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Global proteomic characterisation of uterine histotroph recovered from beef heifers yielding good quality and degenerate Day 7 embryos

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Abstract

The objective was to analyse the proteomic composition of uterine flushes collected from beef heifers on Day 7 post-insemination. Oestrus was synchronised in cross-bred beef heifers using a Controlled Intravaginal Drug Releasing device (CIDR) protocol. Heifers detected in standing oestrus (within 24-48 h post CIDR removal) were inseminated (oestrus=Day 0) with frozen-thawed semen from a single ejaculate of a bull with proven fertility. Heifers from which an embryo was recovered (following slaughter on Day 7) were classified as either having a viable embryo (morula/blastocyst stage) or a degenerate embryo (arrested at the 2- to 16-cell stage). The overall recovery rate (viable and degenerate combined) was 64%. Global LC-MS/MS proteomic analysis of the histotroph collected identified 40 high confidence proteins present on Day 7; 26 proteins in the viable group, 10 in the degenerate group and four shared between both groups. Five proteins (Platelet-activating factor acetylhydrolase IB subunit gamma (PAFAH1B3), Tubulin alpha-1D chain, Tubulin beta-4A chain, Cytochrome C and Dihydropyrimidinase-related protein-2) were unique or more abundant in the histotroph collected from animals with a viable embryo and one protein (S100A4) was more abundant in the histotroph collected from animals with a degenerate embryo. Of interest, PAFAH1B3, detected only in histotroph from the group yielding viable embryos, belongs to the group of platelet activating factors that are known to be important for the development of the pre-implantation embryo in other species. To our knowledge this is the first report of PAFAH1B3 in relation to bovine early embryonic development.

Keywords: Proteomics, histotroph, embryo development

1. Introduction

Embryo mortality in cattle, reflected in reduced conception rate/calving rate per service, is a major cause of economic loss for the farming industry. In heifers, only 60% of single inseminations lead to a successful full term pregnancy despite a fertilisation rate of 90-95% [1]. Despite the fact that the period of greatest reproductive wastage in cattle occurs before Day 16 [1,2], the underlying molecular events that regulate early conceptus development up to the time of maternal recognition of pregnancy in cattle have not been clearly elucidated. It is clear, however, that the uterine endometrium plays a central role in early conceptus-maternal communication for establishment and maintenance of pregnancy. This involves dynamic changes in the uterine epithelium that are tightly regulated by changes in steroid hormones. The embryo leaves the oviduct and enters the uterus between Day 4 and 5 post fertilisation at the 8- to 16-cell stage [3]. From this point onwards until the start of implantation, which occurs around Day 19, the embryo is not attached in the uterus and is completely dependent on the uterine secretions for its further development [4,5]. As such it is of vital importance that the composition of the histotroph meets the requirements of a developing embryo. Indeed, changes in endometrial gene expression around this time, under the influence of progesterone, can lead to changes in the composition of the histotroph to which the developing conceptus is exposed [6,7]. The importance of histotroph for conceptus development has been demonstrated in the uterine gland knockout (UGKO) model in sheep in which embryos developed to Day 9 of gestation but then failed to develop beyond the blastocyst stage, i.e., Day 14, in adult UGKO ewes [8,9]. The process of conceptus elongation post-hatching is regulated mainly by histotroph-derived factors as evidenced by the fact that despite attempts to artificially induce this process, hatched bovine blastocysts fail to elongate *in vitro*, but will do so if transferred to the uterus of a recipient female [10,11].

The histotroph is composed of proteins, carbohydrates, sugars, lipids and ions produced by the endometrial glands that are necessary to sustain the conceptus. The protein components of the histotroph are important for conceptus-maternal interaction, specifically the processes of elongation of the trophoblast, recognition of pregnancy, implantation, and placentation [12,13]. Many components of the histotroph are secreted under the influence of progesterone and in the early luteal phase [14,15] also by oestradiol [16] but the optimum biochemical composition of the histotroph that supports the development of a healthy embryo/conceptus is not yet known. Recent studies have described the proteomic composition of uterine histotroph during the oestrous cycle and in comparison with plasma [17-19].

