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From Peptide Masses to Pregnancy Maintenance: A Comprehensive Proteomic Analysis of The Early Equine Embryo Secretome, Blastocoel Fluid, and Capsule

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Early pregnancy in the mare is a poorly understood, high risk period during which the embryo communicates its presence to the maternal endometrium. Remarkably, the maternal recognition of pregnancy signal is unknown in the horse. This study aimed to profile the proteins secreted by equine blastocysts into their immediate environment, along with proteins contained in the blastocoel and within the acellular embryo capsule. Embryos were recovered on day 8 after ovulation and cultured for 48 hours. Secretomes of day 9 and day 10 embryos were analyzed by LC-MS/MS and supported by analysis of blastocoel fluid and embryo capsule. Analyses revealed 72 (24 h) and 97 (48 h) unique protein IDs in the embryo secretome, 732 protein IDs in blastocoel fluid, and 11 proteins IDs in the embryo capsule. Novel findings of interest include secretion of a pregnancy specific proteinase (PAG) by the equine embryo at day 10, along with detection of a prostaglandin receptor inhibiting protein (PTGFRN) and a progesterone potentiating factor (FKBP4) in blastocoel fluid. This is the first comprehensive proteomic analysis of the equine embryo secretome, and provides new insights into the unique physiology of early pregnancy in this species.

1. Introduction

Early pregnancy in the horse remains the most enigmatic among the domesticated livestock species. Characterized by several unique features, including a long pre-implantation period, a highly mobile, spherical conceptus, and an acellular tertiary coat encasing the post-hatching embryo, it represents a critical but poorly understood period in gestation. The risk of pregnancy loss is higher during these few weeks (prior to definitive placental interdigitation around day 40) than the entire remaining 10 months of gestation.[1,2] In addition, while fertilization rates in experimental studies are very high (>90%: [1]), in the field pregnancy rates are much lower (65%: [2]), and the inability to detect pregnancy prior to day 9 makes it impossible to distinguish between failure of fertilization and embryo loss during this period. Given the monetary and labor inputs invested in achieving pregnancy in the equine breeding industry, early embryo loss presents a significant area of economic inefficiency, and our limited understanding of the biology of early pregnancy in this species demands further investigation of the mechanisms underlying success or failure of embryo survival.

Maternal recognition of pregnancy (MROP) is a term first coined by Roger Short,[3] and refers to the biochemical signal emitted by a conceptus and recognized by the maternal system to ensure the prolonged survival of the primary corpus luteum, instead of its lysis and a consequent return to estrus. Pregnancy recognition signalling molecules are striking in their diversity across species, ranging from single chain proteins to steroid hormones.[4] Discovery of the human MROP, human chorionic gonadotrophin (hCG),[5,6] facilitated the development of a widely used pregnancy test and ushered in a new era in fertility intervention, based on luteal phase support for IVF pregnancies and those at risk of spontaneous abortion.[7] Remarkably, the equine MROP has remained elusive, despite decades of research and sophisticated experiments, along with a series of breakthroughs in our understanding of the downstream effects of the purported signal (reviewed extensively in [8–10]). Defining the assortment of proteins

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Significance of the study

Equine breeding industries contribute substantially to economies in many parts of the world. However, there are many inefficiencies within these industries, a major concern being high rates of idiopathic early pregnancy loss. Ultimately, these inefficiencies stem from the lack of in depth knowledge of the processes underlying fertility in equids, which lags behind research in other livestock, and many species-specific features of reproductive function. Remarkably, the maternal recognition of pregnancy signal remains unknown in the horse, unlike other domesticated species. Researchers have previously attempted to identify proteins secreted by early equine embryos but have been unable to detect and identify more than a handful of proteins, due to deficiencies in available technologies, experimental design, contamination of media, and other factors. In this study we present the first comprehensive mass spectrometry-driven analysis of components of the equine pregnancy, including embryo-secreted factors, blastocoel fluid and embryo capsule. Careful micro-dissection of cultured embryos allowed us to examine the sub-components of equine embryos individually, further distinguishing this study from previous attempts at protein analysis of the equine conceptus. We also report, for the first time, successful culture of embryos in protein-free media (i.e. embryos exhibiting normal growth rates), paving the way for future proteomic analysis and functional studies. We believe this study will contribute to an improved understanding of early pregnancy in the mare, and will facilitate novel research directions in idiopathic early embryo loss, pregnancy monitoring and diagnosis, and in vitro embryo culture.

that the early embryo releases into its immediate environment will not only bring us closer to identifying the MROP, but has the potential to expand our clinical capacity in pregnancy diagnosis, assessment of embryo developmental potential, early pregnancy support, and breeding herd management.

In the current study, we set out to profile the proteins that are secreted by early equine embryos, and those contained within the blastocoel cavity. We also examined the proteins of the acellular embryo capsule. These proteomic data were integrated with a thorough examination of the existing literature to identify proteins of interest and their potential contributions to early equine pregnancy, culminating in a series of clinically focused recommendations and novel hypotheses for future research.

2. Experimental Section

2.1. Sample Collection and Preparation: Embryo-Conditioned Medium, Embryo Capsules and Blastocoel Fluid

Institutional and state government approval was obtained for use of animal material in this study (University of Sydney Animal Ethics Committee, Project #541). Standardbred mares were housed on pasture with Supporting Information feeding. Mares' reproductive cycles were monitored by transrectal palpation and ultrasonography. Upon signs of impending ovulation, mares were inseminated with a dose of chilled semen containing at least 5×10^8 motile spermatozoa, obtained from one of three fertile stallions. Where necessary, inseminations were repeated daily until ovulation was confirmed to have occurred.

