THESIS

EXOSOMAL MICRORNAS AS A BIOLOGICAL MARKER OF MATERNAL RECOGNITION OF PREGNANCY IN THE MARE

Submitted by

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ABSTRACT

EXOSOMAL MICRORNAS AS A BIOLOGICAL MARKER OF MATERNAL RECOGNITION OF PREGNANCY IN THE MARE

Pregnancy maintenance mandates a signal from the embryo or by-product of the presence of the embryo in the uterus of the mare. This signal must occur between days 12 and 16 post-ovulation. A mobile conceptus prior to day 16 is obligatory for maintenance of pregnancy. However other communication between the embryo and suppression of endometrial prostaglandin F2α has been thoroughly researched yet remains enigmatic. Exosomes, cell secreted vesicles of endocytic origin, have been associated in a wide variety of important physiological cell-to-cell communication. Exosomes are secreted from numerous cell types and have been isolated from a variety of biological samples including; urine, breast milk, serum, plasma, and semen. Exosome presence in peripheral fluids makes them attractive, non-invasive biomarkers. Exosomes contain specific cargo including messengerRNA (mRNA), microRNA (miRNA), and proteins. Exosomes containing miRNA have been implicated in normal and complicated pregnancies as well as signature miRNAs associated with pregnancy status in women. We hypothesized that exosomal miRNA profiles between pregnant and non-pregnant mares would differ due to the changing uterine environment during maternal recognition of pregnancy. To test this hypothesis exosomes were isolated from serum samples in a simple cross-over design before (n=8) during (n=5) and after (n=3) maternal recognition of pregnancy. Pregnancy was confirmed using trans-rectal ultrasonography and embryo collection. Exosome isolation was

performed using Exoquick™ (System Biosciences, Inc.) and total RNA was isolated using TriReagent BD (Molecular Research Center, Inc.). The first experiment profiled 380 human miRNAs to identify differences by quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) between pregnant and non-pregnant mares after maternal recognition of pregnancy (n=3) on day 16 post-ovulation. For the second experiment, we designed 340 equine miRNAs primers to assess differences with qRT-PCR between pregnancy status. Samples from day 9 and 11 (n=8) and day 13 (n=5) were profiled to represent time points before and during maternal recognition of pregnancy in the mare. Pathway analysis of significantly different ($P<0.05$) miRNAs was performed employing Diana mirPath software.

qRT-PCR identified six miRNAs differentially expressed on day 16 post-ovulation; two only in pregnant, two only in non-pregnant, and two significantly up-regulated in non-pregnant mares. qRT-PCR analysis revealed one, four, and seven miRNAs differentially expressed on days 9, 11, and 13 respectively with only day 9 samples present at higher levels in samples from pregnant mares. miRNAs significantly different in day 16 samples were not found to be significant in earlier time points. Pathway analysis indicated focal adhesion molecules as the primary pathway of miRNAs that were expressed higher in non-pregnant samples on days 11 and 13. Therefore we conclude that exosomal miRNAs from serum do demonstrate differential expression, and can be used as biomarkers for pregnancy and maternal recognition of pregnancy. Predicted targets of these miRNAs may be biologically relevant for determining the signal for maternal recognition of pregnancy in the mare.

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Introduction

Pregnancy must strike a delicate balance of tolerance and support, to protect both the fetus and the mother. The mechanisms at play to sustain fetal life are intricately linked to alerting the female that she is pregnant while not raising enough alarm to cause complications in pregnancy. The signal that informs the mother that she is pregnant thereby extending the normal life of the ovarian corpus luteum (CL) is referred to as maternal recognition of pregnancy. Normal pregnancy in the horse has been well reserached but the mechanism underlying certain events in pregnancy in the context of embryo-uterine interaction, precise mechanisms of CL maintenance and uterine function have not yet been clarified. Embryo loss in early pregnancy in the mare (ovulation [day 0]-day 40) may be a result of improper signaling. Early embryonic loss has a significant cost in equine reproduction. Understanding the signal to maternal recognition of pregnancy may aid in curbing this loss. Signals *in vitro* and *in vivo* have been demonstrated by the exposure of cells to exosomes, small vesicles of endocytic origin. These exosomes are considered bioactive and contain various proteins, mRNAs, and miRNAs. miRNAs are known to degrade or silence the translation of mRNAs into protein by binding to the 3'UTR of mRNAs. miRNAs have been described and implicated in various biological and physiologic pathways including cancer, prion diseases, and most importantly to this study; pregnancy. miRNAs contained in exosomes may act as a signaling system in the mare during pregnancy due to tight regulation of protein expression and careful balance of symbiosis.