The objective of this study was to analyse the proteomic composition of uterine flushes of inseminated beef heifers with normal and degenerate embryos on Day 7 post insemination in order to elucidate what potential proteins are present in the uterus to support the embryo until blastocyst development. Our hypothesis is that there will be differences in composition between the animals with different types of embryos.

1. Materials and Methods

All experimental procedures involving animals were approved by the Animal Research Ethics Committee of University College Dublin and were licensed by the Department of Health and Children, Ireland, in accordance with the cruelty to animals act (Ireland 1876) and European Community Directive 86/609/EC.

2.1 Animal management and treatments

The experimental design used for this study has been described previously [20]. Cross-bred beef heifers, approximately 2 yrs old and weighing 524 ± 5.5 kg housed in a slatted floor

facility in a commercial feedlot were used. All heifers were housed under the same management conditions with *ad-libitum* access to a total mixed ration designed to achieve an average live-weight gain of 1.3 kg/heifer/day. Oestrus (Day 0) was synchronised by insertion of a Controlled Internal Drug Release (CIDR, 1.36g Progesterone, Pfizer UK) device placed per vaginum for 8 days with a 2 mL injection of PGF2α analogue (Prosolvin, Intervet Ireland Ltd., Dublin, Ireland) given on Day 7. Heifers were checked for signs of oestrus 4 times per day commencing 36 h after CIDR removal. Twelve to eighteen hours after onset of oestrus (Day 0) only those heifers recorded in standing oestrus within a narrow window were inseminated with frozen-thawed semen from a single ejaculate of a bull with proven fertility. Jugular blood samples were collected on Days 4, 6 and 7 post-oestrus from all heifers. Blood samples were stored at room temperature for 1 h and at 4°C for a further 16 h. Serum was decanted after centrifugation for 20 minutes at 1,600 x g and stored at -20°C until subsequent analyses. All heifers were slaughtered on Day 7 of pregnancy.

2.2 Progesterone assay

Serum progesterone concentrations were measured in all heifers on Days 4, 6 and 7 post oestrus using a time-resolved fluorescenceimmunoassay (FIA) with an AutoDELFIA™ Progesterone kit (Perkin Elmer, Wallac Oy, Turku, Finland), as previously described (Carter *et al.* 2008). All samples were assayed within a single assay with a sensitivity of 0.01 ng/mL for the progesterone assay. The intra-assay coefficients of variation (% CV) were 4.6, 5.5 and 4.6% for high, medium and low progesterone quality control sera, respectively.

2.3 Flush collection

Heifers from which an embryo was recovered were assigned to either (i) the viable group when the embryo was at the correct developmental stage for age (i.e. morula/early blastocyst), or (ii) the degenerate group when the embryo was arrested at the 2- to 16-cell stage. Heifers from which an unfertilised oocyte was recovered or from which no structure was recovered were omitted from the study. Within 30 min of slaughter the reproductive tract of all heifers was flushed with 20 mL of 10 mM Tris (pH 7.2, Sigma, Dublin, Ireland) by injecting this volume into the tip of the uterine horn and collecting it at the caudal end of the uterine body. All flushes were subsequently transported on ice to the laboratory and flushes were centrifuged at 4000 x g for 30min at 4°C prior to snap freezing in liquid nitrogen and storage at -80°C until further analysis.

2.4 Protein extraction from uterine flushes

Proteins were extracted from six samples (three heifers per group) that were visibly free from blood (no red colour) using acetone precipitation as described previously [14]. Each sample was thawed on ice, split into 2 aliquots of 10 mL. Four volumes of ice cold acetone were added and samples stored at – 80 °C overnight. Samples were then thawed, centrifuged at 4,000 x g for 30 min at 4 °C and the supernatant removed. The pellets were resuspended in 100 µl of 100 mM Tris buffer, transferred into 1.5 mL Eppendorf tubes and sonicated briefly to aid resuspension. Samples were centrifuged at 12,000 x g for 30 min at 4 °C to remove insoluble material and the supernatant decanted and frozen at -80 °C for proteomic analysis. Total protein concentration of extracted uterine flush samples was determined using the Bradford assay, according to the manufacturers' protocol (Sigma Aldrich, Ireland).

