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Changes in various endometrial proteins during cloprostenol-induced failure of early pregnancy in mares

M.A. Hayes[1], B.A. Quinn[1], B.N. Lillie[1], O. Côté[1], D. Bienzle[1], R.O. Waelchli[2], K.J. Betteridge[2]

[1]Departments of Pathobiology and [2]Biomedical Sciences, Ontario Veterinary College,
University of Guelph, Guelph, Ontario, Canada.

Abstract

Various major proteins in uterine secretions flushed from mares on day 20 of gestation have been identified and related to levels of gene expression in endometrial biopsies. Pregnancy-related changes were determined by comparison with samples from mares on day 20 after being given a luteolytic IM dose of cloprostenol on day 18, and with cycling mares that were not pregnant on days 17-20. Proteins in cell-free uterine flush samples were identified by LC-MS/MS of trypsin-digested peptides. Levels of endometrial expression were estimated from raw signal strength in Agilent 21322 *E. caballus* expression microarray. In this paper we describe a subset of pregnancy-related endometrial proteins with potential roles in embryonic development and/or mucosal innate immunity. Endometrial proteins that increased greatly during pregnancy included GM2 activator (GM2A), lipocalin 2 (LCN2), stanniocalcin 1 (STC1) and uterine serpin (SERPINA14). Endometrial proteins that decreased in normal pregnancy included secretory phospholipase A2 (sPLA2), secretoglobin 1A1 (SCGB1A1), and vanin 1 (VNN1). Cloprostenol-induced failure of pregnancy resulted in increased endometrial expression of SPLA2, cathepsin L1 (CTSL1), interleukin-1 receptor antagonist (IL1RN), SCGB1A1 epididymal secretory protein E1 (NPC2), connective tissue growth factor (CTGF), retinol binding protein 4 (RBP4), uteroferrin/TRAP5 (ACP5), SERPINA14, annexin A1 (ANXA1), annexin A3 (ANXA3), and vanin 3 (VNN3). Expression of P19 uterocalin (P19) was similarly high in pregnant and non-pregnant endometrium, and virtually undetectable in the yolk-sac wall. High levels of expression of ANXA2, GM2A, NPC2, CTSL1 and RBP4 in both endometrium and yolk-sac wall suggests that these have important roles on both sides of the maternal:conceptus interface. The properties and distribution of GM2A and NPC2 suggest that they are important in the transport of phospholipids and sterols. SPLA2, LCN2, SCGB1A1 and IL1RA have potential roles in mucosal innate immunity but their production is reduced during early pregnancy. These studies provide an inventory of many proteins in the uterine lumen during early pregnancy, and a subset for which local endometrial expression is altered when pregnancy is compromised by cloprostenol-induced luteolysis.

Keywords: endometrial proteins, lipid transport, lipocalins, phospholipase, trophoblast, yolk-sac.

Introduction

When the equine conceptus is first detectable in the uterus by transrectal ultrasonography (around day 10; ovulation = day 0), it is a small fluid filled vesicle comprised of the embryonic disk associated with an avascular bilaminar yolk-sac wall that consists of the trophectoderm and endoderm (Ginther *et al*., 1985; Betteridge, 2007; Allen and Wilsher, 2009). As the conceptus enlarges, the mesoderm separates these two layers to form the trilaminar yolk-sac wall (omphalopleure), and the allantois emanates from the embryo proper to establish chorioallantoic placentation. Of equine pregnancies diagnosed at about day 15, 16-17% are subsequently lost, most between days 15 and 35 (Morris and Allen, 2002). Early pregnancy is thus a period of economically important embryonic loss in mares so various laboratories have been characterizing the biological and molecular events involved in success or failure of early pregnancy.

During the second and third weeks of pregnancy, the conceptus is enclosed within a thin translucent acellular capsule that is essential for embryonic survival (Betteridge, 2000; Stout *et al*., 2005). The conceptus expands rapidly and moves freely within the uterus until it is immobilized ('fixed') around day 16 at the site of subsequent placentation. The capsule is composed of various proteins and glycans that are modified during the process of fixation, and it is largely degraded by day 21. The capsule of the mobile conceptus loses sialic acid during fixation in a process that evidently contributes to its initial attachment to the endometrium (Oriol *et al*., 1993; Arar *et al*., 2007). However, the immobile equine conceptus is still not firmly attached after fixation so the intact conceptus can be readily recovered by uterine lavage. This and various other features of equine pregnancy provide useful comparative research avenues for analysis of events involved in the success or failure of early pregnancy (Betteridge, 2000; Betteridge *et al*., 2012).

Recently available technologies, especially protein mass spectrometry and expression microarrays, combined with the release of the equine genome, have lead to more detailed knowledge of the molecular

Corresponding author: ahayes@uoguelph.ca
Phone: +1(519)824-4120 ext 54637
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components and events of the conceptus-endometrial interaction (Klein *et al*., 2010; Merkl *et al*., 2010; Klein and Troedsson, 2011). However, there are still important knowledge gaps relating to the manner by which the conceptus is recognized and retained, and the roles of various uterine proteins in the establishment of pregnancy. Our laboratory has been analyzing the composition of proteins in the uterine flush fluids, capsule, and yolk-sac fluids to better understand this interaction during the third week of successful pregnancy. For comparison, to learn how these components change as pregnancy fails, we induce failure in some mares by administering a single dose of cloprostenol at various times (Betteridge *et al*., 2012).

This report provides an overview of the more abundant identified proteins produced in and secreted by the equine uterus during the establishment of pregnancy. We also consider some for which endometrial gene expression changes the most during cloprostenol-induced pregnancy failure. Some of these have been well studied in recent years, but there is still much to learn about the physiological functions of most proteins secreted by the endometrium.

Materials and Methods

Animals

These studies involve 14-16 mares maintained at the University of Guelph according to procedures approved within standards of the Canadian Council of Animal Care. Mares in estrus were monitored ultrasonographically for ovulation (day 0.5 ± 0.5) and inseminated as described previously (Betteridge *et al*., 2012). After diagnosis of pregnancy at day 11.5, conceptus development was monitored by daily ultrasound imaging. On day 18.5, pregnant mares were given a single intramuscular injection of either saline (control pregnancies n = 5) or cloprostenol (250 µg Estrumate, Schering Canada, Inc.) to induce luteolysis and compromise pregnancy (n = 6).

Samples

Samples used in this report were processed as described previously (Quinn *et al*., 2007; Hayes *et al*., 2008). Conceptuses were collected by transcervical uterine lavage with 1 liter of phosphate-buffered saline on day 20 postovulation. After flushing, an endometrial biopsy was collected, half of which was snap frozen in liquid nitrogen or RNAlater® for proteomic studies or RNA analysis, and half fixed in methacarn or paraformaldehyde for histological studies. On recovery, the conceptus was rinsed in saline and yolk sac fluid aspirated and frozen at -70°C for proteomic studies. The capsule, embryo proper, and trilaminar and bilaminar portions of the yolk-sac wall were dissected and stored for various analyses as described. Uterine flush fluid was centrifuged to remove particulate debris and frozen at -20°C pending subsequent thawing and concentration 20-fold by ultrafiltration in 10 kDa cutoff centrifugal concentrators.

Protein analysis

Proteins in uterine flush, embryonic capsule and yolk-sac fluid or fragments of capsule were analysed by LC-MS/MS after in-solution trypsin digestion (performed at the Advanced Protein Technology Centre, Toronto, ON). Identified proteins were ranked by numbers of trypsin-digested peptides identified by LC-MS/MS using Scaffold 2 software (Proteome Software Inc., Portland, OR; Searle, 2010).

Various proteins including serum albumin, immunoglobulins and other plasma proteins that were present in uterine flush fluids, but not locally expressed (i.e. low signal on endometrial microarray) were not considered in this report. This exclusion was not an assumption that they are not secreted or important in the endometrial milieu; rather it related to our major interest in proteins produced locally from genes expressed in endometrium and/or yolk-sac wall.

For the locally-expressed and most-abundant proteins in flush fluid, we identified some that differed depending on whether pregnancy was proceeding normally (in saline-injected control mares) or had been compromised by cloprostenol treatment. The ratios of gene expression in endometrium or yolk-sac wall in these two categories were used to further identify pregnancy-associated genes with relatively high levels of expression in endometrium or yolk-sac wall. We also compared levels of endometrial expression control and cloprostenol-treated mares with levels in non-pregnant cycling mares (days 17 to 20 postovulation).

Immunohistochemistry (IHC)

Methacarn-fixed tissues were embedded in paraffin and 6 µm sections attached to positively charged glass slides (Animal Health Laboratory, University of Guelph). Sections were deparaffinized and rehydrated, then quenched in 3% hydrogen peroxide to reduce non-specific peroxidase, after which IHC was performed using Dako Envision + System-HRP kits for rabbit or mouse primary antibodies. Negative controls included omission of or substitution for the primary specific antibody used. The Pierce Metal Enhanced DAB Substrate kit (Thermo Fisher Scientific, Nepean, ON) was used for detection and the solution was prepared according to the instructions provided. The stained sections were dehydrated through ethanol (70 to 100%) and then xylene and mounted in Permount.

Gene expression

RNA was isolated from endometrium and yolk-sac walls using QIAGEN RNeasy Fibrous Tissue Mini

Kit according to the instructions of the manufacture (QIAGEN, Inc., Mississauga, ON). The RNA concentrations were determined (NanoDrop, Thermo Scientific, Wilmington, DE) and quality assessed (Bioanalyser, Agilent Technologies, Mississauga, ON) prior to transcriptome-wide microarray analysis using the Agilent Horse 4x44K single color gene expression 21322 array (performed at the University Health Network Microarray Centre, Toronto, ON). Data for the gene expression set were checked for overall quality using R (v2.14.0) with the Bioconductor framework package installed. Comparisons were based on log_2-transformed signals from a QC filtered set of 34328 probes (20-100th percentiles for raw signal) in the Agilent 21322 *E. caballus* array.

