DISSERTATION

EVALUATING THE EQUINE ENDOMETRIAL TRANSCRIPTOME DURING MATERNAL RECOGNITION OF PREGNANCY

Submitted by

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ABSTRACT

EVALUATING THE EQUINE ENDOMETRIAL TRANSCRIPTOME DURING MATERNAL RECOGNITION OF PREGNANCY

The goal of this dissertation was to evaluate endometrium during maternal recognition of pregnancy (MRP) in the mare. Maternal recognition of pregnancy is a process that is still not well defined. In a non-pregnant mare, endometrial prostaglandin $F_{2\alpha}$ (PGF) is released on day 14 post ovulation (PO) to cause regression of the corpus luteum, resulting in the loss of progesterone production and the mare will begin the next follicular cycle. In a pregnant mare MRP occurs prior to day 14 to decrease PGF production. Previous studies have failed to identify a clear signal candidate for MRP. Studies in our laboratory have shown that focal adhesions are present in endometrium during maternal recognition of pregnancy, gene expression varies between pregnant and non-pregnant mares, and serum exosomes, which are cell secreted vesicles from pregnant and non-pregnant mares differ in miRNA content. These miRNA were predicted to target focal adhesions, which are complex molecules that can transduce biomechanical signals from outside the cell to biochemical signals within the cell. We hypothesized that embryo contact with endometrium will cause focal adhesion assembly resulting in transcriptional changes in the endometrium and ultimately a decrease in PGF secretion. The objectives of this dissertation were: (1a) identify focal adhesion abundance in endometrium with the contact of an embryo, mechanical contact through spherical beads and peanut oil; (1b) determine PGF secretion from endometrium after 24 hours of contact with these stimuli; (2) evaluate endometrial gene expression changes based upon pregnancy status via RNA-seq; (3) evaluate the small noncoding RNA content in

endometrium and endometrial secreted exosomes during maternal recognition of pregnancy via RNA-seq.

All mares for these projects were used in a cross-over design with each mare having a pregnant and non-pregnant (non-mated) control cycle. For objective 1, days 9 and 11 were chosen for analysis (n = 3/day) with 3 mares per day. Mares were randomly assigned to a collection day and provided endometrial samples and embryos (from a pregnant cycle) on the specified day. Biopsy samples were divided into five pieces, four for culture for 24 hours and one was immediately snap frozen. Endometrial biopsies for culture were placed in an incubator for 24 hours with one of four treatments: (1) an embryo in contact on the luminal side of the endometrium, (2) beads in contact on the luminal side of the endometrium, (3) peanut oil in contact on the luminal side of the endometrium or (4) endometrium by itself. After twenty hours, biopsies and culture medium were frozen for further analysis. PGF assays were performed on culture medium to determine the concentration of PGF in the media from the endometrium. RNA and protein were isolated from the endometrial biopsies for PCR and Western blot analysis for focal adhesion molecules (FAM). For objective 2, days 9, 11, and 13 PO (n=3/status/day) were selected for analysis. Mares were randomly assigned to a collection day and each provided endometrial samples for a pregnant and non-mated cycle. Pregnancy was confirmed by terminal uterine lavage at the time of endometrial biopsy. Biopsy samples were snap frozen and stored for RNA isolation. Total RNA was isolated and cNDA libraries prepared using the Illumina TruSeq RNA Sample Preparation kit and sent to the University of California-Berkeley for RNA-seq. Reads were mapped and annotated using CLC Genome Workbench. Annotation details were based on Ensembl and NCBI models combined with publicly available RNA-Seq data. Expression values for genes and transcript variants were summarized as reads per kilobase per million reads (RPKM). All transcripts considered for analysis if the average RPKM for the three samples within a group were ≥ 0.25. Differential gene expression was analyzed with SAS Proc Mixed and application of the Benjamini-Hochberg correction for multiple testing ($P \leq 0.05$ was considered significant).

On day 9, 11 and 13 there were 357, 410 and 130 genes and 402, 452 and 396 corresponding transcript variants, respectively. Five genes were in common across all three days. Interestingly, there were multiple genes that had splice variants that had different expression patterns within the same day. For objective 3, days 11 and 13 were chosen for analysis. (n = 3/day). Each mare was randomly assigned a day, and on that day endometrial biopsies were obtained via trans-cervical biopsy punch, and the embryo was collected via terminal uterine lavage to confirm pregnancy. Endometrial biopsies were divided into two parts. Half was immediately snap frozen and the other half was cultured for 24 hours to collect secreted exosomes. Exosomes were isolated from the culture medium after 24 hours. Total RNA was isolated from the endometrial and endometrial exosome samples. Sequencing libraries were prepared for small RNA-seq at University of California- Davis and sequenced at University of California- Berkeley on a HiSeq 2000. Reads were mapped and annotated using CLC Genome Workbench. EquCab2 was used as the reference genome and annotation details were provided by ncRNA EquCAB2.72.ncrna. SAS was used for statistical analysis.

The results of this dissertation show that culturing endometrium with the selected treatments for 24 hours results in very few changes in focal adhesions but does impact PGF secretion. On day 11, the presence of peanut oil actually increased PGF secretion in samples from non-pregnant mares. On day 9, 11 and 13 there were 360, 407 and 367 genes and 401, 450 and 393 corresponding transcript variants, respectively. Five genes were in common across all three days. Interestingly, there were multiple genes that had splice variants that had different expression

patterns within the same day. Overall there were 998 small noncoding RNA (ncRNA) identified in equine endometrium across days 11 and 13. These ncRNA represented miRNA, snRNA, snoRNA, Mt rRNA, RRNA and misc RNA. Of the endometrial secreted exosomes, there were 11 ncRNA on day 11 and 21 ncRNA on day 13 that differed. These ncRNA represented miRNA, snoRNA, misc RNA and snRNA. There were many novel miRNAs identified in the endometrium and endometrial secreted exosomes. Interestingly, a large percentage of the ncRNA identified were snRNA. Further analysis of biological processes and the targets of the ncRNAs could lead to the identification of the communication pathway for maternal recognition of pregnancy. In conclusion, these were the first set of experiments designed to evaluate the effect of embryo contact versus mechanical force and the entire transcriptome, large and small RNA, in endometrium during the time frame of maternal recognition of pregnancy in the mare. These studies have led to a more in depth understanding of transcriptional changes occurring in endometrium during MRP and will aid in understanding the communication occurring between embryo and endometrium during this crucial time of early pregnancy in the mare.

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Summary

In the horse, maternal recognition of pregnancy (MRP) is not well understood. In a non-pregnant mare, prostaglandin $F_{2\alpha}$ (PGF) production results in luteal regression of the corpus luteum on day 14 post-ovulation. The presence of an embryo in the uterus results in a halt in PGF production. The mechanism by which the endometrium identifies the presence of an embryo remains unknown. It is necessary that an embryo comes into contact with at least 80% of the uterus in order to signal MRP. Previous data has indicated that focal adhesions are present in the endometrium during this time frame. Focal adhesions are involved in mechanotransduction, a process where a cell converts an external force into a biochemical signal within the cell. Focal adhesions impact cell mobility, cell growth, cell proliferation, gene expression, development, tissue repair and invasion. Their mechanism of action are continuing to be studied and understood. The primary components of focal adhesions are integrins. Research has shown that focal adhesions are active during pregnancy, especially at the fetal-maternal interface. This review aims to describe the roles of focal adhesion molecules during pregnancy and potentially maternal recognition of pregnancy in mammalian species.

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Introduction

In the horse, fertilization and early pregnancy is well understood. There is a lack of knowledge during the specific time frame of maternal recognition of pregnancy (approximately day 12), more specifically, the mechanism by which it occurs. Maternal recognition of pregnancy is necessary in order to halt prostaglandin $F_{2\alpha}$ (PGF) production from the endometrium, which causes luteal regression of the corpus luteum (CL) (Douglas and Ginther, 1972; Roberts et al., 1996; McCracken et al., 1999). The CL is the primary source of progesterone during early pregnancy in the mare, a hormone necessary to sustain pregnancy (Allen and Stewart, 2001). On day 14, a non-pregnant mare produces PGF from the endometrium, which goes into circulation, causes luteal regression resulting in the mare continuing cyclicity to the next estrous cycle (McCracken et al., 1970). In a pregnant mare, once the embryo enters the uterus it is mobile due to endometrial contractions (Ginther, 1983). It is known that the embryo must come into contact with at least 80% of the uterus in order to signal MRP, resulting in decreased PGF production (McDowell et al., 1988). The embryo ceases mobility by day 16, with peak mobility between days 11-14, indicating that MRP occurs during this time frame and is antiluteolytic (Ginther, 1983; Leith and Ginther, 1984; Stout and Allen, 2001).

Previous research has failed to identify a specific candidate gene for MRP during this time frame (Klein et al., 2010; Klein and Troedsson, 2011; Klohonatz et al., 2015). In our laboratory, research has identified the presence of focal adhesions in the endometrium during maternal recognition of pregnancy (Klohonatz et al., 2016). Specifically, exosomes, which are cell derived vesicles containing bioactive material (RNA or proteins), in the sera of non-pregnant mares contain miRNA targeting focal adhesions (Valadi et al., 2007; Camussi et al., 2011; Lasser et al., 2011). These data have led us to further evaluate focal adhesions and their potential role during maternal recognition of pregnancy in the mare. The focus of this review was to explain the function and role of focal adhesions during pregnancy and possibly maternal recognition of pregnancy in mammalian species.

Focal Adhesion Function

It is well documented that cells are capable of translating biomechanical force into biochemical signals, which direct and regulate cell function through a process termed mechanotransduction (Wang and Ingber, 1995; Geiger et al., 2001; Chen et al., 2004; Wozniak et al., 2004). The external force is detected by integrins, which are heterodimeric transmembrane adhesion receptors for extracellular matrix proteins and the biochemical signals are translated by generators such as protein kinase 2/focal adhesion kinase (PTK2/FAK) that are activated based upon conformational-dependent changes (Geiger et al., 2001; Giannone and Sheetz, 2006). Mechanotransduction occurs at specialized structures that link cells to the extracellular matrix (ECM) that are termed focal adhesions (Burridge et al., 1988). Force-dependent phosphorylation of adapter, cytoskeletal, and signaling proteins within focal adhesions imply that focal adhesions sense and transduce mechanical forces (Vogel, 2006). Studies have shown that mesenchymal cells *in vitro*, when grown on two- or three-dimensional scaffolds of the ECM, will develop focal adhesions to link cells to the ECM (Izzard and Lochner, 1976; Burridge et al., 1988). Mechanotransduction can also occur across cell-cell junctions or "adherans junctions," but this review will focus primarily on focal adhesions (Rodriguez-Boulan and Nelson, 1989; Gumbiner, 1996). Focal adhesions serve as signaling centers for the cell and a large number intracellular pathways regulating cell growth, proliferation, gene expression, development, tissue repair, migration and invasion stem from these cell adhesion sites (Burghardt et al., 2009).

Focal adhesions are preceded by smaller focal complexes (Wozniak et al., 2004). Focal complex assembly is regulated by Rac and Cdc42 (Nobes and Hall, 1995). These complexes are located at the periphery of spreading or migrating cells (Nobes and Hall, 1995). Larger focal adhesion assembly is regulated by Rho activity (Ridley and Hall, 1992; Hotchin and Hall, 1995; Chrzanowska-Wodnicka and Burridge, 1996). Table 1.1 describes the differences between focal complexes and focal adhesions (Geiger et al., 2001).

Table 1.1. Features of the different types of cell-matrix adhesions.
This table explains the differences between focal complexes and focal adhesions. This table was modified from Geiger et. al., 2000.

Property/Structure	Focal Complexes	Focal Adhesions
Location	Edge of lamellipodium	Cell periphery
Morphology	Dot-like	Elongated, oval
Size	1 μm	2-5 μm
Typical Constituents	Paxillin Vinculin Tyrosine-phosphorylated proteins	α_v integrin Paxillin Vinculin α-actinin Talin Focal adhesion kinase Tyrosine-phosphorylated proteins
Induced By	Rac	Rho

Focal adhesions are comprised of multiple protein-to-protein interactions (Wozniak et al., 2004). While focal adhesions can have multiple different formations, the two commonalities between all are that they are mediated by integrins and they interact with the actin cytoskeleton at the cell interior (Geiger et al., 2001). These integrins are heterodimers of α and β subunits (Figure 1.1) that bind to the ECM through a large extracellular domain (Geiger et al., 2001). Integrins span the membrane and contain a cytoplasmic region that interact with intracellular proteins (Liu et al., 2000). In the cytoplasm, several integrin-associated multimolecular domains have been identified which interact directly and indirectly with the actin cytoskeleton (Lin and Lin, 1996; Zamir and Geiger, 2001b). Most of these proteins interact with β integrins (Geiger et al., 2001). Interestingly, paxillin, which has a crucial role in anchoring host proteins, can tightly interact with two closely related α integrins (α_4 and α_9), which actually inhibits cell spreading and the formation of focal adhesions (Liu and Ginsberg, 2000; Liu et al., 2001). Another group of proteins that interact with focal adhesions are adapter proteins that interact with actin-bound and integrin-bound components and link them together (Geiger et al., 2001). Many of these adapter proteins are also enzymes including tyrosine kinases (Src and FAK), serine/threonine kinases (Integrin-linked kinase, ILK; protein kinase C, PKC; p21-activated kinase, PAK), tyrosine phosphatases (protein tyrosine phosphatase, non-receptor type 11, SHP-2; leukocyte common antigen-related, LAR), inositol 5-phosphatases (SH-2 containing inositol 5'-phosphatase-2, SHIP-2), modulators of GTPases (ArfGAP with SH3 domain, ankyrin repeat and PH domain 1, ASAP1; 180-kDa protein downstream of CRK, DOCK180), phosphatidylinositol 3-kinase (PI3K) and protease calpain II (Prasad et al., 2001; Zamir and Geiger, 2001b). The intricacy with focal adhesions is that focal-adhesion components can interact with many different partner molecules, and not necessarily simultaneously (for example, FAK can bind more than ten different molecules (Geiger et al., 2001). The different combinations of integrins and partner molecules results in different intracellular processes.

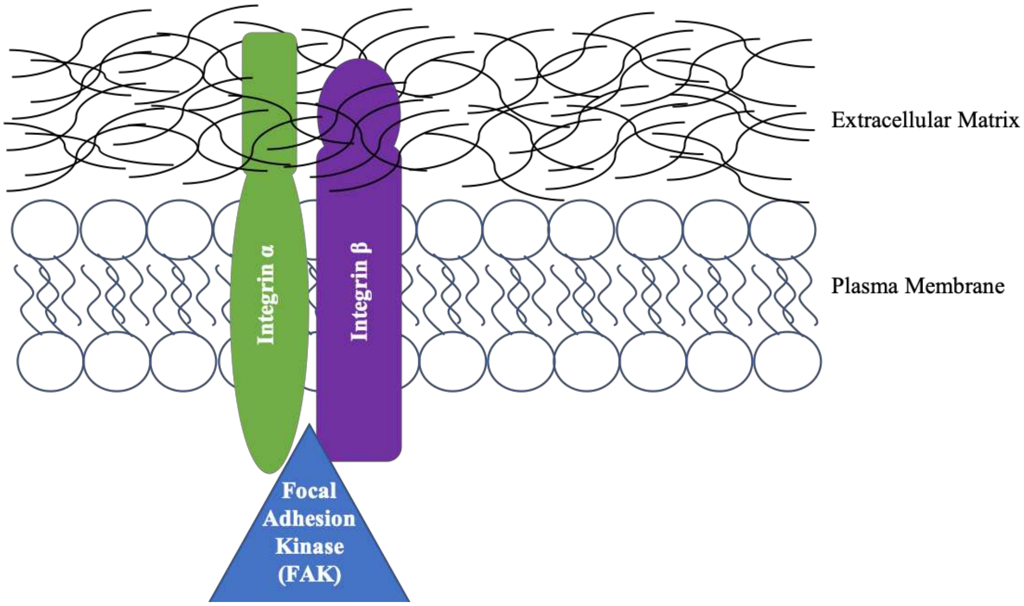

Figure 1.1 Integrin assembly for focal adhesions.
This figure demonstrates the assembly of focal adhesions with α and β integrins in a heterodimeric arrangement. The integrins bind to the extracellular matrix through their large extracellular domain. This binding results in the translation of the biomechanical signal outside the cell to a biochemical signal inside the cell, usually trough the aid of focal adhesion kinase.

The focal adhesion pathway is initially activated by clustering of integrins, growth factors or applying force on cells resulting in focal complexes (Burridge et al., 1992; Kornberg et al., 1992; Rankin and Rozengurt, 1994; Miyamoto et al., 1995a; Miyamoto et al., 1995b; Yano et al., 1996; Li et al., 1997; Casamassima and Rozengurt, 1998; Sai et al., 1999). The stimulation of focal complexes to form focal adhesions can be applied internally or externally. Internal stimulation is from the myosin-II-driven contractility or microtubule disruption (Bershadsky et al., 1996; Chrzanowska-Wodnicka and Burridge, 1996). External stimulation comes from simple mechanical pressure (Riveline et al., 2001). Data has demonstrated that actomyosin inhibitors and Rho-kinase inhibitors reduce contractility and result in the rapid loss of focal adhesions (Volberg et al., 1994; Chrzanowska-Wodnicka and Burridge, 1996; Uehata et al., 1997; Rottner et al., 1999).

A mediator of protein-protein interactions is the binding of proto-oncogene tyrosine-protein kinase (Src) homology 2 (SH-2) domains to tyrosine-phosphorylated partners (Geiger et al., 2001). Focal adhesions have many SH-2 containing components and tyrosine-phosphorylated molecules (Geiger et al., 2001). As the force on the cell increases, phosphotyrosine content in focal adhesions increases, prior to any apparent changes in focal adhesion structure (Zamir and Geiger, 2001a). This activity results in recruitment of FAK and vinculin to the focal adhesion, where FAK is autophosphorylated at tyrosine 397 (p-FAK/Y397) (Parsons et al., 2000; Kirchner et al., 2003; Wozniak et al., 2004). Src is recruited to the SH-2 domain of the phosphorylated FAK (Parsons et al., 2000). These enzymes then phosphorylate other focal adhesions creating more docking sites for SH2-containing proteins and regulating additional activation of kinases and phosphatases (Wozniak et al., 2004). In fact, inhibitors of tyrosine phosphorylation block adhesion-complex formation and recruitment of larger focal adhesions (Burridge et al., 1992; Miyamoto et al., 1995b). Some literature states that FAK and Src seem to have principal roles in promoting turnover and dynamics rather than assembly of focal adhesions (Geiger et al., 2001). For example, cells that are deficient in FAK or Src have enlarged focal adhesions (Ilic et al., 1995; Volberg et al., 2001). The phosphorylation of tyrosine residues on both FAK and Src appear to have dual purposes in the regulation of focal adhesions. They can both promote molecular interactions and aid in focal adhesion assembly and be involved in focal adhesion turnover (Volberg et al., 2001). If FAK is hyperphosphorylated, it is also phosphorylated at Y925, a region within the focal adhesion targeting (FAT) domain (Katz et al., 2003). When FAK is phosphorylated at Y925 it is actually lost from focal adhesions (Katz et al., 2003). These observations explain that FAK molecules dynamically cycle in and out of focal adhesion complexes based upon phosphorylation status.

The type of external force applied to a cell will dictate which focal adhesions are formed (Geiger and Bershadsky, 2002). Specifically, Wang and Ingber, 1995, demonstrated that mechanical force only applied to integrins resulted in focal adhesion assembly. The ability of focal adhesions to be maintained as stable structures depends upon either continuous application of force or continuous application of contractile machinery within the cell (Bershadsky et al., 2006).

Focal adhesions have been implicated in numerous processes throughout the body from gravitationally compressive forces controlling deposition of bone, isotonic tension causing muscle growth, normal hydrostatic pressure in blood vessels promoting maturation of vascular smooth muscle to hypertensive pressure causing arterial walls to thicken (Thompson, 1942; Folkow, 1982; Guyton and Hall, 2006). Another area of research that focal adhesions have been implicated in is pregnancy (Burghardt et al., 2002). This review focuses specifically on focal adhesions and their roles in pregnancy in mammalian species.

Rodents

Focal adhesions have been identified in rodent endometrium during the window of receptivity, which is when the blastocysts enters the uterine cavity. (Psychoyos, 1976). Specifically, focal adhesions form at the uterine-conceptus surface (Burghardt et al., 2002). Interestingly, treatments infused into the uterus of rodents that block integrin attachment, such as echistatin (a disintegrin, integrin β_1 and β_3 inhibitor) and arginine-glycine-aspartic acid (RGD)-containing hexapeptides, reduce the number of implantation sites, and ultimately litter size (Illera et al., 2000). Many integrins have been identified in the rodent in relation to pregnancy (Table 1.2). Blastocysts express α_{6B}, α_v, α_2, α_7, α_{6A}, α_5, β_1 and β_3 (Sutherland et al., 1993; Thorsteinsdottir et al., 1995; Schultz et al., 1997). Specifically, $\alpha_v\beta_3$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ heterodimers are located on the cell surface of the blastocyst (Sutherland et al., 1993). Developmental regulation of integrin expression on the blastocyst along with spatial and temporal distribution and modulation of several subunits in rodent uteri during the reproductive cycle and early pregnancy suggest direct integrin involvement in implantation (Damsky et al., 1993; Coutifaris et al., 1998; Lessey, 2000). In rats, development of smooth muscle cell focal adhesions lining the uterus (myometrium) increases as pregnancy progresses (Williams et al., 2005). These focal adhesions increase due to changes in endocrine signaling and mechanical stretching of the uterine wall. This leads to increased expression of ECM components as the myometrium prepares to expel a fetus at full term (Macphee and Lye, 2000; Li et al., 2007). In the rodent uterus, highly organized focal adhesion complexes in myometrial cells are restricted to a period during late pregnancy when pregnancy hormones and mechanical stretch upregulate expression of fibronectin, and ECM mechanosensor (Shynlova et al., 2004). During late pregnancy and labor, it is critical for adhesion of myometrial cells and ECM to occur for coordinated contractions of labor (Macphee and Lye, 2000). Focal adhesion signaling and assembly is rapidly terminated following labor (Macphee and Lye, 2000).

Humans

Very similar to rodents, focal adhesions are identified during the window of receptivity at the endometrial and conceptus surface (Psychoyos, 1976; Burghardt et al., 2002). Three integrins are of particular interest for marking the window of receptivity in humans: α_v and β_3, which coincide with embryo attachment, which occurs around day 9, and α_4, which disappears when the window of receptivity closes (Lessey et al., 1994; Lessey et al., 1995; Wilcox et al., 1999). Several integrins have been identified in human endometrium in relation to pregnancy (Table 1.2). Human trophectoderm cells express α_3, α_5, β_1, β_3 and β_5 subunits (Campbell et al., 1995). When trophoblasts enter the outgrowth stage they express α_1, α_2 and α_1 (Campbell et al., 1995). In fact, when villous explants are cultured with antisense FAK, there is a dramatic reduction in trophoblast outgrowth (MacPhee et al., 2001). Interestingly, endometrium exhibits both constitutive and cycle-dependent expression of integrins and appears to be the only tissue known to exhibit hormone-dependent integrin expression (Lessey et al., 1995). Estrogen stimulation results in rapid FAK phosphorylation and enhances motility of endometrial cells (Flamini et al., 2011).

Cows

Very little research has been performed on focal adhesions and their roles during pregnancy in cattle. Interestingly though, a recent study evaluated the circulating miRNA in serum during early pregnancy. MicroRNA are single-stranded short RNAs that destabilize mRNA, usually resulting in mRNA degradation (Chendrimada et al., 2007; Kim et al., 2009). MicroRNAs in sera of pregnant cows on days 19 and 24 had predicted targets of adherans junctions and ECM-receptor interaction respectively, implying focal adhesions may have a role in early pregnancy (Gebremedhn et al., 2018).

Sheep

Endometrium from pregnant ewes have a dramatically increased abundance of protein molecules involved in ECM-receptor interaction and focal adhesions compared to endometrium from non-pregnant ewes (Zhao et al., 2015). Both the conceptus trophectoderm and uterine luminal epithelium express integrins $\alpha_v, \alpha_4, \alpha_5, \beta_1, \beta_3$ and β_5 (ITGAV, ITGA4, ITGA5, ITGB1, ITGB3 and ITGB5) during the preimplantation period of pregnancy (Table 1.2) (Gray et al., 2004). They also both express integrin binding proteins secreted phosphoprotein 1 (SPP1, also known as osteopontin) and galectin 15 (LGALS15) (Gray et al., 2004). Studies have shown that focal adhesion organization begins during the first trimester of pregnancy, which is significantly earlier compared to rodents (Burghardt et al., 2009). Focal adhesions are present in the gravid horn by day 40 of pregnancy and become more pronounced by day 80 (Burghardt et al., 2009). The assembly and complexity of focal adhesions is highly dependent upon force (Macphee and Lye, 2000; Williams et al., 2005; Li et al., 2007; Shynlova et al., 2007). It is thought that as the fetus develops and accumulates fetal fluid in the allantois in amnion, the mechanical stretch of the uterine wall is sufficient to signal focal adhesion assembly (Burghardt et al., 2009). Prior to day 120, focal adhesion subunits are inconsistent throughout the maternal-conceptus interface. This is thought to occur due to movement of the animal and fetus within the uterus (Burghardt et al., 2009). Focal adhesions are highly dynamic and can rapidly respond to force by assembling focal adhesions or relaxation by disassembling focal adhesions (Riveline et al., 2001; Bershadsky et al., 2006). As pregnancy progresses, size and extensiveness of focal adhesions along the fetal-maternal interface increase and by day 120 extensive focal adhesions are present along most of the interface (Burghardt et al., 2009). In contrast to the preimplantation period, the non-gravid horn has very little apical staining of ITGAV, ITGA4, ITGA5, ITGB1, ITGB3 and ITGB5 in caruncular and intercaruncular regions (Burghardt et al., 2009).

Pigs

Both conceptus and uterine luminal epithelium express α_1, α_3, α_4, α_5, α_v, β_1, β_3 and β_5 subunits (Table 1.2). More specifically, α_4, α_5, α_v, β_1, β_3 and β_5 subunits are detected at the sites of initial attachment between the luminal epithelium and trophoblast cells on days 12-15 of pregnancy (Bowen et al., 1996). Data have also shown that miRNAs in porcine endometrium during implantation, placentation and mid-gestation specifically target cell adhesion (Su et al., 2014).

Horses

Very little information is known regarding focal adhesions and their roles during pregnancy in the horse. Our laboratory has shown that exosomes, which are cell secreted vesicles capable of transferring bioactive materials, isolated from sera from non-pregnant mares target the focal adhesion pathway during early pregnancy (Valadi et al., 2007; Camussi et al., 2011; Lasser et al., 2011; Klohonatz et al., 2016). In the horse, the embryo is mobile due to uterine contractions until day 16, where it will fixate at a location, without implanting (Ginther, 1983; Allen and Stewart, 2001). It is proposed that this mobility is necessary for the endometrium to activate maternal recognition of pregnancy (MRP) and possibly promote focal adhesion assembly (McDowell et al., 1988). Data have shown that focal adhesions are upregulated in endometrial samples from pregnant mares compared to non-pregnant mares (Klohonatz et al., 2016). The functionality of these focal adhesions and their role during early pregnancy is unknown.

Table 1.2. Integrin subunits that have been identified in each species.

Species	In the Uterine Endothelium	In the Conceptus
Rodents	α_v, β_3, α_4	α_{6B}, α_v, α_2, α_7, α_{6A}, α_5, β_1, β_3
Cows	Unknown	Unknown
Sheep	α_v, α_4, α_5, β_1, β_3, β_5	α_v, α_4, α_5, β_1, β_3, β_5
Pigs	α_1, α_3, α_4, α_5, α_v, β_1, β_3	α_1, α_3, α_4, α_5, α_v, β_1, β_3
Horses	α_2, α_4, α_5, α_6, α_{10}, α_{11}, α_v, α_X β_1, β_3, β_4, β_8	Unknown
Humans	α_v, β_3, α_4	α_3, α_5, β_1, β_3, β_5

Conclusion

Maternal recognition of pregnancy in the horse is a process that is not well defined. The recognition of an embryo by at least 80% of the endometrium is necessary to signal MRP and attenuate PGF production. Research has indicated that focal adhesions are present in the endometrium during this time frame and may have an active role in signaling MRP through mechanotransduction. Mechanotransduction is the mechanism by which cells respond to an external force, resulting in a biochemical signal within the cell. The mechanism by which this occurs is through focal adhesions. While the mode of action for focal adhesions is becoming clearer, the true complexity of the focal adhesion network is being realized. With FAK alone capable of interacting with at least ten different molecules, the extensiveness of this network is continuing to grow. The network becomes more complex due to the variability of focal adhesions based upon species, location in the body, external stimulation and amount of phosphorylation.

It is certain that focal adhesions play an integral role in the endometrium during pregnancy and possibly maternal recognition of pregnancy. The exact functions of focal adhesions vary based upon the species and timing of the pregnancy. Research is scratching the surface on the roles of focal adhesions during pregnancy, but much still remains a mystery. The objective of this dissertation was to describe focal adhesions in the endometrium during maternal recognition of pregnancy in the horse and their potential role in attenuating PGF production.

