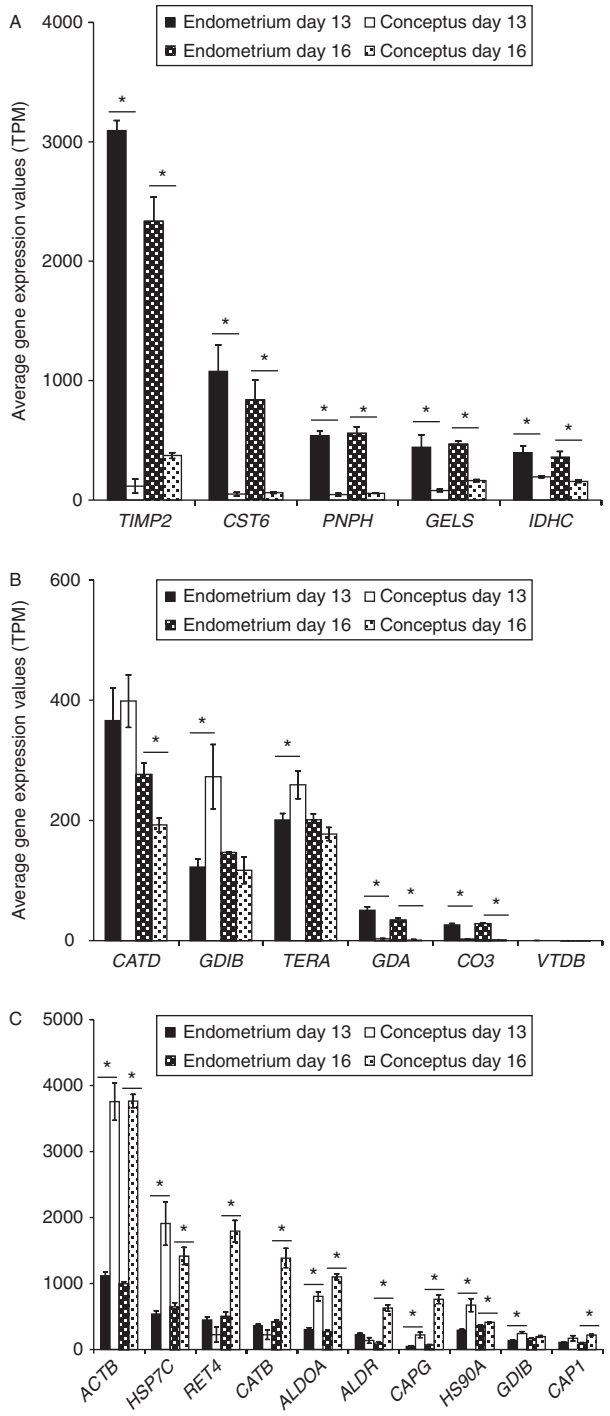

Figure 5 Abundance of RNAs whose protein products were detected in the uterine luminal fluid by nano-LC MS/MS analysis during pregnancy recognition (day 16) in cattle. Data are displayed as mean abundance in transcripts per million (TPM ± S.E.M.) in pregnant endometrium on day 13 (Solid black bars: $n=5$); conceptus tissue on day 13 (solid white bars: $n=5$); pregnant endometrium on day 16 (black bars, white stipple: $n=5$) and conceptus tissue on day 16 (white bars, black stipple: $n=5$). (A) Genes predominantly expressed in the endometrium, (B) genes affected on one day only and (C) genes predominantly expressed in the conceptus. An asterisk (*) denotes a significant ($P<0.05$) difference among tissue types on a given day for a specific gene.

CST6, *GDA*, *GELS*, *IDHC PNPH* and *TIMP2* mRNAs indicates that their protein products are predominantly derived from the endometrium, while the expression of *ACTB*, *ALDOA*, *ALDR CAP1*, *CATB*, *CATG*, *GD1B*, *HSP7C*, *HSP90A*, *RET4* and *TERA* suggests that they may be predominantly conceptus derived.

The proteomic content of the ULF on day 16 of pregnancy reflects the physiological processes that occur both in overrepresented molecular functions and in biological processes. During this time period, IFNT, along with other conceptus-derived factors such as PGs (Dorniak *et al.* 2011*a*), acts on the endometrium to further stimulate the expression of genes (and their protein products) that drive conceptus elongation in ruminants (Gray *et al.* 2004, Song *et al.* 2005, 2006, 2008, Simmons *et al.* 2009). In cattle, by day 16 conceptus-derived IFNT acts on the endometrium to induce the expression of both classical (Mansouri-Attia *et al.* 2009, Forde *et al.* 2011*a*, 2011*b*, 2012*a*, 2012*b*) and non-classical (Bauersachs *et al.* 2012) IFN-stimulated genes and it is likely the protein products of some of these IFNT-induced genes are secreted into the ULF and contribute to the composition observed.