Statistical analysis

Microarray data for 43,663 probes were imported into Genespring v11.5.1 and normalized for spatial detrending for data under comparison. Comparisons were performed on a filtered subset of 34328 probes in the top 80th percentile of intensities in all samples. All analyses were performed on log_2-transformed data. Endometrial gene expression differences between control and cloprostenol-treated pregnant mares, and non-pregnant mares were first compared by ANOVA, whereas differences between yolk-sac walls from normal and cloprostenol-treated mares yolk-sac wall were compared by T-tests. In each case, differences were considered significant at $P < 0.05$ after Benjamini and Hochberg multiple testing correction. After the identification of a priority set of 21 proteins found in the uterine flush, expression values (see Table 2) were compared by ANOVA with a Benjamini and Hochberg multiple testing correction threshold of $P < 0.05$.

Results and Discussion

Table 1 lists the proteins identified in uterine flush by LC-MS/MS analysis for which there was enough signal in microarrays to indicate that they were produced in the endometrium. Uterine flush samples usually contained large amounts of proteins (e.g. serum albumin, transferrin, immunoglobulins, various other plasma globulins and hemoglobins) that were not major expression products in the endometrium; these are identified by their low rank for mRNA expression. Microarray studies also revealed differences in numerous genes for which the expressed protein was below the proteomic detection limit.

Table 1 lists 185 uterine flush proteins identified with >95% confidence by MS/MS of uterine flush samples from day-20 pregnant control mares, pregnant mares 48 h after cloprostenol treatment, and non-pregnant cycling mares (Table 1). These are ranked according to the average number of unique peptides identified in one or more flush samples from pregnant mares. These rankings are approximations of the amount present; the numbers of peptides detected also depends on the levels of post-translational modification and other factors. Thus, some proteins known to be abundant in SDS-PAGE were ranked lower than expected. From the proteins known to be in the flush fluid, we selected a subset of proteins (Table 2) as locally produced factors with putative innate immune defence or histotroph functions. These included some with high microarray expression signals and some innate immune factors with significant treatment- or pregnancy-related differences. The following describes the genes listed in Table 2A (endometrium) and Table 2B (yolk-sac wall) in decreasing order of the ratio of treated/control pregnant endometrium, and discusses some of their known or putative roles (Table 3) in normal pregnancy or in cloprostenol-induced pregnancy failure.

Secretory phospholipase A2 (sPLA$_2$)

Amounts of sPLA$_2$ protein detected in normal pregnant uterine flush were low but were ranked higher after cloprostenol. *SPLA2* gene expression was very low in normal pregnant endometrium, and 66-fold higher after cloprostenol, similar to levels in non-pregnant endometrium. *SPLA2* expression was low in the yolk-sac wall samples, indicating that sPLA$_2$ bound to the embryonic capsule originates in the endometrium. IHC with anti-human PLA$_2$-Type IIA (human *PLA2G2A* is most homologous with equine *SPLA2*) demonstrated that sPLA$_2$ is present in, and secreted by, the endometrial glands (Fig. 1A).

Secretory PLA$_2$ (PLA2 type IIA) is recognized as an extracellular phospholipid hydrolase that releases arachidonic acid (Boyanovsky and Webb, 2009). Hydrolysis of cell membranes of degenerate cells or microorganisms is apparently important in innate immune defence in the endometrial lumen (Nevalainen *et al*., 2008). *SPLA2* expression was high in non-pregnant mares in our study. Phospholipase activity and *PLA2G2A* expression increases after luteolysis around estrus in cycling mares (Ababneh and Troedsson, 2012). The reasons for marked reduction in *SPLA2/PLA2G2A* expression during the establishment of equine pregnancy have not been established. The potential to release arachidonic acid for prostaglandin production or lyse yolk-sac wall membranes or nutrient phospholipids could harm the conceptus. Previous studies have shown that sPLA$_2$ accumulates in the embryonic capsule after cloprostenol treatment (Quinn *et al*., 2007; Hayes *et al*., 2008), likely because it is a highly cationic protein that avidly interacts with capsule glycan which is acidic

(Arar *et al*., 2007) and targets the phospholipids of the trophoblast as the capsule starts to deteriorate.

Human $sPLA_2$ mediates various non-enzymatic functions that involve phospholipid binding (Birts *et al*., 2010). $sPLA_2$ is an acute phase protein that increases with inflammation and is therefore a potential contributor to early pregnancy failure if it is increased asynchronously in endometritis. Accordingly, $sPLA_2$ activity is a potential target for therapeutic inhibitors being developed. Inhibitors of cell-associated PLA2s do not target secretory phospholipases in the same way (Birts *et al*., 2010).

Interleukin receptor antagonist (IL1RA)

IL1RA, the product of the *IL1RN* gene, was found in low amounts in flush fluids from some pregnant mares. The baseline levels of *IL1RN* expression in the endometrium from day-20 pregnant mares, as well as non-pregnant mares, were very low. However, after cloprostenol treatment, *IL1RN* expression in the pregnant endometrium increased ~60 fold. Others have recently reported that equine endometrial *IL1RN* expression is progesterone-related and preferentially increased near the conceptus at day 25 (Haneda *et al*., 2009). This suggests that regulation of IL-1 by IL1RA might be important in the establishment of pregnancy and that the IL-1 activity could be detrimental. However, the increases in *IL1RN* expression that we observed at day 20 in mares after cloprostenol-induced luteolysis suggests that progesterone is not responsible for induced *IL1RN*. An alternative explanation is that *IL1RN* expression after cloprostenol is part of an inflammatory response elicited by PGF2α. Cloprostenol also increases expression of endometrial *SAA1* (not shown), which encodes an acute phase protein that is increased locally in mares with endometritis (Christoffersen *et al*., 2010; Berg *et al*., 2011).

Cathepsins

We detected various cathepsins in uterine flush fluids, especially cathepsins A, B, L, Z. High levels of expression of *CTSL1*, *CTSL2*, *CTSA* and *CTSZ* were observed in the pregnant endometrium and in the yolk-sac wall, but we detected virtually no *CTSB* expression signal. This suggests that cathepsin B in the flush fluids mostly originated from blood or had been preformed in the uterus. *CTSZ* was variable but ~2.3-2.8-fold higher (not significant) in pregnant endometrium. *CTSL1* expression was low in normal pregnant endometrium but ~20-fold higher in mares that had been treated with cloprostenol, and levels in the non-pregnant uterus were much higher than during normal pregnancy. This suggests that *CTSL1* expression is reduced during early pregnancy, following an expression profile similar to that of $sPLA_2$, raising the possibility that cathepsin L1 is actually harmful during the establishment of pregnancy. Horses have two forms of cathepsin L1-like proteins, from separate *CTSL1*-like genes (LOC100061532 and LOC100061234) on chromosome 23; these are designated *CTSL1-1* and *CTSL1-2*, respectively.

Our observation for *CTSL1* expression in mares contrasts with responses reported in ruminants and pigs. Endometrial *CTSL* is highly expressed and progesterone-dependent during early pregnancy in sheep and is induced by progesterone and IFN-tau which is essential for pregnancy development in ruminants (Song *et al*., 2005, 2010). Cathepsins like CTSL are also important in placentation in mice (Screen *et al*., 2008).

Connective tissue growth factor (CTGF)

In the present studies, small amounts of CTGF protein were identified in the uterine flush. Endometrial *CTGF* expression was present at moderate levels during pregnancy, but increased ~7-fold after cloprostenol treatment. Thus, the reduced *CTGF* expression during pregnancy resembled the gene expression responses of *SPLA2* and *CTSL1*.

CTGF has been previously observed to accumulate in the embryonic capsule and to decline in expression in the endometrium after cloprostenol treatment (Lillie *et al*., 2010). It is cationic which likely explains its propensity to associate with the capsule. Our findings indicate that CTGF is important in the non-pregnant uterus but is markedly reduced during early pregnancy. Others have reported that endometrial *CTGF* expression decreases during early pregnancy (Klein *et al*., 2010). In some species, CTGF is progesterone-dependent and increases as pregnancy is established (Moussad *et al*., 2002). However, CTGF is a well-recognized endometrial cytokine that is important in endometrial repair and not necessarily related to pregnancy, at least in humans (Maybin *et al*., 2012).

Uterine serpin (SERPINA14)

Uterine serpin, now referred to as SERPINA14 (Padua and Hansen, 2010), was among the more abundant locally expressed proteins in uterine flush from pregnant mares, but it was also found in lower amounts in non-pregnant cycling mares. Correspondingly, endometrial expression of *SERPINA14* was increased in pregnant, compared with non-pregnant mares. Interestingly, cloprostenol treatment resulted in a 6.2 fold increase in endometrial gene expression over that seen in normal control pregnant endometrium. It is notable that there was a substantial expression of *SERPINA14* in the yolk-sac

wall at day 20. Being highly cationic, uterine serpin binds to the capsule as do $sPLA_2$ and uterocalin (Hayes *et al*., 2008; Lillie *et al*., 2010).

SERPINA14 is a major secreted protein in the pregnant endometrium in animals with epitheliochorial placentation, especially horses, pigs and ruminants (Padua *et al*., 2010). The *SERPINA14* gene belongs to the large family of serine proteinase inhibitors (serpins), but it lacks most of the ability to inhibit serine proteases. While *SERPINA14* is induced in endometrial glands by progesterone during the luteal phase in sheep, in cattle it is estrogen-dependent and increases during estrus (Ulbrich *et al*., 2009). Various immunoreactive forms of uterine serpin have been described in uterine flushes from mares, and these are increased in ovariectomized mares treated with progesterone and estrogen separately or in combination (Padua *et al*., 2010). Uterine serpin has immunosuppressive functions in some species and nutrient transport functions (Padua *et al*., 2010) but its function(s) in equine pregnancy are still unclear.