2.5 Protein digestion

Extracted uterine proteins were vacuum dried and subjected to reduction and alkylation and proteolytic digestion as described by [21]. Cysteine residues were reduced by using 10 mM DTT in 100 mM ammonium bicarbonate for 1 h at 56 °C. The samples were alkylated with 50 mM iodoacetamide in 100 mM ammonium bicarbonate for 30 min at room temperature and digested with sequencing grade modified porcine trypsin 100 ng (Sigma-Aldrich, Ireland) on a rotary shaker at 37 °C for 8 h. Samples were subsequently dried down and stored at -80 °C until further analysis.

2.6 Chromatography

Proteolytic peptides were resuspended in 1 mL of strong cation exchange (SCX) buffer A (10 mM K_2PO_4, pH 3.0 25% MeCN) and separated offline by strong cation exchange using a Dionex/LC Packings UltiMate. Samples were loaded onto a polysulfoethyl A column (The Nest Group, Southboro, MA, USA) and eluted with an increasing linear gradient (0% - 40%) of SCX buffer B (10 mM K_2PO_4, pH 3.0 + 25% MeCN with 600 mM KCl) over 70 min at a flow rate of 200 µl / min. Seventy SCX fractions of approximately 200 µl were collected into 96-well microtitre plates. Eluted peptide fractions were pooled into 10 to 12 fractions according to the UV activity (214 nm) and desalted using Silica C18 columns (The Nest Group, Southborough, MA, USA), dried under vacuum and stored at -80 °C until further analysis.

2.7 Proteomic analysis

Tryptic peptides from pooled SCX fractions were resuspended in 1% ACN, 0.1% FA and analyzed on a Thermo Scientific LTQ linear ion trap mass spectrometer (Thermo Fisher

Scientific, Rockford, IL, USA) connected to a Surveyor, (Thermo Scientific) chromatography system with auto-sampler. Each sample was loaded onto a Biobasic C18 Picofrit™ column (100 mm length, 75 mm ID) and was separated by an increasing ACN gradient. Chromatography buffer solutions (Buffer A, 1% ACN, 0.1% formic acid; Buffer B, 100% ACN and 0.1% formic acid) were used to deliver a 72-min gradient (5 min sample loading, 32 min to 40% Buffer B, 2 min to 80%, hold 11 min, 1 min to 0%, hold for 20 min, 1 min flow adjusting). A flow rate of 150 μl/min was used at the electrospray source.

2.8 Database search and protein identification

Protein identification was carried out using PEAKS (v 5.3). Parameters: enzyme, trypsin; two missing cleavages allowed; parent tolerance ± 0.8 Da and ± 0.5 Da for fragment ion masses; methionine oxidation and carbamidomethylation of cysteines were specified as variable modifications. MS/MS spectra were searched against the Uniprot-Swissprot/TrEMBL *Bos Taurus* v 7.6 database (25/04/12) containing 35,297 sequences.

Estimation of false positives was conducted by searching all spectra against decoy databases. The cut-off false discovery rate (FDR) for peptide spectrum matches was <1% and the maximum FDR observed for peptide sequences was 2.6 %. Only proteins with a PEAKS score of $-10 \log P \geq 20$, containing at least one unique peptide and only peptides containing an unbroken “b” or “y” ion series of a minimum of 4 amino acid residues were considered for further analysis.

2.9 Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA)

GO analysis was carried out using AMIGO [22] (v1.8) and the DAVID (v6.7) bioinformatics resource [23,24] with Benjamini corrected and EASE score *P* values of < 0.05 were considered significant, respectively.