Embryos ($n = 4$) were obtained by transcervical uterine lavage 8 days after confirmation of ovulation, using a 34 French-gauge silicone Foley catheter with a 100cc balloon and Y-tube (MAI Animal Health, Elmwood, WI, USA). Warmed Emcare Complete Ultra flushing medium (1000–2000 mL per flush; ICPbio Reproduction, Auckland, New Zealand) was used for flushing uteri and collected through an Em-Con® embryo filter (MAI Animal Health). Filter contents were transferred to search dishes, and embryos were recovered under a dissecting microscope and transferred to transport media. The transport medium consisted of Hepes-buffered DMEM/F12 (11330-032; Gibco, Grand Island, NY, USA) supplemented with 0.5% w/v fatty acid-free bovine serum albumin (BSA; ICPbio), 10 units/mL penicillin-G and 10 μg/mL streptomycin sulphate. After transport (<30 min) to the laboratory, each embryo was rinsed several times by moving the embryo through dishes of BSA-containing culture medium (bicarbonate-buffered DMEM/F12 (11320-033; Gibco) with 0.5% w/v BSA, 10 units/mL penicillin-G and 10 μg/mL streptomycin sulphate), deposited in a 50 μL droplet of BSA-containing culture medium under oil, and incubated for 2–3 h at 37 °C in a humidified atmosphere of 5% O_2, 6% CO_2 and 89% N_2. Embryos were then washed twice in BSA-free culture medium (bicarbonate-buffered DMEM/F12 with 0.1% polyvinyl alcohol (PVA), 10 units/mL penicillin-G and 10 μg/mL streptomycin sulphate), with the second wash extended to 30 min to allow dissociation of BSA. Finally, embryos were transferred to a 50 μL droplet of BSA-free culture medium under oil, and incubated for 24 h at 37 °C in a humidified atmosphere of 5% O_2, 6% CO_2 and 89% N_2.

After 24 h of culture, embryos were examined to verify continued blastocyst development and expansion, transferred to a droplet of fresh BSA-free culture medium, and incubated for a further 24 h. The spent culture medium (henceforth termed embryo conditioned medium) was collected and transferred to a cryovial, plunged in liquid nitrogen, and stored at −80 °C until further analysis. At the end of the culture period (48 h), the conditioned medium was again collected; embryos were transferred to fresh medium, washed once, placed in a new droplet and collapsed using a microblade. Droplets in which the embryos were collapsed were also collected for analysis (blastocoel fluid or 'BF' samples), while collapsed embryos were transferred to dishes containing 0.5 mL BSA-free culture medium and gently aspirated several times until the embryo capsule could be seen to separate completely from the trophoblast. The capsule and embryonic tissues were transferred to separate cryovials, plunged into liquid nitrogen, and stored at −80 °C until further analysis. Embryos were examined and manipulated under a stereomicroscope (SMZ1500; Nikon Corporation, Kawasaki, Kanagawa, Japan).

Prior to trypsin digestion, embryo-conditioned medium and BF samples were precipitated using methanol/chloroform as previously described[13] to produce a protein pellet. Embryo capsules were subjected to trypsin digestion directly. Protein pellets and capsules were incubated overnight at 37 °C with 25 mM ammonium bicarbonate solution containing 200 ng Trypsin and 0.04% ProteaseMAX surfactant (both from Promega, Madison, WI, USA). Trifluoroacetic acid was added to a final concentration

of 1% to inactivate Trypsin and the digests were centrifuged at 14 000 × *g* for 10 min to remove any undigested material and debris. Supernatants were transferred to Waters Autosampler vials for analysis.

2.2. Mass Spectrometry and Bioinformatics

Peptides from embryo-conditioned media were analyzed by Ion Trap MS/MS as follows:

Peptides were sequenced by nanoflow reverse phased Liquid Chromatography (Dionex Ultimate 3000 RSLCnano, Dionex, Idstein, Germany) coupled directly to an ESI 3D Ion Trap Mass Spectrometer (AmaZon ETD, Bruker GmbH, Preston, VIC, Australia) operating in MS/MS (CID) mode. Peptides were loaded at 5 μL/min onto a 5 μm C18 nanoViper trap column (100 μm × 2 cm, Acclaim PepMap100, Thermo) for desalting and pre-concentration. Peptide separation was then performed at 300 nL/min over an Acclaim nanoViper analytical column (2 μm C18, 75 μm × 15 cm) utilizing a gradient of 2–40% Buffer B (80% Acetonitrile, 0.1% Formic Acid) over 60 min. The peptides were eluted directly into the nanoflow ESI Ion source of the MS system for MS/MS analysis. The AmaZon Ion Trap system was set to perform MS/MS on the top 5 ions present in each MS scan with an Ion exclusion time of 30 s. Raw MS Files were converted into MASCOT Generic Format using DataAnalysis 4.1 and imported into ProteinScape 2.1 platform (both Bruker, Bremen, Germany) for database searching. Searches were performed against the UniProt (Horse) and SwissProt (mammalian) databases using an in-house licensed MASCOT server (version 2.3.02, Matrix Science). The number of allowed trypsin missed cleavages was set to 2. Deamidation of Asparagine and Glutamine, Oxidation of Methionine and Phosphorylation of Serine, Threonine and Tyrosine were set as variable modifications. The parent ion tolerance was set to 1.2 Da with fragment ion tolerance set to 0.7 Da. Peptide thresholds were set requiring False Positive Rate less than 0.05% with a low stringency MASCOT score greater than 35.

Peptides from embryo capsules and blastocoel fluid samples were analyzed by Quadrupole-Orbitrap MS/MS as follows: Peptides were sequenced by nanoflow reversed phased Liquid Chromatography (Dionex Ultimate 3000 RSLCnano, Thermo Fisher Scientific) coupled directly to a High Resolution mode equipped, Q-Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific). Samples were loaded at 10 uL/min (2% Acetonitrile, 0.1% Trifluoroacetic acid in Water) for 5 min onto a 5 μm C18 nanoViper trap column (100 μm × 2 cm, Acclaim PepMap100, Thermo Fisher Scientific) for desalting and pre-concentration. Separation was then performed at 300 nL/min over an EASY-Spray PepMap column (3 μm C18, 75 μm × 15 cm) utilizing a gradient of 2–40% Buffer B (80% Acetonitrile, 0.1% Formic Acid) over 120 min the gradient was then increased to 90% Buffer B briefly to wash the column before allowed to re-equilibrate at 2% Buffer B for a further 15 min (145 min total run time per sample). The compounds were eluted directly into the EasySpray nano-ESI Ion Source (Thermo Fisher Scientific) for high resolution MS mass determination and MS/MS analysis of the top 20 ions in each MS scan. MS analysis scanned the mass range from 370 to 1400 m/z in positive ion mode with resolution set to 70 000 at m/z 200 (equals approximately 50 000 at m/z 400), an AGC target of 1e6 and maximum injection time of 50 ms. Ubiquitous Polysiloxane was utilized as a lock mass at m/z 445.12002. Top 20 MS/MS was performed on ions with intensities greater than 1.8e4 at a resolution of 17 500 with an AGC target of 2e5 and maximum injection time of 110 ms. HCD collision energy was set to a value of 30 and dynamic exclusion time was set at 30 s. Singularly charged peptides were excluded from MS/MS selection.