Lipocalin 2

Lipocalin 2, also known as neutrophil gelatinase-associated lipocalin (NGAL), is the product of the *LCN2* gene. At day 20, amounts in uterine flush fluids were higher in pregnant than in non-pregnant mares, and *LCN2* was expressed at a 2.3-fold higher level. However, expression during pregnancy is increased 4.57-fold by cloprostenol treatment on day 18. *LCN2* was minimally expressed in the yolk-sac wall but was detected in yolk sac fluid and in the capsule (not shown).

Lipocalin 2 is prominent as a 24 kDa protein in mouse uterine secretions (Kuo *et al*., 2009) but it is unlikely that lipocalin 2 is critical for reproductive success because *lcn* –/– mice are viable and apparently reproduce normally (Chan *et al*., 2009). Lipocalin 2 is inducibly expressed in various mucosal surfaces and is constitutively expressed in neutrophils from which it co-purifies in association with MMP9 (gelatinase; Li and Chan, 2011; Chakraborty *et al*., 2012). Its function has become considered to be more important since the recognition that it facilitates binding of bacterial siderophores and thereby impedes iron acquisition by microbes (Li *et al*., 2011). Lipocalin 2 is being developed as a biomarker to monitor inflammatory responses because it is induced in various tissues undergoing infection or inflammation (Li and Chan, 2011). Recent studies have shown *LCN2* expression to be elevated in pregnant endometrium (Kikuchi *et al*., 2011) and in recurrent uveitis in horses (Hofmaier *et al*., 2011). Various bacterial pathogens express lipocalin 2-resistant siderophores (Li and Chan, 2011) so it is expected that bacteria involved in chronic endometritis will exhibit such properties.

Secretoglobin 1A1 (SCGB1A1)

Secretoglobin 1A1 (SCGB1A1) is also known as uteroglobin, as it is a major secretory protein in the uterus, or Clara Cell Secretory Protein (CCSP), as it is also a major protein secreted in small pulmonary airways (Mukherjee *et al*., 2007). Endometrial *SCGB1A1* expression in pregnant endometrium was significantly increased ~4.5 fold after cloprostenol treatment, returning towards the levels of expression observed in the non-pregnant controls.

SCGB1A1 has various innate immune functions that are likely important in mucosal defence in the two mucosal surfaces in which they are most abundant. Among the various anti-inflammatory and immunomodulatory functions proposed are its ability to inhibit $sPLA_2$ (PLA2G2A) and to sequester hydrophobic molecules including retinol, progesterone, phospholipids and prostaglandins (Mukherjee *et al*., 2007). Equine SCGB1A1 also has anti-inflammatory effects such that reduced production in airways occurs in horses with chronic recurrent airway obstruction (Katavolos *et al*., 2009, 2011). On this basis, it is expected that SCGB1A1 is important in reducing the inflammatory environment in the uterus, as may be required for the establishment of pregnancy. Alternatively, the various factors involved in phospholipid and sterol transport during early pregnancy suggest that it might also facilitate these functions. It is unknown whether the two forms of equine SCGB1A1 differ in their functions. Most mammals have a single *SCGB1A1* gene but horses have a cluster of three *SCGB1A1* genes on chromosome 12; of these, only the *SCGB1A1-2* and *-3* are expressed in the lung and uterus (Côté *et al*., 2012; University of Guelph, Canada; unpublished). These correspond to the different uterine flush proteins with pI ~5 or ~6 respectively that are distinguishable by MS/MS (Hayes *et al*., 2008). IHC with anti-horse CCSP (Katavolos *et al*., 2009), which does not distinguish the two forms, show SCGB1A1 expressed in some endometrial glands (Fig. 1B), in accordance with reports of others (Ellenberger *et al*., 2008; Hoffmann *et al*., 2009). The *SCGB1A1* probe in the Agilent 21322 microarray recognizes a sequence common to both so the expression results are a composite of both.

Retinol-binding protein 4 (RBP4)

While only small amounts of RBP4 were identified in the uterine flush samples, RBP4 was a major expressed product of the uterus and particularly of the yolk-sac wall. Expression was increased 4.3 fold in the endometrium after cloprostenol. RBP4 has been found previously in the embryonic capsule, likely as an incidental association of an abundant protein (Lillie *et al*., 2010). RBP4 is a lipocalin with a major role in transport of retinoids in the plasma in which it

associates with transthyretin. In pigs, RBP4 has been previously recognized as a progesterone-induced gene involved in early pregnancy (Schweigert *et al*., 1999). However, it is an ubiquitous lipocalin, present also in the non-pregnant cycling uterus, so it is likely to have other functions besides retinoid supply to the conceptus.

Annexins

Proteomic analysis of uterine flush fluids identified various annexins, namely A1 to A5 and A8. Of these, annexin A1 and A2 were more substantial components of the uterine flush and were also found in embryonic capsules. Levels of endometrial gene expression and protein in uterine flush at day 20 were mostly unaffected by pregnancy status or prior cloprostenol treatment, except that endometrial expression of annexins A3 and A5 was ~3-fold higher in normal pregnancy. Annexin A2 was one of the most highly expressed proteins in the yolk-sac wall, regardless of cloprostenol exposure, and small amounts of the protein were identified in the yolk-sac fluid.

Substantial amounts of annexin A1 and A2 in our cell-free uterine fluid preparations indicate that they likely have important functions in the uterine lumen. Annexins are Ca^{2+}-dependent phospholipid-binding proteins with various roles relating to their location on the cytoplasmic face of cell membranes (Gerke *et al*., 2005). Annexins play various roles in endocytosis and exocytosis so it is plausible that they might be released during the exocrine production of histotroph. Abundant annexin A2 in the endometrium and yolk-sac wall is likely to be involved in some transport processes and the maternal:conceptus interface. By comparison, annexin A1 has well-defined innate immune functions.

Annexin A1, also known as lipocortin-1, is the product of the *ANXA1* gene and is a central regulator of the inflammatory and immune response. Glucocorticoids mediate their physiological and pharmacological abilities to reduce and resolve inflammation, largely through their induction of expression of annexin 1, formerly known as lipocortin-1 (Perretti and D'Acquisto, 2009). Cortisol induces the externalization of annexin A1 and, in the presence of >1mM Ca^{2+}, it undergoes a conformational change that exposes and cleaves an internal N-terminal sequence that can signal through a formyl peptide receptor (FPR2 or ALXR; Perretti and D´acquisto, 2009). The ALXR is unusual in its ability to respond to various ligands including lipoxins and SAA with pleiotropic anti-inflammatory and sometimes pro-inflammatory functions. The cleaved N-terminal peptide (Ac2-26) from equine annexin A inhibits the migration and activation of equine neutrophils but under some conditions can activate neutrophils (Brooks *et al*., 2012).

Glucocorticoids inhibit annexin A1 expression in T-cells and this contributes to the immunosuppressive effects of glucocorticoids. Annexin A1 has various roles in enhancing the resolution of inflammatory responses through the activation of monocytes. Uterine production of annexin A1 is well recognized but its role in early pregnancy is still unclear (Hutchinson *et al*., 2011). These dual roles in the innate and adaptive immune responses might be important in providing a hospitable uterine environment for the establishment of pregnancy.

Vanins (VNN)

The equine endometrium expresses substantial amounts of three vanin genes (vascular non-inflammatory molecules), namely *VNN1*, *VNN2* and *VNN3*, and all three proteins were identified in large amounts in the uterine flush. *VNN1* expression was reduced in the pregnant endometrium, but increased 2.3-fold (i.e. towards levels found in the non-pregnant endometrium) after cloprostenol. Expression in the yolk-sac wall was low for all three genes so it appears that vanins are major endometrial products regardless of pregnancy status.

Vanins have various known functions, especially pantetheinase activity that is involved in recycling acetyl CoA activity. They are therefore involved enzymatically in fatty acid metabolism by producing pantothenic acid and also cysteamine which can denature proteins by reducing disulfides (Kaskow *et al*., 2012). Epithelial vanins are induced near the epidermal surface in inflammatory skin disease (Jansen *et al*., 2009). Horses have three *VNN* genes in a cluster on chromosome 10 and code proteins with high amino acid sequence homology, but the tissue distribution, and splice variant forms, of equine vanins are still unknown. Immunoreactive VNN3 is located in the surface epithelium of the equine endometrium (Fig. 1C). Given the ability of vanins to denature proteins and participate the inflammatory response in various ways, it is expected that vanins have innate immune functions and might contribute to tissue injury in endometritis.

Uteroferrin (Tartrate-resistant acid phosphatase [TRAP] or ACP5)

We observed that uteroferrin is much increased in uterine flush fluids in pregnant mares with a corresponding increase in gene expression in the endometrium. Cloprostenol treatment resulted in a significant 2.7-fold increase in endometrial expression. Almost no signal was found in the yolk-sac wall. Uteroferrin has long been established as a major secretory product of the pregnant uterus in various species including the horse (Padua *et al*., 2012), where it is secreted by endometrial glands in a progesterone-dependent manner (Ellenberger *et al*., 2008; Hoffmann *et al*., 2009; Lehmann *et al*., 2011).