For IPA (v9.0) analysis, the enriched protein data set containing Uniprot ID's and corresponding spectral count values was uploaded into the application. Each identifier was mapped to its corresponding object in the Ingenuity® Knowledge Base (build 171496; content version 14197757, release date 11-8-2012). These molecules, called Network Eligible molecules, were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. Only IPA networks with a score of 4 or greater, equivalent to a significance value of $P < 0.001$ were reported [25].

2.10 Statistics

Spectral counts (the number of mass spectra assigned to each protein) were normalised using the average total spectra obtained for each sample (4,228), an approach described and validated by [26] and utilised by several other studies [19, 27-30]. Due to non-normal distribution of spectral count data and to facilitate the use of parametric statistical analysis a fixed integer of 1 was added to all data values and subsequently log transformed. Both groups were then compared with each other using PROC MIXED (SAS v. 9.1; SAS Institute, Cary, NC, USA). Probability values less than or equal to 0.05 were considered significant. Correction for multiple testing was carried out using the Benjamini and Hochberg FDR as described previously [31].

3 Results

3.1 Progesterone

The progesterone concentration on Day 4 was 0.43 ± 0.05 ng/mL for the degenerate group and 0.52 ± 0.04 ng/mL for the viable group. On Day 5 the concentrations were 0.95 ± 0.09 ng/mL and 0.99 ± 0.06 ng/mL respectively and on Day 7 they were 1.03 ± 0.1 ng/mL versus 1.21 ± 0.1 ng/mL. There were no differences in area under the curve (AUC) for serum progesterone concentrations between both groups of heifers from Day 4 to Day 7.

3.2 Protein identification

To discern the most biologically pertinent proteomic profiles, and considering the low number of animals per group (n=3), thresholds were applied to characterise high confidence targets present in the viable or degenerate groups. These included: 1) signal presence in all three animals in either group; or 2) identification with at least one unique peptide per sample (as mentioned above); similar criteria as utilized in previous studies [30, 32, 33]. This classification identified a total of 40 high confidence proteins; 26 proteins in the viable group, 10 in the degenerate group and 4 common to both groups (Table 1; Supplemental Table 1).

Five proteins were identified as more abundant in the viable compared with the degenerate group (Table 1; Supplemental Table 1) which included Platelet-activating factor acetylhydrolase 1b, catalytic subunit 3 (PAFAH1B3), Tubulin, beta 4A class IVa (TUBB4A), Tubulin, alpha 1d (TUBA1D), Cytochrome c-1 (CYC1) and dihydropyrimidinase-like 2 (DPYSL2). One protein, S100 calcium binding protein A4 (S100-A4), was significantly increased in histotroph from the degenerate group but failed to maintain significance after multiple testing correction. However, it was retained in the subsequent analysis and

discussion to avoid loss of pertinent information and as prior information on its abundance in uterine flushes on Day 7 in cattle has been reported [19].

3.3 Characterization of Day 7 histotroph proteomes

GO slim analysis of the n=40 high confidence targets identified in histotroph on Day 7 showed all GO terms identified, with the exception of cell death, were associated with more proteins in the viable compared with the degenerate group. The biological processes with the largest number of proteins associated were response to stress, cellular component assembly and macromolecular complex assembly (n=9), the latter two processes only associated with proteins identified from the viable group. In addition, three other processes were unique to the viable group and include protein complex assembly, cytoskeletal organization and cell cycle (Figure 1).

3.4 IPA analysis

IPA identified one network associated with proteins differentially expressed in histotroph between the viable and degenerate groups (n=6) on Day 7 involved in cellular assembly and organisation; cellular function and maintenance; and cell morphology (Figure 2).