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CHAPTER II: THE ROLE OF EMBRYO CONTACT AND FOCAL ADHESIONS DURING MATERNAL RECOGNTION OF PREGNANCY[2]

Summary

Maternal recognition of pregnancy (MRP) in the mare is still a widely unknown process. In a non-pregnant mare on day 14 post-ovulation (PO), prostaglandin $F_{2\alpha}$ (PGF) is secreted by the endometrium causing regression of the corpus luteum (CL). Regression of the CL halts progesterone production allowing the mare to begin cycle again. Prior to day 14, MRP must occur in order to attenuate the production and secretion of PGF. The embryo is mobile throughout the uterus due to uterine contractions from day 9 to day 14. During this time frame, it is necessary that the embryo comes in contact with at least 80% of the uterus. It is unknown what signaling is occurring to signal MRP. Literature has stated that infusing oil into the mare's uterus or placing a glass marble into the mare's uterus prolongs luteal lifespan. Literature also states that in non-pregnant mares, serum exosomes contain miRNA that are targeting the focal adhesion (FA) pathway. The hypothesis of the current study is that the presence of an embryo contacting endometrium causes a change in the abundance of focal adhesion molecules (FAM) in the endometrium leading to decrease in PGF secretion. Mares (n = 3/day) were utilized in a cross-over design with each mare serving as a pregnant and non-pregnant (non-mated) control on days 9 and

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11 PO. Mares were randomly assigned to a collection day and endometrial samples and embryos (if it was a pregnant cycle) were collected on the specified day. Biopsy samples were divided into five pieces, four for culture for 24 hours and one was immediately snap frozen. Endometrial biopsies for culture were placed in an incubator for 24 hours with one of four treatments: (1) an embryo in contact on the luminal side of the endometrium, (2) beads in contact on the luminal side of the endometrium, (3) peanut oil in contact on the luminal side of the endometrium or (4) the endometrium by itself. Biopsies and culture medium were frozen for further analysis. RNA and protein were isolated from the endometrial biopsies for PCR and Western blot analysis for FAM. PGF assays were performed on the culture medium to determine the concentration of PGF in the medium from the endometrium. Statistics were all performed using SAS and $P \leq 0.05$ indicated significance. The presence of beads on day 9 mainly impacted samples from pregnant mares more than non-pregnant mares and had very little impact on day 11. The presence of oil decreased α-ACTININ, CAV1 and CCND1 abundance in samples from pregnant mares on day 9. On day 11, oil decreased *ACTN4* and *CAV1* abundance in sample from non-pregnant mares and increased BCL-2 in samples from pregnant mares. The presence of an embryo caused multiple changes in RNA and protein abundance in endometrium from pregnant and non-pregnant mares. In samples from non-pregnant mares on day 9, *BCL-2, ITGA4* and *SLCO2A1* were increased in the presence of an embryo. ITGB3 was also increased in the presence of an embryo. In samples from pregnant mares on day 9, *RAF1* was the only gene that was increased with the presence of an embryo. α-ACTININ, CAV1 and CCND1 were decreased with the presence of an embryo. On day 11, BCL-2 was the only protein with higher abundance in the presence of an embryo in samples from pregnant mares and PAK6 was the only protein increased in the presence of an embryo from non-pregnant mares. *ACTN1, ACTN4, CAV1,* and *ITGA6* were all decreased in the presence of an

embryo from non-pregnant mares. The next step was to evaluate PGF secretion after 24 hours with each treatment. On day 9, there was no change in PGF secretion compared to any of the treatments. On day 11, the presence of peanut oil increased PGF secretion in samples from non-pregnant mares. Most importantly, in samples from non-pregnant mares, the presence of an embryo decreased PGF secretion compared to the control samples from non-pregnant mares. Results revealed that while beads and peanut oil may impact the abundance of FAM RNA and protein in endometrial samples, it doesn't appear to impact PGF secretion. Conversely, the presence of an embryo for 24 hours in contact with endometrium from a non-pregnant mare was enough to cause a decrease in PGF secretion. These results suggest that it is not just the contact of any substance/object causing PGF secretion to decrease, but it is the embryo itself necessary to attenuate PGF secretion.

Introduction

Maternal recognition of pregnancy (MRP) refers to the mechanism by which endometrium identifies the presence of an embryo resulting in the continued secretion of progesterone (P_4) by the corpus luteum (CL) (1). In non-pregnant mares on day 14 post-ovulation (PO), oxytocin is released from the endometrium into the uterine lumen, binding endometrial receptors, causing the release of more oxytocin and production and release of prostaglandin $F_{2\alpha}$ (PGF) (2). In pregnant mares, the conceptus enters the uterus on day 6, and by day 9 is surrounded by a glycoprotein rich capsule, a characteristic unique to the horse and rabbit (3). Once the conceptus is in the uterus, uterine contractions move the embryo throughout the uterus reaching peak mobility between days 11 and 14 PO (4, 5). This mobility is necessary to mitigate the secretion of PGF. The mobility ceases by day 16, indicating that MRP occurs between days 11 to 14 PO and is antiluteolytic (4, 6, 7).

The CL must be protected from endometrial PGF in order to sustain P_4 production (8-10). Maternal recognition of pregnancy is vital to pregnancy success yet differs in the mare compared to other species. Interferon tau and estradiol are the MRP signals in ruminants and pigs, respectively, but they do not have any impact on luteal function in the horse (11-14). The equine conceptus secretes prostaglandin E_2 on day 4, but it has no effect if infused into the uterus of non-pregnant mares (12). Interestingly, when coconut or peanut oil was infused into the uterus of a NP mare on day 10 PO, luteostasis occurred, indicating that a component in the oils impacted the luteolytic pathway (15). Literature also states that placing a glass ball, or marble, into the diestrous uterus of a mare will prolong the lifespan of the CL, suggesting it is the physical contact on the endometrium that prevents PGF secretion (16, 17).

Exosomes, which are cell secreted vesicles, have been identified in equine serum (18). Exosomes are capable of storing and transferring bioactive material, such as RNA and protein, between cells (19). Recent literature has indicated that serum exosome content varied between pregnant and non-pregnant mares (20). Specifically, it was found that differential miRNA in exosomes from non-pregnant mares potentially target the focal adhesion (FA) pathway (20). Focal adhesions are macromolecular complexes that are comprised of heterodimeric transmembrane integrin receptors that regulate effects in extracellular matrices in endometrium (21). It has also been reported that FA sense and transduce mechanical forces (22). It has been suggested that mechanotransduction is the reason intrauterine devices resulted in luteal persistence in mares (16).

The present study was designed to test if the presence of an embryo is necessary to attenuate PGF secretion, or if PGF secretion can be attenuated by the presence of oil or noncellular contact with the epithelium. The hypothesis of this study is that the contact of an embryo with equine endometrium at the epithelium will cause a change in FAM abundance and result in a decrease in PGF section.

Materials and Methods

Care and Management of Mares

All horse use was approved by the Colorado State University Animal Care and Use Committee (Approval Number 13-4293A). Mares ($n = 6$) were housed in group pens at the Colorado State University Equine Reproduction Laboratory (Fort Collins, CO) and maintained on a dry lot and fed grass-alfalfa mix with free choice mineral and salt supplement. Mares were used in a cross-over design in which each mare had a pregnant and non-pregnant (non-mated) cycle. Mares were teased with a stallion daily and transrectal ultrasonography was used to monitor to track their follicular development every other day. Once a follicle reached 35 mm in diameter, or greater, the mare was inseminated with 500×10^6 progressively motile sperm from a single stallion with proven fertility. After insemination, mares were evaluated every day with transrectal ultrasonography and inseminated every other day until ovulation (day 0) was detected.

Mares were randomly assigned to one collection day (day 9 or 13) for their pregnant and non-pregnant cycles. On the assigned day, the mare was evaluated via transrectal ultrasonography to confirm pregnancy by the visualization of an embryonic vesicle. Endometrial samples were obtained non-surgically via a trans-cervical biopsy punch (23). Embryos were collected via terminal uterine lavage on the same day as biopsy to be used in culture. After embryo and biopsy collection the mare received a luteolytic dose of prostaglandin $F_{2\alpha}$ (PGF; Estrumate, Merck Animal

Health, 250 mcg per dose). The subsequent estrous cycle was utilized for the non-pregnant (non-mated) control cycle. Another herd of mares (n = 3) were monitored and bred with the same protocol in order to provide embryos to be cultured with endometrial samples from non-pregnant mares on corresponding days.

After endometrial biopsy samples were obtained, each sample was rinsed in DPBS/Modified 1X (Hyclone Laboratories, Logan, UT). Special care was taken to ensure the sample was not inverted in order to keep the luminal side of the endometrium facing upwards. Biopsy samples were cut into five pieces. Four pieces were then washed three times in incubation medium [(Medium 199 (Life Technologies, Carlsbad, CA) containing 5% antibiotic-antimycotic (Life Technologies, Carlsbad, CA)] prior to culture for 24 hours (explained in the next section). The fifth piece was immediately snap frozen in liquid nitrogen and transferred to -80°C.

Endometrial Biopsy Culture

After endometrial biopsy and embryo collection, samples were immediately transferred to clean culture dishes for incubation for 24 hours with the corresponding treatment. Biopsy samples were cultured in one of four conditions: (1) direct contact with an embryo from the corresponding day (EE), (2) direct contact with plastic beads (utilized routinely to teach embryo flushing and transfer) (EB) (Cospheric, Product Number: UVPMS-BR-1-5, Santa Barbara, CA), (3) direct contact with peanut oil (EO) and (4) endometrial biopsy alone (control)(E-). Figure 2.1 contains a diagram of the culture method. Tissues were incubated in a humidified atmosphere at 5% CO_2, 95% air at 37°C according to Watson and Sertich 1989 (24) for 24 hours. After 24 hours endometrial biopsies and their corresponding medium were immediately placed at -80°C until further analysis.

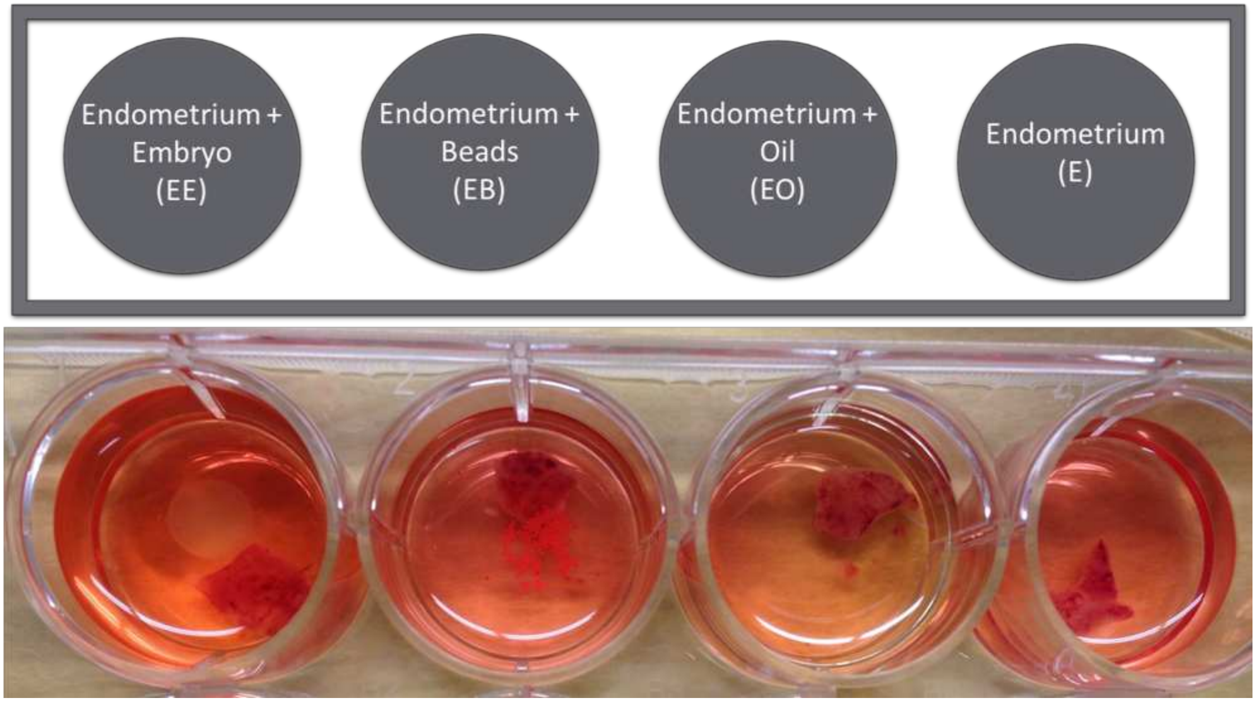


Figure 2.1 Endometrial Culture Layout
This figure represents the culture layout for each sample. Special care was taken to ensure the luminal side of the endometrium was in contact with the treatment for the entire 24 hours.

RNA Isolation and Quantification

Total RNA was isolated from endometrial biopsies, both cultured and snap frozen, using TRI Reagent (Molecular Research Center, Cincinnati, OH). Frozen tissue was homogenized in TRI Reagent and left at room temperature for ten minutes. After the incubation, chloroform was added to the homogenate, vortexed and incubated for an additional eight minutes at room temperature. The sample was then centrifuged at 13,200 revolutions per minute (RPM; 16,100 x g) for 15 minutes in order to separate the sample into three distinct phases (RNA, DNA and protein). The top, aqueous phase RNA phase was transferred to a new 1.7 mL Eppendorf tube for RNA isolation. DNA and protein phases were frozen at -80°C until further analysis.

RNA was isolated from samples utilizing RNeasy Mini Kit (Qiagen, Valencia, CA). The protocol was followed according to manufacturer's recommendations. Following isolation, all samples were treated with an RNase-Free DNase set (Qiagen, Valencia, CA) to remove any DNA contamination. RNA purity and quantification were assessed using the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE). Samples were used for PCR analysis if they had 260/280 and 260/230 values above 1.7.

Real Time Quantitative Polymerase Chain Reaction

The following genes were selected for evaluation within the endometrium based upon literature and previous data in our laboratory: *ACTN1, ACTN2, ACTN3, ACTN4, AKT3, BCL-2, CAV1, CCND1, ITGA10, ITGA4, ITGA5, ITGA6, ITGAV, ITGAX, ITGB1, ITGB3, PAK6, PTGS2, PTK2 (FAK), RAF1, SLCO2A1* and *TLN1* (20, 21). Equine specific forward and reverse primers were designed using Primer3 (http://primer3.wi.mit.edu/). Primers were designed specifically to have a product size between 115-135 bp, a primer length between 19-27 bp, a primer Tm between 60-65°C, and a GC% content between 40-60. The genes, designed primer sequences and amplicon length for each of the genes can be found in Supplemental Table S1. Prior to PCR analysis, primer specificity, via DNA sequencing of PCR products was evaluated. PCR analysis for specificity was performed using an endometrial cDNA pool and GoTaq (Promega, Madison, WI) following the manufacturer's protocol. PCR products were electrophoresed on a 2% agarose gel to confirm presence of an amplicon with the expected size. The product band was excised from the gel for DNA isolation with Qiaquick Gel Extraction (Qiagen, Valencia, CA). Isolated DNA was then sent to the University of California-Davis DNA Sequencing Facility, Davis, CA, to be sequenced. The resulting sequences were confirmed using NCBI BLAST for gene specificity.

For qRT-PCR analysis, total RNA was processed for reverse transcription using iScript cDNA Synthesis (Bio-Rad, Hercules, CA). One µg of total RNA was added to each reverse transcription reaction with 4 µL of 5x iScript reaction mix, 1 µL of iScript reverse transcriptase, and nuclease-free water to reach a total reaction volume of 20 µL. Reverse transcription was performed following the manufacturer's specifications with 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, and then holding at 4°C for immediate use as cDNA template in qRT-PCR. For each real time PCR reaction 5 µL of SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA) was added to 2.5 µL of nuclease-free water, 1 µL of cDNA at a concentration of 50 ng/µL, and 1.5 µL of primer mix at a concentration of 10 µM to reach a final volume of 10 µL. Samples were loaded into 384 well LightCycler 480 plates (Roche, Indianapolis, IN) and analyzed in duplicate using a LightCycler 480 PCR System (Roche, Indianapolis, IN). Real Time PCR cycle conditions were per manufacturer's protocol; 30 seconds at 95°C, and 40 cycles of denaturing at 95°C for 5 seconds and annealing and extension at 60°C for 30 seconds. PCR analysis followed by melt peak analysis occurring at 0.5°C increments from 65-95°C, holding for 2 seconds at each increment. Cq values were normalized to the geometric mean of *GAPDH*, *TUBA1B, B2M* and *ACTB* by subtracting the geometric mean from the Cq value (Microsoft Excel). These normalized values were used for the statistical analyses. For theses analyses, the treated sample was compared to the endometrial sample from the same day cultured by itself (control). Statistical analysis was performed with SAS 9.4 (SAS Institute Inc.). Proc Mixed was used to fit a mixed model separately by gene. Fixed effects included day (9 or 11), pregnancy status (pregnant or non-pregnant) and treatment (EE, EB, EO, E) plus all interactions. Horse ID and horse ID by pregnancy status were included as random effects to account for the repeated measures design. Tukey adjusted pairwise comparisons were used and significance was assessed at $P \leq 0.05$.

Protein Isolation and Quantification

Protein was isolated from all endometrial tissue utilizing RIPA buffer containing nuclease-free water, Tris pH 8.0, NaCl, glycerol, Nonidet P-40, SDS, deoxychlorate, ethylenediamine tetraacetic acid, HCl, 0.01% proteinase inhibitor PIC, and 0.05% PMSF. Frozen tissue samples were homogenized in RIPA lysis buffer while on ice, then sonicated on ice for 30 seconds and centrifuged at 9,300 RPM (10,000 x g) at 4°C for 10 minutes. The supernatant was placed into a new tube and PIC and PMSF were added. The protein content in samples was quantified using Pierce BCA Protein Assay Kit (Thermo Scientific, Wilmington, DE) following the manufacturer's protocol. Briefly, standards were prepared using manufacturer provided BSA at 2.0 mg/mL. Working reagent utilizing manufacturer's reagents A and B was prepared. A microplate was loaded with 25 µL of standard or sample and 200 µL of working reagent and the microplate was incubated at 37°C for 30 minutes. Samples were quantified using the Synergy 2 Multi-Mode Microplate Reader (Biotek, Winooski, VT). Sample concentrations were determined based on a standard curve prepared with the standards.

Western Blot Analysis

Western blots were performed for proteins of interest based upon PCR results for all cultured and snap frozen samples. Western blots were normalized to Cytochrome C (primary antibody: sc-7159, rabbit polyclonal, Santa Cruz Biotechnologies, Santa Cruz, CA; secondary antibody: ab6721, goat anti-rabbit IgG with HRP, Abcam, San Francisco, CA) and all gels were run on a 4-15% gradient gel (Bio-Rad Laboratories, Hercules, CA, catalog number 4561086). Proteins of interest were (Table 2.1): FAK (1:200 primary antibody: sc-558, rabbit polyclonal, Santa Cruz Biotechnologies, Santa Cruz, CA; 1:2000 secondary antibody: ab6721, goat anti-rabbit IgG with HRP, Abcam, San Francisco, CA), p-FAK (1:200 primary antibody: sc-16563-R, rabbit polyclonal, Santa Cruz Biotechnologies, Santa Cruz, CA; 1:2000 secondary antibody: ab6721, goat anti-rabbit IgG with HRP, Abcam, San Francisco, CA), Integrin β3 (1:200 primary antibody: sc-6627, goat polyclonal, Santa Cruz Biotechnologies, Santa Cruz, CA; 1:2000 secondary antibody: sc-2345, mouse anti-goat IgG with HRP, Santa Cruz Biotechnologies, Santa Cruz, CA), α-actinin (1:200 primary antibody: sc-7453, goat polyclonal, Santa Cruz Biotechnologies, Santa Cruz, CA; 1:2000 secondary antibody: sc-2345, mouse anti-goat IgG with HRP, Santa Cruz Biotechnologies, Santa Cruz, CA), PAK6 (1:500 primary antibody: ab37749, rabbit polyclonal, Abcam, San Francisco, CA; 1:2000 secondary antibody: ab6721, goat anti-rabbit IgG with HRP, Abcam, San Francisco, CA), CCND1 (1:200 primary antibody: ab7958, rabbit polyclonal, Abcam, San Francisco, CA; 1:2000 secondary antibody: ab6721, goat anti-rabbit IgG with HRP, Abcam, San Francisco, CA), Bcl-2 (1:200 primary antibody: sc-492, goat polyclonal, Santa Cruz Biotechnologies, Santa Cruz, CA; 1:2000 secondary antibody: sc-2345, mouse anti-goat IgG with HRP, Santa Cruz Biotechnologies, Santa Cruz, CA) and CAV1 (1:200 primary antibody: sc-894, rabbit polyclonal, Santa Cruz Biotechnologies, Santa Cruz, CA; 1:2000 secondary antibody: ab6721, goat anti-rabbit IgG with HRP, Abcam, San Francisco, CA).

Table 2.1 Proteins and antibody information used for Western blot analyses.

Protein	Primary Antibody				Secondary Antibody			
	Dilution	Type	Company	Catalog Number	Dilution	Type	Company	Catalog Number
Cytochrome C	1:200	Rabbit Polyclonal	Santa Cruz Biotechnologies	sc-7159	1:2000	Goat anti-rabbit IgG with HRP	Abcam	ab6721
FAK	1:200	Rabbit Polyclonal	Santa Cruz Biotechnologies	sc-558	1:2000	Goat anti-rabbit IgG with HRP	Abcam	ab6721
p-FAK	1:200	Rabbit Polyclonal	Santa Cruz Biotechnologies	sc-16563-R	1:2000	Goat anti-rabbit IgG with HRP	Abcam	ab6721
Integrin β3	1:200	Goat Polyclonal	Santa Cruz Biotechnologies	sc-6627	1:2000	Mouse anti-goat IgG with HRP	Santa Cruz Biotechnologies	sc-2345
α-actinin	1:200	Goat Polyclonal	Santa Cruz Biotechnologies	sc-7453	1:2000	Mouse anti-goat IgG with HRP	Santa Cruz Biotechnologies	sc-2345
PAK6	1:500	Rabbit Polyclonal	Abcam	ab37749	1:2000	Goat anti-rabbit IgG with HRP	Abcam	ab6721
CCND1	1:200	Rabbit Polyclonal	Abcam	ab7958	1:2000	Goat anti-rabbit IgG with HRP	Abcam	ab6721
BCl-2	1:200	Goat Polyclonal	Santa Cruz Biotechnologies	sc-492	1:2000	Mouse anti-goat IgG with HRP	Santa Cruz Biotechnologies	sc-2345
CAV1	1:200	Rabbit Polyclonal	Santa Cruz Biotechnologies	sc-894	1:2000	Goat anti-rabbit IgG with HRP	Abcam	ab6721

For each Western blot, 20 μg of endometrial protein was loaded into each well. Samples were incubated with a 4:1 6x buffer to DTT mix for 10 minutes at 90°C. Samples were then transferred to wells and run for 35 min at 200 V and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, Catalog #1620213) for 1 hour at 100 V at 4°C. Membranes were blocked in 5% blocking buffer (5% non-fat dried milk in 1X TBST) for 1 hour at room temperature and washed with 1X TBST. Membranes were incubated with primary antibody overnight at 4°C on a rocker. The next day membranes were washed three times in 1X TBST for 5 min and incubated with horseradish peroxidase conjugated secondary antibody for one hour at room temperature. Membranes were washed three times in 1X TBST and ECL Plus Western Blotting Detection Reagent (GE Healthcare Life Sciences, Pittsburgh, PA, Catalog #RPN2232) was added for 5 minutes. Membranes were imaged for analysis on a Molecular Imager ChemiDoc XRS+ System (Bio-Rad, Hercules, CA).

For theses analyses, the treated sample was compared to the endometrial sample from the same day cultured by itself (control). Statistical analysis was performed with SAS 9.4 (SAS Institute Inc). Proc Mixed was used to fit a mixed model separately by gene. Fixed effects included day (9 or 11), pregnancy status (pregnant or non-pregnant) and treatment (EE, EB, EO, E) plus all interactions. Horse ID and horse ID by pregnancy status were included as random effects to account for the repeated measures design. Tukey adjusted pairwise comparisons were used and significance was assessed at $P \leq 0.05$.

Prostaglandin $F_{2\alpha}$ Hormone Assay

In order to quantify the amount of PGF secreted by the endometrium after culture, a $PGF_{2\alpha}$ EIA kit from Enzo Life Sciences was utilized (Enzo Life Sciences, Farmingdale, NY, catalog number ADI-901-069). This is a competitive immunoassay utilizing a polyclonal antibody to PGF.

Briefly, standards with known concentrations of PGF were prepared utilizing the incubation medium. 100 μL of standards and samples were pipetted into the corresponding wells. Samples were evaluated in duplicate. Assay buffer, conjugate, and antibody were added to each well. After a two-hour incubation at room temperature, the wells were washed with wash solution. Substrate solution was added to each well and incubated for 45 minutes at room temperature. After stop solution was added to each well the plate was read at an optical density of 405 nm.

Unknown concentrations were determined utilizing a four-parameter logistic fit. Once the concentration was determined, it was adjusted based upon an adjustment factor equal to the weight of the original tissue divided by the mL of medium. There were varying amounts of medium in the wells due to the need to ensure the entire embryo was covered. For theses analyses, the treated sample was compared to the endometrial sample from the same day cultured by itself (control). Concentrations were analyzed for statistical significance using SAS 9.4 (SAS Institute Inc.). Proc Mixed was used to fit a mixed model separately by gene. Fixed effects included day (9 or 11), pregnancy status (pregnant or non-pregnant) and treatment (EE, EB, EO, E) plus all interactions. Horse ID and horse ID by pregnancy status were included as random effects to account for the repeated measures design. Tukey adjusted pairwise comparisons were used and significance was assessed at $P \leq 0.05$.

Results

Focal Adhesion Molecules in Snap Frozen Endometrium

There were very few transcripts differentially expressed on days 9 or 11. On day 9, *TLN1* was increased ($P = 0.011$) in endometrial samples from pregnant mares. Also, in samples from pregnant mares on day 9, CAV1 was more abundant ($P = 0.004$). On day 11, *ACTN3* was increased ($P = 0.050$) in endometrial samples from pregnant mares and *CAV1* was increased ($P = 0.011$) in endometrial samples from non-pregnant mares. Figure 2.2 shows the transcript levels on days 9 and 11. There were no differences identified in the protein abundance on day 11 (Figure 2.2).

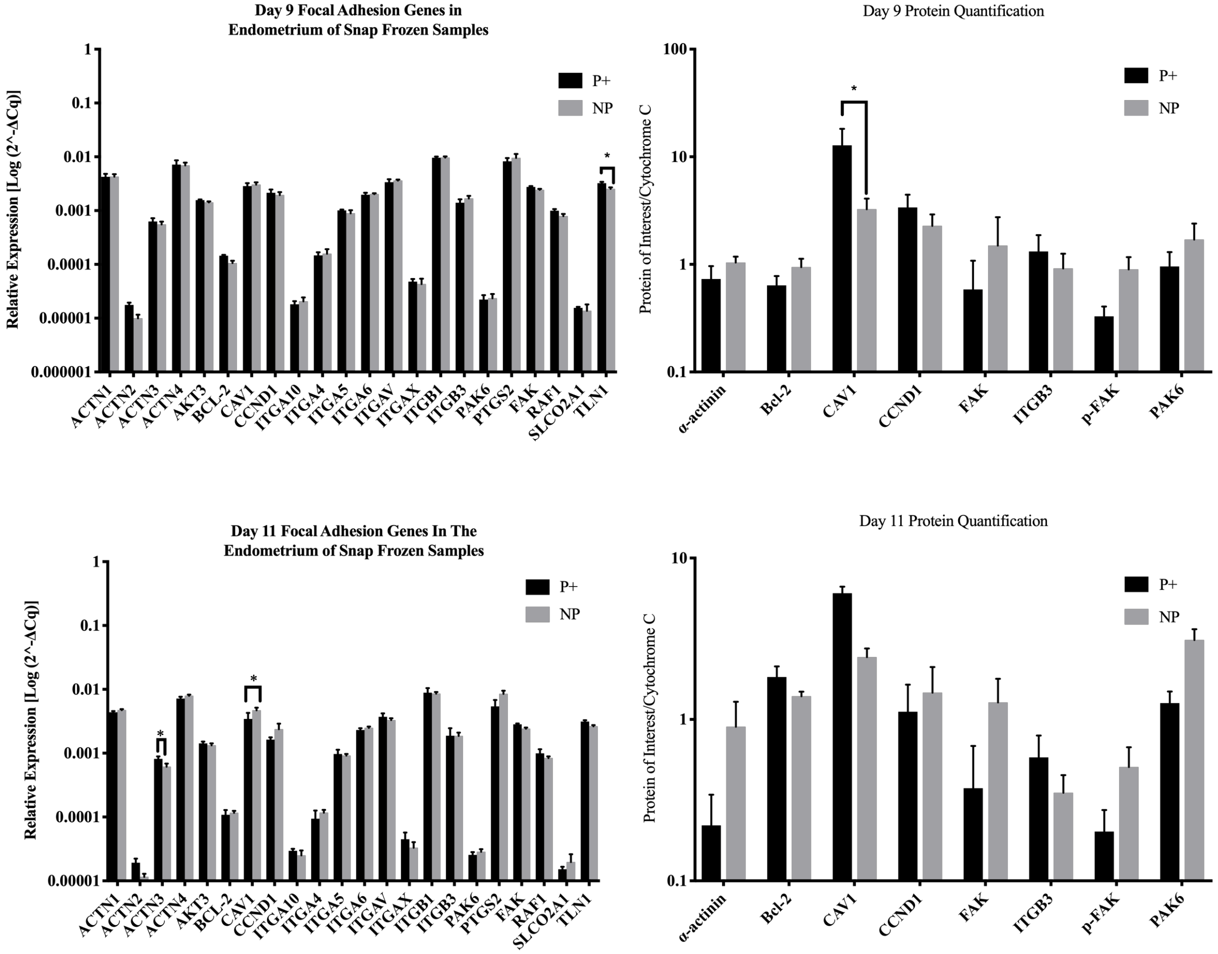


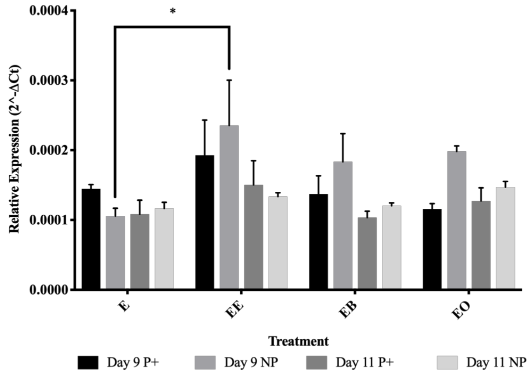
Figure 2.2 Endometrial gene and protein abundance in snap frozen endometrial samples from pregnant (P+) and non-pregnant (NP) mares.

This figure shows the gene and protein abundance of targets for this study. * = $P \leq 0.05$. Graphs are mean ± SEM

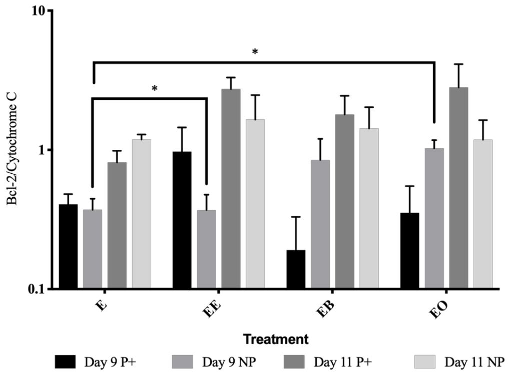
Endometrium Co-Cultured with Mechanical Pressure

On day 9 from endometrial samples collected from pregnant mares, *CCND1* was more abundant ($P = 0.015$) in control tissue (Figure 2.3). In endometrial samples collected from non-pregnant mares, *ITGAV* was more abundant ($P = 0.013$) in control tissue (Figure 2.4). When evaluating protein levels, the samples from pregnant mares had different protein abundance. α-actinin (Figure 2.5) and CCND1 (Figure 2.3) were more abundant ($P = 0.001$ and $P = 0.001$ respectively) in control tissue and p-FAK was more abundant ($P < 0.001$) in endometrial samples co-cultured with beads (Figure 2.6).