NPC2 (Epididymal secretory protein E1)

NPC2 was detected in the uterine flush from control and treated pregnant mares, and non-pregnant mares. Expression signals were high in the endometrium, with a significant 2.7-fold increase after cloprostenol. *NPC2* was among the most highly expressed genes in the yolk-sac wall. Our observations suggest that NPC2 is also secreted by the endometrium, and there are interesting parallels between its functional roles and those of GM2A. NPC2 is a protein involved in cholesterol transport, mainly in lysosomes, and loss-of-function deficiencies in NPC2 result in Niemann-Pick Type C disease in which glycosphingolipids accumulate. However, it also has extra-lysosomal transport functions because it is secreted by the mammary gland, epididymis and biliary system, suggesting that it is also an extracellular sterol transporter (Vanier and Millat, 2004). Therefore, NPC2 might be important in the transport of cholesterol or other lipophilic molecules in the aqueous luminal milieu and through the capsule to the conceptus. Cholesterol is a particularly important metabolic resource in equine pregnancy because the equine yolk-sac wall produces substantial amounts of estrogens that are implicated in the establishment of pregnancy (Raeside *et al*., 2004).

GM2 Activator

The GM2 activator protein (GM2A) was increased in uterine flush and GM2A gene expression was much higher in the endometrium from pregnant than from non-pregnant mares. However, cloprostenol treatment had only a minor influence on these values. *GM2A* expression was also high in yolk-sac wall samples regardless of treatment. GM2A is one of the most abundant proteins in the equine yolk-sac wall (Quinn *et al*., 2006) and is present in endometrial glands (Fig. 1D). The unusually high degree of GM2A expression in the equine endometrium and the yolk-sac wall suggest a major role in lipid transport, and it might be necessary for the transfer of micelle-forming lipids across the embryonic capsule.

GM2A is best known as a lysosomal protein that is defective in one form of Tay-Sachs disease, a ganglioside lysosomal storage disease (Mahuran, 1998). GM2A has structural properties that allow it to bind to phospholipid micelles and then extract glycolipids to form a water soluble complex with the lipid domain enclosed into a hydrophobic pocket of GM2A (Kolter and Sandhoff, 2010). In the lysosome, this is necessary for hexosaminidase hydrolysis of terminal saccharides of GM2 sphingolipids. However, similar properties likely facilitate glycolipid and phospholipid transport in extracellular compartments (Mahuran, 1998). Structural modeling of equine GM2A demonstrates the conserved hydrophobic pocket (Quinn *et al*., 2006).

Stanniocalcin (STC1)

Stanniocalcin was increased in uterine flush samples from pregnant mares and endometrial expression was ~4.5 fold higher than non-pregnant levels in both saline- and cloprostenol-injected pregnant mares. These results are in accordance with recent reports that *STC1* expression increases in the endometrium during early pregnancy in mares (Klein et al., 2010) in which it is produced in endometrial glands (Kikuchi *et al*., 2011). STC1 is a pregnancy-related endometrial product in various species including pigs (Song *et al*., 2009) and ruminants (Song *et al*., 2006, 2009). Stanniocalcins are implicated in calcium and phosphate metabolism but their role in early pregnancy is not yet understood. *STC1* expression was minimal in the yolk-sac wall but it is detectable in substantial amounts in the capsule and yolk-sac fluid (unpublished) so a transport function is possible.

P19 lipocalin (Uterocalin)

P19 lipocalin was present in similar amounts in uterine flushes from control and treated day-20 pregnant mares. Of the genes listed in Table 2, P19 had the highest expression signal in the endometrium in all three groups and was unaffected by cloprostenol treatment. It is known to have an important role as a secreted lipocalin that transports small lipophilic substances from the endometrium through the embryonic capsule to the embryo (Stewart *et al*., 2000b; Suire *et al*., 2001). No *P19* expression was apparent in the yolk-sac wall, but P19 lipocalin is present in the capsule and yolk-sac fluid during early pregnancy (Crossett *et al*., 1998; Quinn *et al*., 2007). It is interesting that cloprostenol treatment did not reduce *P19* expression in the endometrium, and that the levels of expression in non-pregnant mares at day 20 postovulation were similar to those in the pregnant mares. Equine *P19* is a progesterone-regulated gene that is also expressed in cycling mares especially in the luteal phase, but it can be expressed asynchronously in cycling mares with endometrosis so it may have other functions in the non-pregnant uterus (Stewart *et al*., 2000a; Ellenberger *et al*., 2008).

Table 1. List of proteins identified (>95% probability) in uterine flush fluids, ranked in order of estimated amount in flush fluid, compared with rank for amount of mRNA expression signal in microarrays.

Gene	Protein or Predicted (P)		Accession	kDa	Pregnant		Pregnant + PGF		Non-Pregnant		Flush Protein Rank	mRNA Rank
					Mean	SD	Mean	SD	Mean	SD		
ALB	Serum Albumin		gi|126723507 (+1)	69	85.4	± 8.7	84.2	± 20.8	49.5	± 10.6	1	116
TF	Serotransferrin		gi|126352628	78	36.0	± 9.0	32.6	± 17.6	11.5	± 4.9	2	74
A2M	Alpha-2-macroglobulin–like	P	gi|194211675	164	22.8	± 17.1	21.8	± 21.0	3.0	± 4.2	3	158
LCN2	Neutrophil gelatinase-assoc lipocalin (NGAL)–like	P	gi|338720560	23	21.0	± 4.1	19.2	± 4.1	6.0	± 1.4	4	14
VNN3	Vascular non-inflammatory molecule 3 (vanin 3)–like	P	gi|149722957	58	19.2	± 3.8	17.2	± 5.7	10.5	± 2.1	5	65
ANXA1	Annexin A1 (Lipocortin 1)		gi|126352349	39	12.0	± 1.9	7.6	± 4.3	16.5	± 3.5	6	24
HP	Haptoglobin–like	P	gi|149699777	38	12.0	± 7.2	6.4	± 5.5	4.0	± 1.4	7	159
HBB	Hemoglobin subunit beta		gi|122614 (+1)	16	11.8	± 1.5	8.0	± 5.1	7.0	± 4.2	8	50
PIGR	Polymeric immunoglobulin receptor	P	gi|194210251	83	11.6	± 5.6	9.2	± 4.0	16.5	± 6.4	9	136
GM2AP	GM2 activator		gi|126352460	21	10.0	± 2.1	9.8	± 3.6	0.5	± 0.7	10	40
APOA1	Apolipoprotein A-I–like	P	gi|149716548	30	10.0	± 4.4	10.4	± 6.2	5.0	± 2.8	11	110
IGHA	Immunoglobulin alpha constant heavy chain		gi|32331167	37	9.8	± 7.3	10.2	± 3.8	9.5	± 3.5	12	ND
IGHG4	Immunoglobulin gamma 4 heavy chain		gi|42528293	36	9.8	± 3.8	8.2	± 4.7	2.5	± 0.7	13	ND
ANXA2	Annexin A2		gi|183227696	39	9.0	± 2.7	5.2	± 3.3	7.5	± 3.5	14	10
A1BG	Alpha-1B-glycoprotein–like	P	gi|338710440	60	9.0	± 1.2	7.4	± 4.0	6.0	± 2.8	15	144
HBA	Hemoglobin subunit alpha		gi|122411 (+1)	15	8.6	± 1.7	5.2	± 3.0	5.5	± 2.1	16	103
ACP5	Uteroferrin (TRAP5)		gi|350536031	38	8.4	± 2.7	10.0	± 0.7	2.5	± 0.7	17	16
VNN2	Vanin 2	P	gi|149722959	58	8.4	± 4.8	12.0	± 7.6	6.5	± 7.8	18	30
VNN1	Vanin 1 (Pantetheinase)	P	gi|194216453	52	8.4	± 2.1	8.2	± 2.6	12.5	± 4.9	19	73
SERPINA1-2	Alpha-1-antiproteinase 2–like	P	gi|194225326	47	8.2	± 1.3	7.4	± 4.3	2.0	± 1.4	20	113
CP	Ceruloplasmin	P	gi|149729967	122	7.8	± 2.6	4.0	± 2.5	4.0	± 1.4	21	59
TUBA1A	Tubulin alpha-1A chain–like	P	gi|338726213	46	7.4	± 5.3	2.6	± 5.8	8.0	± 1.4	22	12
IGL	Lambda-immunoglobulin		gi|291474	17	7.4	± 0.9	8.0	± 0.7	4.0	± 2.8	23	ND
IGL	Lambda-immunoglobulin		gi|291464	23	6.8	± 3.3	5.4	± 1.8	2.0	± 0.0	24	ND
P19	P19 lipocalin (uterocalin)		gi|126723126	21	6.4	± 3.8	10.4	± 3.0	4.0	± 4.2	25	1
LOC100054939	Vitamin D-binding protein (GC)–like	P	gi|149701606	54	6.4	± 2.9	5.6	± 5.1	0.0	± 0.0	26	162
HSP90A	Heat shock protein 90 alpha		gi|12082136 (+1)	83	6.2	± 1.8	2.6	± 3.6	6.5	± 2.1	27	46
A2ML1	Alpha-2-macroglobulin–like protein 1	P	gi|338726035	160	6.2	± 2.5	3.0	± 3.7	0.5	± 0.7	28	97