4. Discussion

The aim of this study was to analyse the proteomic composition of uterine flushes of beef heifers with normal and degenerate embryos on day 7 post insemination in order to provide insights into what proteins are present in the uterus to support the embryo until its successful development as blastocyst. In this study we identified 40 proteins in histotroph collected on

Day 7 and these proteins were associated with a range of biological processes amongst which response to stress, cellular component assembly and macromolecular complex assembly. The latter two processes were only associated with proteins identified from the viable group. In addition, three other processes were unique to the viable group and include protein complex assembly, cytoskeletal organization and cell cycle. This was not unexpected given the number of proteins identified in the viable group was over double that identified in the degenerate group and suggests a greater provision of functionality in histotroph supportive of early embryo development. The results of the GO analyses were consistent with the network analysis identifying a significant relationship with cellular assembly and organisation; cellular function and maintenance; and cell morphology. GO analysis also identified immune system processes associated with the Day 7 proteome which could be associated with the fact that at a later stage of pregnancy embryo development is dependent on a tight regulation of the maternal immune system [34-36] with expression of components of the immune system in the endometrium and uterine histotroph implicated with successful or unsuccessful embryonic development. We recently found that a decreased expression of genes involved in the regulation of the immune response in the endometrium of heifers from which a viable embryo was recovered [20, 37], which is also supported by the suggestions and findings of Hansen [38] that the regulation of the uterine immune response is precise and that subtle changes can change the outcome of the developing embryo. The establishment of receptivity of the uterine luminal epithelium (LE) to the developing conceptus and the key role in regulating differentiated functions of the uterine glandular epithelium (GE) is very much regulated via indirect effects of progesterone on the endometrium [6, 39, 40], with the up and down regulation of the different genes being tightly regulated and a minor disruption of this regulation having major consequences on conceptus survival.

Although only six proteins were identified as differentially expressed between the groups, their presence in histotroph, during this critical time period may indicate an important role during early embryo development. Indeed, PAFAH1B3, unique to histotroph recovered from the viable group, belongs to the group of the Platelet-activating factors (PAF) that are one of the most potent phospholipids involved in a variety of physiological events including biological processes pre- and post-fertilisation such as spermatozoal function, fertilization, embryo development and implantation [41, 42]. In male reproduction PAF increases the sperm motility and improves the acrosome reaction [43], while in female reproduction the protein is secreted by pre-implantation embryos of a number of species and its secretion appears to be positively correlated with the viability of human embryos produced by IVF [44] PAF antibody inhibits mouse pre-implantation embryo development [45] and platelet activating factor produced by the rabbit embryo has been shown to increase during the pre-implantation phase [46].

The function of Tubulins, a group of proteins to which both TUBB4A and TUBA1D belong, in reproduction is not clear although these proteins have been found in the flagellum of mouse sperm where it appears to be located in the midpiece and terminal piece, as well as in the testes [47]). Tubulin, however, is more widely described in relation to cancer research. The protein can be found in the nucleus of cells and in mitochondria and downstream events that result from tubulin binding are critical events for the generation of apoptosis in malignant cells [48]. The identification of two members of the tubulin family unique to histotroph recovered from the viable embryo group during a stage of rapid embryo growth may support a role for these proteins in the regulation of cell proliferation and successful blastocyst development. Indeed, TBA1D has previously been identified in histotroph from highly fertile dairy cattle on Day 7 [19].

CYC1, also previously identified in histotroph on both Day 7 and Day 13 post oestrus by Mullen *et al.* [19], is a component of the electron transport chain in mitochondria. The protein is associated with the inner membrane of the mitochondrion and is involved in initiation of apoptosis when it is released. Li et al (2000) [49] report that cells lacking CYC1 show reduced caspase-3 activation and are resistant to the proapoptotic effects of UV irradiation and serum withdrawal. However, cells lacking CYC1 appear to demonstrate increased sensitivity to cell death signals triggered by TNFα. As such lack of CYC1 can lead to an altered stress induced apoptotic response. CYC1 is an essential component of an apoptotic pathway responsive to DNA damage and other forms of cell stress and interestingly mouse embryos that lack CYC1 die *in utero* by mid gestation [49]. The fact that this protein was more abundant in flushes from uteri that yielded a viable embryo indicates this protein may be important for early embryo survival. While CYC1 is typically classified as non-secretory, bioinformatic analysis using Secretome P predicted CYC1 to be secreted non classically (data not shown).