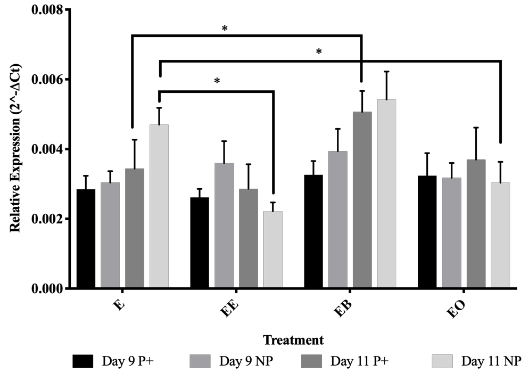
BCl-2 Transcript Expression



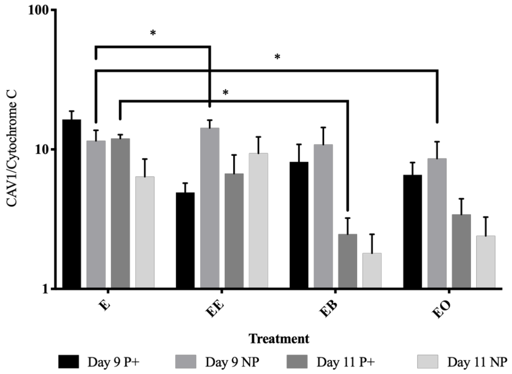
Bcl-2 Protein Quantification



CAV1 Transcript Expression

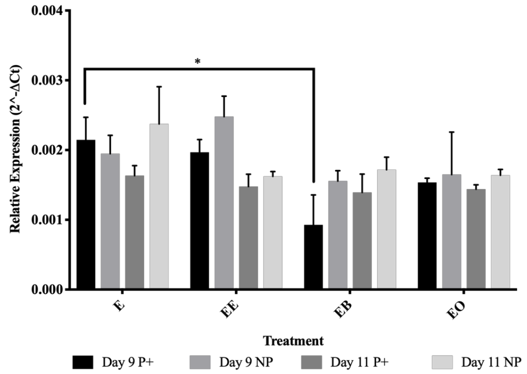


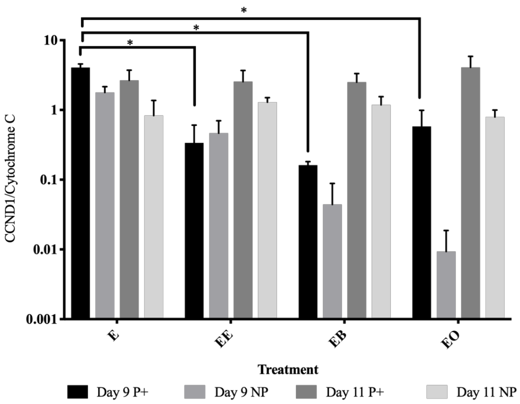
CAV1 Protein Quantification



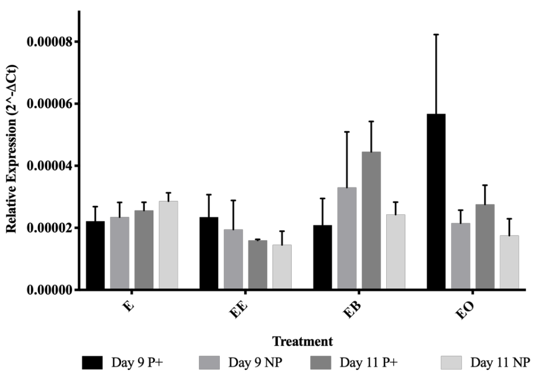
CCND1 Protein Quantification

CCND1 Transcript Expression





PAK6 Transcript Expression



PAK6 Protein Quantification

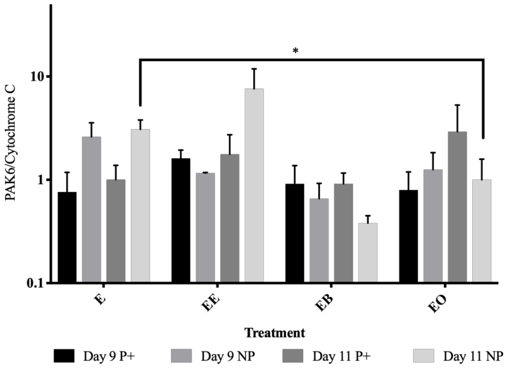


Figure 2.3 Gene and protein abundance for Bcl-2, CAV1, CCND1 and PAK6 for all treatments in endometrial samples from pregnant (P+) and non-pregnant (NP) mares.

This figure contains all of the gene expression and protein quantification data for Bcl-2, CAV1, CCND1 and PAK6. E = no treatment (control); EE = endometrium with an embryo in contact with the luminal side of the biopsy sample; EB = endometrium with beads in contact with the luminal side of the biopsy sample; EO = endometrium with beads in contact with the luminal side of the biopsy sample* = $P \leq 0.05$. Graphs are mean ± SEM.

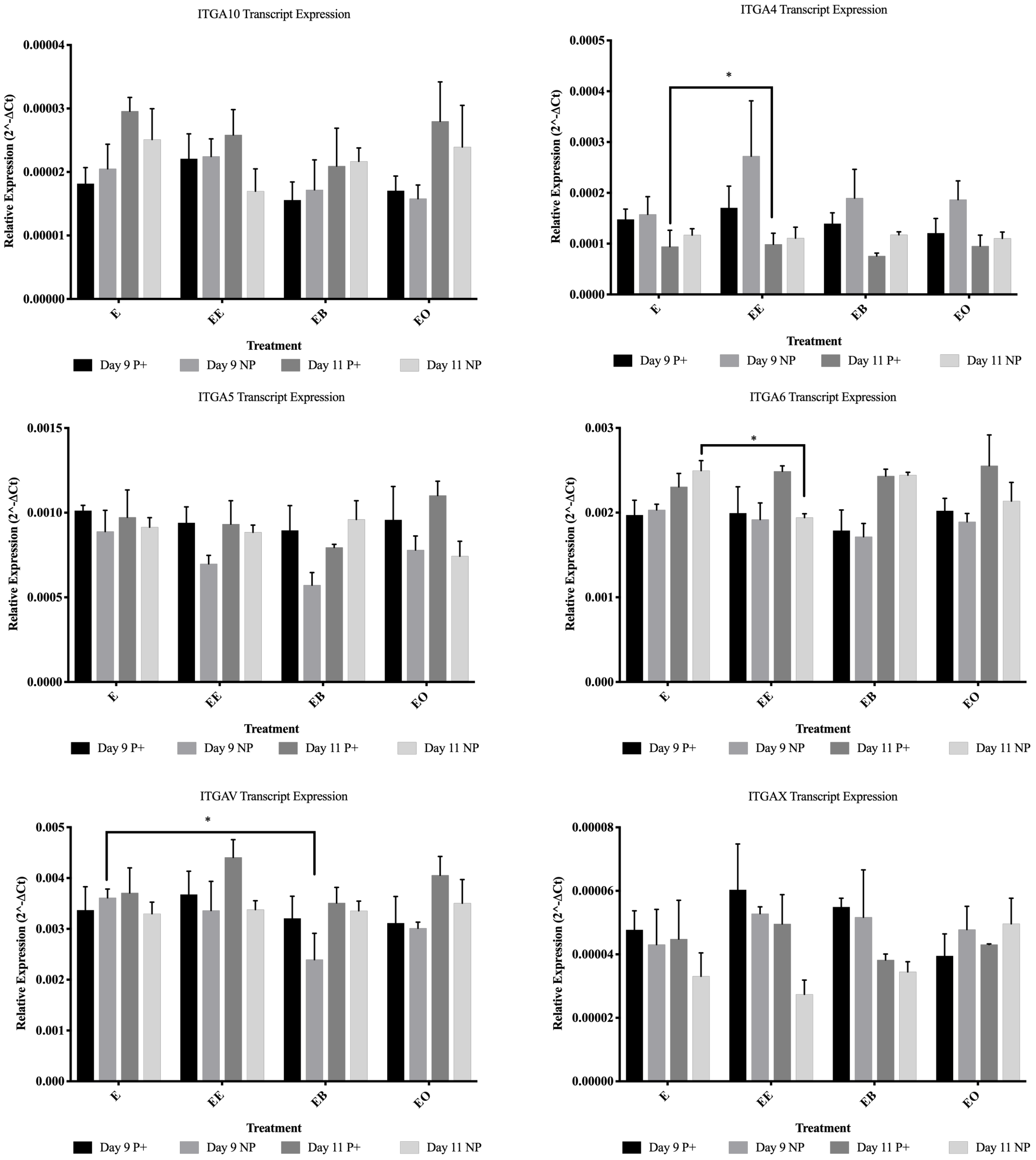
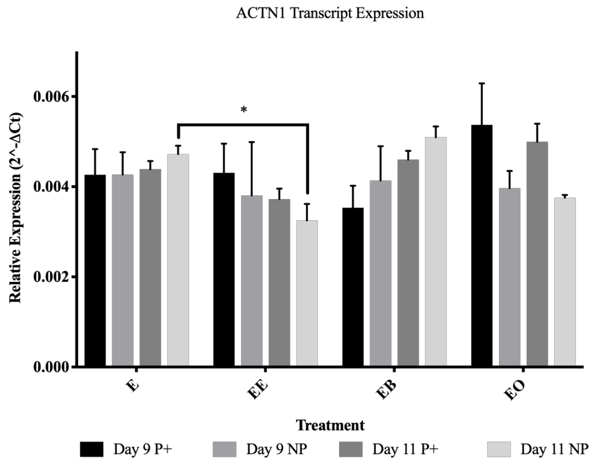
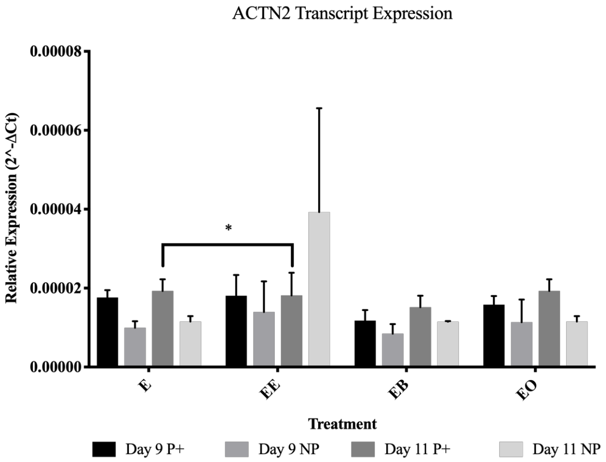


Figure 2.4 Gene abundance for α-integrins for all treatments in endometrial samples from pregnant (P+) and non-pregnant (NP) mares.

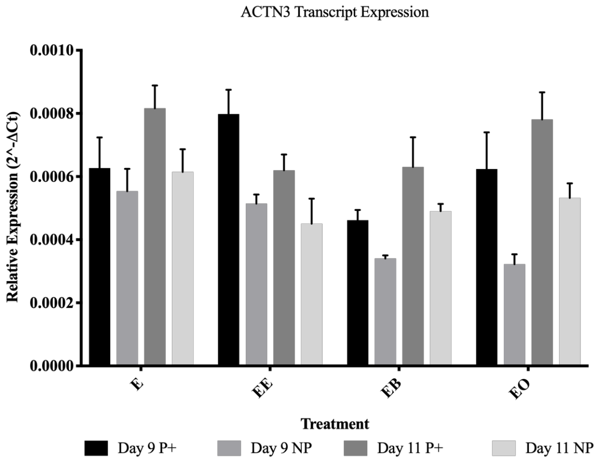
This figure contains all of the protein and gene expression data that was analyzed in this project. E = no treatment (control); EE = endometrium with an embryo in contact with the luminal side of the biopsy sample; EB = endometrium with beads in contact with the luminal side of the biopsy sample; EO = endometrium with beads in contact with the luminal side of the biopsy sample* = $P \leq 0.05$. Graphs are mean ± SEM.



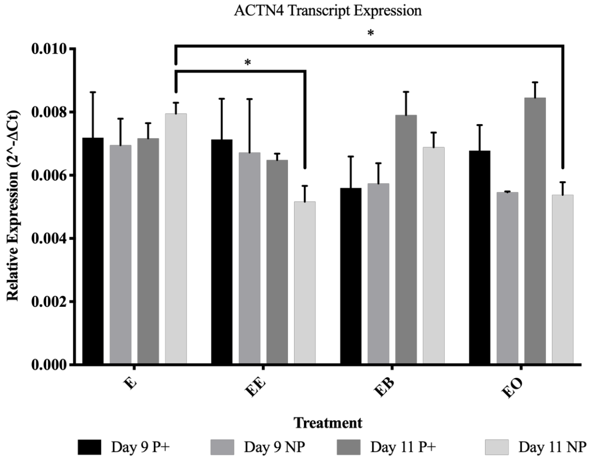
ACTN1 Transcript Expression



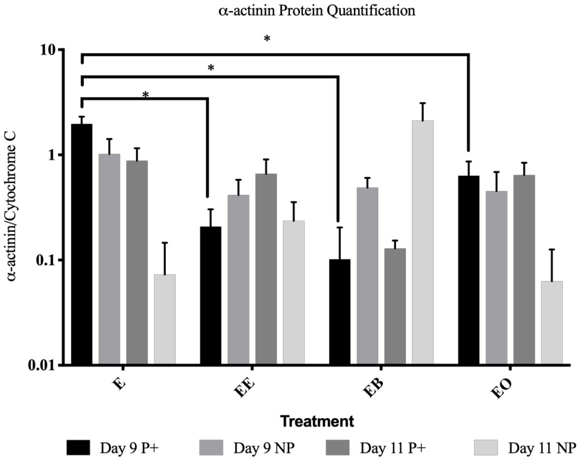
ACTN2 Transcript Expression



ACTN3 Transcript Expression



ACTN4 Transcript Expression



α-actinin Protein Quantification

Figure 2.5 Actin genes and protein abundance for all treatments in endometrial samples from pregnant (P+) and non-pregnant (NP) mares.

This figure contains all of the gene expression and protein quantification for ACTN1-4 and α-actinin protein data. E = no treatment (control); EE = endometrium with an embryo in contact with the luminal side of the biopsy sample; EB = endometrium with beads in contact with the luminal side of the biopsy sample; EO = endometrium with beads in contact with the luminal side of the biopsy sample* = $P \leq 0.05$. Graphs are mean ± SEM.

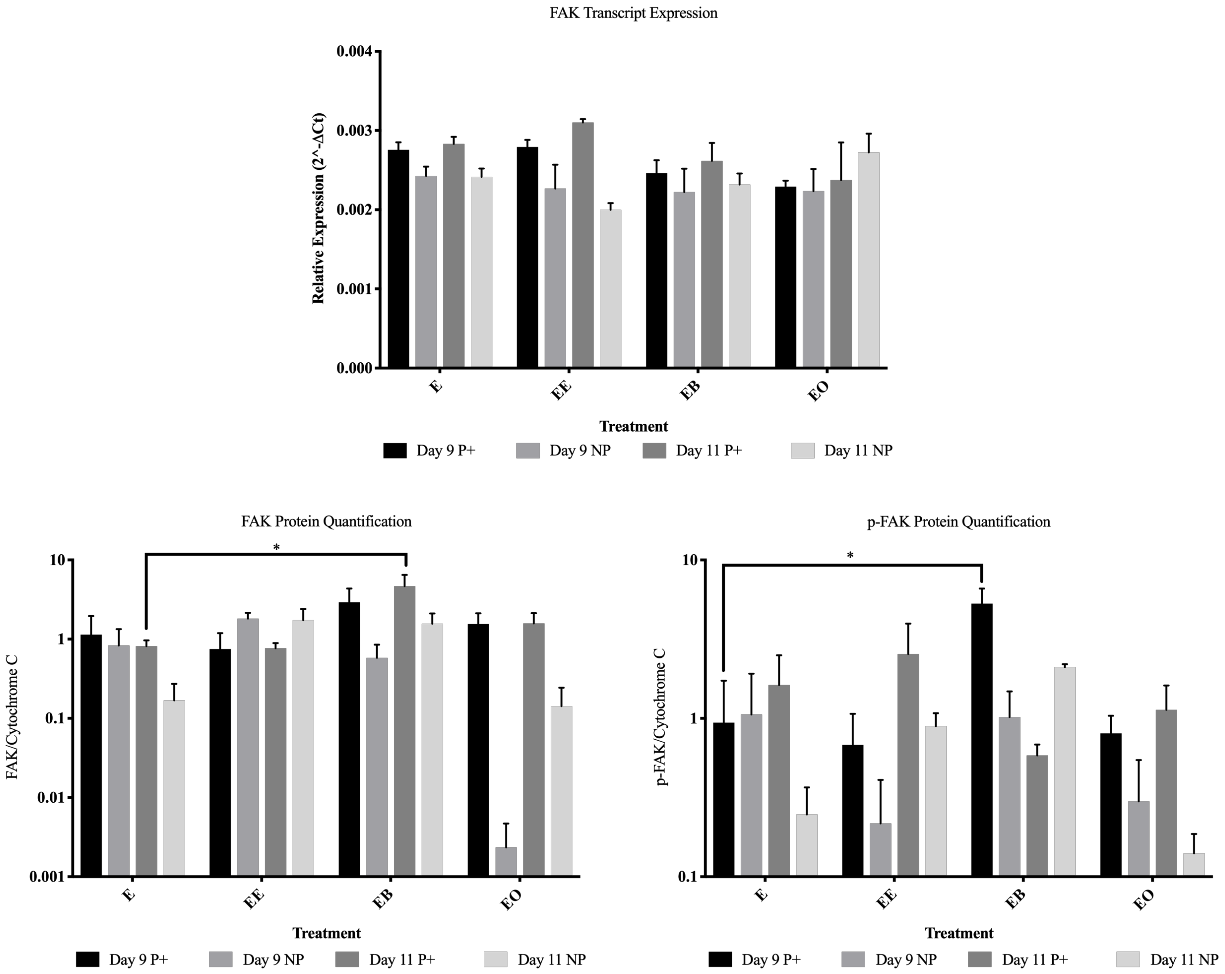


Figure 2.6 FAK and p-FAK expression and protein abundance for all treatments in endometrial samples from pregnant (P+) and non-pregnant (NP) mares.

This figure contains the gene and protein levels for FAK and p-FAK in the endometrium on days 9 and 11. E = no treatment (control); EE = endometrium with an embryo in contact with the luminal side of the biopsy sample; EB = endometrium with beads in contact with the luminal side of the biopsy sample; EO = endometrium with beads in contact with the luminal side of the biopsy sample. * = $P \leq 0.05$. Graphs are mean ± SEM.

On day 11, *CAV1* was more abundant ($P = 0.001$) in samples from pregnant mares with mechanical pressure on the endometrium. FAK more abundant ($P = 0.004$) in samples with mechanical force from pregnant mares and CAV1 was more abundant ($P = 0.030$) in control samples from pregnant mares (Figures 2.3 and 2.6). α-ACTININ was more abundant ($P < 0.001$) in samples with mechanical force from non-pregnant mares (Figure 2.5).

Endometrium Co-Cultured with Peanut Oil

On day 9, no transcript or protein levels in endometrial samples from non-pregnant mares were affected by co-culture with oil. *PAK6* was more abundant (*P* = 0.012) in samples from pregnant mares co-cultured with peanut oil. Only protein levels in samples from pregnant mares were impacted by the presence of oil. α-ACTININ (*P* = 0.026), CAV1 (*P* = 0.024) and CCND1 (*P* = 0.005) were all in higher abundance in control samples compared to the samples co-cultured with peanut oil (Figures 2.3 and 2.5).

On day 11, transcript differences were only identified in samples from non-pregnant mares. *ACTN4* (*P* = 0.011) and *CAV1* (*P* = 0.001) were more abundant in control tissue samples compared to samples co-cultured with peanut oil (Figures 2.3 and 2.5). There were no changes in protein abundance in endometrial samples collected from non-pregnant mares. BCL-2 was the only protein that was higher in abundance (*P* = 0.007) in the presence of oil in samples collected from pregnant mares (Figure 2.3).

Endometrium Co-Cultured with an Embryo

On day 9 both transcript and protein changes were identified based upon the presence of an embryo. Only one gene, *RAF1*, was more abundant (*P* = 0.047) in endometrial samples co-cultured with an embryo from samples from pregnant mares (Figure 2.7). *BCL-2* (*P* = 0.004), *ITGA4* (*P* = 0.028) and *SLCO2A1* (*P* = 0.001) were all higher in abundance levels in samples from non-pregnant mares after culture with an embryo for 24 hours (Figures 2.3, 2.4 and 2.7). In samples from pregnant mares, α-ACTININ (*P* = 0.002), CAV1 (*P* = 0.006) and CCND1 (*P* = 0.002) were more abundant in control samples (Figures 2.3 and 2.5). ITGB3 was more abundant (*P* = 0.004) in the presence of an embryo in samples from non-pregnant mares (Figure 2.8).

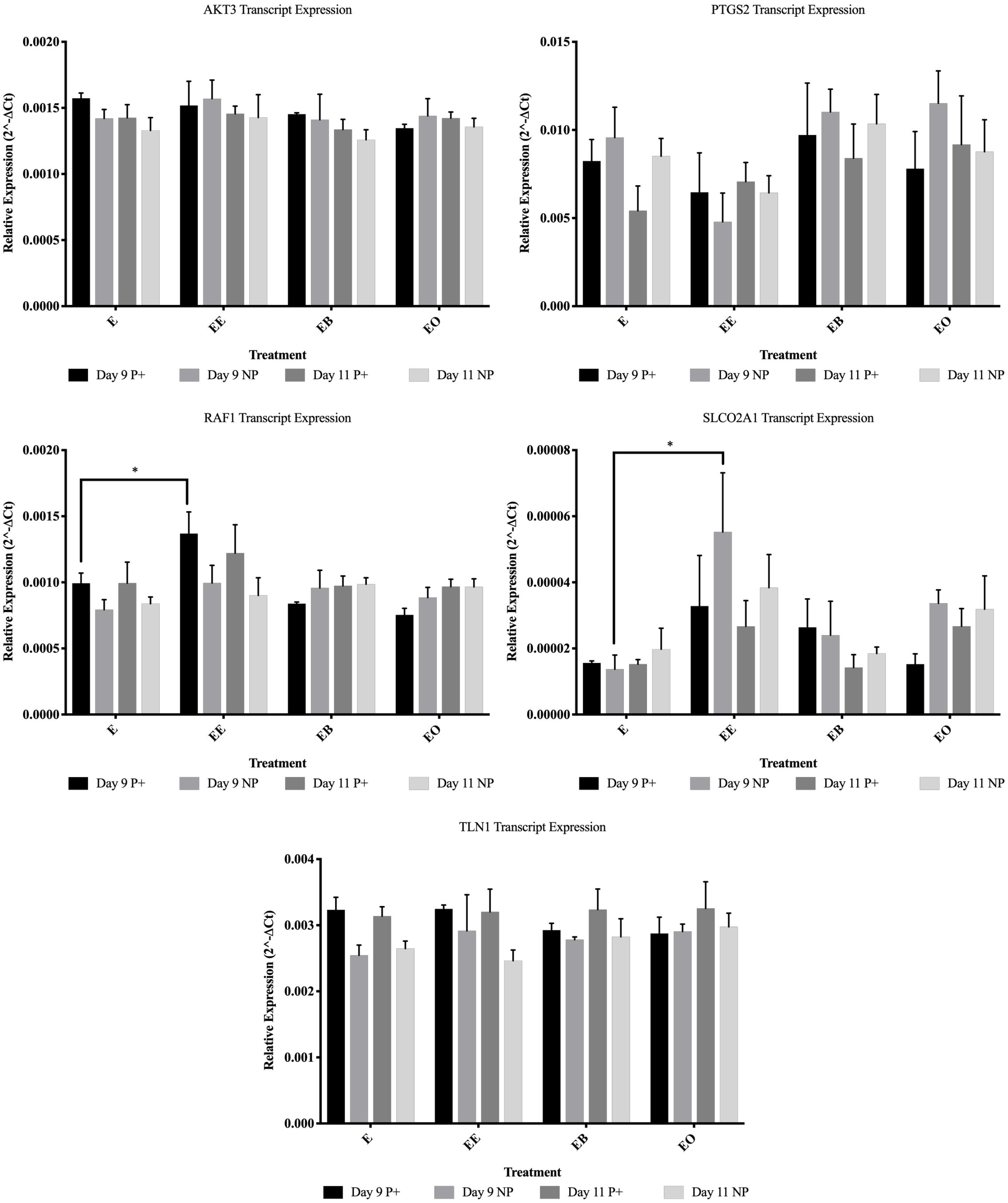


Figure 2.7 Transcript abundance levels for AKT3, PTGS2, RAF1, SLCO2A1 and TLN1 for all treatments in endometrial samples from pregnant (P+) and non-pregnant (NP) mares. This figure contains the gene expression data for AKT3, PTGS2, RAF1, SLCO2A1 and TLN1. E = no treatment (control); EE = endometrium with an embryo in contact with the luminal side of the biopsy sample; EB = endometrium with beads in contact with the luminal side of the biopsy sample; EO = endometrium with beads in contact with the luminal side of the biopsy sample* = $P \leq 0.05$. Graphs are mean ± SEM.

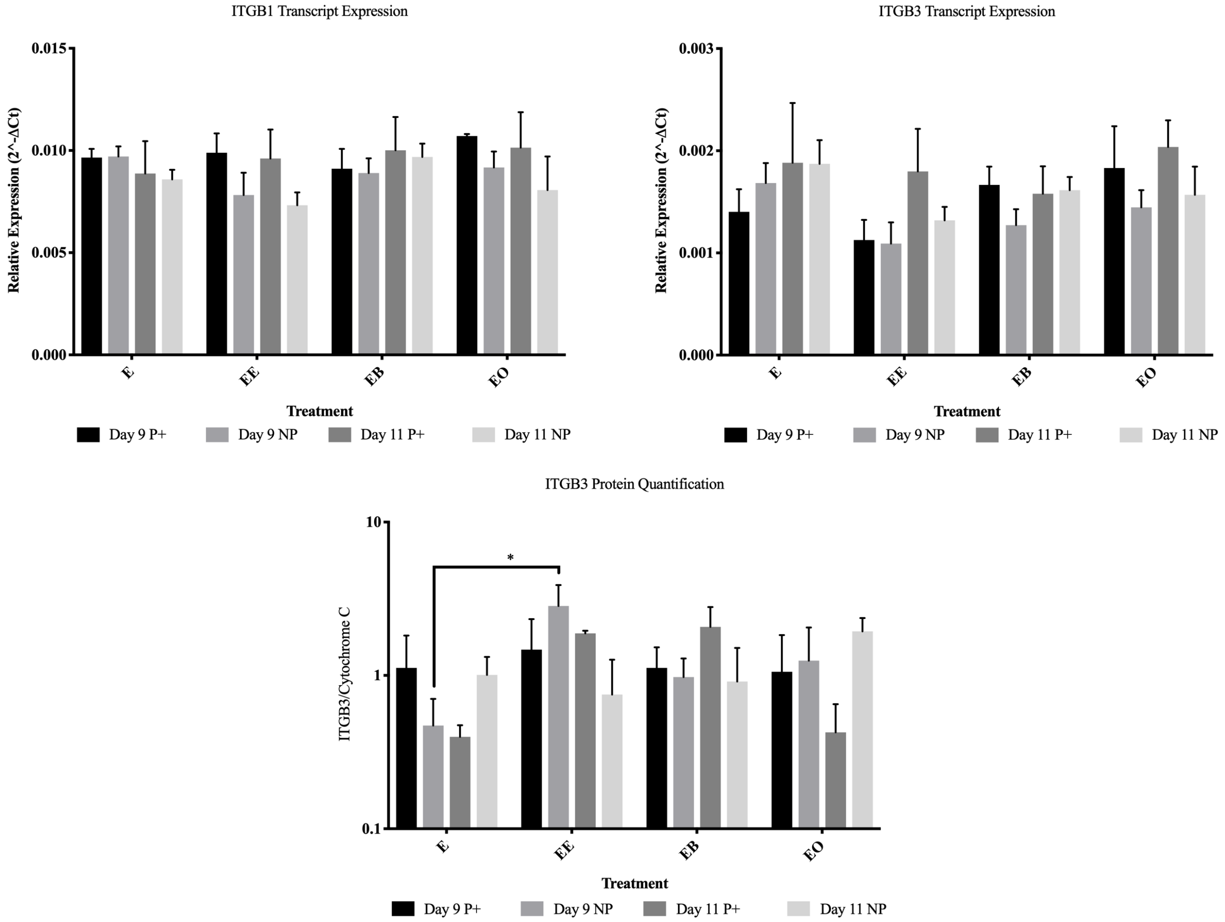


Figure 2.8 Gene and protein abundance for β-integrins for all treatments in endometrial samples from pregnant (P+) and non-pregnant (NP) mares.

This figure contains all of the protein and gene expression data that was analyzed in this project. E = no treatment (control); EE = endometrium with an embryo in contact with the luminal side of the biopsy sample; EB = endometrium with beads in contact with the luminal side of the biopsy sample; EO = endometrium with beads in contact with the luminal side of the biopsy sample* = $P \leq 0.05$. Graphs are mean ± SEM.

On day 11, no transcripts were changed due to the presence of an embryo in samples from pregnant mares. In samples from non-pregnant mares, *ACTN2* ($P = 0.050$) was the only gene that was higher in abundance in the presence of an embryo (Figure 2.5). *ACTN1* ($P = 0.007$) , *ACTN4* ($P = 0.005$)*, CAV1* ($P < 0.001$) and *ITGA6* ($P = 0.035$) were all more abundant in control samples from non-pregnant mares (Figures 2.3-5). Only one protein, BCL-2, was more abundant ($P = 0.009$) in samples from pregnant mares in the presence of an embryo (Figure 2.3). PAK6 was the only protein more abundant (*P* = 0.047) in samples from non-pregnant mares in the presence of an embryo (Figure 2.3).

PGF Secretion after Co-Culture

PGF concentration in the medium was evaluated to determine if the presence of beads, oil or an embryo would alter PGF secretion. On day 9 there was no change in PGF secretion due to any of the treatments. On day 11, the presence of oil increased PGF secretion in samples from pregnant mares compared to control samples (*P* = 0.043). Most importantly, in samples from non-pregnant mares, the presence of an embryo decreased PGF secretion (*P* = 0.003) compared to the sample that was not in the presence of an embryo (591.73 pg/mL versus 3282.96 pg/mL respectively). Interestingly, when comparing samples from pregnant and non-pregnant mares after 24 hours of culture without the presence of an embryo, PGF secretion was lower in samples obtained from pregnant mares (*P* = 0.036). Figure 2.9 shows the concentration of PGF in each treatment group.

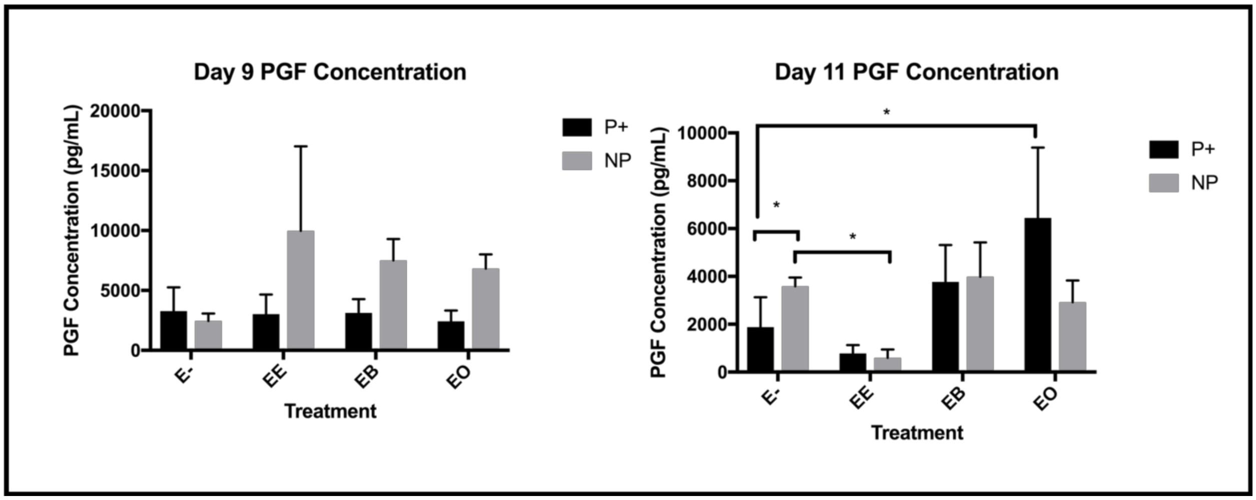


Figure 2.9. PGF concentration in the medium after 24 hours of co-culture for all treatments for endometrial samples from pregnant (P+) and non-pregnant (NP) mares.