Thermo .raw MS Files were imported into Proteome Discoverer 2.0 platform (Thermo Fisher Scientific) for database searching. Searches were performed against the UniProt (horse) and SwissProt (mammalian) databases using the search algorithm SEQUEST HT. The number of allowed trypsin missed cleavages set to 2. Carbamidomethylation of Cysteine was set as a static modification while Deamidation of Asparagine and Glutamine, Oxidation of Methionine and Phosphorylation of Serine, Threonine and Tyrosine were set as variable modifications. The parent ion tolerance was set to 10 ppm with fragment ion tolerance set to 0.02 Da. Protein Identification thresholds were set requiring False Discovery Rate less than 0.05% with a Maximum Delta Cn of 0.05. Blastocoel fluid data were used to crosscheck and validate the proteins of interest identified in embryo-conditioned media.

Gene ontology analyses were performed using the DAVID online bioinformatics database.[14] STRING database was employed to assist with data mining and for detection of putative functional networks.[15]

3. Results

Embryo development in vitro was well supported under the conditions used, as demonstrated by continued growth and expansion of all blastocysts throughout the culture period (**Figure 1**). The diameters (mean ± SEM) of the blastocysts on the day of collection (Day 8 after ovulation), after 24 h of culture (Day 9), and after 48 h of culture (Day 10), were 1.05 ± 0.24 mm, 1.44 ± 0.24 mm and 2.29 ± 0.09 mm, respectively. The percent increases in mean blastocyst diameter from Day 8 to 9, and from Day 9 to 10, were 45.1 ± 12.7% and 38.2 ± 4.7%, respectively. The overall percent increase in mean blastocyst diameter from Day 8 to 10 was 87.5 ± 20.0%.

Embryo conditioned medium was analyzed to investigate the secretome of early embryos. Conditioned medium analysis identified a total of 72 and 97 unique IDs with MASCOT scores >35, at 24 and 48 h of culture, respectively (File 1, Supporting Information). Protein IDs were mapped to 58 (24 h) and 80 (48 h) gene IDs recognized by the DAVID database and these were used to perform gene ontology analysis comparing the profiles of 24 h and 48 h time-points (**Figure 2**). Lipid-, lipoprotein- and cholesterol-associated processes were overrepresented across both time-points, while cellular component GO analysis indicated the extracellular region (along with vesicles, vacuoles and lysosomes) to be the dominant putative origin of these proteins. Among molecular functions, enzyme activity and in particular, peptidase activity, were clearly enriched at both time-points, with enrichment of several binding functions emerging in the 48 h profile. Proteins of interest were identified through literature searches and data mining, and

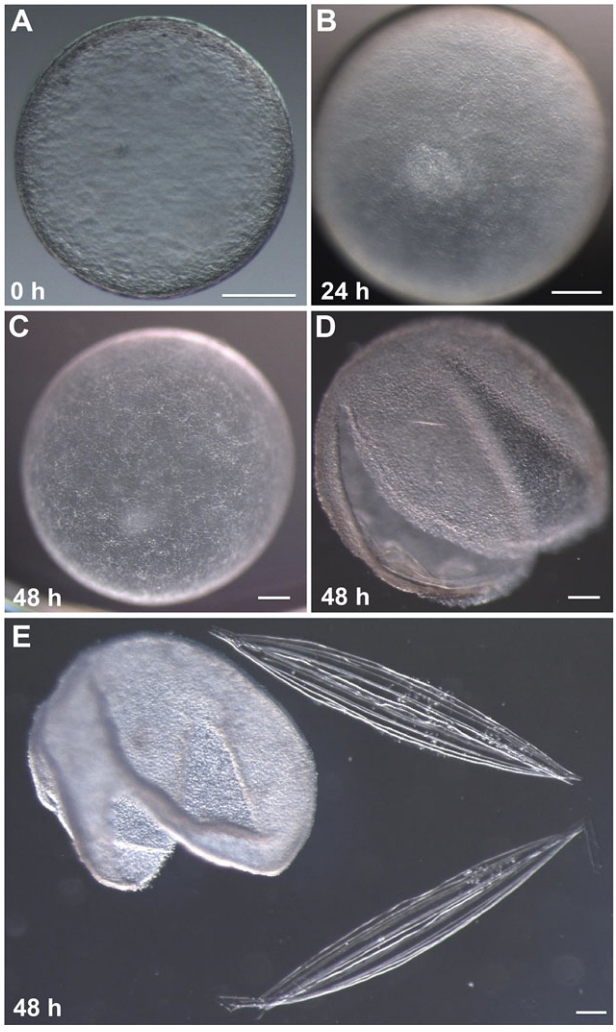

Figure 1. Embryos recovered by uterine lavage and cultured in protein-free media were photographed immediately after recovery (A), and following 24 h (B) and 48 h (D) of culture. At the end of the culture period embryos were washed and dissected with a microblade (D) to release blastocoel fluid and isolate the embryo capsule, visible as a clear acellular structure detached from the trophoblast in (E). Scale bar = 0.25 mm.

subsequently validated by crosschecking against blastocoel fluid proteome; these are presented in **Table 1**.

Blastocoel fluid analysis by Orbitrap MS/MS was used to validate proteins of interest identified in embryo culture media analysis and to further characterize proteins produced by the conceptus; this revealed a pooled total of 732 protein IDs. Of these, 404 presented with two or more unique peptides and these proteins are listed in File 2, Supporting Information. Gene ontology analysis (**Figure 3**) supported translation and regulation of cellular metabolic processes as key biological processes, cytosol and intracellular organelles as cellular compartments of origin, and nucleotide binding as a dominant molecular activity. Literature searches and text mining were further used to identify proteins pertinent to equine maternal recognition of pregnancy and associated mechanisms, yielding FK506-binding protein 4 (FKBP4), follistatin, follistatin-like 1, hydroxysteroid (17-beta) dehydrogenase 1, prostaglandin F2 receptor negative regulator (PTGFRN); annexins A2 and A4, phospholipase A2 (PLA2G7), heat shock 10 kDa protein (HSPE1), cadherins 1 and 2, insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1), insulin-like growth factor 2 receptor (IGF2R), and insulin-like growth factor binding protein 2 (IGFBP2).