The remaining protein more abundant in the flushes of uteri of which a viable embryo was recovered was DPYSL2. DPYSL2 has been described in relation to neuronal development and axon growth, the family of dihydropyrimidinases is known to have a role in growth and development and deficiency of this protein in humans can lead to abnormalities of both a neurological and gastrointestinal nature [50]. DPYSL2 has also been identified as one of the proteins that are part of a group that appears to be a specific target of protein carbonylation. In the brain the carbonylation of DPYSL2 leads to shortening of dendritic length with consequent decreased interneuronal communication. In general, protein carbonyl content is the most general and well-used biomarker of severe oxidative protein damage in many human diseases. The role of reactive oxygen species (ROS) that cause this protein damage has

become more apparent in many disease processes and ROS have the potential to induce significant biological damage to cells [51]. Under physiological conditions, there is an established balance between formation and neutralisation of ROS, but this fine balance is disrupted, for instance by disruption in the anti-oxidant defence mechanism of the cell, oxidative stress and hence damage to the cell can occur. Protein carbonyls have been found in both placentas and decidua of women that suffered from pre-eclampsia, suggesting a role of damage done by ROS in this disorder [52]. The increased abundance of DPYSL2 in heifers from which subsequently a viable embryo was recovered suggests it might play a role in embryo protection regulating the oxidative damage at this stage of early pregnancy. Indeed, DPYSL2 has been reported approximately 3-fold more abundant on Day 7 compared with Day 13 [19], which may indicate a stage specific requirement for this protein during early embryo development.

Only one protein was identified as more abundant in the histotroph of heifers yielding degenerate embryos compared with viable embryos, S100-A4. We have previously identified S100-A4 unique to Day 7 uterine flushes compared with those on Day 13 [19]. S100A4 is a protein involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation [53]. The protein belongs to a group of calcium binding proteins that tend to be highly expressed in pathological conditions. The group of S100 calcium binding proteins has been associated with a number of aspects of the interaction between cancer cells and stromal cells, and contributes to the formation of an inflammatory tumor microenvironment [54]. It has also been associated with cancer cells and appears to contribute to the motility of tumor cells and as such the progression of metastasis [55, 56]. The relative increase in abundance of S100-A4 in the flushes of animals that yielded a degenerate embryo may indicate a negative impact of excess S100-A4 on early embryo

development perhaps through the anti- and pro-inflammatory processes that occur during the pre-implantation phase as we previously hypothesised [37].

In conclusion, we identified 40 proteins in histotroph collected from the uterus on Day 7 of pregnancy from heifers that were inseminated and included five proteins more abundant in histotroph collected from animals with a viable embryo and one protein more abundant in the histotroph collected from animals with a degenerate embryo. While we are aware of the limited numbers per group and difficulties associated with any secretome analysis such as the potential for cellular contamination, in our opinion, these data may consist of markers of successful early embryo development and warrant further investigation. In support of these findings several proteins have previously been identified as expressed in the endometrium [6, 57] and histotroph [19] by our group on Day 7 in cattle. Of particular interest, one protein PA1B3, belongs to the groups of platelet activating factors which are known to be very important for the development of pre-implantation embryos in other species, but to our knowledge has not been reported in relation to bovine early embryonic development and may warrant further investigation.

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544 Table 1. 40 proteins identified on Day 7 of pregnancy in histotroph from beef heifers yielding viable or degenerate embryos.