This is a graphical representation of the concentration of PGF in the medium after 24 hours of culture with the specific treatment. E- = no treatment (control); EE = endometrium with an embryo in contact with the luminal side of the biopsy sample; EB = endometrium with beads in contact with the luminal side of the biopsy sample; EO = endometrium with beads in contact with the luminal side of the biopsy sample. * = $P \leq 0.05$. PGF concentration is in pg/mL, mean ± SEM.

Discussion

Previous studies reported that focal adhesions play an important role at the maternal-fetal interface in many species, but none have identified their potential role in equine endometrium (21). While it is known that focal adhesions are present in equine endometrium, their impact on maternal recognition of pregnancy and PGF secretion have not been studied to date (20). In the present study, we evaluated the presence of specific focal adhesion molecules in equine endometrium that were immediately snap frozen after collection, but also after the sample had been challenged for 24 hours with one of four treatments (an embryo, beads/mechanical force, peanut oil, or with no contact). The genes tested in this study were derived from previous studies in our laboratory and studies on focal adhesions in other species (20, 21). Others have hypothesized that placing a glass bead in the uterus of a mare will extend luteal function (25). This suggested that MRP is triggered based upon the presence of a round object moving throughout the uterine body. Even though this finding is highly disputed, the beads used in the present study mimicked the contact of a glass marble on endometrium (16). The peanut oil was used because previous studies have indicated that infusing a mare's uterus with oil will prolong diestrous (15).

Mechanical force on the endometrium (beads) induced changes in focal adhesion molecule abundance. This could be due to the fact that focal adhesion molecules are mechanical sensors, so just the contact of something on these molecules will induce a change but may not illicit a response (21). Studies have shown that focal adhesions and their downstream effects are dependent upon the composition and rigidity of the ECM (26, 27). Therefore, while the beads are making contact and causing a change in the focal adhesions, they may not be eliciting a true response. This lack of response was further validated when the PGF concentration was measured in the medium. On both days 9 and 11, the PGF concentration in the medium was similar between samples with and without bead co-culture. The beads actually resulted in a larger amount of PGF ($P = 0.004$) being secreted into the medium than the embryo (3987.4 pg/mL and 591.7 pg/mL respectively) on day 11 in samples from non-pregnant and pregnant mares ($P = 0.019$; 3771.9 pg./mL and 775.8 pg/mL respectively). This data shows *in vitro* that the presence of beads making physical contact with the endometrium is not enough to signal maternal recognition of pregnancy, ultimately leading to a decrease in PGF secretion.

Contrary to previous reports, this study showed that the application of peanut oil on endometrial samples does not cause a decrease in PGF secretion (15). This was true for both days 9 and 11. Interestingly, on day 9, no FAMs were changed based upon the presence of peanut oil on endometrial samples from non-pregnant mares and *PAK6* was the only transcript more abundant ($P = 0.012$) in samples from pregnant mares. α-ACTININ ($P = 0.026$), CAV1 ($P = 0.024$) and

CCND1 (*P* = 0.005) were all decreased due to the presence of peanut oil on day 9 in samples from pregnant mares. On day 11, only *ACTN4* (*P* = 0.011) and *CAV1* (*P* = 0.001) were decreased due to the presence of peanut oil and BCL-2 was more abundant in samples from pregnant mares. Peanut oil actually caused the endometrium from pregnant mares on day 11 to secrete more PGF compared to the control (6444.4 pg/mL and 3586.0 pg/mL respectively).

The presence of an embryo altered many FAMs and PGF secretion. It was expected that samples from non-pregnant mares would be altered in the presence of an embryo, but samples from pregnant mares would not. This is because once focal adhesion molecules are activated, they can stay active due to internal contractility in cells (28). It was hypothesized that once the endometrial samples from pregnant mares were exposed to an embryo in utero, even once the embryo was removed for culture, the focal adhesions would remain active for the 24 hour culture. On day 9, samples from both pregnant and non-pregnant mares were altered by the presence of an embryo. Only *RAF1* was more abundant (*P* = 0.047) in samples from pregnant mares due to the presence of an embryo. α-ACTININ (*P* = 0.002), CAV1 (*P* = 0.006) and CCND1 (*P* = 0.002) were less abundant in samples from pregnant mares. In samples from non-pregnant mares on day 9, *BCL-2* (*P* = 0.004)*, ITGA4* (*P* = 0.028), *SLCO2A1* (*P* = 0.001) and ITGB3 (*P* = 0.004) were more abundant due to the presence of an embryo. Even with these alterations, on day 9 there was no change in PGF secretion in the presence of an embryo. It is thought that although the embryo is mobile, it does not reach maximum mobility until days 11-14, therefore the signaling has not occurred for maternal recognition of pregnancy and there is no change in PGF secretion (4).

On day 11, only BCL-2 was more abundant (*P* = 0.009) due to the presence of an embryo in samples from pregnant mares. All other alterations occurred in samples from non-pregnant mares. *ACTN1* (*P* = 0.007) *, ACTN4* (*P* = 0.005)*, CAV1* (*P* < 0.001) and *ITGA6* (*P* = 0.035) were less abundant in the presence of an embryo and *ACTN2* (*P* = 0.050) and PAK6 (*P* = 0.047) were more abundant in the presence of an embryo in samples from non-pregnant mares. PGF secretion was dramatically decreased (*P* = 0.003) in samples from non-pregnant mares in the presence of an embryo (591.7 pg/mL versus 3586.0 pg/mL) on day 11. Another interesting observation was when comparing the PGF secretion in control samples between pregnant and non-pregnant mares, although the sample from a pregnant mare was not in the presence an embryo for 24 hours, the amount of PGF secreted remained lower than the samples from non-pregnant mares (1877.8 pg/mL and 3586.0 pg/mL respectively). These data further validate the idea that once the machinery is activated, it remains active for a period of time without external pressure (28).

An interesting observation from this dataset was that α-ACTININ, CAV1, and CCND1 were altered due to all treatments. These three proteins may play a crucial role in sensing force in the extracellular matrix and relaying that to the cell. Caveolin-1 (CAV1) is a mechano-mediator that has been identified in rat endometrium and increases during pregnancy (29). It is located apically in uterine epithelial cells and human Ishikawa cells (30). α-ACTININ is a Ca^{+2} sensitive actin filament cross-linking protein (31). It is a cytoskeletal protein, and in contrast to actin, is located specifically on the apical plasma membrane of rat uterine epithelial cells (32). It was hypothesized to be involved in actin filament reorganization during early pregnancy, especially the period of receptivity (32). Cyclin D1 (CCND1) plays an important role in the progression of the cell cycle (33). CCND1 acts as an oncogene in many different human neoplasia's when overexpressed (34). More specifically, CCND1 overexpression has been reported in many endometrial carcinomas (35). Overall, these three proteins can be playing a distinct role in sensing and reacting to external stimuli from the extracellular matrix but are not transducing the signal into the cell to signal maternal recognition of pregnancy leading to a decrease in PGF.

In conclusion, we determined that the contact of beads, peanut oil, and an embryo cause changes in focal adhesion molecules in endometrium from pregnant and non-pregnant mares. In contrast, the contact with an embryo on day 11 on endometrial samples from non-pregnant mares was the only treatment capable of decreasing PGF secretion. These results suggest the contact of an embryo alone, and only for 24 hours, is enough to alter focal adhesions and decrease PGF secretion. Based upon this data we hypothesize that a mobile embryo in the uterus activates focal adhesions, which lead to a decrease in PGF secretion (Figure 2.10). Future studies will need to evaluate what portion of the embryo is responsible for this change in PGF secretion and the mechanism by which that message is being relayed to the endometrial cell. In order to study the genes that are altered during maternal recognition of pregnancy, we performed RNA sequencing on endometrium during this time frame (Chapter III).

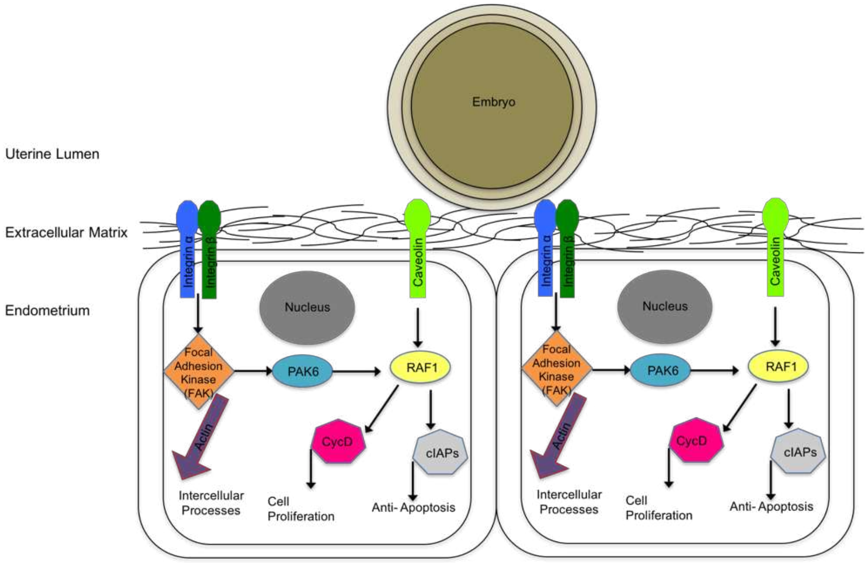


Figure 2.10 A schematic of a mobile embryo and focal adhesions within the endometrium. As the embryo is being bounced around the uterine lumen from days 9-16 it is causing the assembly and activation of focal adhesions. Once activated, these focal adhesions intracellularly can be impacting multiple processes, including the prevention of PGF release.

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CHAPTER III: RNA SEQUENCING OF EQUINE ENDOMETRIUM DURING MATERNAL RECOGNITION OF PREGNANCY[3]

Summary

Equine maternal recognition of pregnancy (MRP) is a process whose signal remains unknown. During MRP, the conceptus and endometrium communicate to attenuate prostaglandin $F_{2\alpha}$ (PGF) secretion, thus sparing the corpus luteum and maintaining progesterone production and secretion. Recognition of a mobile conceptus by the endometrium is critical prior to days 14-16 post-ovulation (PO), when in the non-pregnant mare, endometrium produces and secretes PGF, which initiates luteal regression. Previous gene expression analyses have failed to robustly reveal possible candidates involved in MRP. Therefore, we evaluated equine endometrial genes via RNA-seq during MRP in order to detect changes in the entire transcriptome. The objective of this study was to evaluate endometrial gene expression changes based upon pregnancy status. This experiment utilized a cross-over design with each mare serving as a pregnant and non-mated control on days 9, 11, and 13 PO (n=3/status/day). Mares were randomly assigned to a collection day and each provided endometrial samples for a pregnant and non-mated cycle. Pregnancy was

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confirmed by terminal uterine lavage at the time of endometrial biopsy. Samples were snap frozen and stored until RNA isolation. Total RNA was isolated and libraries prepared using the Illumina TruSeq RNA Sample Preparation kit and sent to the University of California-Berkeley for RNA-seq. Reads were mapped and annotated using CLC Genome Workbench. Annotation details were based on Ensembl and NCBI models combined with publicly available RNA-seq data. Expression values for genes and transcripts were summarized as reads per kilobase per million reads (RPKM). All transcripts considered for analysis were present in all three samples from a group with an RPKM ≥ 0.25. Differential gene expression was analyzed with SAS comparing pregnancy status within following calculation and application of the Benjamini-Hochberg correction for multiple testing ($FDR \leq 0.05$ was considered significant). On day 9, 11 and 13 there were 360, 407 and 367 genes and 401, 450 and 393 corresponding transcript variants, respectively, that were differentially abundant between pregnancy statuses. Five genes were in common across all three days. These five genes are: PJVK, XLOC_001772, XLOC_034312, XLOC_041999 and XLOC_047417. Interestingly, there were multiple genes with splice variants that had different abundance patterns within the same day. These findings imply that transcript variants differ between endometrium from pregnant and non-pregnant mares as well as over the time of MRP.

Introduction

Maternal recognition of pregnancy (MRP) in the mare is a complex process that involves communication between the conceptus and maternal endometrium. Interestingly, in the horse, the conceptus does not attach to endometrium until around day 35 post-ovulation (PO), which means communication is occurring without attachment to prevent the endometrium from secreting prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which causes luteal regression of the corpus luteum, ultimately eliminating the source of progesterone (1, 2).

In both pregnant and non-pregnant mares, hormonal profiles stay the same until day 14 PO (3). In non-pregnant mares, oxytocin is released from both the posterior pituitary gland and endometrium. Oxytocin binds to endometrial receptors, which causes release of more oxytocin and stimulates $PGF_{2\alpha}$ production and secretion (4). Fertilization occurs in the oviduct, but the embryo does not enter the uterus until day 6. At this time, the embryo is surrounded by the zona pellucida, which remains intact until day 9, followed by an acellular, glycoprotein capsule, which surrounds the embryo until day 16, the day of fixation (5). The glycoprotein rich capsule is necessary for embryo survival and if it is removed prior to day 16 there is no establishment of pregnancy (6). Upon entering the uterus, the embryo is mobile, caused by uterine contractions, reaching peak mobility between days 11-14 (5, 7). This mobility is necessary to delay secretion of $PGF_{2\alpha}$ (7, 8). By day 16, embryo mobility ceases and the embryo fixes in a single location but does not attach or invade (7). Maternal recognition occurs between days 11-14, when the embryo reaches max mobility, and is also still surrounded by the glycoprotein rich capsule, acting as an anti-adhesive (7, 9, 10). Because MRP results in the delay of production and secretion of endometrial $PGF_{2\alpha}$, the signal acts in an anti-luteolytic manner.

The signals for MRP in other species, such as interferon tau and estradiol, have been tested in the horse, but do not impact on luteal function (11, 12). Unique to the horse, prostaglandin E_2 is secreted by the conceptus on days 4 to 6 in order for it to enter the uterus, but when infused into the uterus of non-pregnant mares there is no evidence of luteostasis (1, 12-14). Coconut or peanut oil have been infused into the uterus on day 10 of non-pregnant mares and luteostasis occurred (15). This suggests that a component in the oil impacts the luteolytic pathway. Previous research on MRP has evaluated transcriptional differences in the endometrium during and after MRP utilizing a microarray (16). This research suggested that there was a transcriptional difference occurring by day 14, but previous research has failed to reveal possible candidates involved in MRP (16).

Maternal recognition of pregnancy is a multifaceted process that is still not well understood. All that is known is that the embryo must come into contact with at least 80 of endometrium to illicit the anti-luteolytic signal (17). The objective of this study was to evaluate total transcriptome endometrial gene expression changes based upon pregnancy status before and during MRP with the goal to identify candidate genes involved in MRP.

Materials and Methods

Care and Management of Mares

All horse use was approved by the Colorado State University Institutional Animal Care and Use Committee (Approval Number: 11-2795A). Mares (n=9) were housed in group pens at Colorado State University Equine Reproduction Laboratory (Fort Collins, CO). Mares were maintained on a dry lot and fed grass-alfalfa mix with free choice mineral and salt supplement. Mares were used in a cross-over design in which each mare had a pregnant and non-pregnant (non-mated) cycle. Mares were monitored via transrectal palpation and ultrasonography to track follicular development every other day. When a follicle reached 35 mm in diameter, or greater, the mare was inseminated with at least 500 x 10^6 progressively motile sperm from stallions with proven fertility. Mares were monitored via transrectal ultrasonography every day and inseminated every other day until ovulation (day 0).

Mares were randomly assigned to collection day 9, 11, or 13 post-ovulation (PO) for both their pregnant and non-pregnant cycles. On assigned day, each mare was evaluated via transrectal ultrasonography to confirm pregnancy status by visualization of an embryonic vesicle and terminal uterine lavage was completed. Endometrial samples were obtained non-surgically via a trans-cervical biopsy punch prior to uterine lavage (18). After biopsy and embryo collection each mare received a luteolytic dose of $PGF_{2\alpha}$ (Estrumate, Merck Animal Health, 250 mcg per dose). For the non-pregnant (non-mated) control cycle, the subsequent estrous cycle was utilized.

For the non-pregnant (non-mated) control, the subsequent estrous cycle was utilized. After endometrial samples were obtained, each sample was rinsed in DPBS/Modified 1X (Hyclone Laboratories, Logan, UT), snap frozen in liquid nitrogen and stored at -80°C until further analysis.

RNA Isolation and Quantification

Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) for lysis and extraction and RNeasy Mini Kit (Qiagen, Valencia, CA) for isolation. About 30 mg of frozen tissue was homogenized in TRI Reagent and left at room temperature for 10 minutes. Chloroform was added to the homogenate, vortexed and left at room temperature for 8 minutes. Samples was centrifuged at 13,200 revolutions per minute (RPM; 16,100 x g) for 15 minutes, which separated the sample into three distinct phases (RNA, DNA and protein). The aqueous RNA phase was transferred to a new 1.7 mL tube for isolation. RNA was isolated using Qiagen RNeasy Mini Kit according to the manufacturer’s recommendations. All samples were treated with an RNase-Free DNase kit (Qiagen, Valencia, CA) to remove DNA contamination. RNA purity and quantity were assessed using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE). Samples were used for analysis if they had 260/280 and 260/230 values above 1.7 for RNA-seq library preparation.

RNA-seq

RNA-seq (cDNA) library preparation was done using an Illumina TruSeq Sample Preparation Kit v2 (Illumina, San Diego, CA) and 1 µg of Total RNA from each sample. The manufacturer's protocol was used for library preparation. Briefly, adapters were ligated to the 3' and 5' ends of the RNA samples. Samples were then reverse transcribed to form cDNA with adapters. Libraries were sent University of California-Davis for quality control assessment and then sent to University of California-Berkeley for sequencing on an Illumina HiSeq 2000 (Illumina, San Diego, CA). Reads were 50 basepair single end reads utilizing three lanes, with six samples per lane.

Bioinformatic and Statistical Analyses

After sequencing, reads were mapped and annotated using CLC Genomics Workbench 9 (Qiagen, Valencia, CA). Equine genome EquCab2.0 was used as the reference genome. Criteria used to filter unique sequence reads was: minimum length fraction of 0.7; minimum similarity fraction of 0.8; maximum numbers of two mismatches. Data were normalized by calculating the reads per kilobase per million mapped reads (RPKM = total exon reads/mapped reads in millions x exon length in kb) (19). Annotation details were based on Ensembl and NCBI models combined with publicly available RNA-Seq data. Annotation was based upon genes and transcripts. A histogram was analyzed to evaluate mapping distribution in order to determine a threshold of detection. To be considered for analysis, the average RPKM for all three samples within a group was ≥ 0.25. Statistical analyses were conducted using SAS 9.4 (SAS Institute Inc.). Proc Mixed was used to fit a mixed model separately by gene. Fixed effects included day (9, 11, 13) and pregnancy status (pregnant or non-pregnant) plus day by pregnancy status interaction. Horse ID was included as a random effect to account for the repeated measures design. Benjamini-Hochberg

FDR adjustment was used. Significance was assessed at *FDR* ≤ 0.05. Panther was used to analyze the biological pathways associated with our dataset (20). For this analysis a gene indicates a specific region in the genome which can support multiple transcripts (transcript variants). Novel genes and transcript variants are defined as sequences that are known to be genes but have not been further evaluated to determine function and given a name.

Results

Overall, 14,197 genes and 29,314 transcript variants were expressed in endometrium across days 9, 11, 13. When evaluating within days, on day 9 there were 360 differentially abundant genes and 401 differentially abundant transcript variants (*FDR* ≤ 0.05). Interestingly, 128 transcript variants were only expressed in samples from pregnant mares and 227 transcripts variants were only in samples from non-pregnant mares (Table 3.1, Figure 3.1). Of the transcript variants present in both samples from pregnant and non-pregnant mares, 12 were more highly abundant (*FDR* ≤ 0.05) in endometrium from pregnant mares and 24 were more highly abundant (*FDR* ≤ 0.05) in endometrium from non-pregnant mares. The most highly abundant gene in day 9 endometrial samples from pregnant mares was Unc-93 homolog B1, TLR signaling receptor (UNC93B1; RPKM = 10.6) and from endometrial samples from non-pregnant mares it was coiled-coil domain containing 15 (CCDC15; RPKM = 6.2). Interestingly, 157 novel genes were identified on day 9. All significant genes for Day 9 are in Appendix II.

Biological pathway analysis was performed for transcripts that were more highly expressed in samples from pregnant mares and for transcripts that were highly expressed in samples from non-pregnant mares. The genes that were unique to each group were also included in this analysis. There were a few biological pathways in common between groups such as EGF signaling and FGF signaling. Genes with higher expression in samples from pregnant mares were enriched uniquely for angiogenesis and hedgehog signaling pathway (Table 3.2).

Table 3.1 Genes and transcript variants with FDR ≤ 0.05 within days 9, 11 and 13 in endometrial samples.

	Genes	Transcript Variants	Novel Transcript Variants
Day 9	360	401	128
Day 11	407	450	187
Day 13	367	393	143

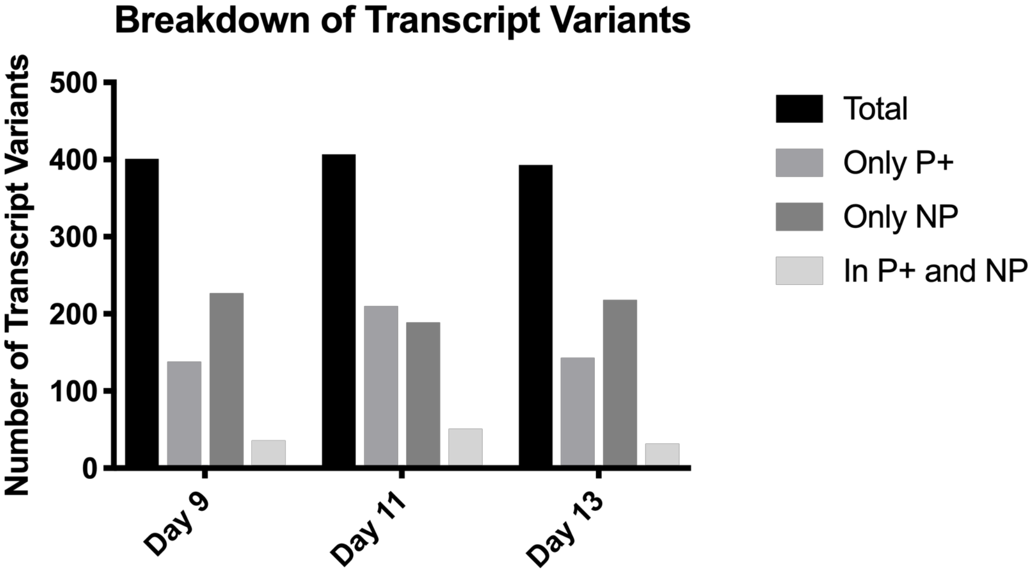

Fig 3.1 Breakdown of significant transcripts within days 9, 11 and 13 in endometrial samples from pregnant (P+) and non-pregnant (NP) mares.

This is a breakdown of the expression patterns of significant transcript variants within each day.

Table 3.2 Biological pathways associated with genes that were differentially abundant (FDR ≤ 0.05) on day 9 in endometrium from pregnant (P+) and non-pregnant (NP) mares.

Higher Abundance in P+	Higher Abundance in NP
Alzheimer disease-presenilin pathway	Alzheimer disease-amyloid secretase pathway
Angiogenesis	Alzheimer disease-presenilin pathway
Blood coagulation	Axon guidance mediated by netrin
CCKR signaling map	B cell activation
Dopamine receptor mediated signaling pathway	Blood coagulation
EGF receptor signaling pathway	Dopamine receptor mediated signaling pathway
FGF signaling pathway	EGF receptor signaling pathway
Fructose galactose metabolism	FGF signaling pathway
Hedgehog signaling pathway	Gonadotropin-releasing hormone receptor pathway
Inflammation mediated by chemokine and cytokine signaling pathway	Huntington disease
Ionotropic glutamate receptor pathway	Inflammation mediated by chemokine and cytokine signaling pathway
Muscarinic acetylcholine receptor 2 and 4 signaling pathway	Interferon-gamma signaling pathway
Nicotine pharmacodynamics pathway	Ionotropic glutamate receptor pathway
Nicotinic acetylcholine receptor signaling pathway	Metabotropic glutamate receptor group III pathway
Notch signaling pathway	Nicotine pharmacodynamics pathway
O-antigen biosynthesis	Notch signaling pathway
Oxidative stress response	Oxidative stress response
p53 pathway	p38 MAPK pathway
Parkinson disease	p53 pathway feedback loops 2
Plasminogen activating cascade	Parkinson disease
Wnt signaling pathway	PDGF signaling pathway
	Ras Pathway
	T cell activation
	TGF-beta signaling pathway
	Wnt signaling pathway

On day 11,1 there were 407 differentially abundant genes and 450 differentially abundant transcript variants (*FDR* ≤ 0.05). Of the identified transcript variants, 210 were only expressed in samples from pregnant mares and 189 were only expressed in samples from non-pregnant mares (Table 3.1, Figure 3.1). Of the transcript variants that were in both samples from pregnant and non-pregnant mares, 25 more highly expressed (*FDR* ≤ 0.05) in endometrium from pregnant mares and 26 were more highly expressed (*FDR* ≤ 0.05) in endometrium from non-pregnant mares. On day 11 there were 178 novel genes identified, 28 of which were in common with day 9. The transcript with the highest abundance in sample from pregnant and non-pregnant mares was Indian Hedgehog (IHH; RPKM = 326.0 and 537.8 respectively), which was more abundant in samples from non-pregnant mares. All significant genes for Day 11 are in Appendix III.

When evaluating the enriched biological pathways for transcripts identified in samples from non-pregnant mares, the pathways included FGF signaling and TGF-beta signaling. The transcripts in samples from pregnant mares were enriched for integrin signaling and gonadotropin-releasing hormone receptor pathway (Table 3.3).

Table 3.3 Biological pathways associated with genes that were differentially abundant (FDR ≤ 0.05) on day 11 in endometrium from pregnant (P+) and non-pregnant (NP) mares.

Higher Abundance in P+	Higher Abundance in NP
Alpha adrenergic receptor signaling pathway	FGF signaling pathway
Alzheimer disease-presenilin pathway	Gamma-aminobutyric acid synthesis
Angiotensin II-stimulated signaling through G proteins and beta-arrestin	General transcription regulation
B cell activation	Inflammation mediated by chemokine and cytokine signaling pathway
CCKR signaling map	Ionotropic glutamate receptor pathway
EGF receptor signaling pathway	Nicotine pharmacodynamics pathway
Endothelin signaling pathway	Nicotinic acetylcholine receptor signaling pathway
Gonadotropin-releasing hormone receptor pathway	PDGF signaling pathway
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	TGF-beta signaling pathway
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	Transcription regulation by bZIP transcription factor
Histamine H1 receptor mediated signaling pathway	Wnt signaling pathway
Huntington disease	
Inflammation mediated by chemokine and cytokine signaling pathway	
Integrin signaling pathway	
Metabotropic glutamate receptor group I pathway	
Muscarinic acetylcholine receptor 1 and 3 signaling pathway	
Nicotinic acetylcholine receptor signaling pathway	
PDGF signaling pathway	
T cell activation	
Wnt signaling pathway	

On day 13, there were a total of 367 differentially abundant genes and 393 differentially abundant transcript variants (*FDR* ≤ 0.05; Table 3.1, Figure 3.1). Within these genes, 132 were novel and 8 were in common with all three days. Overall on day 13 there were 143 transcript variants that were expressed only in samples from pregnant mares and 218 transcript variants that were expressed only in samples from non-pregnant mares. When analyzing the transcript variants present in both samples closer, 14 were more highly expressed (*FDR* ≤ 0.05) in endometrium from pregnant mares and 18 were more highly expressed (*FDR* ≤ 0.05) in endometrium from non-pregnant mares. The transcript with the highest abundance in endometrial samples from both pregnant and non-pregnant mares was carboxypeptidase vitellogenic like (CPVL; RPKM = 52.8 and 33.0 respectively), but it was more abundant in samples from pregnant mares. All significant genes for Day 13 are in Appendix IV.

Biological pathways also revealed that genes that were more highly abundant in samples from pregnant mares were enriched for hedgehog signaling and cholesterol biosynthesis. Genes that were more highly expressed in samples from non-pregnant mares were enriched for apoptotic signaling (Table 3.4). When evaluated across days, there were 5 genes that were present on days 9, 11 and 13 (Tables 3.5 and 3.6). Gene XLOC_001772, transcript variant 3 was present only in samples from non-pregnant mares across all three days.

Table 3.4 Biological pathways associated with genes that were differentially abundant (FDR ≤ 0.05) on day 13 in endometrium from pregnant (P+) and non-pregnant (NP) mares.

Higher Abundance in P+	Higher Abundance in NP
2-arachidonoylglycerol biosynthesis	Angiogenesis
5-Hydroxytryptamine biosynthesis	Apoptosis signaling pathway
Adrenaline and noradrenaline biosynthesis	B cell activation
Cholesterol biosynthesis	CCKR signaling map
Circadian clock system	Dopamine receptor mediated signaling pathway
Dopamine receptor mediated signaling pathway	EGF receptor signaling pathway
EGF receptor signaling pathway	FAS signaling pathway
FGF signaling pathway	Gonadotropin-releasing hormone receptor pathway
Gonadotropin-releasing hormone receptor pathway	Hedgehog signaling pathway
Hedgehog signaling pathway	Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	Histamine H1 receptor mediated signaling pathway
Nicotine pharmacodynamics pathway	Huntington disease
Notch signaling pathway	Inflammation mediated by chemokine and cytokine signaling pathway
Transcription regulation by bZIP transcription factor	Integrin signaling pathway
Wnt signaling pathway	Interleukin signaling pathway
	Nicotine pharmacodynamics pathway
	Nicotinic acetylcholine receptor signaling pathway
	p38 MAPK pathway
	p53 pathway
	p53 pathway feedback loops 2
	PDGF signaling pathway
	PI3 kinase pathway
	Pyruvate metabolism
	Ras Pathway
	T cell activation
	TGF-beta signaling pathway
	Toll receptor signaling pathway
	VEGF signaling pathway
	Vitamin D metabolism and pathway

Table 3.5 Number of genes and transcript variants (FDR ≤ 0.05) in common between days.

	Number of Genes	Number of Transcript Variants
Days 9 and 11	**52**	**55**
Days 9 and 13	**32**	**33**
Days 11 and 13	**49**	**53**
Days 9, 11 and 13	**5**	**5**

Table 3.6 Transcripts present in days 9, 11 and 13.