Early Pregnancy in the Mare

Fusion of the two pronuclei defines fertilization. Cell cleavage and formation of the embryo allow it to reach the 2, 4, and 8-cell stages about 24, 48, and 72 hours after ovulation respectively (Bezard *et al*. 1989). The embryo progresses through the morula and blastocyst stages from 4-5 and 5-6 days respectively (McKinnon and Voss, 2005). Figure 1 illustrates describes different cell stages. On day 6 of gestation the embryo reaches a threshold of prostaglandinE_2 (PGE_2) production and drives oviductal patency and transport of the embryo into the uterus (Weber *et al*. 1991). The blastocyst is defined by cell differentiation of the inner cell mass (ICM) separated by the fluid-filled blastocoel and surrounding trophoblast cells. The glycoprotein-rich capsule underneath the zona pellucida forms on day 6-7 after ovulation (Tremoleda *et al.* 2003) and the zona is subsequently shed. The capsule is a structure unique to equine and leporine pregnancies and complicates understanding of early embryonic loss and cell-cell signaling in the embryo-maternal interface (Betteridge 1989 and Betteridge 2007).

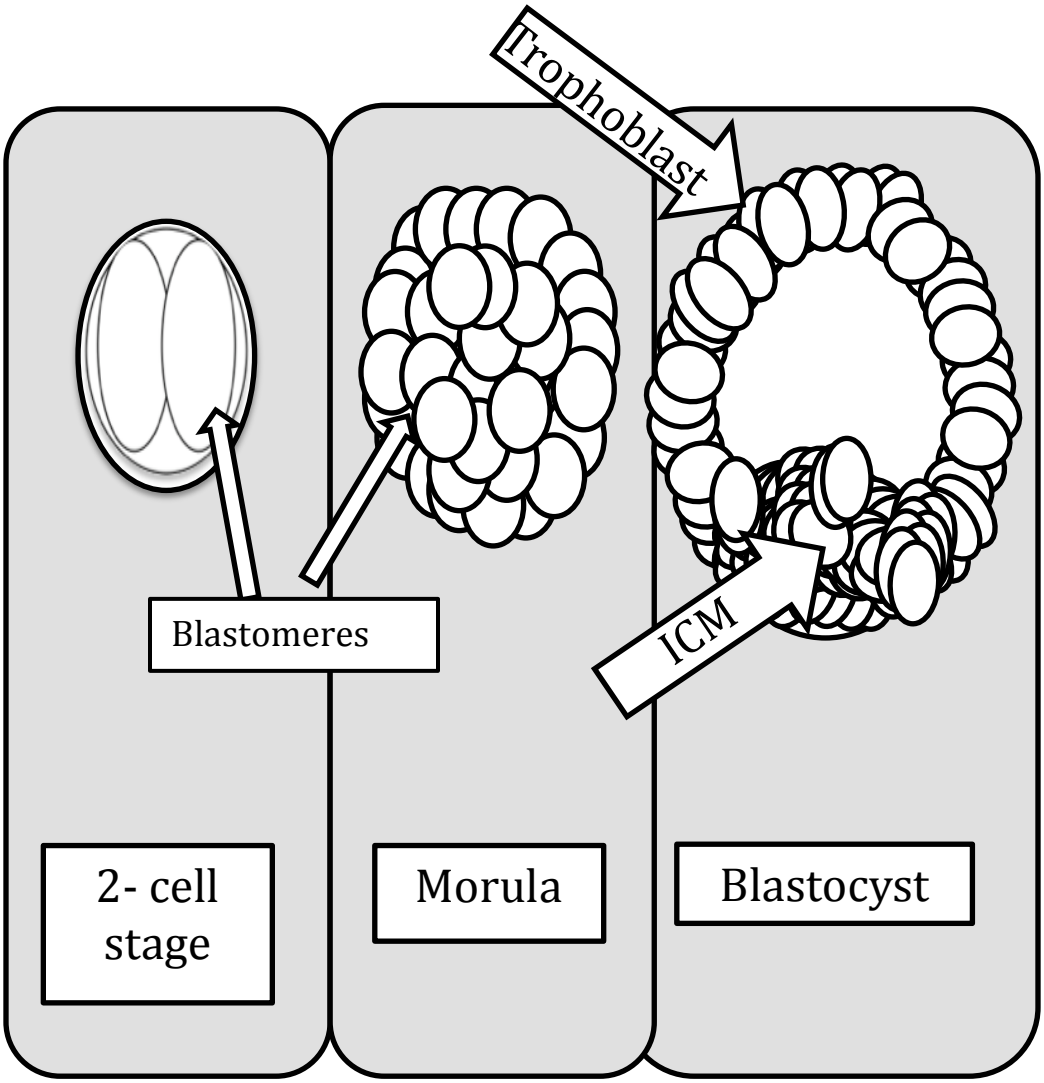

Figure 1: Depiction of cell orientation in early embryology

Figure 1: The above displays an illustrated view of the appearance of embryos at different stages of development. The 2-cell to 32 cell stage have a similar appearance inside the zona pellucida, while the morula has a characteristic 'scalloped' appearance. Blastocysts are characterized by the ICM that will become the embryo proper and the differentiating trophoblast cells.

Trans uterine migration of the embryo, maximal between days 11 and 14, has been clearly described in early pregnancy in the mare and is necessary for maintenance of