One of the key morphological events that characterizes early pregnancy in ruminants is rapid growth of the conceptus trophectoderm; therefore, changes in the protein content of ULF during these key stages are implicated in driving the process of elongation. It is hypothesized that proliferation of certain cells is driven by aerobic glycolysis despite the presence of sufficient oxygen, known as the Warburg effect (Vander Heiden *et al.* 2009). The premise of this type of cellular metabolism is that, despite the fact that the citric acid cycle produces more ATP than glycolysis in proliferating cells, energy, i.e. ATP, is only one of the requirements of the cells. Therefore, despite the fact that aerobic glycolysis is less efficient in terms of ATP production, it allows production of all types of cellular components, not just energy in the form of ATP. These cellular components are required by proliferating cells as their major function is to double all the components of the cell. There is some evidence in the literature that the Warburg effect may be utilized by both bovine (Cagnone *et al.* 2012) and porcine (Redel *et al.* 2012) blastocysts under different *in vitro* culture conditions; however, no data are available on the utilization of this pathway by the elongating conceptus. Given that some of the most abundant proteins in the ULF (IDHC), in addition to proteins in the overrepresented pathways of glycolysis/glyconeogenesis (LDHA, ALDOA, ALDOC, ENO1, ENO2, ENO3, PGAM1 and TPIS), are intermediaries involved in the Warburg effect (Krisher & Prather 2012), we hypothesize that secretion of these proteins into the uterine lumen may be required to aid cellular metabolism in order to drive rapid proliferation during the elongation process.

Table 2 Overrepresented pathways associated with proteins identified by nano-LC MS/MS analysis of uterine luminal fluid of pregnant heifers ($n=4$) on day 16 of pregnancy. Overrepresented pathway analysis was performed by DAVID (http://david.abcc.ncifcrf.gov/) on the list of proteins identified on day 16 in at least three or four biological replicates.