Gene Symbol	Transcript Variant Number	Expression Status Day 9	Expression Status Day 11	Expression Status Day 13
PJVK	1	Only NP	Only P+	Only NP
XLOC_001772	3	Only NP	Only NP	Only NP
XLOC_034312	7	Only NP	Only P+	Only NP
XLOC_041999	3	Only NP	Only P+	Only P+
XLOC_047417	4	Only P+	Only NP	Only P+

Discussion

This is the first collection of data to evaluate the equine endometrial transcriptome across this time frame of maternal recognition of pregnancy (MRP). This study utilized days 9, 11 and 13 in order to evaluate the transcriptome before, during and after MRP. What is also unique about these data is that we evaluated genes and transcript variants (splice variants) within those genes. In addition to identifying known genes, the results revealed a population of novel genes/transcript variants.

An interesting phenomenon that was noted during this analysis was the change in expression status between different variants of a gene. Multiple genes had many transcripts that were significant within a day, but sometimes the transcripts would vary in whether there they were more highly abundant in samples from pregnant mares or non-pregnant mares. An example is MPHOSPH8, M-phase phosphoprotein 8, which is a factor that is part of an epigenetic silencing complex (21). On day 9 transcript variants 1, 2, 3, 5 and 6 were all differentially abundant (*FDR*

≤ 0.05) in samples from pregnant and non-pregnant mares. Transcript variants 1, 2, 3 and 5 were identified only in samples from non-pregnant mares, but transcript 8 was identified in both samples, and was in higher abundance in samples from pregnant mares. This occurred with many genes throughout the dataset indicating that alternative splicing is occurring in the endometrium during this time frame. Alternative splicing is the combination of different splice sites joining together to form the gene (22). Literature has shown that unbalanced splice variants can result in tumors within tissues (23). Introns are removed by a protein known as a spliceosome (24). Spliceosomes are an assembly of five small nuclear ribonucleoproteins (snRNP) These snRNP are referred to as U1, U2, U4, U5 and U6 small nuclear RNA (snRNA) (24). More work needs to be done to understand the significance of this alternative splicing and determine if snRNA are present in the endometrium, resulting in alternative splicing.

Another observation from this study was the large number of genes/transcripts that were present in only samples from pregnant or non-pregnant mares (Figure 3.1). During this time frame, pregnant and non-pregnant mares have the same hormonal profile (3). Therefore, this gene expression in one sample versus the other has to occur due to the presence of an embryo, indicating an active role in maternal recognition of pregnancy. Further research and validation of these genes are required to understand the significance of the genes being exclusively in one sample group.

Although present in both samples on day 11, IHH, Indian hedgehog, was the most abundant transcript in both groups. Progesterone has been found to induce IHH expression in mouse and human endometrium, resulting in cell proliferation and differentiation (25, 26). This would explain why IHH is in such high abundance, since the mare is under high progesterone influence at this time. Indian hedgehog is also thought to have a role in implantation, explaining why it is in higher abundance in samples from pregnant mares (25, 27). Biological pathway analysis also suggested that hedgehog signaling was a pathway enriched by the genes in this dataset. This was to be expected since IHH is progesterone stimulated, and both pregnant and non-pregnant mares were under high progesterone influence.

Integrin signaling is a biological pathway of interest in MRP. Previous research revealed that focal adhesions are present in equine endometrium during the time of MRP and serum samples from pregnant and non-pregnant mares contain exosomes, which are cell secreted vesicles, that transport microRNA (miRNA) targeting focal adhesions (28, 29). MicroRNA are small non-coding RNA that bind mRNA causing it to destabilize (30, 31). Focal adhesions are integrin receptors on the plasma membrane that sense and transduce mechanical forces from the extracellular matrix to a biochemical signal within the cell (32, 33). In the current study, integrin signaling was enriched in genes that were more highly expressed in samples from pregnant mares on day 11. This enrichment on day 11 corresponds to the data from chapter II in this dissertation, that integrin signaling is increased due to the presence of an embryo. This corresponds to when the embryo is at maximum mobility throughout the uterus (7). Integrin signaling was also enriched by the genes that are more highly abundant in samples from non-pregnant mares on day 13, but the gene responsible for this enrichment is RAPGEF, rap guanine nucleotide exchange factor 1. This gene is more closely related to actin dynamics within the cell and adheres to focal adhesions versus signaling from focal adhesions (34).

Although not observed to be significant between groups, another interesting observation was the identification of CATSPERD, CATSPERG and CATSPERB, all subunits of CATSPER that are required to form a functional ion channel, within in the endometrium (35). Previously CATSPER, a sperm calcium transporter, was thought to be unique to the sperm (36). To our knowledge, this is the first study to identify CATSPER outside of the sperm, and more specifically within the endometrium.

In conclusion, this is the first study to evaluate the equine endometrial transcriptome before, during and after maternal recognition of pregnancy utilizing RNA-seq. A large number of genes/transcripts were identified that are unique to pregnancy status and day, including many novel transcripts with unknown functions. Interestingly, alternative splicing was identified in this dataset and the significance of that needs to be determined in order to understand if it is having a functional role in MRP. Further research is needed to determine the role of the genes identified in this dataset in order to elucidate the signaling that is occurring during maternal recognition of pregnancy. To attempt to understand how these genes were being regulated, we evaluated the presence of small non-coding RNA in the endometrium (Chapter IV).

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CHAPTER IV: SMALL RNA SEQUENCING OF EQUINE ENDOMETRIUM AND ENDOMETRIAL SECRETED EXOSOMES DURING MATERNAL RECOGNITION OF PREGNANCY IN THE MARE[4]

Summary

Maternal recognition of pregnancy (MRP) in the mare is a process that is not well defined. In a non-pregnant mare, prostaglandin $F_{2\alpha}$ (PGF) is released by the endometrium on day 14 post ovulation (PO) to cause regression of the corpus luteum (CL), resulting in a loss of progesterone production and the mare will begin the next follicular cycle. In a pregnant mare, MRP occurs prior to day 14 to halt PGF production and secretion. Previous gene studies have failed to identify a clear gene candidate for MRP. Studies in our laboratory revealed that serum exosomes, which are cell secreted vesicles, from pregnant and non-pregnant mares vary in miRNA content, so attention has turned to small noncoding RNA. The objective of this study was to evaluate the small RNA content in endometrium and endometrial exosomes during MRP via RNA-seq. Mares were used in a cross-over design with each mare having a pregnant and non-pregnant (non-mated) control cycle. Days 11 and 13 were chosen for analysis (n = 3/day). Each mare was randomly assigned a day, and on that day, endometrial biopsies were obtained via trans-cervical biopsy punch, and the

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embryo was collected via terminal uterine lavage to confirm pregnancy. Endometrial biopsies were divided into two equal parts. Half was immediately snap frozen and the other half was cultured for 24 hours to collect secreted exosomes. Exosomes were isolated from the culture medium after 24 hours. Total RNA was isolated from endometrium and endometrial exosome samples. Sequencing libraries were prepared for small RNA-seq at University of California-Davis and sequenced at University of California-Berkeley on a HiSeq 2000. Reads were mapped and annotated using CLC Genome Workbench. EquCab2 was used as the reference genome and annotation details were provided by ncRNA EquCAB2.72.ncrna. SAS was used for statistical analysis. Overall there were 998 small noncoding RNA (ncRNA) identified in equine endometrium across days 11 and 13. On days 11 and 13, there were 85 and 5, respectively, ncRNA that differed (*FDR* ≤ 0.05) between pregnancy statuses. These ncRNA represented miRNA, snRNA, snoRNA, Mt rRNA, RRNA and misc RNA. In endometrial secreted exosomes there were 11 ncRNA on day 11 and 21 ncRNA on day 13 that differed (*FDR* ≤ 0.05) between samples from pregnant and non-pregnant mares. These ncRNA represented miRNA, snoRNA, misc RNA and snRNA. There were many novel miRNAs identified in the endometrium and endometrial secreted exosomes. Interestingly, a large percentage of the ncRNA identified were snRNA. This study was the first to describe the ncRNA transcriptome in equine endometrium and endometrial secreted exosomes. Identifying the targets of these ncRNAs could lead to determining MRP in the mare.

Introduction

Early pregnancy in the horse is a process that is still not well defined. Specifically, the signal for maternal recognition of pregnancy (MRP) remains a mystery. During the estrous cycle, if a mare is not pregnant the endometrium produces prostaglandin $F_{2\alpha}$ (PGF) on day 14 post ovulation (PO) in order to cause regression of the corpus luteum (CL) (1, 2). The corpus luteum is produces and secretes progesterone in order to sustain pregnancy. If a mare is pregnant, MRP occurs and the endometrium will not produce PGF, therefore causing the CL, and progesterone production, to be sustained. In a pregnant mare, the conceptus enters the uterus on day 6 by releasing prostaglandin E_2 (PGE) around day 4 (3). The embryo enters the uterus and is surround by a glycoprotein rich capsule, a feature that is unique to equine and rabbit embryos (4, 5). Once in the uterus, the embryo is mobile throughout the entire uterus due to uterine contractions (6). This mobility is necessary in order to signal MRP (7, 8). The embryo reaches maximum mobility between days 11-14 (6). The embryo then ceases mobility by day 16, which indicates that MRP occurs between days 11-14 (9).

Maternal recognition of pregnancy is crucial for the success a pregnancy, but the signal in the horse differs from other species. In pigs and ruminants, the signal for MRP are estrogen and interferon tau, respectively, but neither have any impact on luteal function in the mare (10-13). Since the equine conceptus secretes PGE on day 6, studies have infused the uterus with PGE, which resulted in no luteostasis (11). More recent studies infused coconut and peanut oil into the uterus of a diestrus mare and were able to prolong diestrus, but the mechanism by which this occurred remains unknown (14).

Studies have progressed to evaluating gene expression within the endometrium, but no obvious gene candidates have been delineated. Recent studies in our laboratory suggested that the content, specifically miRNAs, of serum exosomes differ between pregnant and non-pregnant mares during MRP (15). Exosomes are cell-secreted vesicles that are capable of transferring bioactive materials, such as RNA and protein from cell to cell (16-18). Exosomes can contain small noncoding RNAs (ncRNAs) (19). Small ncRNAs, which are less than 200 nucleotides long, do not code for protein and are typically involved in the regulation of RNA processing and function

(20). MicroRNAs are a family of ncRNAs that are approximately 25 nucleotides in length (21). MicroRNAs are involved in crucial biological processes including development, apoptosis and proliferation (22, 23). They can regulate messenger RNA (mRNA) through imperfect pairing resulting in transcriptional or post-transcriptional regulation of the mRNA expression (24-26). The objective of this study is to identify all small ncRNA present in equine endometrial tissue and endometrial secreted exosomes during maternal recognition of pregnancy via RNA-seq.

Materials and Methods

Care and Management of Mares

All horse use was approved by the Colorado State University Institutional Animal Care and Use Committee (Approval Number: 11-2795A). Mares (n = 6) were housed in group pens at the Bud and Jo Adams Equine Reproduction Laboratory (Fort Collins, CO), maintained on a dry lot and fed a grass-alfalfa mix with free choice mineral and salt supplement. Mares were used in a cross-over design where each mare had a pregnant and non-pregnant (non-mated) cycle at days 11 and 13 post-ovulation. Mares were randomly assigned to a collection day. Mares were monitored via transrectal palpation and ultrasonography to track their follicular development every other day. Once a follicle reached 35 mm in diameter, or larger, the mare was inseminated with at least 500 x 10^6 progressively motile sperm from stallions with proven fertility. Mares were then monitored via transrectal ultrasonography every day and inseminated every other day until ovulation (day 0).

On the mare’s assigned day, she was evaluated via transrectal ultrasonography to confirm pregnancy status by visualization of an embryonic vesicle. Endometrial samples were obtained via a trans-cervical biopsy punch, followed by a terminal uterine lavage to again confirm pregnancy status (27). After endometrial sample collection, the mare received a luteolytic dose of PGF (Estrumate, Merck Animal Health, 250 mcg per dose) and the subsequent estrous cycle was used for the non-pregnant (non-mated) cycle. After endometrial sample collection, the sample was rinsed in DPBS/Modified 1X (Hyclone Laboratories, Logan, UT) and split into two equal parts. Half was immediately snap frozen in liquid nitrogen and stored at -80°C until further analysis. The other half was cultured for 24 hours in an incubator with a humidified atmosphere at 5% CO_2, 95% air at 37°C in incubation medium [(Medium 199 (Life Technologies, Carlsbad, CA) containing 5% antibiotic-antimycotic (Life Technologies, Carlsbad, CA)]. After 24 hours, the tissue sample was frozen at -80°C and exosomes were isolated from the medium.

Exosome Isolation

Exosomes were isolated from medium samples using ExoQuick (System Biosciences, Inc., Palo Alto, CA). Media samples were spun at 300 x g for 10 min and 2,000 x g for 10 minutes to remove residual cells and debris. A final centrifugation of 10,000 x g for 30 minutes was performed to remove microparticle contaminants according to Théry et al. (2006) prior to exosome isolation (28). After removal of contaminants, 400 µl was transferred to a 1.5 ml sterile microcentrifuge tube after which 100 µl ExoQuick was added. Gentle inversion was performed to ensure proper mixing of serum and ExoQuick, which was then incubated at 4°C overnight per manufacturer's instructions. Exosomes were pelleted following the incubation step with centrifugation at 1500 x g for 30 minutes at room temperature. Supernatant was carefully removed and discarded. The resultant exosome pellet was re-suspended in phosphate buffered saline (PBS, HyClone, Logan, UT) and processed for RNA isolation.

RNA Isolation and Quantification

Total RNA from the endometrium was extracted using mirVana miRNA Isolation kit (Invitrogen, Carlsbad, CA) at the University of California-Davis. Briefly, samples were homogenized in lysis/binding buffer. miRNA homogenate additive was added to the lysate and incubated for 10 minutes on ice. Acid-Phenol: Chloroform was added to the lysate, vortexed and centrifuged at 10,000 x g for five minutes. The sample was then separated into three distinct phases (RNA, DNA and protein). The aqueous, RNA phase was transferred to a new 1.7 mL Eppendorf tube for RNA isolation. Ethanol was added to the RNA and the RNA was bound to a spin filter provided by Invitrogen. The filters were washed and miRNA was eluted with elution solution. RNA purity and quantity were assessed using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE). Samples were used for PCR analysis if they had 260/280 and 260/230 values above 1.7.

Total RNA was isolated from endometrial biopsies using TRI Reagent (Molecular Research Center, Cincinnati, OH) for lysis and extraction at Colorado State University. Samples were homogenized in TRI Reagent and Polyacryl Carrier. After a five minute incubation at room temperature, chloroform was added to the sample and incubated for another five minutes. The samples were then centrifuged at 12,000 x g for 15 minutes to separate the sample into three distinct phases (RNA, DNA and protein). The top, aqueous phase RNA phase was transferred to a new 1.7 mL tube for RNA isolation. Isopropanol was added to the RNA phase to precipitate the RNA. After a 10 minute incubation the samples were centrifuged for 12,000 x g for 8 minutes. The samples were then washed two times with 75% ethanol and air-dried. Nuclease free water was used for resuspension then all samples were treated with RNase-Free DNase (Life Technologies, Carlsbad, CA) to remove any DNA contamination. RNA purity and quantification were assessed using the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE). Samples were used for PCR analysis if they had 260/280 and 260/230 values above 1.7.

RNA Sequencing

Sequencing libraries were generated at University of California-Davis using Illumina TruSeq Small RNA Library Preparation kit (Illumina, San Diego, CA) and 1 μg of Total RNA from each sample. The manufacturer's protocol was followed for preparation. Briefly, adapters were ligated to the 5' and 3' ends of the RNA samples. Polymerase chain reaction (PCR) was performed with the following protocol: 98°C for 30 seconds, 11 cycles of 98°C for 10 seconds, 60°C for 30 seconds and 72°C for 15 seconds followed by 72°C for 15 minutes. Samples were then pooled and run out on a DNA gel and the region of 147-157 nucleotides was excised and isolated from the gel. This represented the small RNA that was to be sequenced. After gel extraction the samples were suspended in 10 mM Tris-HCl, pH 8.5. Libraries were checked for quality with a Bioanalyzer (Agilent, Santa Clara, CA) and sent to University of California-Berkeley for sequencing using a HiSeq 2000 sequence analyzer (Illumina, San Diego, CA).

Bioinformatic and Statistical Analyses

After sequencing, reads were mapped and annotated using CLC Genomics Workbench 9 (Qiagen, Valencia, CA). Adapters were trimmed from the reads and mapped to EquCab2.0. The criteria used to filter the unique sequence reads was: minimum length fraction of 0.7; minimum similarity fraction of 0.8; maximum of two mismatches. Annotation details were based on ncRNA EquCAB2.72.ncrna (NCBI). Expression values were normalized using global expression. A histogram was analyzed to evaluate mapping distribution in order to determine a threshold of detection, which was 0.25. To be considered to analysis, the average of the three replicates in a group was ≥ 0.25. Statistical analysis was conducted using SAS 9.4 (SAS Institute Inc.). Proc Mixed was used to fit a mixed model separately by small RNA. Fixed effects included Day (11 and 13) and pregnancy status (pregnant or non-pregnant) plus day by pregnancy status interaction. Horse ID was included as a random effect to account for the repeated measures design. Benjamini-Hochberg FDR adjustment was used, and significance was assessed at *FDR* ≤ 0.05.

Results

Endometrial Small RNA

Overall there were 998 small noncoding RNAs (ncRNAs) identified in equine endometrium across days 11 and 13. Of these 998, 167 were unique to endometrium on day 11 and 8 were unique to day 13. On day 11, there were 85 ncRNA that were differentially abundant (*FDR* ≤ 0.05) between samples from pregnant and non-pregnant mares. These 85 ncRNA represented multiple families of ncRNA including: miRNA, snoRNA, snRNA, Mt rRNA, RRNA and misc RNA (Figure 4.1). The largest family of ncRNA represented in these data were snRNA, followed by miRNA (35 and 18 respectively). Interestingly, of the 85 ncRNA on day 11 (*FDR* ≤ 0.05), 34 were present only in samples from non-pregnant mares and five were present only in samples from pregnant mares (Table 4.1).

Table 4.1 Noncoding RNA (*FDR* ≤ 0.05) in endometrium on day 11 from pregnant (P+) and non-pregnant (NP) mares.

ncRNA Name	Feature ID	ncRNA Description	Day 11P+ Average	Day 11NP Average
eca-mir-1244	ENSECAG00000025242	MiRNA	0	2.64
eca-mir-514	ENSECAG00000025318	MiRNA	1.99	0
eca-mir-767	ENSECAG00000025350	MiRNA	0.68	0
Novel	ENSECAG00000025594	MiRNA	0	2.13
Novel	ENSECAG00000025608	MiRNA	0	0.44
Novel	ENSECAG00000026216	MiRNA	0.43	0
Novel	ENSECAG00000026329	MiRNA	0	0.63
Novel	ENSECAG00000026474	MiRNA	0	1.09
Novel	ENSECAG00000026665	MiRNA	0.55	0
Novel	ENSECAG00000027021	MiRNA	4.81	6.27
Novel	ENSECAG00000027128	MiRNA	0	1.40
Novel	ENSECAG00000027178	MiRNA	0	0.71
Novel	ENSECAG00000027245	MiRNA	0	0.52
Novel	ENSECAG00000027392	MiRNA	0.00	0.56
Novel	ENSECAG00000027427	MiRNA	0	0.52
Novel	ENSECAG00000027494	MiRNA	6.40	15.53
Novel	ENSECAG00000027648	MiRNA	0.00	0.57
RF00006	ENSECAG00000026611	Misc RNA	2214.03	5358.63
RF00019	ENSECAG00000025402	Misc RNA	5349.88	16451.49
RF00100	ENSECAG00000025524	Misc RNA	72.53	161.91
RF00100	ENSECAG00000025549	Misc RNA	1.06	3.09
RF00100	ENSECAG00000025642	Misc RNA	68.29	158.28
RF00100	ENSECAG00000025898	Misc RNA	26.04	61.99
RF00100	ENSECAG00000025928	Misc RNA	84.93	208.99
RF00100	ENSECAG00000026156	Misc RNA	555.98	1601.90
RF00100	ENSECAG00000026233	Misc RNA	2.90	4.85
RF00100	ENSECAG00000026314	Misc RNA	245.24	1055.84
RF00100	ENSECAG00000026534	Misc RNA	7.11	20.36
RF00100	ENSECAG00000026629	Misc RNA	6.79	27.49
RF00100	ENSECAG00000026703	Misc RNA	6.64	14.51
RF00100	ENSECAG00000026745	Misc RNA	1.56	3.02
RF00100	ENSECAG00000027435	Misc RNA	0.88	1.83
Novel	ENSECAG00000027676	Mt rRNA	2282.79	7176.71
RF00001	ENSECAG00000026227	RRNA	0	1.17

RF00001	ENSECAG00000026627	rRNA	0	0.60
RF00002	ENSECAG00000025308	RRNA	0.62	0
RF00067	ENSECAG00000026456	SnoRNA	753.41	1608.37
RF00087	ENSECAG00000025944	SnoRNA	743.08	2618.05
RF00099	ENSECAG00000026073	SnoRNA	258.86	664.83
RF00108	ENSECAG00000025263	SnoRNA	43.66	107.80
RF00108	ENSECAG00000026473	SnoRNA	44.24	108.42
RF00108	ENSECAG00000027106	SnoRNA	5.02	12.75
RF00138	ENSECAG00000025690	SnoRNA	92.56	199.40
RF00410	ENSECAG00000025306	SnoRNA	3.63	4.36
RF00553	ENSECAG00000027354	SnoRNA	25.25	123.18
RF00554	ENSECAG00000027063	SnoRNA	0.68	1.37
RF00567	ENSECAG00000025199	SnoRNA	350.25	616.65
RF00586	ENSECAG00000026528	SnoRNA	0	0.52
RF01191	ENSECAG00000027545	SnoRNA	0	6.13
RF01278	ENSECAG00000027507	SnoRNA	0	0.99
RF00003	ENSECAG00000025300	SnRNA	0	0.71
RF00004	ENSECAG00000026009	SnRNA	746.15	1409.77
RF00004	ENSECAG00000026168	SnRNA	628.78	1324.91
RF00015	ENSECAG00000026152	SnRNA	4.06	9.43
RF00015	ENSECAG00000026796	SnRNA	4.43	7.64
RF00015	ENSECAG00000027180	SnRNA	0	8.98
RF00026	ENSECAG00000025238	SnRNA	3.87	8.22
RF00026	ENSECAG00000025278	SnRNA	43.98	91.98
RF00026	ENSECAG00000025405	SnRNA	9.95	21.16
RF00026	ENSECAG00000025406	SnRNA	0	1.23
RF00026	ENSECAG00000025508	SnRNA	49.82	101.44
RF00026	ENSECAG00000025522	SnRNA	0	1.41
RF00026	ENSECAG00000025575	SnRNA	11.48	22.62
RF00026	ENSECAG00000025613	SnRNA	46.13	106.63
RF00026	ENSECAG00000025625	SnRNA	0	3.53
RF00026	ENSECAG00000025638	SnRNA	0	4.03
RF00026	ENSECAG00000025640	SnRNA	0	0.74
RF00026	ENSECAG00000025665	SnRNA	51.41	102.87
RF00026	ENSECAG00000025710	SnRNA	0	1.11
RF00026	ENSECAG00000025811	SnRNA	0	0.85
RF00026	ENSECAG00000025816	SnRNA	41.28	86.82
RF00026	ENSECAG00000025877	SnRNA	0	0.44
RF00026	ENSECAG00000025984	SnRNA	0	3.88

RF00026	ENSECAG00000026065	SnRNA	0	2.97
RF00026	ENSECAG00000026084	SnRNA	0	1.12
RF00026	ENSECAG00000026089	SnRNA	39.05	93.36
RF00026	ENSECAG00000026158	SnRNA	0.00	1.23
RF00026	ENSECAG00000026382	SnRNA	23.11	57.89
RF00026	ENSECAG00000026446	SnRNA	13.19	27.70
RF00026	ENSECAG00000026452	SnRNA	23.76	44.89
RF00026	ENSECAG00000026453	SnRNA	0	3.94
RF00026	ENSECAG00000026557	SnRNA	0	0.71
RF00026	ENSECAG00000026565	SnRNA	0	1.35
RF00619	ENSECAG00000025321	SnRNA	0	7.03
RF00619	ENSECAG00000025947	SnRNA	55.23	112.51

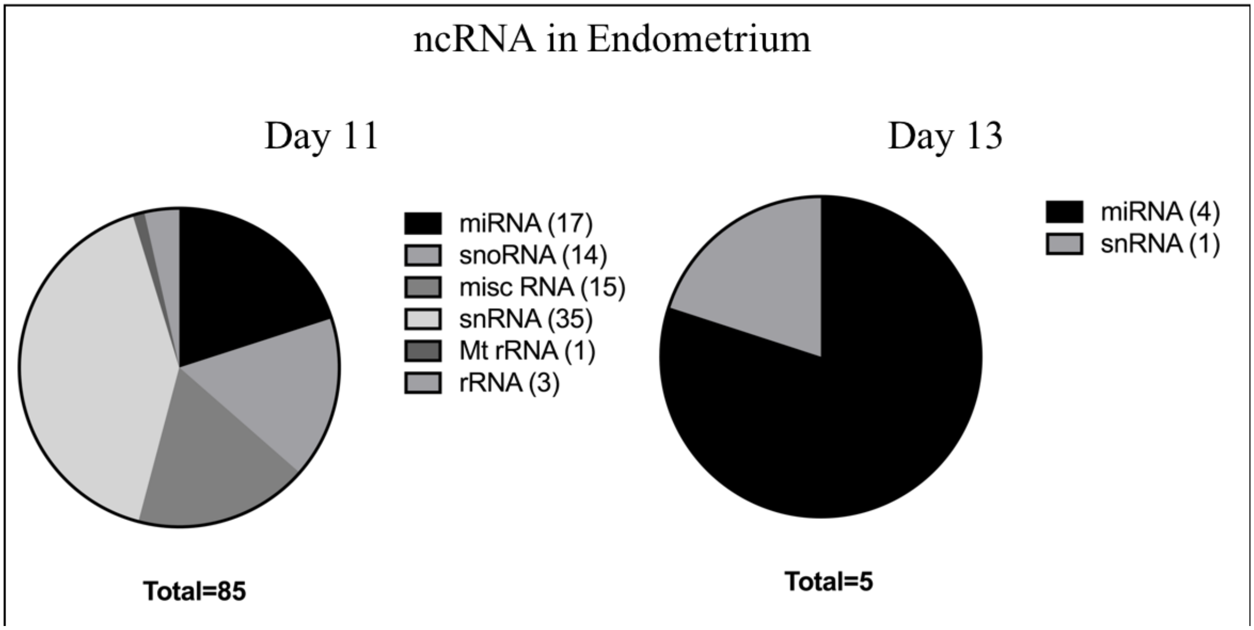

Figure 4.1 Distribution of families of noncoding RNA in endometrium from pregnant and non-pregnant mares

This figure represents the proportions of each family of ncRNA in endometrium on days 11 and 13.

On day 13, there were fewer small ncRNA (*FDR* ≤ 0.05). These data identified five ncRNA that were differentially abundant (*FDR* ≤ 0.05). These five only represented two families, miRNA and snRNA (four and one respectively; Figure 4.1). Small nuclear RNA (snRNA) were observed only in samples from non-pregnant mares. Of the four miRNA, two were observed in samples from non-pregnant mares only and one was only present in samples from pregnant mares Table 4.2. Interestingly, this novel miRNA is the only ncRNA that was significant (*FDR* ≤ 0.05) in both days 11 and 13 and was observed only in samples from non-pregnant mares on both days.

Table 4.2 Noncoding RNA (*FDR* ≤ 0.05) in endometrium on day 11 from pregnant (P+) and non-pregnant (NP) mares.

ncRNA Name	Feature ID	ncRNA Description	Day13P+ Average	Day 13NP Average
eca-mir-1185	ENSECAG00000026218	MiRNA	0.54	0
eca-mir-1197	ENSECAG00000025241	MiRNA	0.36	0.51
eca-mir-20b	ENSECAG00000025857	MiRNA	0	0.64
Novel	ENSECAG00000026329	MiRNA	0	0.64
RF00026	ENSECAG00000026293	SnRNA	0	0.51

Endometrial Secreted Exosomal Small RNA

Overall there were 551 ncRNAs identified in equine endometrial secreted exosomes across days 11 and 13. Of these 551, 49 were unique to day 11 and 39 were unique to day 13. On day 11, eleven ncRNA were differentially (*FDR* ≤ 0.05) present in the endometrial secreted exosomes. These ncRNA represented miRNA, snoRNA and misc RNA families (5, 3 and 3 RNA respectively; Figure 4.2). Of these 11 ncRNA, none were present in samples from both pregnant and non-pregnant mares. Instead, eight were observed only in samples from pregnant mares and three that were present only in the samples from non-pregnant mares. Of the five miRNA, four were present in only samples from pregnant mare (Table 4.3).

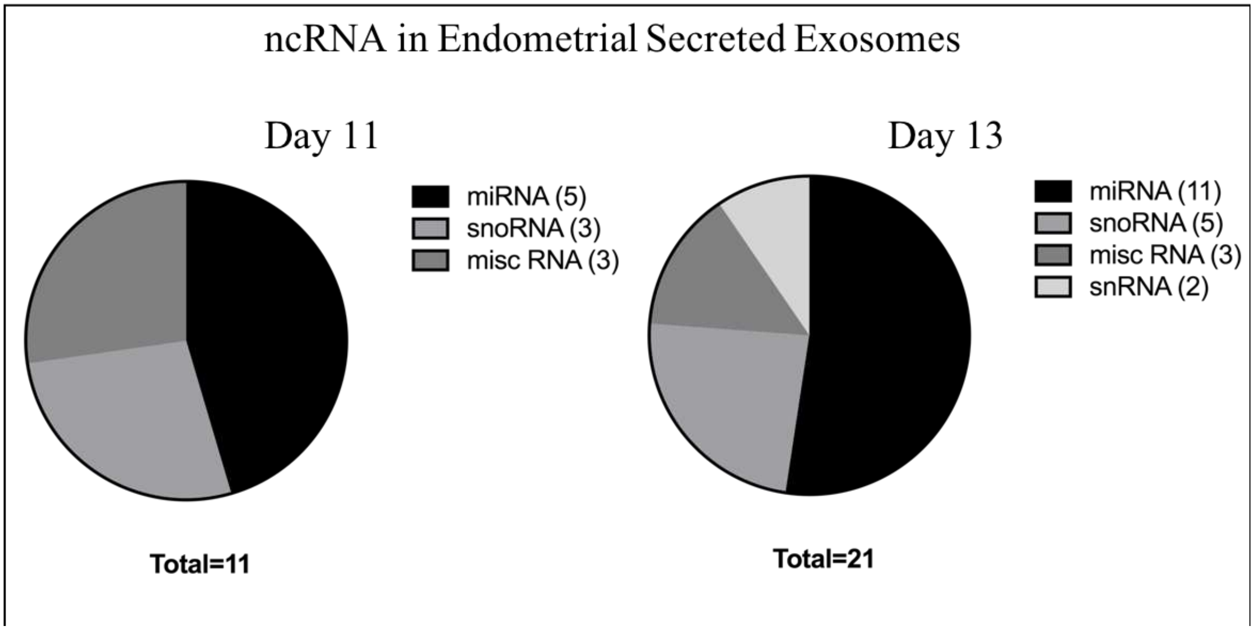

Figure 4.2 Distribution of families of noncoding RNA in endometrial secreted exosomes from pregnant and non-pregnant mares

This figure represents the proportions of each family of ncRNA in endometrial secreted exosomes on days 11 and 13.