IGHG1	Ig gamma 1 heavy chain constant region		gi|15020816 (+1)	37	6.2 ± 1.6	5.2 ± 1.3	3.0 ± 0.0	29	ND
IGGH	Immunoglobulin G heavy chain		gi|9858135	47	6.0 ± 3.5	5.0 ± 4.4	3.5 ± 0.7	30	ND
STC1	Stanniocalcin-1–like	P	gi|149746187	27	5.6 ± 1.8	9.0 ± 3.5	1.5 ± 0.7	31	7
CAPS1	Calcyphosin–like isoform 1	P	gi|149716705	21	5.4 ± 1.5	1.6 ± 2.1	6.5 ± 2.1	32	95
PADI2	Protein-arginine deiminase type-2		gi|255308904	76	5.2 ± 1.6	2.4 ± 2.9	0.0 ± 0.0	33	141
IGHM	Ig mu heavy chain constant secreted form		gi|51831151	49	5.2 ± 3.5	6.4 ± 5.4	4.0 ± 1.4	34	ND
DMBT1	Deleted in malignant brain tumors 1 protein	P	gi|338716410	190	4.4 ± 2.6	2.2 ± 2.7	3.0 ± 1.4	35	160
LAO	L-amino-acid oxidase –like	P	gi|338721758	52	4.2 ± 3.4	8.4 ± 1.1	2.0 ± 2.8	36	84
HRG	Histidine-rich glycoprotein –like	P	gi|338715996	49	4.2 ± 4.9	6.8 ± 5.0	0.0 ± 0.0	37	154
Ezrin	Ezrin	P	gi|194227470	69	4.0 ± 3.7	2.4 ± 2.3	2.5 ± 2.1	38	17
C3	Complement C3 –like	P	gi|194212541	186	4.0 ± 4.2	3.2 ± 3.4	0.5 ± 0.7	39	122
CTBS	Di-N-acetylchitobiase	P	gi|338725488	43	4.0 ± 2.0	1.6 ± 1.1	0.0 ± 0.0	40	83
CTSB	Cathepsin B	P	gi|149698064	38	4.0 ± 2.5	5.2 ± 1.8	1.0 ± 0.0	41	163
S100A6	S100-A6 (calcyclin)		gi|126352590	10	3.8 ± 0.4	2.6 ± 0.9	3.0 ± 0.0	42	3
HSPA8	Heat shock 70kDa protein 8		gi|5729877	71	3.8 ± 1.5	0.8 ± 0.8	3.0 ± 0.0	43	11
ACTB	Actin, cytoplasmic 1		gi|4501885 (+1)	42	3.8 ± 1.5	3.2 ± 1.9	3.5 ± 4.9	44	23
SERPINA14	Uterine serpin		gi|334877907	49	3.8 ± 3.4	5.2 ± 4.0	0.0 ± 0.0	45	43
YWHAE	14-3-3 protein epsilon		gi|5803225	29	3.8 ± 3.6	1.2 ± 2.2	5.5 ± 2.1	46	51
CD109	CD109 antigen	P	gi|194216195	171	3.8 ± 4.4	7.8 ± 7.6	11.5 ± 9.2	47	98
PRDX1	Peroxiredoxin-1	P	gi|149693696	22	3.6 ± 1.3	2.4 ± 2.1	3.5 ± 2.1	48	63
AFP	Alpha-fetoprotein		gi|126352653	68	3.6 ± 3.2	0.4 ± 0.5	0.0 ± 0.0	49	83
TUBB5	Tubulin beta-5		gi|7106439	50	3.6 ± 1.8	2.2 ± 3.3	5.5 ± 2.1	50	94
IGJ	Immunoglobulin J chain–like	P	gi|338723570	18	3.6 ± 1.1	2.8 ± 0.4	4.0 ± 1.4	51	ND
TPPP3	Tubulin polymerization-promoting protein 3 –like	P	gi|194208704	19	3.4 ± 2.9	1.4 ± 2.6	1.0 ± 1.4	52	47
APOA2	Apolipoprotein A-II –like	P	gi|149759787	11	3.2 ± 2.6	3.6 ± 2.9	0.0 ± 0.0	53	92
LGALS3BP	Galectin-3-binding protein	P	gi|149723277	63	3.2 ± 2.9	1.2 ± 2.2	10.5 ± 2.1	54	111
FGA2	Fibrinogen alpha chain isoform 2	P	gi|338722639 (+1)	68	3.0 ± 3.4	3.4 ± 4.1	0.0 ± 0.0	55	140
HPX	Hemopexin	P	gi|338727082	51	3.0 ± 2.2	2.4 ± 2.5	2.5 ± 3.5	56	145
IGHG6	Ig gamma 6 heavy chain constant region		gi|18996197	36	3.0 ± 1.9	5.0 ± 4.6	2.0 ± 1.4	57	ND
IGHG7	Ig gamma 7 heavy chain		gi|42528291	36	3.0 ± 0.0	2.4 ± 0.9	2.0 ± 0.0	58	ND
GSTP1	Glutathione S-transferase P –like	P	gi|149725485	23	2.8 ± 1.1	1.4 ± 1.7	3.0 ± 0.0	59	21
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase		gi|255522848	36	2.8 ± 1.5	1.8 ± 2.7	4.0 ± 2.8	60	22

ANXA3	Annexin A3 –like	P	gi|194209040	36	2.8	± 2.7	0.8	± 1.8	1.5	± 0.7	61	60
THY1	Thy-1 membrane glycoprotein	P	gi|149716599 (+1)	18	2.6	± 0.9	2.6	± 1.1	3.5	± 0.7	62	101
AHSGP	Alpha-2-HS-glycoprotein –like	P	gi|194222675	39	2.6	± 2.4	2.8	± 1.8	0.0	± 0.0	63	143
KRT19	Keratin, type I cytoskeletal 19 –like	P	gi|194216917	45	2.6	± 2.5	1.0	± 0.7	0.0	± 0.0	64	x
GSN	Gelsolin		gi|126352530 (+1)	81	2.4	± 1.1	1.6	± 1.5	0.0	± 0.0	65	39
GZB	Granzyme B –like	P	gi|194238649	30	2.4	± 2.1	1.8	± 1.6	0.0	± 0.0	66	50
PEBP1	Phosphatidylethanolamine-binding protein 1 –like	P	gi|149720563	21	2.4	± 1.3	1.2	± 1.6	2.0	± 0.0	67	61
GSTM1	Glutathione S-transferase Mu 1 isoform 1 –like	P	gi|149708720	26	2.4	± 1.9	0.6	± 0.5	2.5	± 0.7	68	67
CALM	Calmodulin		gi|4502549	17	2.4	± 2.5	0.8	± 1.8	5.0	± 0.0	69	91
CFB	Complement factor B	P	gi|149732066	86	2.4	± 1.7	1.8	± 1.6	1.0	± 1.4	70	105
FGG	Fibrinogen gamma chain	P	gi|338722637	50	2.4	± 3.6	3.0	± 3.5	0.0	± 0.0	71	114
MUC4	Mucin-4	P	gi|338716028	162	2.4	± 2.4	1.6	± 3.0	3.5	± 0.7	72	118
NPC2	Epididymal secretory protein E1 –like	P	gi|149737373	16	2.2	± 1.3	2.8	± 1.6	3.0	± 2.8	73	8
HSPB1	Heat shock protein beta-1 –like	P	gi|149755998	23	2.2	± 0.8	0.2	± 0.4	0.5	± 0.7	74	19
ANXA5	Annexin A5 –like	P	gi|149698420	36	2.2	± 1.3	0.8	± 1.3	1.5	± 2.1	75	57
FN	Fibronectin	P	gi|194211292	272	2.2	± 0.8	2.8	± 1.6	0.0	± 0.0	76	87
YWHAZ	14-3-3 protein zeta/delta isoform 3	P	gi|338728575 (+1)	29	2.2	± 1.8	0.8	± 1.3	2.0	± 1.4	77	104
SERPINA1	Alpha-1-antitrypsin		gi|197631775	47	2.2	± 1.3	3.2	± 2.2	0.0	± 0.0	78	164
IGL	Immunoglobulin lambda		gi|291462	20	2.2	± 0.8	1.4	± 1.1	1.0	± 0.0	79	ND
CLIC1	Chloride intracellular channel protein 1 –like	P	gi|149732344	27	2.0	± 0.7	0.8	± 1.3	1.0	± 1.4	80	32
ACTN1	Alpha-actinin-1 isoform 1 –like	P	gi|194225130 (+2)	104	2.0	± 1.0	0.2	± 0.4	0.0	± 0.0	81	35
PFN1	Profilin-1 –like	P	gi|194217532 (+1)	15	2.0	± 1.2	0.8	± 1.3	2.5	± 0.7	82	55
CTSL1	Cathepsin L1 –like	P	gi|149755237	38	2.0	± 2.5	1.8	± 1.8	0.5	± 0.7	83	75
GDI1	Rab GDP dissociation inhibitor alpha –like	P	gi|338729754	52	2.0	± 0.7	0.2	± 0.4	1.0	± 1.4	84	149
KRT2	Keratin, type II cytoskeletal 8 –like	P	gi|194212042	48	2.0	± 1.0	0.8	± 1.3	0.0	± 0.0	85	x
EEF1A1	Eukaryotic elongation factor 1		gi|126352304 (+1)	50	1.8	± 0.4	0.8	± 1.3	3.0	± 4.2	86	2
CLU	Clusterin		gi|126352584	52	1.8	± 1.1	1.0	± 1.4	3.0	± 0.0	87	6
FGB1	Fibrinogen beta chain isoform 1	P	gi|149698358 (+1)	55	1.8	± 2.5	2.4	± 2.5	0.0	± 0.0	88	123
WDHD1	WD repeat HMG-box DNA-binding 1-1	P	gi|149737109	125	1.8	± 0.4	1.2	± 1.3	1.0	± 1.4	89	133
FETUB	Fetuin-B –like	P	gi|149731458	42	1.8	± 1.6	2.8	± 2.7	0.5	± 0.7	90	157
IGKL1	Ig kappa light chain		gi|488146	25	1.8	± 1.1	2.2	± 1.1	0.5	± 0.7	91	ND
SCGB1A1	Secretoglobin 1A1/Uteroglobin–like	P	gi|194218307	10	1.6	± 0.9	2.0	± 0.0	5.0	± 4.2	92	5