Uniprot ID	Mass (Da)	Protein desciption[a]	Biological Function[b]	Viable				Degenerate				*P* value	
				86	144	177	Mean	18	205	203	Mean	LS	FDR
PA1B3_BOVIN	25865	Platelet-activating factor acetylhydrolase IB subunit gamma	multicellular organism reproduction	6	4	6	5 ± 1	0	0	0	0 ± 0	<.0001	0.000
TBA1D_BOVIN	50283	Tubulin alpha-1D chain	protein complex assembly	6	16	23	15 ± 5	0	0	0	0 ± 0	0.002	0.030
DPYL2_BOVIN	62278	Dihydropyrimidinase-related protein 2	differentiation	17	20	17	18 ± 1	0	3	0	1 ± 1	0.006	0.064
TBB4A_BOVIN	49586	Tubulin beta-4A chain	microtubule cytoskeleton organization	6	45	6	19 ± 13	0	0	0	0 ± 0	0.015	0.095
CYC_BOVIN	11704	Cytochrome c	generation of precursor metabolites and energy	6	57	46	37 ± 16	0	1	0	0 ± 0	0.013	0.102
S10A4_BOVIN	11807	Protein S100-A4	Regulation of kappa kinase cascade	6	8	6	7 ± 1	22	9	29	20 ± 6	0.054	0.293
TKT_BOVIN	67906	Transketolase	regulation of growth	6	8	23	12 ± 6	0	3	5	2 ± 1	0.105	0.418
HBA_BOVIN	15184	Hemoglobin subunit alpha	gas transport	153	201	209	187 ± 17	1458	340	320	706 ± 376	0.102	0.467
TRFE_BOVIN	77753	Serotransferrin	transition metal ion transport	232	197	133	187 ± 29	123	119	158	134 ± 12	0.161	0.470
GDIR1_BOVIN	23421	Rho GDP-dissociation inhibitor 1	intracellular signaling cascade	0	6	17	8 ± 5	45	24	16	28 ± 9	0.135	0.479
PRDX1_BOVIN	22210	Peroxiredoxin-1	response to reactive oxygen species	11	25	17	18 ± 4	0	9	8	6 ± 3	0.153	0.491
ACBP_BOVIN	10044	Acyl-CoA-binding protein	transport	0	142	0	47 ± 47	11	193	76	94 ± 53	0.267	0.534
LDHB_BOVIN	36724	L-lactate dehydrogenase B chain	response to reactive oxygen species	6	4	17	9 ± 4	0	0	11	4 ± 4	0.222	0.545
SERA_BOVIN	56452	D-3-phosphoglycerate dehydrogenase	glutamine metabolic process	6	16	12	11 ± 3	0	12	6	6 ± 3	0.312	0.554
TBA1C_BOVIN	49857	Tubulin alpha-1C chain	protein complex assembly	6	16	23	15 ± 5	0	21	0	7 ± 7	0.212	0.566
ENOA_BOVIN	47326	Alpha-enolase	glycolysis	0	6	57	21 ± 18	22	22	36	27 ± 4	0.336	0.566
TBB5_BOVIN	49671	Tubulin beta-5 chain	microtubule cytoskeleton organization	6	45	6	19 ± 13	0	25	0	8 ± 8	0.301	0.567
A1AG_BOVIN	23182	Alpha-1-acid glycoprotein	acute inflammatory response	45	4	17	22 ± 12	0	9	8	6 ± 3	0.266	0.568
TBA1B_BOVIN	50152	Tubulin alpha-1B chain	microtubule cytoskeleton organization	6	16	23	15 ± 5	0	0	26	9 ± 9	0.251	0.573
TERA_BOVIN	89330	Transitional endoplasmic reticulum ATPase	DNA damage and repair	11	8	12	10 ± 1	0	7	15	7 ± 4	0.396	0.576
NDKB_BOVIN	17316	Nucleoside diphosphate kinase B	apoptosis regulation	0	34	34	23 ± 11	45	44	23	37 ± 7	0.363	0.581