Table 4.3 Noncoding RNA (*FDR* ≤ 0.05) in endometrial secreted exosomes on day 11 from pregnant (P+) and non-pregnant (NP) mares.

ncRNA Name	Feature ID	ncRNA Description	Day 11P+ Average	Day 11NP Average
eca-mir-215	ENSECAG00000025888	MiRNA	0.30	0
eca-mir-329a	ENSECAG00000025999	MiRNA	0.30	0
eca-mir-545	ENSECAG00000026036	MiRNA	0	0.67
Novel	ENSECAG00000026350	MiRNA	0.30	0
Novel	ENSECAG00000027218	MiRNA	0.30	0
RF00100	ENSECAG00000026091	Misc RNA	0.39	0
RF00100	ENSECAG00000026622	Misc RNA	0.30	0
RF00100	ENSECAG00000026629	Misc RNA	0.50	0
RF00056	ENSECAG00000026245	SnoRNA	0	0.67
RF00598	ENSECAG00000026301	SnoRNA	0.30	0
RF00600	ENSECAG00000025779	SnoRNA	0	0.92

On day 13, there were 21 ncRNA that were different (*FDR* ≤ 0.05) between the pregnancy statuses. While the majority (11 ncRNA) are part of the miRNA family, snoRNA, snRNA and misc RNA were also represented in this group (5, 2 and 3 respectively; Figure 4.2). There were no ncRNA that were observed on day 13 that were only in samples from non-pregnant mares, and 17 were present only in samples from pregnant mares (*FDR* ≤ 0.05). All 11 miRNA observed were present exclusively in samples from pregnant mares. One ncRNA that was significant in both days 11 and 13 and was present exclusively in samples from pregnant mares, *RF00100*, a misc RNA (Table 4.4).

Table 4.4 Noncoding RNA (*FDR* ≤ 0.05) in endometrial secreted exosomes on day 13 from pregnant (P+) and non-pregnant (NP) mares.

ncRNA Name	Feature ID	ncRNA Description	Day13P+ Average	Day 13NP Average
eca-mir-330	ENSECAG00000025374	MiRNA	2.03	0
eca-mir-350	ENSECAG00000025677	MiRNA	0.53	0
eca-mir-503	ENSECAG00000025360	MiRNA	0.47	0
eca-mir-769a	ENSECAG00000025352	MiRNA	0.47	0
Novel	ENSECAG00000026171	MiRNA	65.65	0
Novel	ENSECAG00000026396	MiRNA	0.66	0
Novel	ENSECAG00000026226	MiRNA	5.38	0
Novel	ENSECAG00000025220	MiRNA	0.53	0
Novel	ENSECAG00000025806	MiRNA	20.35	0
Novel	ENSECAG00000026119	MiRNA	22.53	0
Novel	ENSECAG00000026535	MiRNA	20.00	0
RF00004	ENSECAG00000025597	SnRNA	2246.49	272.96
RF00026	ENSECAG00000025316	SnRNA	0.40	0
RF00100	ENSECAG00000026091	Misc RNA	0.60	0
RF00100	ENSECAG00000026608	Misc RNA	1.00	0
RF00100	ENSECAG00000026649	Misc RNA	0.77	0
RF00334	ENSECAG00000025580	SnoRNA	30.85	6.21
RF00400	ENSECAG00000026207	SnoRNA	0.40	0
RF00407	ENSECAG00000025644	SnoRNA	5.98	1.48
RF00431	ENSECAG00000026616	SnoRNA	0.40	0
RF00564	ENSECAG00000027043	SnoRNA	22.13	2.47

Discussion

This was the first study to sequence small noncoding RNA (ncRNA) in equine endometrium and endometrial secreted exosomes during MRP. Noncoding RNAs were once considered to be junk DNA and non-functional transcription products. Even though they are not translated into functional proteins, they have a role in RNA interference (RNAi), acting as post-transcriptional modifications impacting gene expression. It is thought that RNAi could control up to 60% of protein coding genes in mammals (29). This study identified many different families of ncRNA including miRNA, snRNA, snoRNA, Mt rRNA, RRNA and misc RNA. Each of these families play an important role in RNA modification.

MicroRNA are single-stranded short RNAs, approximately 22 nucleotides in length (30). These are a highly conserved subset of ncRNA across species. The majority of miRNA are transcribed through RNA polymerase II from the genome to primary-miRNA (pri-miRNA) (31). Drosha and DGCR8 then cleave the pri-miRNA into a 70 nucleotide stem-loop precursor miRNA (pre-miRNA) in the nucleus (32). The pre-miRNA is exported out of the nucleus into the cytoplasm where it is cleaved by the Dicer complex (32). The Dicer complex cleaves the stem-loop into double stranded miRNA which is then loaded onto an AGO protein (33). The AGO protein unwinds the duplex and loads the guide strand of the mature miRNA onto RNA-induced silencing complex (RISC) (33). This guide miRNA guides RISC to the target mRNA (33). In many instances, one miRNA binds multiple different mRNA due to imperfect complementary base pairing to target sites in the 3’ untranslated regions (34). This binding destabilizes mRNA and usually results in degradation of the mRNA (34).

In this study, miRNAs were one of the top families of ncRNA identified in both endometrium and endometrial secreted exosomes. The endometrium on both days 11 and 13 contained 22 miRNA (18 on day 11 and 4 on day 13; *FDR* ≤ 0.05). Interestingly, on day 11, 15 miRNA identified were novel miRNA and have no known function. Specifically of these 15, nine were present only in samples from non-pregnant mares and two were present only in samples from pregnant mares. On day 13, of the four miRNA identified, one was observed only in samples from non-pregnant mares and is a novel miRNA. These miRNA need to be researched further to determine potential mRNA they are binding. It can be proposed that the miRNA found in samples from non-pregnant mares are binding to mRNA within the endometrium and preventing mRNA from signaling MRP. Of the remaining three miRNA in the endometrium on day 11, one (eca-mir-1244) is only in samples from non-pregnant mares. Limited information is known about eca-mir-1244 in the horse except that it has been identified in the stallion as being unique to the distal corpus of the epididymis (35). Interestingly, in human endothelial cells, mir-1244, decreases due to estrogen(36). This family of estradiol-modulated miRNAs are associated with molecular pathways such as extracellular signaling from signal-regulated kinase/mitogen activated protein kinase (ERK/MARPK), integrins and actin cytoskeleton signaling (36). The remining two miRNA were identified only in samples from pregnant mares on day 11 (eca-mir-514 and eca-mir-767). In humans, mir-514 has been highly implicated in ovarian cancer tumors (37). Interestingly, previous data in our laboratory identified eca-mir-767 to be more highly expressed in the serum exosomes from pregnant mares on day 11, corresponding to this study showing it to only be in endometrial samples from pregnant mares on day 11 (15). In humans, mir-767 targets DNMT3A and is highly implicated in carcinogenic cell lines (38). Specifically, mir-767 has been identified to target ECM-receptor interactions, focal adhesions and cell communication (38). Also, present only in samples from pregnant mares, was eca-mir-215 and eca-mir-329a. On day 13, there were four miRNA that were differentially expressed based upon pregnancy status. Two are present only in samples from non-pregnant mares. One of these miRNA is a novel miRNA and was also significant on day 11. The other miRNA was eca-mir-20b. In the horse, it was previously described in epididymal tissue, sperm, bone and liver (35, 39). One miRNA was only in samples from pregnant mares, eca-mir-1185. In the horse it is unique to the epididymal caput (35).

There were five miRNA that were differentially expressed ($FDR \leq 0.05$) in endometrial secreted exosomes on day 11. Two of these miRNA are novel and were observed only in samples from pregnant mares. In horses, eca-mir-215 has only previously been described in epididymal exosomes and sperm (35). In humans, mir-215 has been described to have a tumor suppressor effect in renal cell carcinoma and is associated with endometrial cancer (40, 41). The other miRNA found exclusively in endometrial secreted exosome samples from pregnant mares on day 11 was eca-mir-329a. No information is known about this miRNA in the horse, but it has been identified in cumulus oocyte complexes in cows (42). The only miRNA identified only in endometrial secreted exosomes on day 11 from non-pregnant mares was eca-mir-545. This miRNA has been implicated in cell proliferation, but it can promote or suppress proliferation based upon the cancer cell type (43, 44).

In endometrial secreted exosomes on day 13, there were 11 miRNA observed and more importantly, all 11 were present only in samples from pregnant mares. Seven of these miRNA were novel miRNAs. Eca-mir-350 and eca-mir769a have not been previously described, to our knowledge, in the horse. The mir-503 family, which was identified in endometrial secreted exosomes on day 13, is associated with inhibiting the cell cycle and muscle differentiation (45).

Eca-mir-330 has not been previously described in the horse, but in humans it acts as a tumor suppressor and induces apoptosis in prostate cancer (46).

Another family of ncRNA that was highly represented in these data was small nuclear RNA, or snRNA. They have well defined functions in mRNA splicing and 3'-end formation (47). Small nuclear RNAs are approximately 150 nucleotides in length and are transcribed by RNA polymerase II or III (47). They are also components of the spliceosome in order to participate in pre-mRNA processing (48). In this study, snRNA represented two of the 21 significant ncRNA in endometrial exosomes on day 13, 35 of the 85 endometrial ncRNA on day 11 and 1 of the 4 endometrial ncRNA on day 13. Interestingly, the majority of the snRNA in the endometrium on day 11 are in the family of RF00026, which is a family of U6 snRNA (49). Although not the same ncRNA, members of this family were also identified in endometrium and endometrial secreted exosomes on day 13. Day 11 endometrium and day 13 endometrial secreted exosomes also contained members of the RF00004 family, which are U2 snRNA (50).

The third largest family of ncRNA identified in this study was small nucleolar RNA, or snoRNA. Small nucleolar RNA are mainly involved in controlling ribosome biogenesis by guiding the modification or processing of pre-rRNA (51). There are two main classes of snoRNA, C/D box and H/ACA box. C/D box snoRNAs are primarily associated with methylation modifications and H/ACA box snoRNAs are primarily associated with psuedouridylation (51). Small nucleolar RNAs can also be processed into smaller fragments that participate in RNAi (52). Small Nucleolar RNA were identified in the endometrium on day 11 and the endometrial secreted exosomes on both days 11 and 13. While we identified multiple ncRNA in both the endometrium and endometrial secreted exosomes, there were none in common between the two.

In conclusion, this was the first study to describe the small ncRNA transcriptome in equine endometrium and endometrial secreted exosomes. There were many novel miRNA identified in both groups that need to be evaluated further. Unexpectedly, a large number of snRNA and snoRNA were also discovered in the endometrium of the mare. Small RNA have a robust role in modifying transcription and further understanding these ncRNA in combination with RNA-seq and focal adhesion data in this dissertation could lead to identifying the mechanism for MRP in the horse.

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CHAPTER V: CONCLUDING REMARKS

The research projects reported in this dissertation were designed to help understand the equine endometrium during maternal recognition of pregnancy (MRP) in an effort to delineate communication between the embryo and the endometrium days 9-13 post ovulation. These days were chosen because in the horse, the embryo enters the uterus on day 6 (1). Once in the uterus the embryo is mobile due to myometrial contractions (2). The embryo reaches peak mobility between days 11-14. In cycling (non-pregnant mares), on day 14 endometrium releases oxytocin to the uterine lumen (3, 4). This oxytocin acts in a paracrine fashion and binds to receptors on neighboring endometrial cells (4). Oxytocin binding causes the endometrial cell to synthesize and release more oxytocin into the uterine lumen, but to also synthesize and secrete prostaglandin $F_{2\alpha}$ (PGF) on day 14 (3). Prostaglandin $F_{2\alpha}$ causes regression of the corpus luteum, which is the source of progesterone. This steroid hormone is necessary to maintain a pregnancy. In a pregnant mare, MRP occurs prior to day 14 in order to prevent PGF production. It is known that it is necessary for the embryo to come into contact with at least 80% of the uterus during this time frame in order to prevent oxytocin and PGF production from occurring. Based upon this timing, days 9, 11 and 13 were chosen as the focal point for these studies in order to order to evaluate the endometrium before, during and after MRP.

Based upon previous studies in our laboratory, focal adhesions were an area of interest in regards to pregnancy and potentially maternal recognition of pregnancy. Focal adhesions have been described in the endometrium of many species throughout pregnancy due to their signal translation via mechanotransduction. Focal adhesions are unique molecules that form a protein complex based upon mechanical force on the cell (5). The primary subunits of focal adhesions are integrins (6). In a process known as mechanotransduction, cells can transmit an external force from the extracellular matrix (ECM) into a biochemical signal within the cell. One of the primary focal adhesions within the cell responsible for this signaling is focal adhesion kinase, or phosphorylated focal adhesion kinase (PTK/FAK/p-FAK). Focal adhesions are associated with many processes within the cell including cell motility, intracellular processes, cell proliferation, anti-apoptosis and cell remodeling. We propose that the mobile embryo in the uterus is responsible for focal adhesion formation, which relays the message for MRP within the cell.

In this study (chapter II), focal adhesions were evaluated in the endometrium on days 9 and 11 immediately after biopsy collection. Results showed that focal adhesions were present in endometrium in both pregnant and non-pregnant mares on these days, but very few were differentially abundant between the pregnancy groups on both days. On day 9, talin-1 (*TLN1*) was significantly more abundant ($P \leq 0.05$) in endometrial samples from pregnant mares. Talin-1 is a cytoskeletal protein that colocalizes with activated integrins (7). It is the final element that binds to the integrin β subunit on the cytoplasmic tail to initiate the cascade of cellular signaling (8). On day 11, actinin alpha 3 (*ACTN3*) was more abundant ($P \leq 0.05$) in samples from pregnant mares. Actinin is another cytoskeletal protein that is recruited with *TLN1* (9). These genes indicate that focal adhesions were active in these samples, and more specifically, cytoskeletal remodeling may be occurring. Also on day 11, caveolin-1 (*CAV1*) was more abundant ($P \leq 0.05$) in samples from non-pregnant mares. Caveolin-1 is a membrane adapter that aids in the α integrin subunit formation and is necessary for focal adhesion assembly (6, 10, 11). While there were gene differences in snap frozen endometrial samples, there were no protein level differences between groups on either day.

Part two of the experiment was to culture these endometrial samples with one of four treatments for 24 hours. The treatments were: (1) endometrium with and embryo in contact with the luminal side, EE; (2) endometrium with spherical beads in contact with the luminal side , EB; (3) endometrium with peanut oil in contact with the luminal side, EO; (4) endometrium with no physical contact, E. Spherical beads and peanut oil were chosen based upon previous studies that have stated that a glass spherical object or peanut/coconut oil can prolong diestrus in a non-pregnant mare (12, 13). It was hypothesized that samples from pregnant mares would not change focal adhesion abundance after 24 hours in culture and samples from non-pregnant mares would increase focal adhesion abundance in the presence of an embryo, but not the presence of beads or oil.

The beads and oil caused minor changes in endometrial samples from pregnant and non-pregnant mares. With beads, samples from pregnant mares only on day 9 had any observed changes, with an increase in *CAV1* and a decrease in *CCND1* abundance ($P \leq 0.05$). With peanut oil, day 9 had no genes changes in samples from pregnant or non-pregnant mares. On day 11, peanut oil actually caused a decrease in focal adhesions such as *ACTN4* and *CAV1* ($P \leq 0.05$). These results suggested that beads and peanut oil were not enough to activate focal adhesion assembly complexes.

The presence of an embryo did cause changes in the endometrial samples. As expected, samples from pregnant mares on both days 11 and 13 were very minimally impacted by the presence of the embryo. The reason behind this is focal adhesions can remain active either due to continuous external pressure or contractile pressure within the cell, therefore even though these endometrial samples were not in contact with an embryo for 24 hours, the focal adhesions were still present (14). In samples from non-pregnant mares, *BCL-2* and *ITGA4* on day 9 were both more highly abundant ($P \leq 0.05$) in the presence of an embryo. Interestingly, on day 11, *BCL-2*, was also more abundant in samples from pregnant mares. B-cell lymphoma 2 (BCL-2) is an inner mitochondrial membrane protein that is anti-apoptotic, blocking cell death (15). Interestingly on day 11 the embryo decreases *ACTN1, ACTN4, CAV1,* and *ITGA6* abundance ($P \leq 0.05$) in samples from non-pregnant mares but does not impact protein levels of these genes. This could be due an increase in the turnover of RNA.

The final step of this project was to evaluate PGF concentrations in medium after being cultured for 24 hours with their respective treatments. Day 9 had no changes in PGF concentration after any treatments from both pregnant and non-pregnant mares. On day 11, peanut oil actually increased PGF secretion in samples from pregnant mares. This could indicate that oil caused an inflammatory effect, resulting in PGF secretion. The most interesting result was in samples from non-pregnant mares on day 11. The presence of an embryo decreased PGF secretion ($P \leq 0.05$) into the medium. This indicates that the mechanism for MRP is very fast and the presence of an embryo for only 24 hours was enough to result in a decrease of PGF secretion. Also, when comparing E treatment in samples from pregnant and non-pregnant mares, even after 24 hours with no embryo stimulation, the samples from pregnant mares still had a lower amount of PGF secretion compared to samples from non-pregnant mares. This further validates the idea that once focal adhesions are activated, intracellular contractility can continue signaling keeping the focal adhesions active without the consistent mechanical pressure from the embryo.

This project demonstrated that focal adhesions were present in endometrium and can change based upon different mechanical stressors but did not identify a direct signal for MRP in the mare. It is clear that an embryo is required to decrease PGF secretion versus just mechanical force from beads or oil. It became evident that the entire transcriptome of the endometrium needed to be evaluated to help elicit the signal for MRP and to evaluate additional components of focal adhesions. Numerous studies have been done evaluating transcripts in the endometrium, but none have been able to derive any candidate genes (16-19). The next project in this dissertation (chapter III) aimed to do exactly that by evaluating the entire transcriptome via RNA-seq in the endometrium on days 9, 11 and 13 in pregnant and non-pregnant mares.

RNA-seq of endometrium revealed numerous genes and transcript variants that were of differential abundance between samples from pregnant and non-pregnant mares. A striking observation was the number of transcript variants that resided exclusively in samples from pregnant or non-pregnant mares. On days 9, 11 and 13 there were 128, 210 and 143 transcript variants, respectively, in samples from pregnant mares and 227, 189 and 218 transcript variants, respectively, in samples from non-pregnant mares. The only difference between these groups at this time was pregnancy status and was therefore the reason for these changes. At this time the mares have the same hormonal profile, so an endocrine effect can be ruled out (4). RNA-seq provided more genes to be evaluated in regards to MRP and also many novel genes with unknown function.

Alternative splicing was observed in this data for multiples genes. Alternative splicing is the process where different splice sites join together to form a gene, resulting in a gene with multiple different lengths and forms (20). Research is finding that a balance of alternative splicing is necessary for healthy cells, but if it becomes unbalanced it can result in disease states such as tumors (21). Interestingly, this alternative splicing resulted in transcripts that were more highly expressed in opposite pregnancy status on the same day. Spliceosomes are responsible for alternative splicing and are comprised of multiple small nuclear RNA ribonclearproteins (snRNPs) (22). These snRNPs are formed from small nuclear RNA (snRNA).

After identifying the large RNA transcripts in the equine endometrium, it was also important to define the small noncoding RNA (ncRNA) present in the endometrium and in exosomes secreted by the endometrium on days 11 and 13 in samples from pregnant and non-pregnant mares (Chapter IV). The goal was to identify potential signaling that was occurring within the endometrium and being secreted out of the endometrium through exosomes. Exosomes are cell-secreted vesicles that are capable of transferring bioactive materials, such as RNA and protein from cell to cell (23-25). Specifically, this dataset evaluated small noncoding RNA (ncRNA) within these samples using RNA-seq. This analysis identified many families of ncRNAs. These families include small nuclear RNA (snRNA), microRNA (miRNA), small nucleolar RNA (snoRNA), mitochondrial ribosomal RNA (Mt rRNA), ribosomal RNA (rRNA) and miscellaneous RNA (misc RNA). The small ncRNA could lead us to understand the transcriptional regulation that is occurring during MRP.

The largest family of ncRNA observed in this study was snRNA. Specifically, snRNA were most abundant in endometrial samples. Small nuclear RNA are well defined in mRNA splicing and 3'-end formation (26). These ncRNA are approximately 150 nucleotides in length and are large component of the spliceosomes (26, 27). These snRNA are of particular interest because in the study of large RNA (Chapter III) we identified many genes that had transcript variants with opposing expression patterns. These snRNA could be targeting these genes and explain the splice variants that we identified. The snRNA observed herein were primarily in the family of U6 snRNA and U2 snRNA (28, 29). These snRNA may be responsible for the alternative splicing that occurred in the endometrium that was observed in the large RNA from endometrium.

The second largest family of ncRNA observed in this study was miRNA. Specifically, miRNA were most abundant in endometrial secreted exosomes. MicroRNA are single stranded

RNA approximately 22 nucleotides in length and are highly conserved across species (30). MicroRNA guide RNA-inducing complexes (RISC) to target mRNA for transcription silencing or degradation (31). Because miRNA are short in nucleotide length, they bind mRNA with imperfect complementary base pairing at the target sites of the 3' untranslated regions (32). In many instances, one miRNA can bind multiple different RNA (28). In the endometrium, miRNA were identified on both days 11 and 13. Of all the miRNA across days 11 and 13 , 22 in total, 12 were novel miRNA with an unknown function. Four were present only in samples from pregnant mares and eight were present only in samples from non-pregnant mares. A miRNA of interest was identified only in samples from pregnant mares on day 11, eca-miR-767. This miRNA is of particular interest because previous reports from our laboratory identified this miRNA to be in high abundance in serum exosomes from pregnant mares also on day 11 (33). This miRNA has been predicted to target ECM-receptor interactions, focal adhesions and cell communication (34). Eca-miR-1244 was identified in samples from non-pregnant mares on day 11 and is also associated with extracellular signaling, and integrin and actin cytoskeleton signaling (35). Across days 11 and 13 there was one miRNA that was in the endometrium only from non-pregnant mares. This is a novel miRNA and the function remains unknown. In endometrial secreted exosomes, on day 13 there were 11 miRNA identified, and all were present only in samples from pregnant mares. Seven of these miRNA are novel and have not been described previously in the horse to our knowledge.

The last ncRNA family of interest are snoRNA. This was an unexpected family of ncRNA observed in this study. Small nucleolar RNA are primarily involved in ribosome biogenesis, specifically by guiding the modification or processing of pre-rRNA (36). They can also be processed into smaller fragments that participate in RNA interference (37). Small nucleolar RNA can be divided into two classes. H/ACA box snoRNAs are associated with pseudouridylation and

C/D box snoRNAs are associated with methylation modifications (36). Small nucleolar RNA were identified in endometrial samples on day 11 only and in endometrial secreted exosomes in both days 11 and 13.

In conclusion, this dissertation provided an in-depth overview of the transcriptome of equine endometrium and endometrial exosomes prior to, and during, MRP. It clearly demonstrates that an embryo specifically is required to decrease PGF secretion from endometrium and that focal adhesions are implicated in the anti-luteolytic process of MRP. Multiple novel genes were identified with RNA-seq and these genes could be responsible for MRP. Furthermore, multiple novel miRNAs were identified in the endometrium and endometrial secreted exosomes. This could be the communication that is occurring between the embryo and the endometrium during this time frame.

Future analyses need to continue with this data in order to determine the function and role of the novel genes and ncRNAs that were identified. These transcripts could be the link between an embryo being present in the uterus and PGF production being halted. Further research on the components in the embryo, specifically the capsule, also needs to be performed to determine if it is the mechanical force of the embryo that is activating the focal adhesions or if there is binding that is occurring, even for a brief moment, between the embryo and the endometrium as it is mobile throughout the uterus. Combining this data identified in these studies with future studies will lead to the determination of the process of maternal recognition of pregnancy in the mare.

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APPENDICES

Appendix I: Focal Adhesion Molecules and Primer Design for PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
ACTN1	TGACCCAGAAGCGGAGGGAA	CCCTCCATCCAGTTGTTGAAGGG
ACTN2	CTGACCGAGGGAAAATGCGA	CCAGCGTCATTTTCACATTGCCA
ACTN3	GGAGGAGTACGAGAAGCTTGCCA	AGTCCCGAAAGTCCTCCAGCT
ACTN4	GAGAACATCGACGAGGACTTCC	CAGCGCCTTGTTCACATTGTTGA
AKT3	TGGACAGAAGCTATCCAGGCTGT	TGGAAGCATCCATCTCTTCCTC
BCL2	GACGCTTTGCCACGGTAGTG	ATCTCCCGGTTGACGCTCTC
CAV1	GGCAACATCTACAAGCCCAACA	TTGAGATGCTTGGGGTCGCG
CCND1	CGTGAAGTTCATTTCCAACCCGC	TGTGAGGCGGTGGTAGGACA
ITGA4	TCAACATCACAGTGCATGCCG	CCATGAACAGCTAGCATGACTTCA
ITGA5	AGGGAGAGGAGCCTGTGGAA	AGCGGCTCCTTTTCTGTGCG
ITGA6	TCCCGGCCTGTGATTAACATT	CAGGCCTTAACCTTGAGGCATATC
ITGA10	ACCCACGGCTGAATTTGGAT	CCCCTACAGGGCAGCGATAA
ITGAV	GGTCGCTGCCACTGACATTA	CGAAGCCCTTTGCAGAGACA
ITGAX	CCAGCCAACAACCTTCCGCA	TCACACTGGTAAAGGCCGCC
ITGB1	AGTCTTGGAACCGATCTGATGAATGA	TGCAAGGGTTTCTGAGCTTGGC
ITGB3	AGGAGGTGAAGAAGCAGAGCGT	GGATGCATCATTCCTCCAGCCA
PAK6	GGCTCCTGAAGTGATCTCCAGGT	TGGCTTGCACTGGTGAGTCG
PTGS2	CCTTTTCCAACCTCTCGTATTACACCA	TCCACAATCTCTTTTGAATCAGGAAGC
FAK	AGATCCTCTCCCCAGTGTACAGA	GTTGCAGCCCTTGTCCGTGA
RAF1	CTAAAACCCCTGTGCCGGCA	TGGAGAGCATCACTTCACTCGCT
SLCO2A1	TGAACGGGGCAAGGAAGATGGA	TGGGCCAGGACTGTCAGCAT
TLN1	TGAAGACGATGCAGTTTGAGCCG	TGACAGAAACAGCCCAAAGTCGC
ACTB	CGACATCCGTAAGGACCTGT	CAGGGCTGTGATCTCCTTCT
B2M	GTGTTCCGAAGGTTCAGGTT	ATTTCAATCTCAGGCGGATG
GAPDH	AGAAGGAGAAAGGCCCTCAG	GGAAACTGTGGAGGTCAGGA
RN18S	AACGACACTCTGGCATGCTAACTA	CGCCACTTGTCCCTCTAAGAA

Appendix II: Significant Genes and Transcript Variants Identified in Endometrium on Day 9

Gene Symbol	Transcript Number	Day 9 P-Values	Day 9P+ Avg. RPKM	Day 9NP Avg. RPKM
ABCA10	3	0.0013	0.00	0.43
ABCA13	5	0.0007	0.00	0.31
ACAP3	5	0.0195	0.00	0.25
ACER2	6	0.0394	0.42	0.76
ADAMTS17	2	0.0197	0.00	0.31
AFG1L	2	0.0061	0.00	0.32
AGO2	1	0.0175	0.00	0.36
AGO3	1	0.0038	0.00	0.27
ANKRD33	1	0.0032	0.26	0.00
ANKRD44	3	0.0345	0.25	0.00
ARHGAP28	1	0.0001	0.00	0.31
ARMCX4	3	0.0463	0.27	0.00
ATP2B4	3	0.0396	0.30	0.00
ATP6V1C1	1	<0.0001	0.00	0.30
B3GNT8	1	<0.0001	0.30	0.00
BFSP1	1	0.0204	0.30	0.00
BOC	3	0.0426	0.00	0.25
BUD13	2	0.0323	0.00	0.41
C22H20orf196	3	0.0432	2.10	2.14
C24H14orf1	2	0.0424	5.84	5.79
CA9	1	0.0001	0.32	0.00
CACNA2D1	5	0.0056	0.26	0.00
CAMK2D	2	0.0362	0.29	0.00
CAMK2D	4	0.0283	0.27	0.00
CAMK2D	5	0.0299	0.30	0.00
CAMK2G	2	0.0280	0.00	0.38
CASKIN1	1	0.0117	0.34	0.00
CASZ1	5	0.0546	0.00	0.25
CATSPERG	4	0.0371	0.28	0.00
CCDC15	1	0.0058	0.96	1.70
CCDC15	4	0.0417	0.38	0.62
CCDC15	6	0.0498	6.23	10.40
CCNJ	3	0.0028	0.26	0.00
CCP110	2	0.0108	0.29	0.00

CCR8	1	0.0144	0.00	0.27
CCSAP	1	0.0110	0.00	0.25
CD300LG	7	0.0003	0.58	0.00
CEP250	7	0.0003	0.00	0.39
CITED1	1	0.0390	2.31	4.52
CLEC12A	1	0.0143	0.00	0.34
CMTM4	6	0.0306	0.00	0.25
CPPED1	2	0.0480	0.00	0.27
CPSF2	2	0.0025	0.00	0.25
CPSF4	1	0.0045	0.00	0.25
CRACR2A	3	0.0021	0.00	0.27
CSNK1G1	4	0.0308	0.00	0.32
CXHXorf21	1	0.0303	0.00	0.36
DDX10	2	0.0298	0.28	0.00
DGKQ	1	0.0257	0.26	0.00
DOCK10	2	0.0124	0.00	0.29
DOCK6	1	0.0003	0.00	0.27
DOPEY1	5	0.0380	0.00	0.26
DUSP26	2	0.0118	0.26	0.00
EML6	1	0.0357	0.28	0.00
EPB41	10	0.0297	0.58	0.80
EPB41	1	0.0029	0.49	0.33
EPB41	2	0.0003	0.25	0.00
EQCA-2	1	0.0153	0.00	0.27
ERI3	1	0.0167	0.49	0.00
ERICH1	7	0.0091	0.00	0.34
ERICH1	5	0.0204	0.27	0.00
ERICH6B	1	0.0260	0.00	0.38
F8	2	0.0096	0.00	0.25
F8	8	0.0064	0.00	0.25
FAM172A	3	0.0217	0.00	0.31
FAM212B	1	0.0111	0.00	0.30
FBN1	2	0.0027	0.00	0.36
FBXO2	2	0.0035	0.00	0.35
FBXW7	3	0.0014	0.25	0.00
FGD4	6	0.0103	0.26	0.00
FGD5	1	0.0344	0.00	0.41
FOXP3	1	0.0075	0.31	0.00
FRMD6	1	0.0370	0.00	0.28