TPI	Triosephosphate isomerase –like	P	gi|194211629	31	1.6	±	1.1	0.6	±	0.9	2.5	±	2.1	93	66
PSAP	Proactivator polypeptide (prosaposin)	P	gi|149689950	59	1.6	±	0.9	2.8	±	1.9	0.5	±	0.7	94	68
ANXA4	Annexin A4 isoform 1	P	gi|149727504 (+1)	36	1.6	±	1.1	0.4	±	0.9	1.0	±	1.4	95	70
CFL1	Cofilin 1		gi|5031635	19	1.6	±	2.1	0.8	±	1.1	0.5	±	0.7	96	99
TPP1	Tripeptidyl-peptidase 1 –like	P	gi|194213729	57	1.6	±	1.9	3.4	±	2.2	0.0	±	0.0	97	128
CES1	Liver carboxylesterase–like isoform 1	P	gi|149699085	62	1.6	±	0.9	1.2	±	0.8	1.5	±	0.7	98	x
SERPINB6	Serpin B6–like isoform 1 –like	P	gi|338718301	48	1.4	±	1.3	0.0	±	0.0	0.5	±	0.7	99	79
SERPINB4	Serpin B4 –like	P	gi|194214732	44	1.4	±	1.9	0.0	±	0.0	1.0	±	1.4	100	93
ABHD14B	Abhydrolase domain-containing protein 14B –like	P	gi|149728615	22	1.4	±	1.1	0.6	±	1.3	0.0	±	0.0	101	96
TAGLN2	Transgelin-2 –like	P	gi|149755838	22	1.4	±	0.9	0.4	±	0.5	2.0	±	1.4	102	109
IGHG2	Ig gamma 2 heavy chain constant region		gi|15026999	37	1.4	±	1.5	1.0	±	1.2	0.0	±	0.0	103	ND
HBE2	Hemoglobin epsilon-2 –like	P	gi|149719849	16	1.4	±	0.5	0.2	±	0.4	0.5	±	0.7	104	x
RALDH1	Retinal dehydrogenase 1 –like	P	gi|194224764	53	1.2	±	1.3	1.2	±	2.7	0.5	±	0.7	105	25
CSTB	Cystatin-B –like	P	gi|149742108	11	1.2	±	1.3	0.2	±	0.4	0.0	±	0.0	106	28
VCP	Transitional endoplasmic reticulum ATPase –like	P	gi|338720167	96	1.2	±	0.8	0.6	±	1.3	1.5	±	0.7	107	33
PRDX2	Peroxiredoxin-2 –like	P	gi|194213066	22	1.2	±	1.3	0.0	±	0.0	0.5	±	0.7	108	48
ADH1	Alcohol dehydrogenase [NADP+] isoform 1	P	gi|149693692	37	1.2	±	0.8	0.2	±	0.4	1.0	±	1.4	109	62
SERPINB9	Serpin B9	P	gi|149731759	42	1.2	±	1.8	0.0	±	0.0	0.0	±	0.0	110	79
TXN	Thioredoxin		gi|126352340	12	1.2	±	0.4	0.4	±	0.5	1.0	±	0.0	111	100
ANXA8	Annexin A8	P	gi|149690688	37	1.2	±	2.2	0.2	±	0.4	0.5	±	0.7	112	125
VMO1	Vitelline membrane outer layer protein 1 homolog	P	gi|149724889	22	1.2	±	2.2	0.4	±	0.9	0.0	±	0.0	113	134
KIAA0284	Protein KIAA0284 –like	P	gi|338719910	130	1.2	±	0.4	1.0	±	0.0	0.0	±	0.0	114	146
KRT1	Keratin, type II cytoskeletal 1 –like	P	gi|338726100	65	1.2	±	1.8	1.2	±	0.8	0.0	±	0.0	115	166
IGHG5	Ig gamma 5 heavy chain constant region		gi|18996195	36	1.2	±	0.4	1.6	±	0.9	1.0	±	0.0	116	ND
YWHAB	14-3-3 protein beta/alpha	P	gi|149733297	28	1.0	±	1.7	0.0	±	0.0	0.5	±	0.7	117	18
CTSZ	Cathepsin Z –like	P	gi|338719460	34	1.0	±	1.2	0.6	±	0.5	0.0	±	0.0	118	20
ENO1	Alpha-enolase isoform 1	P	gi|149695415	47	1.0	±	1.2	0.6	±	1.3	2.5	±	0.7	119	29
CTSA	Cathepsin A	P	gi|338719327	54	1.0	±	0.7	1.6	±	0.9	0.5	±	0.7	120	34
TUBA1C	Tubulin alpha-1C chain isoform 1	P	gi|149714278	50	1.0	±	1.0	0.6	±	0.9	3.0	±	0.0	121	41
S100A11	S100-A11 –like	P	gi|149751468	11	1.0	±	0.7	1.2	±	1.1	1.0	±	0.0	122	42
SERPING1	Plasma protease C1 inhibitor –like	P	gi|149758084	53	1.0	±	0.7	0.8	±	1.8	0.5	±	0.7	123	58
TF	Transferrin		gi|3892521	7	1.0	±	1.4	0.8	±	1.8	1.0	±	1.4	124	59

PAG	Pregnancy-associated glycoprotein		gi|126352481	43	1.0 ± 1.2	0.2 ± 0.4	0.0 ± 0.0	125	78
LDHA	L-lactate dehydrogenase A		gi|222080073	37	1.0 ± 0.7	0.6 ± 0.9	2.5 ± 0.7	126	89
WFCD2	WAP four-disulfide core domain protein 2 –like	P	gi|149733303	13	1.0 ± 0.7	1.0 ± 1.4	2.0 ± 1.4	127	90
NAV2	Neuron navigator 2 isoform 1	P	gi|194213909 (+1)	260	1.0 ± 0.0	0.8 ± 0.8	0.5 ± 0.7	128	102
CHIT1	Chitotriosidase-1		gi|219689080	51	1.0 ± 1.2	2.0 ± 2.3	9.5 ± 6.4	129	105
CTGF	Connective tissue growth factor–like	P	gi|194216449	31	1.0 ± 1.7	1.8 ± 1.3	0.0 ± 0.0	130	107
PT	Prothrombin –like	P	gi|338712085	70	1.0 ± 0.7	1.6 ± 1.5	0.5 ± 0.7	131	127
QSOX1	Sulfhydryl oxidase 1	P	gi|338724819	85	1.0 ± 0.7	2.0 ± 1.7	1.5 ± 2.1	132	151
TMSB4	Thymosin beta-4		gi|10946578 (+1)	5	0.8 ± 1.3	0.0 ± 0.0	0.0 ± 0.0	133	13
TALDO1	Transaldolase –like	P	gi|149759693	34	0.8 ± 0.8	0.0 ± 0.0	0.5 ± 0.7	134	26
CAP1	Adenylyl cyclase-associated protein 1	P	gi|338721937	51	0.8 ± 0.8	0.8 ± 1.1	0.0 ± 0.0	135	31
PARK7	Protein DJ-1 –like	P	gi|149695427	20	0.8 ± 0.8	0.4 ± 0.5	1.0 ± 1.4	136	37
PSME2	Proteasome activator complex subunit 2 –like	P	gi|149756146	27	0.8 ± 0.8	0.2 ± 0.4	0.5 ± 0.7	137	44
PRX5_like	Hypothetical protein LOC100055657	P	gi|338712372	51	0.8 ± 0.8	0.2 ± 0.4	0.5 ± 0.7	138	54
FKBP5	Peptidyl-prolyl cis-trans isomerase A–like isoform	P	gi|149704620	18	0.8 ± 0.8	0.2 ± 0.4	2.5 ± 0.7	139	82
PLTP	Phospholipid transfer protein isoform 1	P	gi|149733315 (+1)	55	0.8 ± 1.3	0.4 ± 0.5	0.0 ± 0.0	140	86
YWHAQ	14-3-3 protein theta	P	gi|5803227	28	0.8 ± 0.8	0.2 ± 0.4	0.5 ± 0.7	141	119
IL1RN	Interleukin-1 receptor antagonist		gi|126723134 (+2)	20	0.8 ± 0.4	1.0 ± 1.2	0.0 ± 0.0	142	129
IL1F9	Interleukin-1 family member 9 –like	P	gi|194220476	18	0.8 ± 1.3	0.0 ± 0.0	0.0 ± 0.0	143	130
KIAA1211	Uncharacterized protein KIAA1211 like	P	gi|194209145	139	0.8 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	144	139
SERPINC1	Antithrombin-III	P	gi|149708147	52	0.8 ± 0.4	1.2 ± 1.1	0.0 ± 0.0	145	142
MX2	Interferon-induced GTP-binding protein Mx2	P	gi|194226266	83	0.8 ± 0.4	0.6 ± 0.5	1.0 ± 1.4	146	147
GDI1	Rho GDP-dissociation inhibitor 1 isoform 1	P	gi|149723251 (+1)	23	0.8 ± 0.8	0.6 ± 1.3	1.5 ± 0.7	147	149
HSP70	Heat shock 70 kDa protein 1	P	gi|338718610	70	0.8 ± 0.8	0.2 ± 0.4	0.5 ± 0.7	148	150
ITIH2	Inter-alpha-trypsin inhibitor heavy chain H4	P	gi|338714655	92	0.8 ± 1.3	0.0 ± 0.0	0.0 ± 0.0	149	165
DBI	Acyl-CoA-binding protein	P	gi|149730534	10	0.8 ± 1.1	0.2 ± 0.4	0.0 ± 0.0	150	x
MUC16	Mucin-16 –like	P	gi|338727239	32	0.8 ± 0.8	0.4 ± 0.5	0.0 ± 0.0	151	x
NME2	Nucleoside diphosphate kinase B		gi|325301267	17	0.6 ± 0.9	0.0 ± 0.0	1.0 ± 1.4	152	9
CFI	Complement factor I	P	gi|194208524	64	0.6 ± 0.9	3.4 ± 1.8	0.0 ± 0.0	153	15
ATOX1	Copper transport protein ATOX1 –like	P	gi|194219686	7	0.6 ± 1.3	0.4 ± 0.9	1.0 ± 1.4	154	38
CUTA	Protein CutA isoform 1	P	gi|149732422 (+1)	19	0.6 ± 0.9	0.4 ± 0.5	1.5 ± 0.7	155	45
SPARC	SPARC (osteonectin)	P	gi|221139863	35	0.6 ± 1.3	0.0 ± 0.0	0.0 ± 0.0	156	49