Embryo capsule analysis revealed 11 proteins with two or more peptide spectrum matches. Of these, only four presented with two or more unique peptides; these were transglutaminase 3, chitobiase, uterocalin and TRIM42. Gene ontology analysis was not pursued due to the small number of IDs in this profile and, instead, literature searching was used to assess the relevance of each protein/gene to known and proposed functions of the embryo capsule in early pregnancy. Proteins detected in capsules and their relevant documented functions are presented in **Table 2**.

4. Discussion

This study describes the first comprehensive proteomic analysis of early equine pregnancy, including analysis of the embryo secretome, embryo capsule and blastocoel fluid. Highly sensitive mass spectrometry methods were used to detect low abundance proteins, and several hundred protein identifications obtained.

Gene ontology (GO) analyses provide a 'snapshot' of the protein profile of each sample type and time-point analyzed. For example, the dominance of "translation" and "RNA binding" as biological processes in blastocoel proteins is reflective of the early embryo's rapid growth, as it doubles in size daily and engages in protein synthesis accordingly. The predominance of "extracellular region" as the putative origin of proteins identified in embryo conditioned media provides some reassurance that the proteins detected in this experiment do indeed represent the secretome of the embryo rather than the incidental shedding of structural proteins into culture media. Furthermore, the emergence of specific groups of biological process categories can point to a greater than anticipated involvement of certain processes in early pregnancy; specifically, the incidence of lipid-, glycolipid-, phospholipid-, cholesterol- and lipoprotein-associated biological processes in GO analysis of embryo-conditioned medium proteins was striking and suggests a major role of lipids and protein–lipid complexes in supporting the early embryo. Indeed, a multitude of apolipoproteins, together with related proteins and lipoprotein receptors, were detected in embryo-conditioned medium and blastocoel fluid samples: apolipoproteins A1, A2, B, C2, C3, E, M; low density lipoprotein-related proteins 1 and 2.

Apolipoproteins have been previously reported in transcriptome studies of the equine conceptus and endometrium.[11] Most commonly their presence is attributed to the nutritional demands of the embryo, and the transport of lipids to support these demands. Recent work has also revealed additional roles for apolipoproteins; these include signalling functions,[16] the capacity to bind, transport and deliver microRNAs,[17] and some unique roles in supporting pregnancy.[18] The concurrent detection of apolipoprotein binding receptors in the present study (e.g. ABCA1 in embryo capsule; Table 2) supports the notion that apolipoproteins and their interacting partners form functional networks in the early pregnancy milieu. In a search for

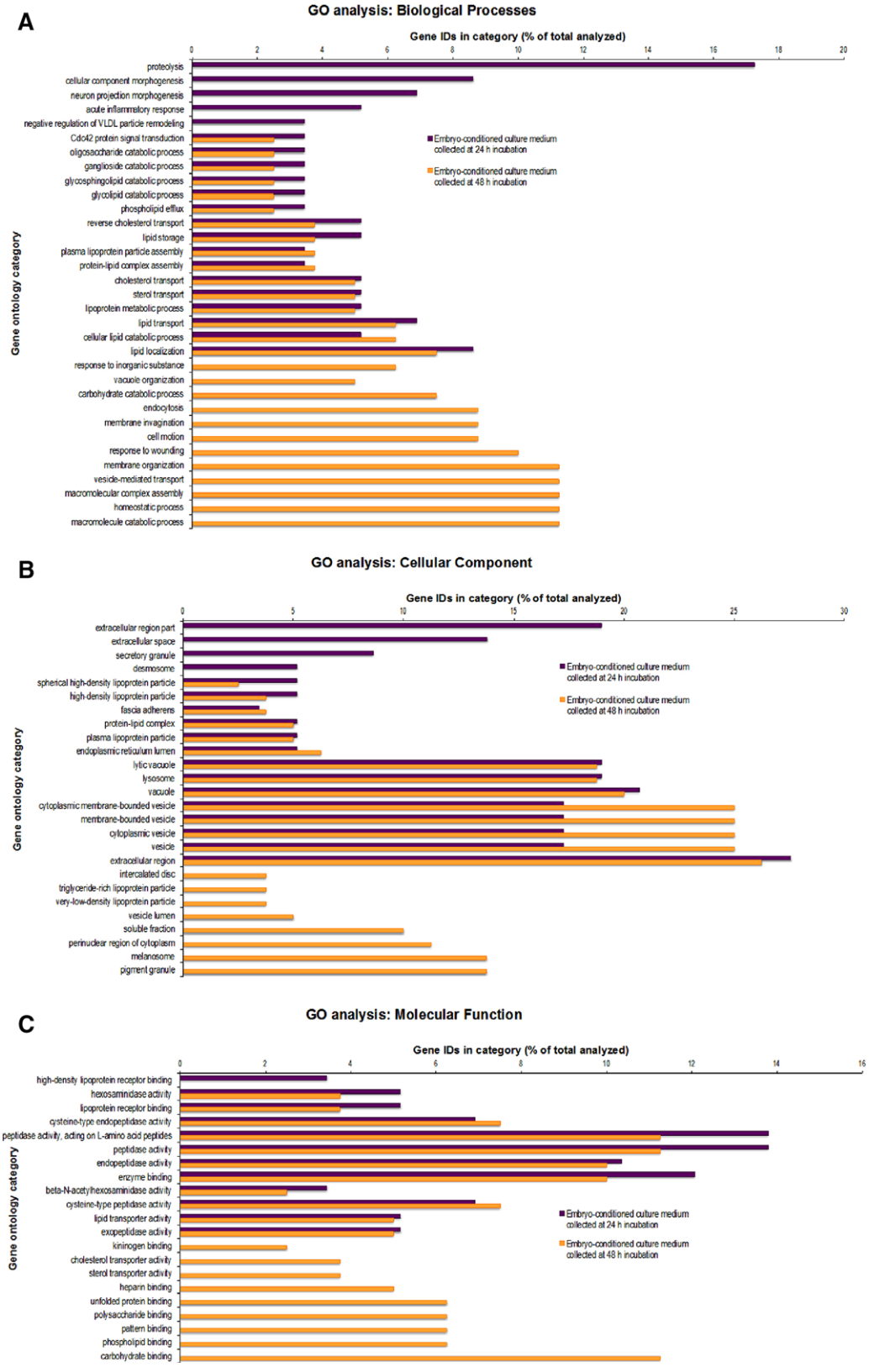

Figure 2. Gene ontology analyses corresponding to proteins detected in embryo conditioned medium, comparing 24 and 48 h of culture. Embryos ($n = 4$) were collected on day 8 after ovulation and cultured in protein-free medium prior to LC-MS/MS analysis of culture media samples. Top categories for biological process (A), cellular compartment (B) and molecular function (C) are shown.