GALE	5	0.0091	0.28	0.00
GBGT1	6	0.0239	0.00	0.26
GPR141	6	0.0064	0.25	0.00
GPR155	1	0.0015	0.28	0.00
GRIK4	1	0.0340	0.00	0.35
HAUS4	1	0.0205	0.00	0.25
HAVCR1	1	0.0258	0.00	0.28
HMGXB4	5	0.0501	0.30	0.00
HMGXB4	6	0.0233	0.25	0.00
HRCT1	4	0.0291	2.94	2.74
IDS	7	0.0118	0.00	0.25
INA	1	0.0428	0.26	0.00
INPP4A	3	0.0351	0.28	0.00
KIAA0040	1	0.0046	0.00	0.28
KIAA0040	2	0.0133	0.00	0.32
KRT2	1	0.0195	0.35	0.00
LHX6	1	0.0017	0.00	0.28
LIMK2	2	0.0251	0.00	0.25
LMNTD1	2	0.0417	0.00	0.27
LOC100058728	1	0.0060	0.00	0.33
LOC100061542	7	0.0217	2.15	3.76
LOC100070509	3	0.0260	0.00	0.27
LOC100072933	1	0.0105	0.29	0.45
LOC100146423	1	0.0267	0.00	0.25
LOC100629339	2	0.0027	0.00	0.31
LOC100629339	5	0.0035	0.00	0.28
LOC100629999	1	0.0189	0.00	0.27
LOC100630083	1	0.0277	0.00	0.39
LOC102147456	4	0.0145	0.00	0.30
LOC102147739	3	0.0005	0.00	0.30
LOC102147761	2	0.0001	0.00	0.30
LOC102148336	2	0.0115	0.00	0.30
LOC102149311	1	0.0091	0.35	0.00
LOC102149354	1	0.0047	0.00	0.25
LOC102149357	1	0.0284	0.00	0.28
LOC102149431	2	0.0480	0.00	0.26
LOC102149524	1	0.0164	0.00	0.26
LOC102149727	1	0.0111	0.30	0.00
LOC102150007	1	0.0093	0.00	0.26

LOC102150380	2	0.0018	0.00	0.30
LOC102150380	2	0.0024	0.27	0.00
LOC102150421	2	0.0388	0.27	0.00
LOC102150925	2	0.0107	0.00	0.41
LOC102150986	2	0.0519	0.00	0.41
LOC102151107	1	0.0391	0.27	0.00
LOC106780959	4	0.0102	0.00	0.26
LOC106780997	14	0.0393	0.38	0.00
LOC106781499	1	0.0206	0.00	0.26
LOC106782451	1	0.0010	0.27	0.00
LOC10678252	1	0.0110	0.34	0.00
LOC106782916	3	0.0252	0.00	0.32
LOC106783229	1	0.0457	0.00	0.33
LOC106783370	6	0.0320	0.41	0.00
LOC106783386	1	0.0276	0.00	0.27
LOC111767592	1	0.0249	0.27	0.00
LOC111768053	1	0.0204	0.45	0.00
LOC111768363	2	0.0150	0.00	0.27
LOC111773132	1	0.0098	0.31	0.00
LOC111773873	2	0.0194	0.25	0.00
LRAT	1	0.0003	0.00	0.33
LRCH3	2	0.0351	0.35	0.00
LRFN5	4	0.0291	0.26	0.00
LRP2	1	0.0026	0.00	0.27
LRRC58	1	<0.0001	0.00	0.34
MAD2L1	1	0.0507	0.28	0.00
MAML1	1	0.0125	0.00	0.26
MAPK12	2	0.0202	0.00	0.36
MBD1	8	0.0076	0.00	0.26
MCTP1	1	0.0027	0.32	0.00
MCTS1	1	0.0423	0.00	0.26
MEST	1	0.0257	0.00	0.25
MFF	1	0.0547	0.26	0.00
MORN4	3	0.0482	0.00	0.32
MPHOSPH8	1	0.0003	0.00	0.27
MPHOSPH8	2	0.0011	0.00	0.31
MPHOSPH8	3	0.0033	0.00	0.28
MPHOSPH8	5	0.0028	0.00	0.29
MPHOSPH8	6	0.0163	0.46	0.30

MPHOSPH9	6	0.0110	0.00	0.25
MTERF1	3	0.0009	0.00	0.27
MXRA5	1	0.0056	0.00	0.27
MYO5C	8	0.0321	0.26	0.00
MYO9A	3	0.0505	0.33	0.00
NARFL	3	0.0122	0.00	0.28
NAT9	3	0.0499	0.00	0.29
NFATC4	2	0.0056	0.00	0.28
NHS	1	0.0001	4.41	4.72
NKAIN2	1	0.0097	0.39	0.00
NLGN3	1	0.0024	0.00	0.28
NOCT	1	0.0249	0.31	0.00
NOTCH4	1	0.0548	0.29	0.00
ENSECAG00000003160	5	0.0100	0.00	0.31
ENSECAG00000003160	6	0.0027	0.26	0.00
ENSECAG00000006760	1	0.0004	0.00	0.29
ENSECAG00000012974	1	<0.0001	0.00	0.38
ENSECAG00000015199	1	0.0477	0.00	0.26
ENSECAG00000021202	1	0.0158	0.00	0.25
ENSECAG00000023794	7	0.0117	0.00	0.31
ENSECAG00000023794	1	0.0158	0.27	0.00
NYNRIN	2	0.0523	0.00	0.26
OTULIN	1	0.0016	0.00	0.26
P4HA2	4	0.0515	0.30	0.00
PARP9	2	0.0396	0.00	0.27
PHF7	1	0.0115	0.00	0.28
PHTF2	1	0.0001	0.26	0.46
PHTF2	6	0.0038	0.00	0.31
PICALM	6	0.0110	0.00	0.27
PJVK	1	0.0144	0.00	0.25
PKD1L3	2	0.0161	0.28	0.00
PKHD1	1	0.0267	0.00	0.31
PNPLA4	1	0.0125	0.00	0.35
PODXL	1	0.0014	0.25	0.00
PPFIBP2	10	0.0303	0.00	0.25
PPHLN1	7	0.0107	0.00	0.27
PPIP5K1	9	0.0158	0.00	0.27
PPIP5K1	14	0.0063	0.00	0.26
PPP2R2C	1	0.0405	0.26	0.00

PPP2R5C	1	0.0138	0.26	0.00
PRSS36	3	0.0035	0.00	0.25
PTPRD	19	0.0425	0.00	0.26
RAP2B	1	0.0328	0.28	0.00
RBBP9	2	0.0091	0.00	0.27
RBM47	8	0.0422	0.00	0.25
RCE1	2	0.0278	0.27	0.00
RHBDL3	2	0.0492	0.00	0.25
RIC3	4	0.0115	0.27	0.00
RIC8B	1	0.0043	0.27	0.00
RICTOR	1	0.0357	0.35	0.58
RTKN	9	0.0543	0.00	0.28
SCMH1	6	0.0200	0.33	0.00
SCN2B	1	0.0427	0.00	0.28
SEC31B	4	0.0070	0.00	0.28
SEMA6C	1	<0.0001	0.61	0.34
SERPINB1	1	0.0027	0.43	0.00
SERPINE1	2	0.0116	0.26	0.00
SHC3	1	0.0021	0.00	0.35
SHE	3	0.0026	0.30	0.00
SLA	1	0.0194	0.53	0.69
SLC12A9	2	0.0216	2.92	2.27
SLC17A8	1	0.0002	0.25	0.00
SLC38A10	1	0.0491	7.57	8.01
SLC45A2	4	0.0346	0.00	0.31
SLFN5	1	0.0507	0.00	0.37
SMPD5	5	0.0170	0.31	0.00
SNAI3	1	0.0502	0.26	0.00
SNRK	3	0.0119	0.00	0.27
SNTA1	4	0.0115	0.26	0.00
SPAST	1	0.0143	0.34	0.00
SPAST	3	0.0143	0.32	0.00
SPIDR	7	0.0192	0.34	0.00
SPTBN1	2	0.0168	0.00	0.34
SPTBN1	3	0.0100	0.00	0.28
SPTBN1	4	0.0007	0.00	0.25
SRBD1	1	0.0002	0.00	0.26
SYNJ1	7	0.0003	0.00	0.29
TAF15	2	0.0103	0.61	1.21

TCTN1	2	0.0543	0.00	0.32
TIMMDC1	2	0.0137	0.26	0.00
TMCO6	3	0.0038	0.25	0.00
TMEM156	2	0.0103	0.00	0.33
TMEM68	11	0.0344	0.00	0.79
TMEM68	12	0.0191	0.30	0.00
TMEM68	14	0.0061	0.28	0.00
TMOD1	2	0.0241	0.26	0.00
TNFRSF19	2	0.0375	0.25	0.00
TRAF5	2	0.0057	0.00	0.25
TRAPPC9	2	0.0320	0.25	0.00
TRIM37	7	0.0103	0.00	0.31
TRMT1	1	0.0306	7.08	6.35
TRMT6	2	0.0235	4.10	2.72
TRPM3	2	0.0501	0.00	0.31
TSC22D1	9	0.0444	0.00	0.25
TTLL11	1	0.0245	0.37	0.48
TUBE1	1	0.0233	0.00	0.33
TXK	3	0.0195	0.00	0.44
UIMC1	2	0.0351	0.00	0.25
UNC93B1	3	0.0518	10.62	8.34
USF3	1	0.0236	0.29	0.53
USP35	1	0.0233	0.00	0.26
VANGL1	1	0.0305	0.00	0.49
VANGL1	2	0.0278	0.38	0.00
VEPH1	1	0.0306	0.00	0.32
WDHD1	1	0.0306	0.00	0.28
WDR77	7	0.0507	0.00	0.25
XKR5	1	0.0003	0.00	0.30
XLOC_000735	1	0.0168	0.29	0.00
XLOC_000968	2	0.0456	0.00	0.31
XLOC_001079	4	0.0047	0.00	0.25
XLOC_001772	3	0.0042	0.00	0.26
XLOC_002093	3	0.0479	0.00	0.25
XLOC_002303	4	0.0028	0.00	0.27
XLOC_002750	4	0.0001	0.00	0.26
XLOC_002848	2	0.0086	0.00	0.41
XLOC_002861	4	0.0340	0.00	0.28
XLOC_003146	1	0.0097	0.00	0.25

XLOC_003278	1	0.0002	0.30	0.00
XLOC_004031	2	0.0293	0.00	0.29
XLOC_004416	8	0.0017	0.00	0.25
XLOC_004483	4	0.0258	0.25	0.00
XLOC_004483	6	0.0133	0.26	0.00
XLOC_004483	9	0.0088	0.30	0.00
XLOC_004688	2	<0.0001	0.27	0.00
XLOC_004694	1	0.0512	0.29	0.00
XLOC_004996	2	0.0239	0.29	0.48
XLOC_005301	1	0.0204	0.32	0.00
XLOC_005555	7	0.0007	0.26	0.00
XLOC_005966	3	0.0028	0.00	0.25
XLOC_007025	3	0.0091	0.00	0.26
XLOC_007369	1	0.0038	0.46	0.00
XLOC_007462	2	0.0122	0.00	0.28
XLOC_008297	6	0.0427	0.35	0.00
XLOC_008309	1	0.0255	0.00	0.28
XLOC_009401	3	0.0517	0.00	0.31
XLOC_009406	1	0.0100	0.46	0.34
XLOC_009408	5	0.0533	0.00	0.29
XLOC_009586	6	0.0255	0.28	0.00
XLOC_009773	3	0.0543	0.00	0.32
XLOC_009938	3	0.0082	0.00	0.28
XLOC_009981	4	0.0505	0.00	0.36
XLOC_009981	1	0.0341	0.27	0.00
XLOC_010036	2	0.0042	0.00	0.30
XLOC_012182	3	0.0026	0.00	0.28
XLOC_012182	6	0.0313	0.00	0.30
XLOC_012519	1	0.0014	0.00	0.34
XLOC_013021	4	0.0144	0.30	0.00
XLOC_013546	2	0.0366	0.00	0.43
XLOC_013840	3	0.0004	0.00	0.33
XLOC_014122	2	0.0163	0.39	0.27
XLOC_014243	3	0.0320	0.00	0.27
XLOC_014353	5	0.0512	0.29	0.00
XLOC_015107	3	0.0114	0.00	0.27
XLOC_015532	3	0.0071	0.25	0.00
XLOC_015835	6	0.0153	0.00	0.29
XLOC_016628	1	0.0514	0.00	0.29

XLOC_017076	5	<0.0001	0.00	0.26
XLOC_017076	9	0.0159	0.35	0.55
XLOC_017525	1	0.0017	0.30	0.00
XLOC_017525	3	0.0026	0.30	0.00
XLOC_017525	4	0.0233	0.39	0.00
XLOC_017833	6	0.0038	0.30	0.00
XLOC_018012	7	0.0412	0.27	0.00
XLOC_019370	5	0.0358	0.32	0.00
XLOC_019673	2	0.0162	0.00	0.34
XLOC_019724	1	0.0091	0.00	0.25
XLOC_019992	3	0.0003	0.00	0.29
XLOC_020574	2	0.0498	0.00	0.28
XLOC_020991	3	0.0152	0.00	0.28
XLOC_021331	1	0.0382	0.29	0.50
XLOC_021392	2	0.0278	0.00	0.48
XLOC_022226	4	0.0002	0.26	0.00
XLOC_022623	1	0.0260	0.00	0.27
XLOC_022749	5	0.0014	0.30	0.00
XLOC_023006	1	0.0001	0.00	0.25
XLOC_023740	1	0.0115	0.55	0.00
XLOC_024609	1	0.0100	0.00	0.28
XLOC_024672	1	0.0303	0.00	0.28
XLOC_025532	1	0.0038	0.48	0.63
XLOC_025532	4	0.0118	0.50	0.60
XLOC_026647	3	0.0268	0.00	0.30
XLOC_028572	5	0.0204	0.00	0.30
XLOC_028572	6	0.0048	0.00	0.32
XLOC_028777	3	0.0497	0.00	0.26
XLOC_028820	1	0.0115	0.29	0.00
XLOC_028859	1	0.0118	0.29	0.00
XLOC_029408	1	0.0303	0.36	0.44
XLOC_030092	2	0.0006	0.00	0.36
XLOC_030180	6	0.0047	0.25	0.00
XLOC_030300	2	0.0005	0.00	0.25
XLOC_030716	3	0.0364	0.00	0.30
XLOC_030732	1	0.0341	0.58	0.00
XLOC_030859	2	0.0133	0.00	0.26
XLOC_031373	1	0.0232	0.27	0.00
XLOC_031373	3	0.0125	0.33	0.00

XLOC_031741	4	0.0093	0.00	0.26
XLOC_032056	6	0.0240	0.26	0.00
XLOC_032345	4	0.0284	0.00	0.42
XLOC_032777	3	0.0306	0.00	0.25
XLOC_032777	4	<0.0001	0.00	0.25
XLOC_034312	7	0.0358	0.00	0.25
XLOC_035147	1	0.0093	0.39	0.00
XLOC_035190	5	0.0484	0.26	0.00
XLOC_035333	5	0.0144	0.00	0.26
XLOC_035333	6	0.0026	0.00	0.28
XLOC_036605	2	0.0075	0.00	0.31
XLOC_036605	4	0.0163	0.00	0.30
XLOC_037518	2	0.0027	0.27	0.00
XLOC_037863	2	0.0004	0.29	0.00
XLOC_037874	1	0.0137	0.25	0.00
XLOC_037988	1	0.0376	0.00	0.32
XLOC_038667	6	<0.0001	0.00	0.30
XLOC_039441	2	0.0441	0.00	0.26
XLOC_039629	3	0.0185	0.00	0.31
XLOC_039656	1	0.0191	0.25	0.00
XLOC_039679	1	0.0038	0.25	0.00
XLOC_039727	1	0.0252	0.00	0.25
XLOC_040085	3	0.0118	0.00	0.26
XLOC_040941	1	0.0100	0.00	0.27
XLOC_041999	3	0.0112	0.00	0.31
XLOC_042180	1	0.0415	0.00	0.32
XLOC_042552	7	0.0270	0.00	0.37
XLOC_042672	1	0.0214	0.87	0.40
XLOC_042800	3	0.0344	0.00	0.44
XLOC_042824	3	0.0003	0.00	0.28
XLOC_043221	2	0.0067	0.25	0.00
XLOC_043278	1	0.0052	0.00	0.26
XLOC_043409	1	0.0282	0.00	0.25
XLOC_043556	12	0.0006	0.00	0.25
XLOC_045920	4	0.0240	0.41	0.00
XLOC_047042	3	0.0506	0.25	0.00
XLOC_047414	4	0.0115	0.00	0.25
XLOC_047417	4	0.0116	0.31	0.00
XLOC_047417	6	0.0265	0.32	0.00

ZBTB17	1	0.0502	0.27	0.00
ZBTB8OS	2	0.0002	0.00	0.25
ZC2HC1B	1	0.0015	0.00	0.27
ZFHX4	2	0.0107	0.27	0.00
ZFHX4	3	0.0049	0.25	0.00
ZFHX4	6	0.0118	0.25	0.00
ZFP82	7	0.0476	0.46	0.78
ZNF274	6	0.0003	0.00	0.29
ZNF529	4	0.0502	0.26	0.00
ZNF594	1	0.0502	0.00	0.32
ZNF609	3	0.0058	0.25	0.00
ZNF618	4	0.0364	0.00	0.25
ZNF618	6	0.0004	0.00	0.31
ZNF784	4	0.0124	0.29	0.00
ZNF891	2	0.0455	0.00	0.36
ZSCAN23	2	0.0062	0.00	0.30

Appendix III: Significant Genes and Transcript Variants Identified in Endometrium on Day 11

Gene Symbol	Transcript Number	Day 11 P-Values	Day 11 P+ Avg. RPKM	Day 11NP Avg. RPKM
ACP6	1	0.0306	0.25	0.00
ADAR	1	0.0204	0.00	0.33
AIDA	1	0.0415	139.87	189.74
AJM1	2	0.0232	0.00	0.25
AKAP8	2	0.0011	0.26	0.00
APBB2	2	0.0299	0.35	0.00
APOBEC3Z2A-Z2	4	0.0424	0.41	0.00
ARAP1	8	0.0497	0.30	0.00
ARHGAP24	1	0.0529	0.36	0.00
ARHGEF4	1	0.0398	1.00	1.77
ARSI	1	0.0298	0.27	0.00
ATP13A3	3	0.0358	19.30	14.72
ATP2B4	4	0.0305	0.00	0.28
ATP2C1	2	0.0011	0.00	0.29
BRD8	4	0.0108	0.43	0.00
C11H17orf75	7	0.0002	0.29	0.00
C11H17orf75	6	0.0350	0.32	0.00
C13H16orf91	2	0.0129	0.35	0.00
C1H10orf88	4	0.0044	0.25	0.00
C2H1orf109	3	0.0200	0.30	0.00
C7H11orf52	2	0.0173	0.00	0.41
CA9	3	0.0064	0.00	0.25
CADM1	4	0.0509	7.34	3.94
CAMK2D	6	0.0069	0.00	0.34
CAMK2D	4	0.0335	0.00	0.25
CAMSAP1	2	0.0139	0.25	0.00
CARS	1	0.0055	0.00	0.38
CASZ1	1	0.0168	0.30	0.00
CBLN3	2	0.0380	0.00	0.32
CC2D2A	9	0.0362	0.26	0.00
CC2D2A	1	0.0507	5.83	3.65
CCM2	5	0.0020	0.26	0.00
CCPG1	3	0.0108	0.00	0.28
CDK5RAP1	1	0.0100	0.00	0.29

CEP68	6	0.0270	0.30	0.00
CHRNA5	1	0.0509	3.36	5.92
CHRNB1	3	0.0020	0.00	0.26
CHRNB1	2	0.0435	0.00	0.30
CIB2	1	0.0300	0.00	0.31
CMTM4	6	0.0261	0.26	0.00
COA3	3	0.0115	1.25	2.05
CPSF4	3	0.0006	0.26	0.00
CPSF4	1	0.0014	0.34	0.00
CPVL	1	0.0034	55.62	91.74
CTIF	2	0.0511	0.00	0.27
CYP4X1	1	0.0278	0.38	0.00
DCX	1	0.0494	0.32	0.00
DDX10	1	0.0275	0.00	0.27
DDX10	2	0.0366	0.26	0.00
DECR1	3	0.0003	0.00	0.27
DGKQ	8	0.0095	0.00	0.29
DGKQ	1	0.0242	0.00	0.27
DHX40	2	0.0505	7.32	4.77
DIDO1	1	0.0463	0.28	0.00
DLC1	5	0.0539	0.00	0.25
DNAJC5	4	0.0497	2.29	3.45
DTX1	1	0.0242	3.22	4.98
ENOX1	1	0.0362	0.00	0.29
ENOX1	5	0.0003	0.00	0.44
ENSECAG00000005643	5	0.0002	0.25	0.00
ENSECAG00000009736	1	0.0494	0.00	0.34
ENSECAG00000011489	1	0.0293	0.36	0.00
ENSECAG00000012514	1	0.0467	0.34	0.00
ENSECAG00000015480	1	0.0545	5.40	3.66
ENSECAG00000021143	1	0.0153	0.00	0.28
ENSECAG00000021996	1	0.0447	0.00	0.30
ENSECAG00000023794	8	0.0163	0.27	0.00
ENSECAT00000010846	5	0.0150	0.29	0.00
EPB41L4B	1	0.0374	23.34	15.62
EPHX4	1	0.0213	0.00	0.27
ERICH1	7	0.0152	0.29	0.00
ESYT3	1	0.0003	0.32	0.00
FAM117A	2	0.0436	0.00	0.26

FAM13A	3	0.0483	4.29	1.51
FAM13B	5	0.0076	0.00	0.25
FAM13B	6	0.0275	0.00	0.26
FAM13B	2	0.0007	0.25	0.00
FAM172A	6	0.0508	0.29	0.00
FAM177A1	1	0.0259	0.28	0.00
FAM83F	2	0.0144	0.00	0.29
FAXC	2	0.0304	0.00	0.28
FBXO2	2	0.0046	0.00	0.29
FBXO32	1	0.0036	9.55	4.52
FGD4	6	0.0014	0.44	0.00
FKBP9	1	0.0361	42.76	59.59
FOXN2	4	0.0027	0.00	0.27
FREM2	3	0.0507	0.00	0.27
FRMD6	1	0.0278	0.30	0.00
FRY	3	0.0038	0.32	0.00
FUZ	3	0.0021	0.38	0.00
GABPB2	3	0.0012	0.00	0.27
GAD2	1	0.0236	0.00	0.25
GAP43	1	0.0144	0.00	0.27
GLRB	2	0.0158	0.00	0.26
GMEB2	2	0.0194	0.28	0.00
HGSNAT	7	0.0109	0.00	0.27
HHAT	1	0.0069	0.00	0.30
HMGXB4	6	0.0231	0.25	0.00
HPSE2	3	0.0007	0.29	0.00
HSD3B7	4	0.0483	10.80	6.66
IDNK	5	0.0494	0.27	0.00
IFT140	3	0.0357	0.00	0.34
IGFN1	4	0.0396	0.37	0.00
IHH	1	0.0273	325.97	537.79
INPP4A	7	0.0101	0.26	0.00
INPP4A	4	<0.0001	0.32	0.00
INPP4A	8	0.0546	0.39	0.00
ITGB8	1	0.0323	16.35	10.82
ITPR1	1	0.0051	0.25	0.00
ITPR1	4	0.0026	0.27	0.00
ITPR1	3	0.0047	0.27	0.00
KBTBD4	4	0.0382	0.00	0.26

KDM5A	6	0.0323	0.00	0.29
KERA	3	0.0057	0.00	0.27
KIAA1217	11	0.0190	0.25	0.00
KIAA1217	14	0.0210	0.25	0.00
KIAA1217	7	0.0043	0.26	0.00
KIAA1217	12	0.0026	0.26	0.00
KIAA1217	20	0.0056	0.26	0.00
KIAA1217	16	0.0030	0.30	0.00
KIAA1217	10	0.0115	0.31	0.00
KIAA1551	4	0.0458	0.00	0.35
KIAA1551	2	0.0204	0.00	0.37
KLHL31	1	0.0497	0.34	0.00
KRT2	1	0.0498	0.26	0.00
KRT3	1	0.0144	0.00	0.51
LIMK2	2	0.0135	0.00	0.30
LMO7	3	0.0063	0.00	0.25
LMOD3	1	0.0183	0.27	0.00
LOC100059149	1	0.0227	0.00	0.25
LOC100060656	1	0.0163	0.00	0.29
LOC100060688	2	0.0013	0.00	0.40
LOC100062286	4	0.0029	0.30	0.00
LOC100070509	3	0.0161	0.31	0.00
LOC100072611	1	0.0185	0.00	0.54
LOC100146266	1	0.0113	0.33	0.00
LOC100629339	4	0.0095	0.25	0.00
LOC100629999	1	0.0214	0.26	0.00
LOC102147510	2	0.0367	0.37	0.00
LOC102147718	1	0.0456	0.27	0.00
LOC102148336	1	0.0354	0.25	0.00
LOC102148459	2	0.0135	0.25	0.00
LOC102148700	1	0.0280	0.27	0.00
LOC102148965	4	0.0259	0.30	0.00
LOC102149354	1	0.0038	0.26	0.00
LOC102149379	2	0.0209	0.26	0.00
LOC102149399	2	0.0168	0.00	0.28
LOC102149586	1	0.0341	0.36	0.00
LOC102150103	14	0.0153	0.00	0.27
LOC102150322	1	0.0115	0.00	0.25
LOC102150544	1	0.0110	0.25	0.00

LOC102150696	3	0.0174	0.00	0.34
LOC102150704	5	0.0507	0.27	0.00
LOC102150925	2	0.0436	0.27	0.00
LOC106781541	1	0.0227	0.37	0.00
LOC106781598	1	0.0217	0.00	0.27
LOC106781751	1	0.0498	0.00	0.30
LOC106781772	1	0.0133	0.35	0.00
LOC106781772	5	0.0132	0.35	0.00
LOC106782324	3	0.0083	0.30	0.00
LOC106782956	1	0.0498	0.00	0.25
LOC106783126	1	0.0494	0.25	0.00
LOC106783229	1	0.0144	0.46	0.00
LOC106783346	1	<0.0001	0.00	0.36
LOC111767496	4	0.0204	0.00	0.28
LOC111767592	1	0.0162	0.00	0.30
LOC111768080	2	0.0227	0.30	0.00
LOC111769102	1	0.0066	0.25	0.00
LOC111771658	1	0.0144	0.00	0.26
LOC111772046	3	0.0323	0.25	0.00
LOC111772731	3	0.0040	0.00	0.33
LOC111773132	1	0.0150	0.27	0.00
LOC111774626	1	0.0318	0.26	0.00
LRRC23	2	0.0257	0.27	0.00
LRRC57	1	0.0120	0.27	0.00
LRRFIP2	13	0.0183	0.63	0.00
LUC7L	2	0.0546	7.36	4.40
LY6G6F	1	0.0421	0.28	0.00
LYL1	1	0.0342	0.26	0.00
MAD2L1	1	0.0346	0.31	0.00
MAPK8IP3	7	0.0012	0.26	0.00
MAU2	1	0.0362	0.00	0.27
MBD1	29	0.0026	0.00	0.25
MBD1	23	0.0047	0.00	0.28
MCTP1	11	0.0473	0.27	0.00
MIER1	4	0.0214	0.36	0.00
MMAB	4	0.0122	0.00	0.32
MSI1	4	0.0012	0.00	0.31
MSS51	1	0.0169	0.27	0.00
MSS51	3	0.0507	0.31	0.00

MSTN	1	0.0135	0.00	0.33
MYO5B	4	0.0258	0.26	0.00
MYO5C	8	0.0324	0.00	0.26
MYO5C	7	0.0009	0.00	0.28
NAT9	3	0.0479	0.00	0.29
NCDN	1	0.0236	0.30	0.00
NDUFAF8	2	0.0439	0.49	0.71
NECAB3	5	0.0154	0.00	0.29
NEDD1	1	0.0277	15.76	10.43
NEDD4L	3	0.0217	8.67	6.04
NFKBIZ	6	0.0479	0.00	0.38
NGF	1	0.0163	0.25	0.00
NKD1	1	0.0519	6.37	18.31
NOXA1	4	0.0255	0.00	0.26
NTF4	1	0.0314	0.00	0.25
NUMA1	4	0.0152	0.00	0.26
P2RX7	1	0.0228	0.28	0.00
PAK1IP1	3	0.0357	0.00	0.25
PAPPA2	4	0.0340	0.00	0.29
PARP9	1	0.0298	12.03	9.94
PHF7	1	0.0161	0.25	0.00
PICALM	6	0.0139	0.25	0.00
PIGU	2	0.0030	0.00	0.26
PIH1D1	2	0.0306	0.00	0.27
PJVK	1	0.0096	0.29	0.00
PLCXD2	3	0.0374	0.00	0.37
PMEL	1	0.0532	0.33	0.00
POC1A	6	0.0309	0.00	0.46
POMT2	6	0.0152	0.00	0.26
PPARGC1A	2	0.0001	0.39	0.00
PPHLN1	7	0.0088	0.00	0.28
PPIP5K1	14	0.0075	0.25	0.00
PPIP5K1	9	0.0167	0.26	0.00
PPP1R9A	6	0.0209	0.33	0.00
PPP1R9A	2	0.0306	0.37	0.00
PPP1R9A	11	0.0028	0.37	0.00
PPP2R2C	1	0.0307	0.00	0.28
PRKDC	2	0.0143	0.30	0.00
PRKDC	3	0.0047	0.31	0.00

PRMT8	3	0.0099	0.00	0.32
PRRC2B	1	0.0104	1.67	0.00
PRSS36	3	0.0026	0.00	0.27
PRSS36	2	0.0025	0.00	0.40
PTPN7	1	0.0010	0.30	0.00
PXDNL	1	0.0075	0.27	0.00
PXK	9	0.0529	0.36	0.00
RAB3IL1	2	0.0352	0.00	0.30
RABGGTB	1	0.0343	0.00	0.35
RAE1	1	0.0435	0.34	0.00
RCC1	12	0.0305	0.00	0.25
RCC1	5	0.0229	0.35	0.00
RCC1L	2	0.0420	0.00	0.34
RCE1	2	0.0306	0.00	0.26
RGS12	1	0.0421	0.37	0.00
RHBDD1	6	0.0428	0.00	0.28
RHBDL3	2	0.0427	0.00	0.26
RICTOR	1	0.0163	0.29	0.00
RIN2	1	0.0413	18.27	14.20
RNF115	3	0.0191	0.00	0.37
RNF43	6	0.0493	5.19	9.67
RPL13A	1	0.0113	0.00	0.28
RSF1	4	0.0202	0.00	0.28
RTKN	9	0.0357	0.32	0.00
RXRG	1	0.0363	0.00	0.35
SAMD14	2	0.0426	0.00	0.29
SAT2	1	0.0303	6.80	10.84
SCUBE1	1	0.0027	0.25	0.00
SEC16A	3	0.0426	0.00	0.27
SENP6	2	0.0008	0.25	0.00
SEPSECS	2	0.0388	0.00	0.25
SERPINE2	2	0.0396	0.00	0.26
SERPINE2	5	0.0463	0.00	0.29
SGMS2	1	0.0485	0.34	0.00
SIPA1L2	3	0.0291	0.30	0.00
SLC20A2	3	0.0355	0.00	0.25
SLC25A39	1	0.0445	0.00	0.38
SLC3A1	8	0.0436	0.28	0.00
SMC3	3	0.0491	0.00	0.27