CNDP2	Cytosolic non-specific dipeptidase isoform 1	P	gi|149721245	53	0.6	±	0.9	0.6	±	1.3	0.5	±	0.7	157	53
PTGES3	Prostaglandin E synthase 3–like isoform 1	P	gi|194212264 (+1)	19	0.6	±	0.9	0.4	±	0.9	0.0	±	0.0	158	56
CKB	Creatine kinase B-type isoform 1 –like	P	gi|149737691	43	0.6	±	0.9	0.4	±	0.9	0.5	±	0.7	159	64
SPLA2	Secretory phospholipase A2		gi|153792451	13	0.6	±	1.3	1.6	±	1.3	3.5	±	4.9	160	71
PLG	Plasminogen		gi|130315	37	0.6	±	0.9	0.4	±	0.9	0.0	±	0.0	161	76
PLS3	Plastin-3 isoform 1	P	gi|149744913	71	0.6	±	0.5	0.6	±	0.9	0.5	±	0.7	162	81
HBE	Hemoglobin subunit epsilon –like	P	gi|149719851	16	0.6	±	0.9	0.8	±	0.8	0.0	±	0.0	163	85
DCAF7	DDB1- and CUL4-associated factor 7	P	gi|149723393	38	0.6	±	0.9	0.0	±	0.0	0.0	±	0.0	164	120
YWHAG	14-3-3 protein gamma –like	P	gi|194218901	27	0.6	±	0.9	0.0	±	0.0	0.0	±	0.0	165	126
TMPRSS13	Transmembrane protease serine 13	P	gi|149716921	56	0.6	±	0.9	0.2	±	0.4	0.0	±	0.0	166	132
FIGNL1	Fidgetin 1 –like	P	gi|149704647	74	0.6	±	0.9	0.6	±	0.5	0.5	±	0.7	167	137
EqUC1-2	Major allergen Equ c 1–like	P	gi|149738827	21	0.6	±	0.9	1.4	±	1.5	3.5	±	2.1	168	138
KRT1	Keratin, type II cytoskeletal 1b isoform 1	P	gi|149714785	64	0.6	±	0.9	0.6	±	0.5	0.0	±	0.0	169	166
KRT4	Keratin, type II cytoskeletal 4	P	gi|149714794	65	0.6	±	0.9	0.4	±	0.9	0.0	±	0.0	170	150
CCDC148	Coiled-coil domain-containing protein 148	P	gi|194222239	72	0.6	±	0.5	1.0	±	0.7	0.0	±	0.0	171	155
AGT	Angiotensinogen –like	P	gi|194206059	70	0.6	±	0.5	0.6	±	0.9	1.5	±	0.7	172	156
IGLC	Ig lambda light chain constant region		gi|356494351	11	0.6	±	1.3	0.0	±	0.0	0.0	±	0.0	173	ND
IGLC	Ig lambda light chain constant region		gi|356494359	11	0.6	±	1.3	0.0	±	0.0	0.0	±	0.0	174	ND
COL1A1	Collagen alpha-1(I) chain	P	gi|338711499	125	0.6	±	0.9	0.0	±	0.0	0.0	±	0.0	175	ND
B2M	Beta-2-microglobulin		gi|126722587 (+1)	13	0.4	±	0.5	1.2	±	0.8	1.0	±	1.4	176	4
EEF2	Elongation factor 2	P	gi|194212460	95	0.4	±	0.5	0.0	±	0.0	1.5	±	0.7	177	27
RBP4	Retinol-binding protein 4		gi|126352389	23	0.4	±	0.9	0.4	±	0.9	0.0	±	0.0	178	36
CGL3	Galectin-3 –like	P	gi|338720072	25	0.4	±	0.9	0.0	±	0.0	0.0	±	0.0	179	72
HBZ1	Hemoglobin subunit zeta		gi|167621441	16	0.4	±	0.9	0.2	±	0.4	0.0	±	0.0	180	77
ANKRD12	Ankyrin repeat-containing protein 12 isoform 1	P	gi|149720807 (+1)	234	0.4	±	0.9	0.0	±	0.0	0.0	±	0.0	181	112
EQUC1	Major allergen Equ c 1		gi|126723762 (+1)	22	0.4	±	0.9	0.8	±	0.8	1.5	±	0.7	182	131
LOC100630813	Hypothetical protein LOC100630813	P	gi|338729340	133	0.4	±	0.9	0.0	±	0.0	0.0	±	0.0	183	x
HIST1H2AB	Histone H2A type 1-B		gi|10645195 (+8)	14	0.4	±	0.9	0.2	±	0.4	0.0	±	0.0	184	x
H4-1	Histone H4	P	gi|194223075 (+1)	22	0.4	±	0.5	0.6	±	0.9	0.0	±	0.0	185	x

ND indicates that immunoglobulin and collagen gene expression signals were excluded from rankings of mRNA expression values.
X indicates that a corresponding annotated gene was not identified in the microarray datasets.

Table 2A. List of selected genes comparing mean (± SD) of average mRNA expression signal in microarrays in the endometrium from saline-injected pregnant mares, cloprostenol-injected pregnant mares, and non-pregnant mares.

Protein or Predicted	Gene Symbol	No. of Probes	Endometrial Expression in Pregnant Control		Flush Protein Rank	mRNA Rank	Endometrial Expression in Pregnant Treated		mRNA Rank	Endometrial Expression in Non-Pregnant		mRNA Rank	Treated/ Control	Treated/ Non-Pregnant	Control/ Non-Pregnant	Corrected P-value
			Mean	SD			Mean	SD		Mean	SD		Fold	Fold	Fold	
Secretory phospholipase A2	SPLA2	5	3676 ±	4777	160	71	243842 ±	41687	4	223572 ±	36403	4	66.42	1.09	0.02	0.000
IL-1 receptor antagonist	IL1RN	10	167 ±	61	142	129	8422 ±	12763	68	138 ±	121	122	59.94	63.15	1.06	0.004
Cathepsin L1	CTSL1-1	1	3388 ±	1414	83	75	72425 ±	43754	18	46343 ±	24875	21	21.37	1.56	0.07	0.000
Connective tissue growth factor	CTGF	5	790 ±	244	130	107	5744 ±	2834	76	1695 ±	2671	91	7.27	3.39	0.47	0.008
Uterine serpin	SERPINA14	15	12045 ±	3119	45	43	74496 ±	20931	15	5566 ±	9569	69	6.25	11.88	1.81	0.002
Lipocalin 2 (NGAL)	LCN2	1	32000 ±	13242	4	14	146266 ±	73581	6	14336 ±	19237	51	4.57	10.20	2.23	0.002
Secretoglobin 1A1 (uteroglobin)	SCGB1A1	8	86843 ±	72853	92	5	380480 ±	55530	1	498485 ±	13241	1	4.42	0.77	0.18	0.000
Retinol-binding protein 4	RBP4	4	15674 ±	9972	178	36	59521 ±	27496	23	13716 ±	18676	53	3.85	4.34	1.13	0.011
Annexin A3	ANXA3	2	5201 ±	1356	61	60	18804 ±	5157	49	7273 ±	3532	63	3.61	2.59	0.72	0.000
Annexin A5	ANXA5	1	5519 ±	1520	75	57	16516 ±	9052	53	14554 ±	467	50	2.99	1.13	0.38	0.002
Vanin 3	VNN3	3	4450 ±	2572	5	65	12278 ±	6643	59	3132 ±	1218	81	2.75	3.97	1.44	0.010
Uteroferrin (TRAP5)	ACP5	1	29184 ±	7602	17	16	79523 ±	27451	12	17261 ±	12176	46	2.72	4.61	1.69	0.000
Epididymal secretory protein E1	NPC2	5	54203 ±	14043	73	8	144822 ±	48145	8	178216 ±	3012	5	2.67	0.81	0.30	0.000
Vanin 1	VNN1	5	3574 ±	873	19	73	8073 ±	3745	71	14325 ±	6977	52	2.26	0.56	0.25	0.000
Annexin A2	ANXA2	3	44929 ±	9691	14	10	77566 ±	22954	13	51751 ±	12475	18	1.79	1.54	0.86	0.014
Annexin A4	ANXA4	1	3738 ±	2174	95	70	6538 ±	4091	72	5964 ±	1009	67	1.75	1.10	0.63	0.386
Annexin A1 (Lipocortin 1)	ANXA1	8	22729 ±	8546	6	24	37482 ±	19668	31	27691 ±	11536	29	1.62	1.35	0.84	0.014
GM2 activator	GM2A	1	12560 ±	9225	10	40	19247 ±	12813	45	2519 ±	1931	85	1.53	7.64	4.99	0.013
Stanniocalcin-1	STC1	3	66459 ±	41758	31	7	67039 ±	34735	19	14675 ±	24232	49	1.01	4.58	4.54	0.009
Vanin 2	VNN2	1	18423 ±	5822	18	30	16885 ±	14790	51	20161 ±	12233	41	0.92	0.84	0.91	0.667
P19 lipocalin	P19	1	399647 ±	31267	25	1	351751 ±	38893	2	368990 ±	31957	2	0.88	0.95	1.08	0.161
Cathepsin Z	CTSZ	3	23553 ±	32899	118	20	18960 ±	10738	46	8367 ±	1582	62	0.80	2.27	2.83	0.530

Table 2B. List of selected genes comparing mean (± SD) of average mRNA expression signal in microarrays in the yolk-sac wall from conceptuses flushed on Day 20 from saline-injected or cloprostenol-injected pregnant mares. For comparison, expression values for control pregnant endometrium are also included.