Uniprot ID	Mass (Da)	Protein desciption[a]	Biological function[b]	Viable[c]				Degenerate[c]				*P* value	
				86	**144**	**177**	**Mean[d]**	**18**	**205**	**203**	**Mean[d]**	**LS**	**FDR**
TPM3_BOVIN	32819	Tropomyosin alpha-3	No information	6	4	6	5 ± 1	0	3	8	4 ± 2	0.382	0.582
PPIA_BOVIN	17869	Peptidyl-prolyl cis-trans isomerase A	protein folding	0	74	51	42 ± 22	56	25	47	43 ± 9	0.528	0.604
APOA1_BOVIN	30276	Apolipoprotein A-I	regulation of cytokine production	6	29	6	13 ± 8	0	14	11	9 ± 4	0.520	0.617
IDHC_BOVIN	46785	Isocitrate dehydrogenase [NADP] cytoplasmic	cellular aldehyde metabolic process	0	23	6	9 ± 7	22	5	10	12 ± 5	0.505	0.622
HS90A_BOVIN	84731	Heat shock protein HSP 90-alpha	regulation of nitric oxide biosynthetic process	40	29	17	29 ± 6	0	46	61	36 ± 18	0.644	0.624
PNPH_BOVIN	32037	Purine nucleoside phosphorylase	nucleoside metabolic process	23	25	12	20 ± 4	0	16	31	15 ± 9	0.454	0.632
HBB_BOVIN	15954	Hemoglobin subunit beta	gas transport	210	135	342	229 ± 61	482	265	153	300 ± 97	0.613	0.633
ALDR_BOVIN	35919	Aldose reductase	oxidation reduction	11	61	81	51 ± 21	0	35	115	50 ± 34	0.595	0.635
B2MG_BOVIN	13677	Beta-2-microglobulin	regulation of leukocyte mediated cytotoxicity	11	0	11	8 ± 4	11	1	27	13 ± 8	0.675	0.635
TPIS_BOVIN	26690	Triosephosphate isomerase	monosaccharide metabolic process	28	12	12	17 ± 6	0	25	31	19 ± 9	0.635	0.635
HSP7C_BOVIN	71241	Heat shock cognate 71 kDa protein	protein foldin	17	25	52	31 ± 11	0	48	45	31 ± 16	0.579	0.639
HS90B_BOVIN	83253	Heat shock protein HSP 90-beta	placenta development	23	33	29	28 ± 3	0	35	44	26 ± 13	0.505	0.647
PRDX2_BOVIN	21946	Peroxiredoxin-2	MAPKKK cascade	0	23	11	11 ± 7	11	12	16	13 ± 2	0.488	0.651
TBB4B_BOVIN	49831	Tubulin beta-4B chain	protein polymerisation	6	45	6	19 ± 13	0	25	24	16 ± 8	0.757	0.654
KCRB_BOVIN	42719	Creatine kinase B-type	Ion homeostasis	23	4	29	19 ± 8	0	17	44	20 ± 13	0.717	0.656
AMPN_BOVIN	109276	Aminopeptidase N	angiogenesis	6	25	41	24 ± 10	0	38	47	28 ± 14	0.749	0.666
HSPB1_BOVIN	22393	Heat shock protein beta-1	response to temperature stimulus	0	40	17	19 ± 12	34	5	6	15 ± 9	0.864	0.728
ZA2G_BOVIN	33852	Zinc-alpha-2-glycoprotein	immune response	102	0	17	40 ± 32	11	4	19	12 ± 4	0.923	0.738
ALBU_BOVIN	69294	Serum albumin	cytolysis by symbiont of host cells	2180	1331	916	1476 ± 372	1693	1090	1294	1359 ± 177	0.905	0.743

[a] Proteins are listed with their Uniprot ID and description. [b]Functional annotation was performed using DAVID. [c]Spectral counts (the number of mass spectra assigned to each protein) reported normalised counts. [d] Mean ± s.e.m.

Figure 1. Gene Ontology (GO) slim terms and the numbers of proteins associated with each term on Day 7 in high confidence datasets (i.e. proteins (n=40) identified in at least all three animals in either viable or degenerate groups).

Figure 2. Ingenuity pathway interaction network analysis. Proteins more abundant in histotroph in viable compared with degenerate groups on Day 7 post insemination involved in cellular assembly and organisation; cellular function and maintenance; and cell morphology (n=6), $P < 10^{-15}$. The network displays nodes (genes/gene products) and edges (the biological relationship between nodes). The colour intensity of the nodes indicates the spectral count increase associated with a particular protein in histotroph from the viable group (red) or the degenerate group (green) on Day 7. A solid line indicates a direct interaction between nodes (genes/gene products) and a dashed line indicates an indirect relationship between nodes. The shape of the node is indicative of its function.