Table 1. Proteins of interest detected in embryo-conditioned culture media and evidence for their potential functional relevance to early pregnancy in the mare

Time	Protein	Gene ID	MASCOT score	Potential role in early equine pregnancy
24 h & 48 h	Apolipoprotein A-I	APOA1	321	Has been detected in secretome of human IVF embryos; lower levels of APOA1 secretion were consistently associated with higher likelihood of viable pregnancies and thus proposed as a useful biomarker of embryo competence.[19] APOA1 can bind, transport and deliver microRNAs[12] in circulation and could be involved in microRNA-mediated signalling between the early embryo and maternal endometrium.
	Cathepsin A Cathepsin D* Cathepsin L1 Cathepsin L2* Cathepsin Z	CTSA CTSD CTSL1 CTSL2 CTSZ	114 103.9 443.5 494.9 151	Cysteine proteases with known functions at maternal-fetal interface, roles in blastocyst hatching (ZP dissolution) and implantation. Cathepsins L, P and B implicated in zona hatching of hamster blastocysts.[58] Cathepsin D is present in extracellular compartment of the maternal-fetal interface (mouse) and its protease activity appears necessary for trophoblast invasion.[59] Cathepsins B and L have potential roles in endometrial/placental remodelling in the pig.[60] Cathepsins B, L and D shown to originate from trophoblast in the mouse.[61] Several cathepsins of endometrial and conceptus origin suggested to regulate endometrial remodelling and conceptus implantation in sheep.[62] Protease activity appears to be important in establishment of the equine pregnancy since numerous members of the cathepsin group, along with other proteases, were identified with high confidence both in embryo conditioned media and blastocoel fluid.
	Chitobiase*	CTBS	380.7	A glycoprotein-degrading enzyme with highly species-dependent expression (expressed in humans and rodents but not ruminants, cats or dogs).[63] Probably involved in degradation of the embryo capsule.
	GM2 activator protein	GM2A	91.7	Lipid carrier protein. Shown to be abundant in equine trophoblast during the encapsulated phase; can be secreted into, and re-captured from, extracellular fluid, with or without bound lipid. Suggested role in acquisition, retention or sequestration of glycolipids or phospholipids that are transported through the capsule.[64]
	Insulin-like growth factor 2 receptor	IGF2R	131	Receptor for both mannose 6-phosphate and insulin-like growth factor 2; potential for multiple roles at feto-maternal interface.[65] IGF2R is a multifunction receptor (e.g. intracellular trafficking of lysosomal enzymes, activation of transforming growth factor beta, and the degradation of insulin-like growth factor 2) and is maternally imprinted in the mouse. IGF2 stimulates human trophoblast cell migration/invasion.[66]
	Pregnancy-associated glycoprotein	PAG	83.1	Belongs to a group of pregnancy-specific proteins; multiple PAGs are present in ruminants and clinically exploited for pregnancy testing via serum.[67] Role in equine pregnancy unknown but may involve immune modulation, luteotrophic activity, and/or proteinase activity in regulation of maternal environment.[21] Previously found to be expressed in equine placenta.[68]
	Transcobalamin II*	TCN2	260	Binds and delivers vitamin B12 to cells; vitamin B12 is important for maintaining pregnancy and other associated molecules, including homocysteine and folic acid. Polymorphisms in TCN2 gene (humans) reported to be a genetic risk factor for idiopathic recurrent spontaneous abortion.[32,33]
24 h	Follistatin-like 1	FSTL1	37	Secreted protein with diverse but poorly understood functions. FSTLs have been implicated in embryo development and BMP signaling.[69]
	Low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor)	LRP1	35.8	Endocytic transmembrane receptor considered essential for early embryo development; highly expressed in human placenta.[70,71] Involved in cellular lipid homeostasis. Involved in the plasma clearance of chylomicron remnants and activated alpha 2-macroglobulin.
	Annexin A2	ANXA2	38.5	Annexins are calcium-dependent phospholipid-binding proteins; they also have roles in endocytosis and exocytosis[72] and can inhibit PLA2 (which liberates arachidonic acid as a limiting step in prostaglandin synthesis). Annexin A2 previously detected in mare endometrium, and yolk-sacs of 20 day old equine conceptuses[73]; possibly involved in embryo-maternal transport/signalling.
	Apolipoprotein E	APOE	46	A major component of fetal high-density lipoprotein and appears to be involved to feto-maternal communication, regulating genes controlling cholesterol biosynthesis and stimulating expression of potent anti-oxidative metallothioneins.[74] Specific polymorphisms of APOE gene in humans associated with increased risk of recurrent pregnancy loss.[75]

(*Continued*)