Overrepresented pathway	No. of proteins	Protein names
Lysosome	18	Acid ceramidase precursor (*Bos taurus*), proactivator polypeptide precursor (*Bos taurus*), NAGA protein (*Bos taurus*), lysosomal α-glucosidase precursor (*Bos taurus*), cathepsin D (*Bos taurus*), deoxyribonuclease-2-α precursor (*Bos taurus*), cathepsin D precursor (*Bos taurus*), arylsulfatase A precursor (*Bos taurus*), N(4)-(β-*N*-acetylglucosaminyl)-L-asparaginase (*Bos taurus*), cathepsin S precursor (*Bos taurus*), β-hexosaminidase subunit β preproprotein (*Bos taurus*), β-galactosidase precursor (*Bos taurus*), clathrin heavy chain 1 (*Bos taurus*), β-hexosaminidase subunit α precursor (*Bos taurus*), *N*-acetylglucosamine-6-sulfatase precursor (*Bos taurus*), cathepsin Z precursor (*Bos taurus*), cathepsin B precursor (*Bos taurus*), legumain precursor (*Bos taurus*), sialidase-1 precursor (*Bos taurus*)
Glycolysis/gluconeogenesis	11	Fructose-bisphosphate aldolase C (*Bos taurus*), triosephosphate isomerase (*Bos taurus*), fructose-bisphosphate aldolase A (*Bos taurus*), PGM2 protein (*Bos taurus*), alcohol dehydrogenase [NADP(+)] (*Bos taurus*), α-enolase (*Bos taurus*), phosphoglycerate kinase 1 (*Bos taurus*), β-enolase (*Bos taurus*), phosphoglycerate mutase 1 (*Bos taurus*), α-enolase (*Bos taurus*), lactate dehydrogenase-A (*Bos taurus*), glucose-6-phosphate isomerase (*Bos taurus*)
Regulation of actin cytoskeleton	10	α-actinin-4 (*Bos taurus*), PREDICTED: actin, β-like 2 (*Bos taurus*), α-actinin-2 (*Bos taurus*), gelsolin isoform b (*Bos taurus*), α-actinin-1 (*Bos taurus*), radixin (*Bos taurus*), actin-related protein 2/3 complex subunit 5-like protein (*Bos taurus*), moesin (*Bos taurus*), ezrin (*Bos taurus*), α-actinin-3 (*Bos taurus*)
Antigen processing and presentation	9	RecName: full=heat-shock 70 kDa protein 1B; AltName: full=heat-shock 70 kDa protein 2; short=HSP70.2, heat-shock protein HSP 90-α (*Bos taurus*), similar to β-2-microglobulin (*Bos taurus*), RecName: full=heat-shock cognate 71 kDa protein; AltName: full=heat-shock 70 kDa protein 8, heat-shock protein HSP 90-β (*Bos taurus*), cathepsin B precursor (*Bos taurus*), legumain precursor (*Bos taurus*), cathepsin S precursor (*Bos taurus*), PREDICTED: heat-shock 70 kDa protein 6 (*Bos taurus*)
Leukocyte transendothelial migration	7	α-actinin-4 (*Bos taurus*), PREDICTED: actin, β-like 2 (*Bos taurus*), α-actinin-2 (*Bos taurus*), α-actinin-1 (*Bos taurus*), moesin (*Bos taurus*), ezrin (*Bos taurus*), α-actinin-3 (*Bos taurus*)
Other glycan degradation	6	β-hexosaminidase subunit β preproprotein (*Bos taurus*), β-galactosidase precursor (*Bos taurus*), β-hexosaminidase subunit α precursor (*Bos taurus*), tissue α-L-fucosidase precursor (*Bos taurus*), N(4)-(β-*N*-acetylglucosaminyl)-L-asparaginase (*Bos taurus*), sialidase-1 precursor (*Bos taurus*)
Pentose phosphate pathway	6	Fructose-bisphosphate aldolase C (*Bos taurus*), fructose-bisphosphate aldolase A (*Bos taurus*), PGM2 protein (*Bos taurus*), transketolase (*Bos taurus*), 6-phosphogluconate dehydrogenase, decarboxylating (*Bos taurus*), transketolase (*Bos taurus*), glucose-6-phosphate isomerase (*Bos taurus*)
Glutathione metabolism	6	Glutathione synthetase (*Bos taurus*), isocitrate dehydrogenase [NADP], mitochondrial precursor (*Bos taurus*), glutathione S-transferase Mu 1 (*Bos taurus*), isocitrate dehydrogenase [NADP] cytoplasmic (*Bos taurus*), 6-phosphogluconate dehydrogenase, decarboxylating (*Bos taurus*), glutathione S-transferase P (*Bos taurus*)
Adherens junction	6	α-actinin-4 (*Bos taurus*), PREDICTED: actin, β-like 2 (*Bos taurus*), α-actinin-2 (*Bos taurus*), α-actinin-1 (*Bos taurus*), α-actinin-3 (*Bos taurus*), Met proto-oncogene precursor (*Bos taurus*)
Complement and coagulation cascades	6	α-1-antiproteinase precursor (*Bos taurus*), complement factor B precursor (*Bos taurus*), prepro complement component C3 (*Bos taurus*), DAF-1 (*Bos taurus*), antithrombin-III precursor (*Bos taurus*), CD59 glycoprotein precursor (*Bos taurus*)
Tight junction	6	α-actinin-4 (*Bos taurus*), PREDICTED: actin, β-like 2 (*Bos taurus*), α-actinin-2 (*Bos taurus*), α-actinin-1 (*Bos taurus*), α-actinin-3 (*Bos taurus*), PREDICTED: membrane-associated guanylate kinase, WW and PDZ domain-containing protein 1 isoform 1 (*Bos taurus*)
Cysteine and methionine metabolism	5	Adenosylhomocysteinase (*Bos taurus*), putative adenosylhomocysteinase 3 (*Bos taurus*), aspartate aminotransferase, cytoplasmic (*Bos taurus*), enolase-phosphatase E1 (*Bos taurus*), lactate dehydrogenase-A (*Bos taurus*)
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	5	α-actinin-4 (*Bos taurus*), PREDICTED: actin, β-like 2 (*Bos taurus*), α-actinin-2 (*Bos taurus*), α-actinin-1 (*Bos taurus*), α-actinin-3 (*Bos taurus*)
Glycosaminoglycan degradation	4	β-hexosaminidase subunit β preproprotein (*Bos taurus*), β-galactosidase precursor (*Bos taurus*), β-hexosaminidase subunit α precursor (*Bos taurus*), *N*-acetylglucosamine-6-sulfatase precursor (*Bos taurus*)
Galactose metabolism	4	Lysosomal α-glucosidase precursor (*Bos taurus*), β-galactosidase precursor (*Bos taurus*), PGM2 protein (*Bos taurus*), RecName: full=aldose reductase; short=AR; AltName: full=20- α-hydroxysteroid dehydrogenase; short=20-α-HSD; AltName: full=aldehyde reductase
Fructose and mannose metabolism	4	Fructose-bisphosphate aldolase C (*Bos taurus*), triosephosphate isomerase (*Bos taurus*), fructose-bisphosphate aldolase A (*Bos taurus*), RecName: full=aldose reductase; short=AR; AltName: full=20-α-hydroxysteroid dehydrogenase; short=20-α-HSD; AltName: full=aldehyde reductase
Citrate cycle (TCA cycle)	4	Isocitrate dehydrogenase [NADP], mitochondrial precursor (*Bos taurus*), malate dehydrogenase, cytoplasmic (*Bos taurus*), isocitrate dehydrogenase [NADP] cytoplasmic (*Bos taurus*), aconitate hydratase, mitochondrial precursor (*Bos taurus*), mitochondrial aconitase 2 (*Bos taurus*)
Sphingolipid metabolism	4	Acid ceramidase precursor (*Bos taurus*), β-galactosidase precursor (*Bos taurus*), arylsulfatase A precursor (*Bos taurus*), sialidase-1 precursor (*Bos taurus*)
Amino sugar and nucleotide sugar metabolism	4	β-hexosaminidase subunit β preproprotein (*Bos taurus*), PGM2 protein (*Bos taurus*), β-hexosaminidase subunit α precursor (*Bos taurus*), glucose-6-phosphate isomerase (*Bos taurus*)
Glycosphingolipid biosynthesis	3	β-hexosaminidase subunit β preproprotein (*Bos taurus*), NAGA protein (*Bos taurus*), β-hexosaminidase subunit α precursor (*Bos taurus*)