SNX24	4	0.0320	0.00	0.25
SOX5	4	0.0276	0.25	0.00
SP7	1	0.0004	0.30	0.00
SPTAN1	1	0.0277	0.00	0.26
SRD5A1	1	0.0260	0.60	0.00
STARD13	6	0.0481	0.25	0.00
SUCNR1	1	0.0402	0.52	1.08
SULF1	8	0.0497	0.25	0.00
SYNJ1	7	0.0003	0.30	0.00
SYNJ1	10	0.0383	0.30	0.00
SYNJ1	3	0.0257	0.30	0.00
SYNJ1	11	0.0230	0.31	0.00
SYNJ1	1	0.0006	0.33	0.00
TAF12	1	0.0352	5.61	6.12
TBC1D15	2	0.0249	3.37	2.57
TBC1D19	2	0.0497	0.37	0.00
TCERG1	1	0.0346	2.37	2.93
TELO2	2	0.0370	0.00	0.36
TIMMDC1	2	0.0161	0.25	0.00
TMCO6	3	0.0040	0.25	0.00
TMEM138	3	0.0450	0.00	0.30
TMEM41B	2	0.0509	0.00	0.30
TOPBP1	2	0.0472	0.29	0.00
TREM2	1	0.0376	0.00	0.40
TRIM35	3	0.0093	0.26	0.00
TRIM37	7	0.0174	0.26	0.00
TSPAN2	1	0.0115	0.00	0.27
TTC16	1	0.0362	2.69	2.35
USP4	8	0.0189	1.31	0.90
VPS45	2	0.0413	0.56	0.00
WASHC1	2	0.0278	0.00	0.26
WDR77	1	0.0303	0.00	0.38
XLOC_000012	3	0.0260	0.42	0.64
XLOC_000075	1	0.0115	0.00	0.31
XLOC_000954	1	0.0082	4.49	6.90
XLOC_001772	3	0.0053	0.00	0.25
XLOC_001885	1	0.0117	3.45	1.24
XLOC_002369	4	0.0132	0.33	0.00
XLOC_002591	2	0.0010	0.27	0.00

XLOC_003179	3	0.0021	0.00	0.32
XLOC_004031	2	0.0417	0.00	0.26
XLOC_004483	4	0.0238	0.25	0.00
XLOC_004483	9	0.0115	0.27	0.00
XLOC_004483	6	0.0082	0.30	0.00
XLOC_004694	1	0.0452	0.00	0.30
XLOC_004982	3	<0.0001	0.00	0.25
XLOC_006174	1	0.0225	0.00	0.25
XLOC_006787	3	<0.0001	0.00	0.26
XLOC_006831	3	0.0093	0.00	0.26
XLOC_006904	2	0.0433	3.20	5.18
XLOC_007091	1	0.0026	0.29	0.00
XLOC_007128	2	0.0194	0.33	0.00
XLOC_007342	5	0.0161	0.00	0.25
XLOC_007369	1	0.0115	0.34	0.00
XLOC_007526	5	0.0005	0.00	0.25
XLOC_007588	3	0.0108	0.40	0.00
XLOC_008405	3	0.0236	0.34	0.00
XLOC_008409	4	0.0104	0.43	0.60
XLOC_009507	4	0.0183	0.00	0.26
XLOC_009632	1	0.0428	0.00	0.31
XLOC_009653	2	0.0008	0.25	0.00
XLOC_010109	3	0.0251	0.00	0.28
XLOC_010618	3	0.0107	0.00	0.28
XLOC_011283	4	0.0022	0.25	0.00
XLOC_011283	1	0.0016	0.27	0.00
XLOC_013009	1	0.0055	13.15	4.56
XLOC_013893	2	0.0499	3.50	1.74
XLOC_014096	2	0.0028	0.26	0.00
XLOC_014405	1	0.0004	0.00	0.25
XLOC_014475	1	0.0102	0.26	0.00
XLOC_014724	1	0.0320	0.00	0.33
XLOC_015038	3	0.0414	0.26	0.00
XLOC_015554	1	0.0278	0.00	0.48
XLOC_015681	1	0.0445	2.29	3.25
XLOC_015835	3	0.0270	0.00	0.44
XLOC_017957	2	0.0003	0.33	0.00
XLOC_018012	7	0.0284	0.00	0.29
XLOC_018040	1	0.0117	0.00	0.27

XLOC_018361	2	0.0519	9.98	3.58
XLOC_018403	4	0.0053	0.00	0.26
XLOC_018652	3	0.0006	0.00	0.26
XLOC_019600	2	0.0100	0.00	0.29
XLOC_020617	1	0.0186	0.00	0.33
XLOC_021138	5	0.0193	0.49	0.00
XLOC_021335	1	0.0300	0.31	0.46
XLOC_022209	1	0.0200	0.25	0.00
XLOC_023597	4	0.0424	0.29	0.00
XLOC_023658	1	0.0529	0.00	0.32
XLOC_023753	2	0.0047	0.27	0.00
XLOC_024260	1	0.0282	0.35	0.00
XLOC_024271	4	0.0139	0.00	0.30
XLOC_024418	1	0.0056	0.26	0.00
XLOC_024581	4	0.0042	0.37	0.00
XLOC_025862	3	0.0026	0.40	0.00
XLOC_025863	2	0.0130	0.00	0.30
XLOC_025863	1	0.0523	0.00	0.33
XLOC_026316	1	0.0204	0.26	0.00
XLOC_026647	3	0.0225	0.31	0.00
XLOC_026781	5	0.0161	0.26	0.00
XLOC_026969	4	<0.0001	0.00	0.26
XLOC_027188	4	0.0088	0.00	0.27
XLOC_027188_4	1	0.0086	0.40	0.00
XLOC_027449	1	0.0005	0.25	0.00
XLOC_027498	2	0.0183	0.00	0.35
XLOC_028199	2	0.0083	0.25	0.00
XLOC_029378	3	0.0306	0.00	0.41
XLOC_029475	5	0.0028	0.25	0.00
XLOC_029558	2	0.0449	0.00	0.46
XLOC_030032	3	0.0064	0.25	0.00
XLOC_030032	2	0.0525	0.26	0.00
XLOC_030744	2	0.0091	0.27	0.00
XLOC_030962	3	0.0302	0.00	0.27
XLOC_031404	1	0.0507	0.27	0.00
XLOC_032131	2	0.0058	0.28	0.00
XLOC_032343	1	0.0115	0.00	0.26
XLOC_032540	1	0.0266	12.31	16.28
XLOC_032586	3	0.0424	0.00	0.64

XLOC_032777	3	0.0119	0.00	0.33
XLOC_033105	6	0.0051	0.27	0.00
XLOC_033552	5	0.0023	0.00	0.25
XLOC_034260	2	0.0520	0.00	0.36
XLOC_034306	3	0.0226	0.29	0.00
XLOC_034312	7	0.0341	0.25	0.00
XLOC_034360	2	<0.0001	0.00	0.25
XLOC_034486	1	0.0051	0.27	0.00
XLOC_034762	3	0.0018	0.00	0.27
XLOC_034895	2	0.0092	0.00	0.27
XLOC_034979	1	0.0397	3.22	1.81
XLOC_035199	3	0.0069	0.80	1.11
XLOC_035334	2	0.0295	0.25	0.00
XLOC_035426	3	0.0278	0.25	0.00
XLOC_035758	1	0.0483	0.00	0.35
XLOC_035999	2	0.0211	0.00	0.33
XLOC_036469	2	0.0008	0.00	0.27
XLOC_036605	1	0.0188	0.28	0.00
XLOC_036766	2	0.0146	0.54	0.37
XLOC_037510	2	0.0476	9.78	15.11
XLOC_037598	1	0.0479	0.36	0.00
XLOC_037639	2	0.0012	0.00	0.37
XLOC_038302	3	0.0195	0.37	0.00
XLOC_038310	2	0.0135	0.25	0.00
XLOC_038825	2	0.0267	0.00	0.30
XLOC_038862	3	0.0278	0.00	0.40
XLOC_039539	1	0.0096	0.00	0.26
XLOC_039629	3	0.0270	0.28	0.00
XLOC_039967	1	0.0069	0.26	0.00
XLOC_040085	1	0.0282	0.34	0.00
XLOC_040434	1	0.0413	0.00	0.38
XLOC_040976	1	0.0194	0.00	0.27
XLOC_041183	2	0.0074	0.28	0.00
XLOC_041240	7	0.0276	21.15	9.79
XLOC_041243	1	0.0051	0.33	0.00
XLOC_041327	6	0.0037	0.00	0.28
XLOC_041992	7	0.0208	0.31	0.00
XLOC_041999	3	0.0217	0.25	0.00
XLOC_042012	1	0.0077	0.25	0.00

XLOC_042824	1	0.0145	0.00	0.35
XLOC_043409	3	0.0144	0.00	0.30
XLOC_043409	2	0.0205	0.00	0.32
XLOC_043409	4	0.0205	0.00	0.36
XLOC_043556	11	0.0052	0.25	0.00
XLOC_046918	1	0.0364	0.00	0.27
XLOC_046919	1	0.0005	0.00	0.26
XLOC_047155	3	0.0003	0.00	0.26
XLOC_047417	4	0.0161	0.00	0.28
XLOC_047457	3	0.0480	0.00	0.25
ZBTB17	1	0.0077	0.47	0.00
ZBTB32	3	0.0255	0.00	0.28
ZBTB8A	3	0.0330	0.25	0.00
ZBTB8OS	2	0.0001	0.28	0.00
ZBTB8OS	7	<0.0001	0.25	0.00
ZC3H18	2	0.0353	3.13	4.34
ZNF133	4	0.0041	0.00	0.32
ZNF135	7	0.0460	0.38	0.00
ZNF346	4	0.0298	0.00	0.29
ZNF367	3	0.0049	0.30	0.00
ZNF584	5	0.0098	0.00	0.28
ZNF624	1	0.0195	0.25	0.00
ZNF691	4	0.0417	0.00	0.27
ZNF821	3	0.0454	0.00	0.30
ZSCAN23	2	0.0105	0.00	0.26
ZZZ3	2	0.0497	0.28	0.00

Appendix IV: Significant Genes and Transcript Variants Identified in Endometrium on Day 13

Gene Symbol	Transcript Number	Day 13 P-Values	Day 13P+ Avg. RPKM	Day 13NP Avg. RPKM
ACAD10	5	0.0539	0.00	0.27
ACKR1	1	0.0258	0.00	0.32
ACPP	1	0.0046	0.00	0.29
ADAR	1	0.0362	0.00	0.28
AKAP7	3	0.0125	0.00	0.26
AKTIP	4	0.0005	0.25	0.00
AKTIP	3	0.0341	0.26	0.00
ALPL	2	0.0003	0.00	0.26
ANKRD13A	2	0.0173	0.00	0.53
ARHGAP9	4	0.0100	0.00	0.32
ARHGEF2	6	<0.0001	0.29	0.00
ARHGEF2	5	0.0237	0.31	0.00
ARMC10	2	0.0299	0.00	0.25
ATM	2	0.0091	0.00	0.25
ATP6V0A4	2	0.0017	0.00	0.28
ATP6V1D	2	0.0502	0.00	0.34
B4GALT7	2	<0.0001	0.25	0.00
BBOX1	1	0.0118	0.00	0.29
BLVRA	3	0.0068	0.00	0.27
BPHL	3	0.0233	0.00	0.26
BRCA2	4	0.0249	0.25	0.00
C10H19orf12	4	0.0003	0.28	0.00
C11H17orf75	7	0.0339	0.31	0.38
C1H14orf28	4	0.0265	0.00	0.28
CADM3	3	0.0139	0.31	1.16
CAPN11	3	0.0108	0.00	0.27
CASP9	2	0.0315	0.00	0.26
CASZ1	1	0.0255	0.00	0.27
CASZ1	5	0.0542	0.00	0.25
CC2D2A	9	0.0319	0.00	0.27
CC2D2A	3	0.0523	0.00	0.28
CCDC15	5	0.0120	0.30	0.00
CCDC159	2	0.0034	0.31	0.00
CD40LG	1	0.0546	0.26	0.00

CHCHD8	2	0.0494	0.26	0.00
CIDEC	1	0.0097	0.00	0.36
CLCN3	1	0.0021	0.00	0.32
CLEC12A	1	0.0309	0.00	0.27
COMP	4	0.0280	0.00	0.26
CPSF2	3	0.0214	5.34	5.91
CPT1C	1	0.0036	0.00	0.35
CPVL	1	0.0275	52.81	32.98
CREB3L1	3	0.0093	0.35	0.00
CSNK1A1	2	0.0520	0.38	0.00
CST7	1	0.0007	0.33	0.00
DAGLA	2	0.0321	0.29	0.00
DDC	1	0.0356	0.34	0.00
DDX51	3	0.0523	1.02	1.48
DGKQ	4	0.0303	0.00	0.26
DHX40	2	0.0496	7.52	4.95
DIS3L2	5	0.0021	0.00	0.27
DLC1	5	0.0310	0.00	0.29
DNASE1L1	4	0.0070	2.30	1.38
DTNBP1	3	0.0217	1.30	0.52
ECHDC2	5	0.0188	0.00	0.27
EDRF1	1	0.0088	0.34	0.00
ENOX1	1	0.0498	0.00	0.27
ENTPD2	3	0.0113	0.28	0.00
EP400	3	0.0046	0.26	0.00
EPB41	5	0.0227	0.25	0.39
EPB41	10	0.0278	0.51	0.70
EPS15L1	2	0.0211	0.00	0.31
ETNPPL	1	0.0082	0.32	0.00
FAM13B	2	0.0008	0.00	0.25
FAM13B	6	0.0214	0.00	0.28
FAM13B	5	0.0067	0.26	0.00
FBXO39	4	0.0003	0.00	0.32
FEZ2	1	0.0328	0.00	0.25
FGD6	3	0.0277	0.26	0.00
FGD6	2	0.0205	0.30	0.00
FIGN	8	0.0058	0.00	0.26
FOXP3	1	0.0161	0.25	0.00
GABRA3	4	0.0227	0.30	0.00

GAS2	6	0.0018	0.25	0.00
GAS2	5	0.0061	0.27	0.00
GAS2	3	0.0188	0.27	0.00
GAS2	2	0.0482	0.30	0.00
GLI3	1	0.0269	0.32	0.00
GRAMD1B	7	0.0195	0.00	0.32
GREB1L	1	0.0192	0.96	0.79
HDHD5	5	0.0193	0.00	0.25
HGF	6	0.0494	0.00	0.39
HGF	5	0.0533	0.00	0.41
HMGXB3	2	0.0410	2.19	3.56
HMGXB4	7	0.0473	0.00	0.26
HRH1	2	0.0479	0.00	1.10
HSF2BP	4	0.0333	0.29	0.00
HSPA12B	4	0.0005	0.00	0.27
IAPP	2	0.0304	0.52	0.00
IDS	7	0.0046	0.00	0.33
IL33	5	0.0143	0.00	0.25
IL34	4	0.0212	0.00	0.30
IPO8	1	0.0114	0.00	0.26
ISG20	3	0.0145	0.00	0.44
KANSL3	2	0.0227	0.00	0.26
KCNK17	1	0.0175	0.00	0.28
KCTD6	3	0.0415	0.32	0.00
KDM5A	6	0.0505	0.25	0.00
KIAA1217	7	0.0026	0.30	0.00
KIF25	1	0.0456	0.00	0.26
KLHL23	2	0.0294	0.27	0.00
KLHL26	2	0.0318	0.00	0.30
LHPP	3	0.0308	0.00	0.27
LMO7	6	0.0001	0.00	0.26
LOC100052339	4	0.0136	0.27	0.00
LOC100053296	3	0.0064	0.40	0.00
LOC100059149	1	0.0063	0.00	0.37
LOC100059529	2	0.0007	0.39	0.00
LOC100062286	3	0.0479	0.00	0.38
LOC100064097	1	0.0249	0.00	0.28
LOC100065011	2	0.0026	0.00	0.34
LOC100065350	2	0.0018	0.27	0.00

LOC100065350	4	0.0001	0.30	0.00
LOC100066125	1	0.0145	0.00	0.28
LOC100066811	1	0.0075	0.00	0.28
LOC100067801	4	0.0108	0.27	0.00
LOC100067882	3	0.0007	0.00	0.26
LOC100068447	1	0.0161	0.00	0.27
LOC100070604	1	0.0121	0.31	0.00
LOC100072605	1	0.0237	0.00	0.25
LOC100146266	1	0.0144	0.00	0.30
LOC100147145	2	0.0223	0.00	0.25
LOC100629739	2	0.0270	0.00	0.52
LOC102147384	1	0.0006	0.29	0.00
LOC102147614	2	0.0069	0.00	0.32
LOC102147722	3	0.0185	0.00	0.33
LOC102147836	2	0.0514	0.00	0.33
LOC102148188	1	0.0328	0.34	0.00
LOC102148316	1	0.0402	0.00	0.27
LOC102148336	2	0.0233	0.25	0.00
LOC102148371	2	0.0280	0.00	0.35
LOC102148467	1	0.0013	0.36	0.00
LOC102148700	1	0.0278	0.27	0.00
LOC102148712	2	0.0143	0.00	0.26
LOC102148781	2	0.0008	0.00	0.28
LOC102148818	1	0.0388	2.32	1.13
LOC102148965	4	0.0070	0.00	0.44
LOC102149237	1	0.0230	0.27	0.00
LOC102149586	1	0.0549	0.00	0.31
LOC102150332	1	0.0241	0.26	0.00
LOC102150421	2	0.0262	0.31	0.00
LOC102151014	4	0.0191	0.00	0.46
LOC106781249	1	0.0036	0.00	0.29
LOC106781611	1	0.0097	0.35	0.00
LOC106781772	5	0.0104	0.00	0.38
LOC106782324	3	0.0138	0.00	0.26
LOC106782691	1	0.0456	0.44	0.00
LOC106783193	1	0.0507	0.25	0.00
LOC106783234	1	0.0184	0.30	0.00
LOC111768319	1	0.0046	0.29	0.00
LOC111774591	5	0.0168	0.00	0.28

LRFN5	4	0.0241	0.28	0.00
LRRC40	2	0.0168	0.00	0.34
LY49F	1	0.0239	0.27	0.00
LYRM1	6	0.0262	0.00	0.25
LYRM1	5	0.0303	0.41	0.00
MAML1	2	0.0010	0.26	0.00
MBD1	17	0.0036	0.00	0.40
MDFI	1	0.0014	0.00	0.46
MECOM	2	0.0519	0.00	0.70
METTL3	3	0.0036	0.00	0.26
MMAB	4	0.0166	0.00	0.29
MORN4	1	0.0064	0.30	0.00
MPHOSPH8	5	0.0032	0.28	0.00
MSANTD1	1	0.0038	0.28	0.00
MTIF2	1	0.0484	0.00	0.47
MYNN	3	0.0105	0.00	0.26
MYO5B	4	0.0291	0.00	0.25
NARFL	3	0.0132	0.00	0.27
NAT9	2	0.0024	0.00	0.26
NCDN	1	0.0284	0.00	0.28
NFRKB	3	0.0235	0.27	0.00
NFX1	2	0.0277	0.00	0.56
NLN	3	0.0340	0.00	0.29
NMRAL1	3	0.0051	0.00	0.29
ENSECAG00000003160	5	0.0153	0.00	0.27
ENSECAG00000003160	7	0.0006	0.00	0.27
ENSECAG00000003160	9	0.0372	0.00	0.26
ENSECAG00000003160	3	0.0437	0.75	0.41
ENSECAG00000008481	6	0.0328	0.00	0.25
ENSECAG00000009404	1	0.0476	0.44	0.00
ENSECAG00000017970	6	0.0432	0.00	0.39
ENSECAG00000023794	1	0.0198	0.25	0.00
ENSECAG00000023794	8	0.0173	0.26	0.00
ENSECAG00000023794	9	0.0377	0.27	0.00
ENSECAG00000012818	1	0.0003	0.00	0.25
NPR1	1	0.0452	0.00	0.32
NUBP2	3	0.0001	0.29	0.00
NUP160	5	0.0116	0.00	0.26
NXPE3	3	0.0266	0.00	0.30

OSMR	3	0.0056	0.00	0.25
P2RX7	1	0.0333	0.25	0.00
PAK1IP1	3	0.0195	0.00	0.30
PARP9	1	0.0472	11.05	12.88
PC	1	0.0034	9.09	9.62
PDSS1	1	<0.0001	0.27	0.00
PER2	2	0.0310	0.28	0.00
PEX11G	3	0.0280	0.27	0.00
PFKFB2	4	0.0488	0.00	0.30
PHC1	3	0.0428	0.00	0.28
PIGU	2	0.0009	0.00	0.36
PIH1D1	2	0.0173	0.00	0.32
PJVK	1	0.0045	0.00	0.35
PKD2	6	0.0268	0.00	0.28
PLPP3	2	0.0037	0.00	0.34
PLXNB3	2	0.0207	0.00	0.37
POC1A	4	0.0480	0.00	0.25
PPARA	4	0.0519	0.96	0.40
PPHLN1	1	0.0397	0.00	0.31
PPP1R9A	6	0.0152	0.37	0.00
PPP1R9A	11	0.0029	0.37	0.00
PPP2R5E	1	0.0030	0.30	0.00
PRPF39	6	0.0456	0.00	0.25
PRPF39	3	0.0130	0.25	0.00
PRRX1	5	0.0302	0.00	0.28
PSD	1	0.0433	0.31	0.00
PSD	2	0.0479	0.32	0.00
PTRH2	1	0.0116	0.26	0.00
PTS	3	0.0134	0.00	0.34
PXDNL	1	0.0100	0.25	0.00
RAB19	4	0.0006	0.29	0.00
RADIL	1	0.0412	0.40	0.00
RALGPS2	4	0.0094	0.32	0.00
RALGPS2	6	0.0291	0.33	0.00
RAPGEF1	3	<0.0001	0.00	0.30
RAPGEF1	7	0.0115	0.00	0.26
RHBDF2	2	0.0426	0.00	0.27
RIF1	1	0.0483	0.00	0.26
RMI1	6	0.0479	0.27	0.00

RNF115	3	0.0395	0.00	0.30
RNF180	1	0.0143	0.26	0.00
RRAS2	4	0.0427	0.00	0.25
RRNAD1	3	0.0237	0.00	0.25
RSRP1	2	0.0359	0.00	0.36
RUSC2	1	0.0494	3.94	5.98
S1PR2	5	0.0068	0.29	0.00
SAP18	2	0.0058	0.26	0.31
SCAPER	7	0.0323	0.00	0.25
SETX	3	0.0014	0.00	0.29
SGTB	1	0.0040	0.00	0.41
SH3GL3	1	0.0016	0.00	0.29
SHISAL2B	1	0.0135	0.00	0.26
SIK1	7	0.0026	0.25	0.00
SKP2	1	0.0092	0.00	0.30
SLC12A9	2	0.0168	2.63	1.93
SLC25A45	1	0.0001	0.50	0.00
SLC27A5	1	0.0239	0.00	0.36
SLC30A2	1	0.0113	0.00	0.31
SLC36A4	4	0.0143	0.33	0.50
SLC52A2	2	0.0002	0.00	0.42
SLC6A9	1	0.0007	0.00	0.25
SLC7A6OS	3	0.0028	0.00	0.26
SMTN	1	0.0392	2.45	3.04
SNX21	1	0.0103	0.31	0.00
SORBS2	2	0.0026	0.00	0.29
SPDYC	1	0.0337	0.59	0.00
SPIDR	7	0.0499	0.00	0.25
SPTAN1	1	0.0161	0.30	0.00
SRF	2	0.0093	0.00	0.30
TAF15	2	0.0111	0.88	1.32
TATDN3	6	0.0394	0.26	0.00
TFAP2C	1	0.0346	0.00	0.52
TICAM1	2	0.0161	0.00	0.27
TM4SF19	2	0.0091	0.27	0.00
TMCC1	3	0.0266	0.25	0.00
TMEM130	6	0.0024	0.40	0.00
TMEM35A	1	0.0506	0.32	0.00
TMEM60	2	0.0300	10.80	8.28

TMEM68	14	0.0091	0.00	0.25
TMOD1	2	0.0082	0.00	0.37
TNFSF13B	6	0.0204	0.00	0.35
TOMM40L	1	0.0009	0.00	0.27
TOMM40L	4	0.0098	0.00	0.28
TOMM40L	2	0.0125	0.00	0.25
TRERF1	10	0.0517	0.00	0.28
TRIL	3	0.0014	0.00	0.35
TRIM27	5	0.0162	0.00	0.28
TRIM37	11	0.0259	0.00	0.25
TRIM66	3	0.0103	0.28	0.00
TRNAU1AP	5	0.0034	0.29	0.00
TSNARE1	8	0.0001	0.25	0.00
TSNARE1	6	<0.0001	0.29	0.00
TTC8	1	0.0116	0.00	0.30
TVP23A	1	0.0168	0.00	0.33
TWISTNB	2	0.0386	0.25	0.00
USP5	1	0.0507	7.52	9.50
VAV2	2	0.0542	0.00	0.27
VDR	1	0.0183	0.00	0.25
VEPH1	5	0.0051	0.27	0.00
VPS51	2	0.0052	0.00	0.41
WEE2	1	0.0093	0.32	0.00
XLOC_000012	3	0.0324	0.59	0.39
XLOC_001079	4	0.0024	0.31	0.00
XLOC_001772	3	0.0036	0.00	0.27
XLOC_001772	5	0.0079	0.00	0.39
XLOC_001885	2	0.0002	0.00	0.25
XLOC_002945	1	0.0152	0.00	0.25
XLOC_003905	1	0.0498	0.46	0.34
XLOC_004838	1	0.0410	0.25	0.00
XLOC_004961	4	0.0489	0.00	0.31
XLOC_005555	6	0.0026	0.26	0.00
XLOC_005555	1	0.0291	0.26	0.00
XLOC_005555	4	0.0050	0.26	0.00
XLOC_006210	3	0.0115	0.27	0.00
XLOC_008309	1	0.0105	0.36	0.00
XLOC_009381	3	0.0245	0.00	0.29
XLOC_009981	1	0.0233	0.00	0.30

XLOC_011283	1	0.0008	0.00	0.32
XLOC_011283	4	0.0009	0.00	0.31
XLOC_011420	1	0.0527	0.00	0.26
XLOC_012578	1	0.0481	0.00	0.34
XLOC_013045	1	0.0518	0.00	0.49
XLOC_013697	2	0.0047	0.32	0.37
XLOC_013738	1	0.0093	0.00	0.27
XLOC_014096	2	0.0373	0.52	0.39
XLOC_014406	3	0.0108	0.34	0.00
XLOC_015038	3	0.0374	0.27	0.00
XLOC_015835	1	0.0022	0.00	0.28
XLOC_016102	1	0.0161	0.29	0.00
XLOC_018609	1	0.0110	0.29	0.00
XLOC_019177	5	0.0007	0.00	0.26
XLOC_019370	5	0.0436	0.00	0.31
XLOC_019570	3	0.0004	0.00	0.27
XLOC_019669	2	0.0113	0.27	0.00
XLOC_021589	1	0.0335	0.27	0.00
XLOC_022284	2	0.0461	0.28	0.00
XLOC_022512	3	0.0026	0.00	0.30
XLOC_022596	1	0.0090	0.00	0.37
XLOC_022773	3	0.0091	0.00	0.26
XLOC_023274	5	0.0062	0.33	0.00
XLOC_023543	1	0.0272	0.85	0.48
XLOC_023597	4	0.0372	0.30	0.00
XLOC_024367	1	0.0407	0.00	0.29
XLOC_024478	1	0.0161	0.00	0.32
XLOC_024606	2	0.0512	0.00	0.33
XLOC_024672	1	0.0456	0.25	0.00
XLOC_025536	1	0.0509	0.53	0.00
XLOC_025862	3	0.0119	0.25	0.00
XLOC_026065	1	0.0052	0.00	0.27
XLOC_026781	5	0.0139	0.27	0.00
XLOC_026868	1	0.0286	1.27	1.96
XLOC_027498	2	0.0479	0.26	0.00
XLOC_028186	1	0.0476	0.00	0.42
XLOC_028951	1	0.0168	0.00	0.28
XLOC_030962	3	0.0192	0.00	0.31
XLOC_031952	4	0.0006	0.30	0.00

XLOC_031966	2	0.0520	0.00	0.29
XLOC_033202	3	0.0154	0.26	0.00
XLOC_033617	1	0.0456	0.00	0.26
XLOC_034312	7	0.0135	0.00	0.33
XLOC_035348	3	0.0026	0.00	0.26
XLOC_035640	2	0.0148	0.26	0.00
XLOC_036695	4	0.0303	0.00	0.26
XLOC_036695	1	0.0302	0.26	0.00
XLOC_036726	1	0.0252	0.00	0.26
XLOC_036786	6	0.0237	0.00	0.30
XLOC_037880	2	0.0075	0.00	0.25
XLOC_037992	7	0.0100	0.00	0.25
XLOC_039656	1	0.0100	0.30	0.00
XLOC_040085	3	0.0125	0.00	0.26
XLOC_041327	6	0.0027	0.00	0.31
XLOC_041343	6	0.0370	0.00	0.29
XLOC_041734	1	0.0507	0.00	0.27
XLOC_041999	3	0.0236	0.25	0.00
XLOC_042558	1	0.0091	0.25	0.00
XLOC_042778	1	0.0027	0.00	0.43
XLOC_042890	2	0.0161	0.00	0.27
XLOC_043159	1	0.0003	0.00	0.27
XLOC_045789	1	0.0414	0.28	0.00
XLOC_045920	4	0.0236	0.00	0.42
XLOC_046470	1	0.0361	0.00	0.26
XLOC_046831	8	0.0455	0.00	0.26
XLOC_047042	3	0.0139	0.00	0.37
XLOC_047216	3	0.0498	0.26	0.00
XLOC_047417	4	0.0117	0.31	0.00
XLOC_047594	4	0.0546	0.00	0.38
ZBTB8A	3	0.0358	0.00	0.25
ZC3H18	2	0.0242	2.36	3.71
ZDHHC19	3	0.0507	0.38	0.00
ZFP41	3	0.0058	0.00	0.35
ZMYM1	5	0.0018	0.00	0.26
ZNF134	2	0.0026	0.00	0.26
ZNF18	3	0.0131	0.27	0.00
ZNF286A	3	0.0035	0.00	0.48
ZNF367	1	0.0001	0.00	0.32

ZNF384	5	0.0221	0.00	0.25
ZNF558	3	0.0533	0.00	0.49
ZNF608	2	0.0135	0.25	0.00
ZNF624	6	0.0354	0.31	0.00
ZNF691	4	0.0463	0.26	0.00
ZNF692	4	0.0010	0.29	0.00
ZNF772	1	<0.0001	0.37	0.00
ZNF784	4	0.0097	0.00	0.31