Table 1. Continued

Time	Protein	Gene ID	MASCOT score	Potential role in early equine pregnancy
	Calumenin	CALU	40.8	Calcium-binding protein; its down-regulation in (human) placental proteome associated with unexplained recurrent pregnancy loss.[76]
	Cathepsin B	CTSB	38.7	As above for cathepsins.
	Chaperonin containing TCP1, subunit 8	CCT8	39.8	Chaperone proteins: major role is to promote cell survival via prevention of incorrect folding of newly synthesized proteins. Chaperone functions are necessary in early embryos due to rapid growth and extensive synthesis of new proteins. Heat shock proteins (HSPs) are involved in the cellular response to stress, defences against heat and oxidative stress, and prevention of cell death. Hsp70 induction during pregnancy proposed to be involved in promoting maternal tolerance to paternal antigens.[77] Excessive hsp70 associated with compromised embryogenesis and adverse pregnancy outcomes[78]; thus HSPs may be useful biomarkers for embryo stress and developmental competence.
	Heat Shock Protein Family A (Hsp70) Member 5	HSPA5	78.5	
	Heat Shock Protein Family A (Hsp70) Member 8	HSPA8	464.5	
48 h	Lectin, galactoside-binding, soluble, 1 (Galectin)	LGALS1	63.1	Lectin with a multitude of demonstrated and proposed roles in pregnancy including maternal fetal tolerance, regulation of growth, differentiation, placentation, and protein-protein interactions within the extracellular matrix.[79] Participates in interactions with progesterone and estrogen (e.g. supplementation with galectin-1 restored progesterone levels in stressed pregnancies). Secreted into culture media by human embryos. Human serum galectin-1 levels are decreased in patients with recurrent spontaneous abortion[80]; galectin-1 circulating levels have been identified as a novel biomarker for predicting pregnancy outcome, being more sensitive than serum β-hCG levels.[81] We propose this protein may have significant potential in clinical diagnosis of pregnancy outcome in the mare.
	Phospholipase A2, group VII	PLA2G7	44.6	Secreted, calcium-independent, lipoprotein-associated phospholipase; member of the arachidonic acid releasing PLA2 family. Potential roles in protection from oxidative stress. Various isoforms of PLA2 previously detected in equine conceptuses[11,82]; likely role in supplying arachidonic acid as substrate for prostaglandin synthesis by the conceptus or endometrium. PLA2G7 is a preeclampsia-associated gene in humans.[83] Potentially an indicator of conceptus at risk of demise and may serve as a biomarker of embryo competence.
	Stress-induced-phosphoprotein 1	STIP1	37.2	An adaptor protein that coordinates the functions of HSP70 and HSP90 in protein folding. Likely to assist in the transfer of proteins from HSP70 to HSP90 by binding both HSP90 and substrate-bound HSP70; thus suggests that these HSPs (also detected in embryo-conditioned media) are functional and play active roles in early embryo survival.
	Transferrin receptor	TFRC	85.9	Cell surface receptor essential for cellular iron uptake across the placenta; located in the trophoblast plasma membranes[84]; required for fetal growth, erythropoiesis and neurologic development.
	Transglutaminase 3*	TGM3	693	Calcium-dependent enzyme that catalyzes covalent crosslinking of proteins. Suspected to be responsible for stabilizing the protein matrix of rabbit blastocyst coats, which share biochemical similarities with equine embryo capsules.[20] High levels of TGM3 transcripts previously detected in trophectoderm of 8 day equine embryos.[85]

All IDs listed above have been validated by crosschecking against protein IDs detected in blastocoel fluid samples. * indicates protein was detected in all biological replicates.

biomarkers of embryo quality undertaken via proteomic analysis of human embryo conditioned media, APOA1 emerged as the major protein quantitatively correlated with pregnancy outcome.[19] Notably, lower levels of secreted APOA1 were predictive of a successful pregnancy, and the authors proposed that the embryo's capacity to bind and/or internalize APOA1 might be reflective of its metabolic competence. Further profiling of embryo-secreted apolipoproteins (including their lipidation status and microRNA cargo) in healthy versus metabolically 'stressed' embryos could lead to development of an embryo screening protocol that would facilitate quality control in embryo transfer programs.

Another prominent protein group identified in embryo-conditioned media was that of the cathepsins (A, B, D, L1, L2, Z). Together with the proteinase PAG (pregnancy-associated glycoprotein), these reflect the dominance of the 'proteolysis' GO category in conditioned medium samples and suggest that proteolytic activity is rampant in the equine embryo's immediate environment. Several cathepsins have documented roles at the feto-maternal interface in other species (see Table 1), and it is likely

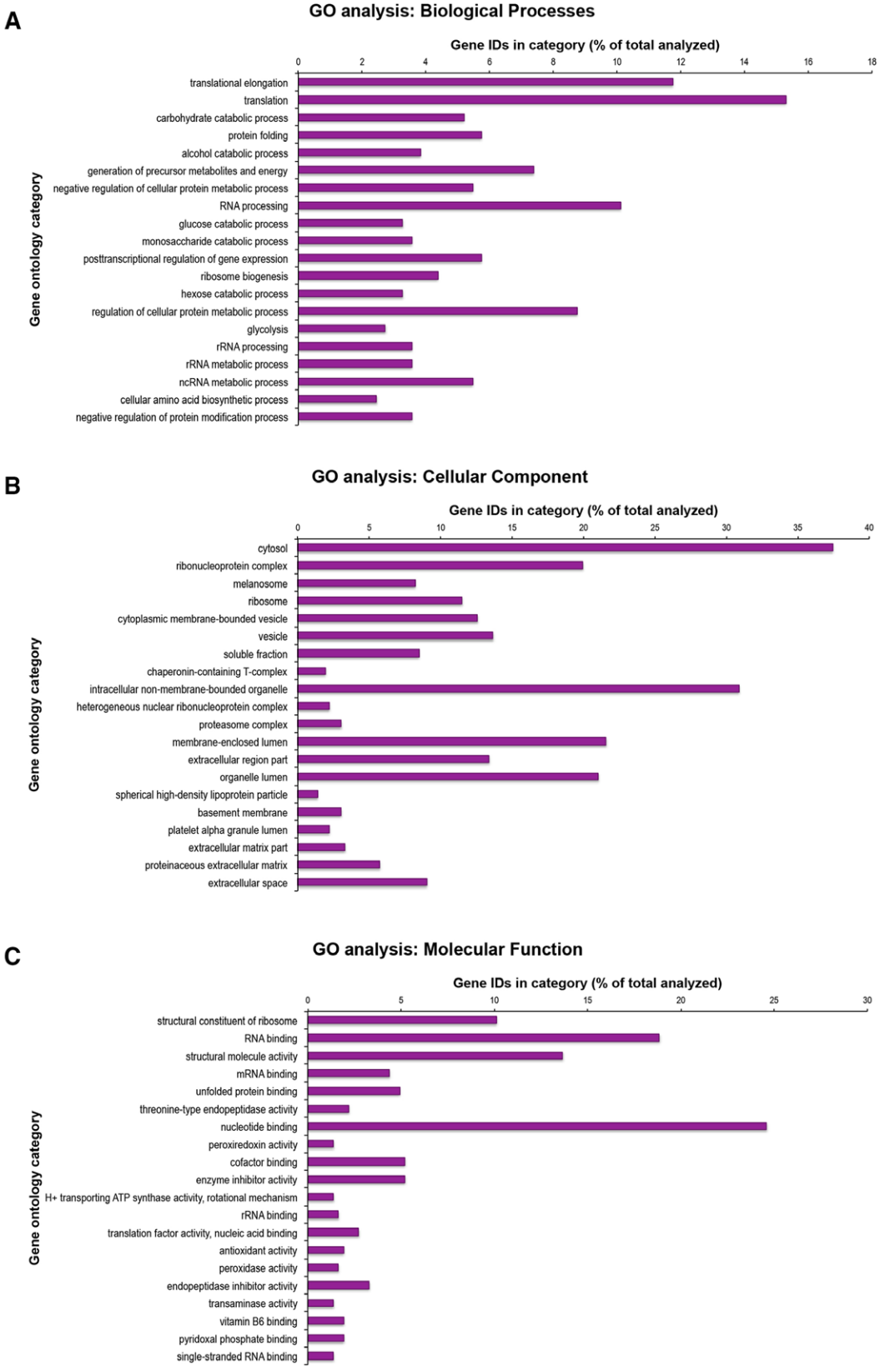

Figure 3. Gene ontology analyses corresponding to proteins detected in blastocoel fluid of embryos ($n = 4$) collected on day 8 after ovulation and cultured for a further 48 h in vitro. Top categories for biological process (A), cellular compartment (B) and molecular function (C) are shown.