Specific protein ontologies are reflective of the morphological events that occur during pregnancy recognition

We utilized overrepresented gene ontology analysis to understand which physiological processes the proteins identified in the ULF on day 16 were involved with in the context of both conceptus elongation (which is an ongoing process during the peri-implantation period of pregnancy) and maternal recognition of pregnancy. Three of the top molecular functions identified on day 16 are proteins that serve as enzymes, i.e. peptidases and endopeptidase inhibition. As an overrepresented group of proteins in the ULF on day 16, these make sense from a biological perspective. In cattle and sheep, endometrial derived proteins, e.g. IGFBP1 (Simmons *et al*. 2009), GRP (Song *et al*. 2008), LGALS15 (Lewis *et al*. 2007), CSF2 (Loureiro *et al*. 2011), actively enhance migration, proliferation and/or attachment of the conceptus trophectoderm. Therefore, inhibitors of protein and peptide cleavage in the ULF would be important to ensure that full-length functional forms of at least some of these proteins, when transported from the endometrium into the uterine lumen, are available for use by the elongating conceptus.

On day 16, a number of GO terms and pathways related to the pregnancy recognition response were identified including response to stimulus, complement and coagulation cascade were associated with ULF proteins. This is consistent with the fact that, by day 16 of pregnancy, sufficient quantities of IFNT must be produced to prevent luteolysis in cattle (Betteridge *et al*. 1980, Northey & French 1980). Moreover, transcriptomic data demonstrated that by days 15–16 of pregnancy, classical interferon-stimulated genes or interferon stimulated gene (ISGs) are significantly up-regulated in the endometrium (Forde *et al*. 2011*a*, 2011*b*, Bauersachs *et al*. 2012), some of which are cytokines and secreted proteins. The fact that IFNT secretion is maximal after day 13 (Farin *et al*. 1990) is consistent with the fact that these proteins are overrepresented on day 16. iTRAQ analysis also detected IFNT in the ULF on day 16 of pregnancy and the detection of these proteins in the ULF during pregnancy recognition demonstrates that protein products associated with the type I IFN response of the endometrium are secreted into the uterus. In addition, on day 16, proteins involved in the process of ion binding were detected in the ULF. Data from sheep indicates the presence of ions in the ULF during the pre-implantation period of pregnancy (Gao *et al*. 2009) and represent a non-protein source of molecules that contribute to the ULF composition. Given that ion-binding proteins are detectable during this period, they may play a role in chaperoning these ions in the ULF and/or enhancing the availability of these ions to the conceptus.