pregnancy (Ginther 1983, McDowell 1988). Impeding conceptus mobility results in embryo death due to luteolysis. Growth of the embryo is very rapid between days 11 and 16 (Ginther 1998). This growth is accompanied by morphological changes and the development. Fixation of the embryo in the caudal end of either horn of the uterus occurs on day 16-17. Correct orientation of the embryo with the embryo proper is oriented caudally ("down") and the yolk sac oriented cranially ("up") occurs around day 18. The complete formation of the amnion follows fixation. The protective capsule is lost around day 20 (McKinnon *et al*. 2011). Capsule rupture seems to coincide with a developing allantois. The allantois completely surrounds the embryo and amnion by day 28 (Allen *et al.* 1973). Organogenesis, development of the circulatory system and body all characterize the growing fetus. Placental trophoblast cells cover most of the conceptus. Specialized trophoblast cells at the vitelline pole will become the chorionic girdle and will invade the endometrium. Girdle cells become a stratified columnar epithelium resulting in troughs and pits and will form the mature endometrial cups (Enders and Liu 1991). At day 37-40 chorion cells appose the endometrium and are especially prolific at the ridges (Allen 2000). Girdle cells extend their processes into the interstitium of the endometrium. These cells will become part of the placental-umbilical unit to support the growing fetus. Additionally the girdle cells produce equine chorionic gonadotropin (eCG) which acts similarly to luteinizing hormone (LH) resulting in an up-regulation of progesterone (P_4) from the CL marking the 'end' of early pregnancy in the mare. Figure 2 depicts a diagrammatic representation of major events in equine pregnancy.