Table 2. Proteins detected in equine embryo capsules of 10-day old embryos

Protein	Gene ID	# Unique Peptides	# PSMs	Potential role in early equine pregnancy
Transglutaminase 3	TGM3	23	369	Calcium-dependent enzyme that catalyzes covalent crosslinking of proteins. Suspected to be responsible for stabilizing the protein matrix of rabbit blastocyst coats, which share biochemical similarities with equine embryo capsules.[20] Possible role in reducing immunogenicity of implanting rabbit embryos.[86] Stabilizes FSH receptor-hormone complexes.[87] High levels of TGM3 transcripts previously detected in trophectoderm of 8 day equine embryos.[85]
Chitobiase	CTBS	8	125	A glycoprotein-degrading enzyme with highly species-dependent expression (expressed in humans and rodents but not ruminants, cats or dogs).[63] Probably involved in degradation of the embryo capsule. Previously detected in 12- and 14-day equine embryo transcriptome.[11]
Uterocalin	P19	2	6	Major component of endometrial secretions during early pregnancy, contributes to capsule formation, helps to deliver lipids to embryo prior to implantation.[88–90]
Tripartite Motif Containing 42	TRIM42	2	2	Biological roles of TRIM family proteins are poorly characterized; function in equine pregnancy unclear.
Potassium voltage-gated channel subfamily A member 3-like protein		1	5	Potassium voltage-gated channels are involved regulation of cell cycle, cellular differentiation and growth.[91]
Phospholipase A2	SPLA2	1	4	Suggested to contribute to the imminent removal and degradation of the capsule.[92]
Amine oxidase [flavin-containing] (Fragment)		1	4	Mitochondrial enzyme; catalyzes the oxidation of monoamines such as dopamine, serotonin and adrenalin. Abundantly expressed in the human CL of pregnancy[93]; suggested as a marker of uterine receptivity at implantation (human and mouse).[94] Inhibitors suppress fertility in rats.
Biorientation Of Chromosomes In Cell Division 1 Like 1	BOD1L1	1	4	Component of the fork protection machinery required to protect stalled/damaged replication forks from uncontrolled DNA2-dependent resection; likely developmental role.
Multiple EGF Like Domains 8	MEGF8	1	4	Regulator of left-right patterning[95]; likely developmental role. Amino acid sequence indicates receptor function but none yet demonstrated.
ATP Binding Cassette Subfamily A Member 1	ABCA1	1	3	Regulates materno-fetal cholesterol transfer; packages cholesterol into APOA1. Implicated in epigenetic alteration of offspring lipid profile through DNA methylation in uterine environment.[96] Necessary for normal placental development in mice.[97]
CD101 Molecule; Cell Surface Glycoprotein V7	CD101	1	2	Cell surface glycoprotein; inhibits T-cell proliferation and reactivity. Likely involved in capsule-endometrium communication to suppress maternal T-cell response. Also is a paralog of PTGFRN, a prostaglandin receptor inhibitor.

Only IDs with two or more peptide spectrum matches are shown, along with documented functions of potential relevance to capsule functions and early pregnancy in the horse. (PSMs: peptide spectrum matches).

that their proteolytic activity contributes to endometrial remodelling and receptivity in the mare.

The major protein detected in the embryo capsule was transglutaminase 3, which stabilizes proteins by facilitating peptide crosslinking, likely contributing to maintenance of the capsule's rigid structure.[20] Other proteins within the capsule may play roles in downregulating the maternal immune response and nutrient shuttling (see Table 2).

Equine pregnancy-associated glycoprotein (PAG) was present in embryo-conditioned media, further validated by consistent detection in blastocoel fluid samples. PAGs are pregnancy-specific proteins produced within the trophoblast and trophectoderm. Initially designated as a unique feature of even-toed ungulates, where they constitute a large and evolutionarily complex family of about two dozen distinct proteins,[21] PAGs and PAG-like proteins have now also been characterized in odd-toed ungulates including equids[22] and many other mammalian orders.[23,24] Some PAGs are proteolytically active while others are not, and the definitive biological function of this protein family is still unclear. Unlike the numerous PAGs of ruminants, only a single PAG has been mapped in the equine genome, yet it appears to be one of the two most enzymatically active of all PAGs studied to date (together with porcine PAG2).[25] Expression of equine PAG has been reported in 25-day placenta[22] and PAG transcripts were detected in the 16-day conceptus.[26] The present study is the first to demonstrate that equine embryos can produce PAG as early as day 10 after conception. This phenomenon demands further investigation in light of the contribution of PAGs to pregnancy testing and monitoring in other species, particularly cattle, where the PAG ELISA is a mainstay of pregnancy detection.[27,28] Moreover, circulating PAG levels can be predictive of embryo mortality at 31–59 days of gestation in cattle.[29] Both the early appearance and proteolytic capacity of equine PAG suggest a functional role rather than an inert vestigial presence, and the wide utilization

of PAGs as pregnancy testing proteins in other species suggests a possible role for equine PAG in future clinical applications in pregnancy monitoring.