Role for predominantly endometrial-derived proteins during the pre-implantation period of pregnancy

Based on RNA sequencing data, we propose that the principal source of CST6, GELS, IDH1, PNP and TIMP2 proteins in the ULF is the endometrium and not the conceptus. Their transcripts were predominantly expressed in the endometrium and their protein products were modulated in the ULF as conceptus elongation progressed. Both TIMP2 and PNP have been previously found in the ULF of pregnant heifers, although at earlier time-points of pregnancy (Ledgard *et al*. 2012). TIMP2 protein was detectable on day 16 in this study and the expression of its mRNA was at least two orders of magnitude greater in the endometrium than the conceptus, suggesting that it may be regulated by the process of pregnancy recognition in cattle. One of the most abundant proteins identified in the ULF during the pregnancy recognition period (day 16) was CST6, a protease inhibitor that is a member of the cystatin family of proteins. In some tissues, CST6 functions as a tumour suppressor (Vigneswaran *et al*. 2006, Ko *et al*. 2010), which seems contradictory in terms of rapid elongation of the conceptus. On the other hand, over-expression or up-regulation of CST6 promotes cell growth and proliferation in other cancer cells (Vigneswaran *et al*. 2003, Hosokawa *et al*. 2008). Despite these seemingly contradictory roles for CST6, previous results indicate a role for the cystatin/cathepsin families in the endometrium and conceptus during early pregnancy in a number of species (Afonso *et al*. 1997, Song *et al*. 2006, 2007, 2010, Baston-Buest *et al*. 2010) and *CST6* has been shown to be up-regulated by IFNT *in vivo* in sheep (Dorniak 2011a). We propose that CST6, predominantly derived from the endometrium, is involved in driving conceptus elongation during the pre-implantation period of pregnancy in cattle. IDH1 is an enzyme involved in the citric acid cycle and has been previously identified as more abundant in both the endometrium (Berendt *et al*. 2005) and the ULF of gravid compared with non-gravid uterine horns on day 18 of pregnancy (Ledgard *et al*. 2009). In this study, we determined that IDH1 is most likely secreted from the endometrium during the initiation of conceptus elongation through to pregnancy recognition; however, by day 19, when implantation is beginning, IDH1 is reduced in abundance in the ULF, which may suggest that it is only required for a short period of time up to pregnancy recognition. Alternatively, the elongating conceptus may be rapidly turning over this protein; hence, we see a decrease in its abundance in the ULF, despite the increased mRNA expression in the endometrium during the peri-implantation period of pregnancy. By contrast, the protein PNP, an enzyme involved in the metabolism of nucleotides, is expressed predominantly by the endometrium and is in the ULF on day 16 of pregnancy as it increases in abundance as pregnancy progresses.

Role for predominantly conceptus-derived proteins during the pre-implantation period of pregnancy

Despite the need for endometrial-derived secretions, without which conceptus elongation does not occur (Gray *et al*. 2002), it is well established that the primary molecule that modulates endometrial expression/production of proteins, which in turn drives conceptus elongation, is conceptus-derived IFNT (Bazer *et al*. 2010). In addition to IFNT, there is emerging evidence that other conceptus-derived products impact on the endometrial transcriptome (Bauersachs *et al*. 2012). For example, in cattle, PGs play a role in modulating the expression of genes in the endometrium involved in the pregnancy recognition signal (Spencer *et al*. 2013), while in sheep they affect the expression of genes that are known to regulate conceptus elongation in sheep (Dorniak *et al*. 2011*a*) as well as affecting the content of the ULF (Dorniak *et al*. 2011*b*). It is therefore reasonable to assume that some proteins detected in the ULF as the conceptus elongates during the pre-implantation period of pregnancy are derived from the conceptus itself. The abundance of proteins such as ALDOA, and HSPA8, increased significantly in the ULF from day 13 to day 19 of pregnancy, coordinate with the considerable increase in conceptus size as it transitions from an ovoid (day 13) through to a fully elongated filamentous conceptus on day 19 which occupies both uterine horns. *ALDOLA* and *HSPA8* mRNA were higher in the conceptus than in the endometrium, raising the possibility that these proteins are predominantly conceptus derived; however, conclusions regarding the source of these proteins on the basis of gene expression should be drawn with caution, considering the total amount of endometrial tissue compared with conceptus tissue present in the uterus in early pregnancy.

In conclusion, this study demonstrates that significant changes in the protein content of the ULF occur during the transition from hatched blastocyst on day 10 to the process of conceptus elongation, pregnancy recognition on day 16 and the peri-implantation period of pregnancy in cattle, a reflection of the morphological changes that occur at these key developmental time-points. In addition, significant changes in the abundance of selected proteins in the ULF occur during the pre-implantation period of pregnancy, some of which are derived predominantly from endometrial or conceptus tissues.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-13-0010.

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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