Protein or Predicted	Gene Symbol	No. of Probes	Endometrial Expression in Pregnant Control			Expression in Yolk-Sac Wall - Control			mRNA Rank	Expression in Yolk-Sac Wall - Treated			mRNA Rank	Treated/ Control	P-value
			Mean		SD	Mean		SD		Mean		SD		Fold	
Secretory phospholipase A2	SPLA2	5	3676	±	4777	56	±	13	132	69	±	34	134	1.27	0.467
IL-1 receptor antagonist	IL1RN	10	167	±	61	132	±	74	121	139	±	36	125	0.96	0.597
Cathepsin L1	CTSL1-1	1	3388	±	1414	26699	±	12354	43	31518	±	11766	49	1.18	0.016
Connective tissue growth factor	CTGF	5	790	±	244	1051	±	775	93	1181	±	807	96	1.13	0.697
Uterine serpin	SERPINA14	15	12045	±	3119	2592	±	1736	83	3310	±	3159	82	1.55	0.888
Lipocalin 2 (NGAL)	LCN2	1	32000	±	13242	360	±	54	108	312	±	27	112	0.87	0.227
Secretoglobin 1A1(uteroglobin)	SCGB1A1	8	86843	±	72853	46	±	9	135	98	±	59	132	2.42	0.021
Retinol-binding protein 4	RBP4	4	15674	±	9972	290297	±	48530	3	301895	±	62794	3	1.05	0.764
Annexin A3	ANXA3	2	5201	±	1356	7332	±	2065	68	15377	±	2986	63	2.09	0.001
Annexin A5	ANXA5	1	5519	±	1520	12106	±	1281	58	20712	±	10754	58	1.71	0.051
Vanin 3	VNN3	3	4450	±	2572	16	±	4	148	16	±	6	144	0.96	1
Uteroferrin (TRAP5)	ACP5	1	29184	±	7602	79	±	22	129	89	±	21	133	1.12	0.436
Epididymal secretory protein E1	NPC2	5	54203	±	14043	105171	±	18958	15	129502	±	13620	15	1.23	0.041
Vanin 1	VNN1	5	3574	±	873	468	±	214	102	435	±	211	109	0.93	0.743
Annexin A2	ANXA2	3	44929	±	9691	131436	±	17707	12	180609	±	33217	11	1.42	0.014
Annexin A4	ANXA4	1	3738	±	2174	2181	±	369	86	4092	±	2657	81	1.88	0.096
Annexin A1 (Lipocortin 1)	ANXA1	8	22729	±	8546	28861	±	8170	41	56901	±	36494	30	1.95	0.063
GM2 activator	GM2A	1	12560	±	9225	190524	±	56060	11	197129	±	14903	9	1.03	0.643
Stanniocalcin-1	STC1	3	66459	±	41758	59	±	15	131	111	±	67	130	1.88	0.104
Vanin 2	VNN2	1	18423	±	5822	501	±	207	100	506	±	165	105	1.01	0.898
P19 lipocalin	P19	1	399647	±	31267	23	±	37	140	4	±	1	158	0.16	0.241
Cathepsin Z	CTSZ	3	23553	±	32899	5290	±	1854	73	7591	±	5464	71	1.44	0.443

Table 3. Summary of some functions of various proteins in equine uterine fluids

Protein	Gene	Some major functions
Annexin A1	ANXA1	Mainly anti-inflammatory
Connective tissue growth factor	CTGF	Promotes cell proliferation and differentiation
GM2 activator protein	GM2A	Glycolipid transport, lysosomal glycan hydrolysis
Epididymal secretory protein E1	NPC2	Cholesterol transport
Cathepsin L1	CTSL1	Protease
IL-1 Receptor antagonist	IL1RN	Regulates IL-1 activity
Lipocalin 2	LCN2	Siderophore binding, antibacterial
p19 Uterocalin	P19	Lipopjhilic molecule transport
Retinol-binding protein 4	RBP4	Retinol transport
Secretoglobin 1A1 (Uteroglobin; CCSP)	SCGB1A1	Anti-inflammatory
Secretory phospholipase A2	SPLA2	Phospolipid hydrolysis to arachidonic acid, antimicrobial.
Serum amyloid A1	SAA1	Acute phase protein induced in inflammation
Stanniocalcin-1	STC1	Calcium and phosphate metabolism
Uterine serpin (UTS)	SERPINA14	Epitheliochorial placentation
Uteroferrin (TRAP5)	ACP5	Phosphate hydrolysis in osteonectin function
Vanins 1-3	VNN1-3	Pantothenic acid recycling, protein disulfide reduction

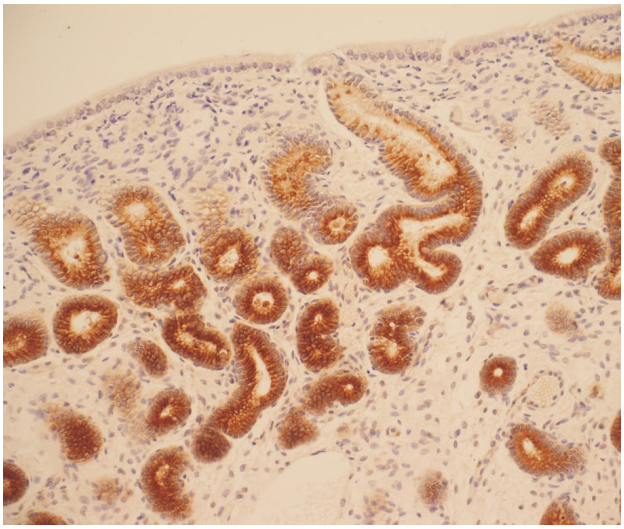

Figure 1A. IHC of sPLA2 in endometrial glands

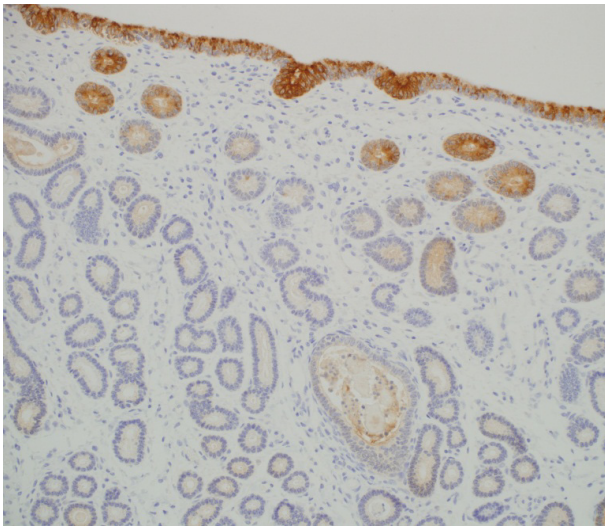

Figure 1C. IHC of vanin 3 in endometrial glands

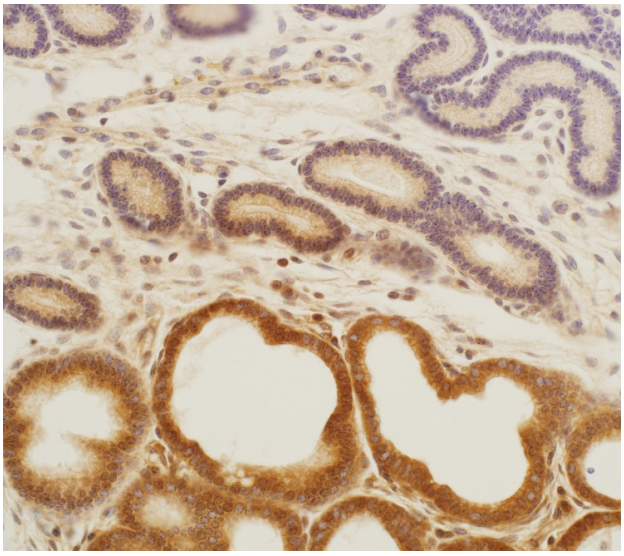

Figure 1B. IHC of SCGB1A1 in endometrial glands

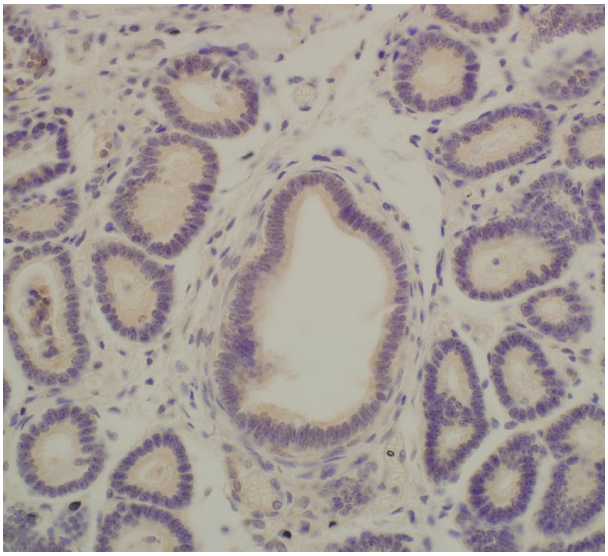

Figure 1D. IHC of GM2A in endometrial glands

Conclusions

These studies describe some of the many genes that are expressed in the pregnant endometrium, with corresponding presence of protein in the uterine secretions. The comparisons with the expression in the endometrium and yolk-sac wall provide some indications of potential roles in mucosal innate immunity or nutritional support of the early conceptus. Further studies similar to what has been reported for P19 to define the distribution of these various carrier proteins might elucidate those with important roles in transport of lipophilic factors across the epitheliochorial maternal:conceptus interface especially while the protective hydrophilic embryonic capsule is intact. It will also be important to learn how many of these proteins change during inflammatory conditions that compromise the establishment or maintenance of early pregnancy to guide further research towards those that are useful diagnostic markers or therapeutic targets.

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