Transcobalamin 2 (TCN2) was among the few proteins to appear consistently in the secretome of all embryos analyzed, and was detected at both 24 h and 48 h of culture. This protein is responsible for the binding and delivery of vitamin B12 (cobalamin) to cells, with fetal origin TCN2 entering the maternal circulation during pregnancy.[30] Vitamin B12 acts as a coenzyme in folate activity to ultimately facilitate DNA methylation/synthesis. Not only is vitamin B12 recognized as vital to a successful pregnancy,[31] but at least two polymorphisms of *TCN2* present a genetic risk factor for idiopathic recurrent spontaneous abortion in women,[32,33] further illuminating the importance of effective vitamin B12 delivery in early pregnancy. In light of the prominent scientific discussion around contribution of folate and vitamin B12 to pregnancy outcome in recent years,[34–36] the paucity of literature on this subject as it relates to equids and other livestock is striking. Vitamin B12 and folate levels in the horse have only briefly been examined in the context of exercise and performance,[37,38] and not at all with regard to the requirements of conception and gestation. Serum levels of folate can vary dramatically between stabled and grazing horses,[39] while vitamin B12 is synthesized by gut microbes,[40,41] rendering it vulnerable to variations of the microbiome between individuals, and to variations in the profile of volatile fatty acids in the hindgut. The consistent, high-confidence detection of transcobalamin 2 in embryo conditioned culture medium (as well as in blastocoel fluid) in the present study supports a requirement of both vitamin B12 and folate for the equine embryo's development, and perhaps suggests that the contribution of their respective deficiencies to early embryo loss has been underestimated in the mare.

Blastocoel fluid analysis was primarily employed to validate protein IDs detected in embryo-conditioned media, ensuring that proteins of interest can be consistently detected by two different MS methods and are likely to originate from the conceptus itself. Due to the higher sensitivity of MS methods used to analyze blastocoel fluid, it is likely that some embryo-secreted factors would be present here despite remaining undetected in conditioned medium analysis, so a stand-alone analysis of blastocoel fluid proteins was performed in order to identify those with potential functional significance in embryo-maternal signaling. A notable finding to emerge from this analysis was the detection of PTGFRN – prostaglandin $F_{2\alpha}$ receptor inhibitor. PTGFRN inhibits the binding of $PGF_{2\alpha}$ to its specific receptor, seemingly by decreasing the receptor number rather than the affinity constant.[42,43] The exact nature of this interaction has not been examined since the 1990s and remains obscure. To our knowledge, our study represents the first report of this protein's presence in day 10–11 embryos, while the corresponding RNA transcripts have been previously identified in day 16 conceptuses.[26] Its discovery is acutely pertinent in light of the role of luteolytic prostaglandin $F_{2\alpha}$ and its receptors in early pregnancy in the mare: suppression of prostaglandin F receptors in the endometrium is one of the hallmarks of the early equine pregnancy,[44,45] and production of PTGFRN by the embryo provides a tantalizingly plausible mechanism by which the conceptus might exert such an effect. Furthermore, PTGFRN has been shown to associate with the plasma membrane[46] and is sorted from the membrane into exosomes,[47] flagging a potential secretory route for the shuttling of this protein from conceptus to endometrium.

Another notable finding to emerge from analysis of blastocoel fluid is the co-chaperone, FK-506-binding-protein-4 (FKBP4). This protein co-operates with HSP90 to form functional steroid receptor complexes, and is essential for activation of the progesterone receptor by progesterone.[48] Effectively a potentiator of progesterone action, its absence in mice causes complete infertility by way of uterine progesterone insensitivity, lack of uterine receptivity and consequent failure of implantation.[49] There is also a reported association between human early pregnancy loss and FKBP4 expression deficit.[50] Progestin supplementation of mares suffering a history of early embryo loss is commonplace in equine breeding practice, but FKBP4 has been largely ignored as a therapeutic target for fertility intervention – even though (together with HSP90) it is now known to be a critical, previously overlooked mechanism in progesterone function. Furthermore, considering the apparent lack of correlation between systemic progesterone levels and pregnancy outcome in the mare, assessment of relative progesterone resistance (and underlying aberrations in chaperone function/expression), rather than absolute deficiency, may be a more fruitful approach for future research into the role of progesterone in early pregnancy failure.

Excitingly, FKBP4 has been hailed as a promising therapeutic target due to the specificity of its interaction with steroid hormone receptors, opening avenues for both contraceptive and fertility-enhancement strategies.[51] In the present study, FKBP4 was found within the blastocoel fluid of 10 day old embryos but it is unclear whether this protein is actively secreted into the uterine lumen, or is capable of interaction with the mare's endometrium. It is tempting to speculate that the embryo itself assists in potentiating the effects of progesterone in the uterus via FKBP4 signalling. However FKBP4 was not detected in the embryo secretome in this instance, perhaps due to the lower sensitivity of the methods used for secretome samples versus blastocoel samples. Thus further studies are necessary to determine if the protein is released into the uterine environment by early embryos, and to establish the functional significance of this critical progesterone receptor co-chaperone in equine pregnancy and mare fertility status.

It should be noted that the culture conditions used in the present study supported considerable growth and development of all embryos, with the mean blastocyst diameter increasing from 1.05 mm to 2.29 mm over the 48 h culture period. While the initial diameter was typical for in vivo-derived equine embryos flushed on Day 8,[52,53] the final diameter was somewhat less than the ≥3 mm diameter reported for in vivo-derived equine embryos flushed on Day 10.[54] Previous studies have described the use of DMEM/F12 medium supplemented with fetal calf serum or bovine serum albumin to effectively culture equine embryos.[55–57] However, to avoid contaminating the samples with an exogenous protein source, which would have confounded the analyses, the DMEM/F12 medium used here was instead supplemented with a chemically defined macromolecule, polyvinyl alcohol. To the best of our knowledge, this is the first report to describe a chemically defined, protein-free medium that effectively supports the development of equine blastocysts and is conducive to proteomic analysis. Further studies are needed to

confirm whether embryos cultured in this medium maintain full developmental potential following embryo transfer.

Taken together, our results represent a significant step forward in understanding the enigmatic equine pregnancy. In particular, detection of prostaglandin-inhibiting and progesterone-potentiating proteins introduces potential new players into the maternal recognition of pregnancy cascade, while molecular chaperones, antioxidant and proteolytic enzymes, apolipoproteins and others provide clues about the demands and challenges experienced by early equine embryos at their most vulnerable time. The present study will serve as a valuable resource to researchers focused on the physiology of early equine pregnancy, including embryo loss and embryo-maternal signaling, and those working toward clinical improvements in pregnancy detection and monitoring, embryo transfer and fertility control.

Abbreviations

BF, blastocoel fluid; FKBP4, FK506-binding protein 4; GO, gene ontology; hCG, human chorionic gonadotrophin; MROP, maternal recognition of pregnancy; PAG, pregnancy-associated glycoprotein; PTGFRN, prostaglandin F receptor negative regulator; TCN2, transcobalamin 2

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

Early pregnancy loss, Equine embryo, Equine reproduction, Fertility, Secretome

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