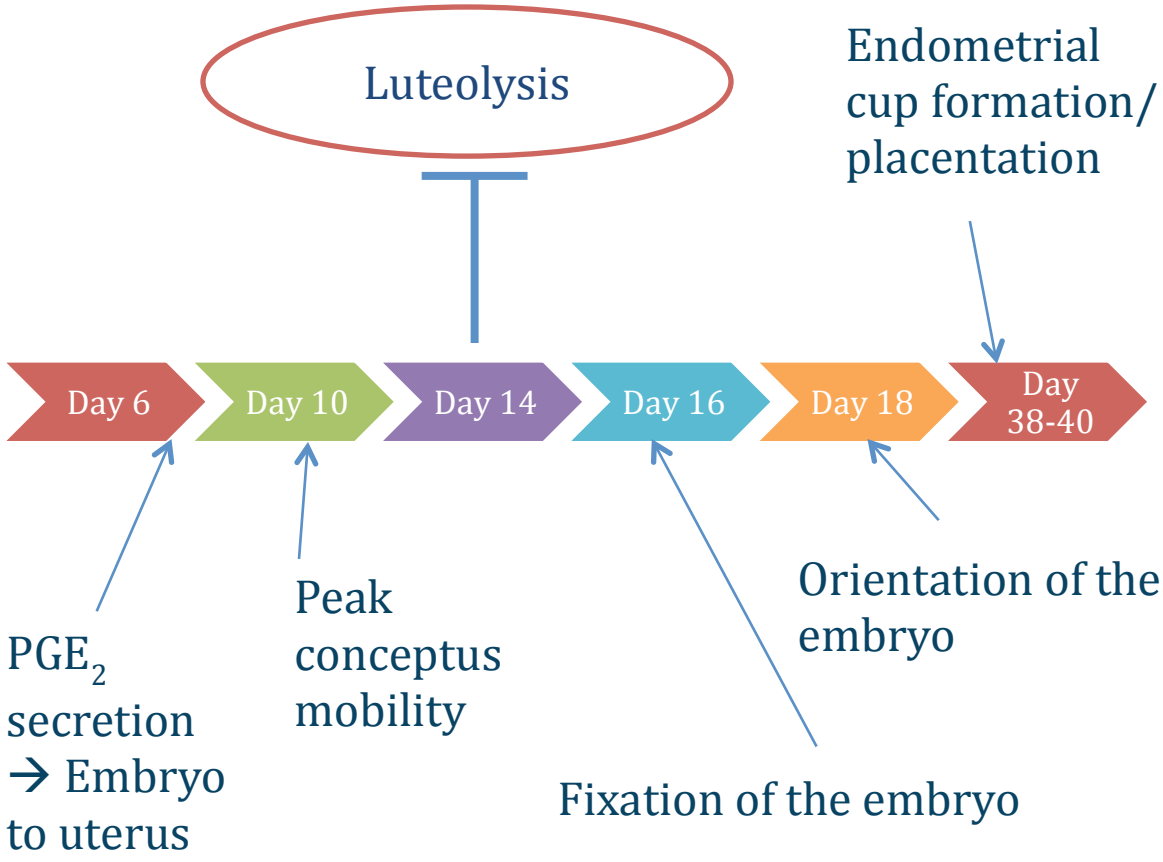

Figure 2: Important Events in Equine Pregnancy

Figure 2: This diagram depicts events in early pregnancy in the mare. Day 0 is defined as ovulation. In a non-pregnant mare luteolysis would begin on day 14 post-ovulation. The embryo is highly mobile throughout the uterus until fixation on day 16. Endometrial cup formation marks the end of early pregnancy in the mare.

Maternal Recognition of Pregnancy

The presences of a fertilized oocyte signals to the female in order to maintain pregnancy. These signals, referred to as maternal recognition of pregnancy, are well documented in many species. The estrous cycle in mares is typically 21 days. This time is

characterized by 5 days of estrus followed by 12-16 days of diestrus. Granulosa and theca cells differentiate into large and small luteal cells producing P_4 (McKinnon and Voss 2005). P_4 is primarily responsible for maintaining pregnancy in the mare during the early stages of pregnancy. Typically a CL is compromised approximately 14 days after ovulation by prostaglandin $F_{2\alpha}$ ($PGF_2\alpha$) from the uterus in the mare. Drops in CL P_4 production results in a non-functional tissue. In order to maintain pregnancy, a signal must exist by the presence of a conceptus. In mice, humans, ruminants and pigs the mechanism to protect the CL and maintain pregnancy is well established (Bazer 1992). A variety of mechanisms to maintain the CL include preventing the secretion of $PGF_2\alpha$ (luteostatic), preventing the distribution of $PGF_2\alpha$ to the CL (luteostatic), or producing a substance that counteracts the luteolytic $PGF_2\alpha$ (luteotrophic or antiluteolytic). The maternal recognition mechanism in horses however remains a mystery.

Pregnancy maintenance in the mare after invasion of the chorionic girdle cells relies on eCG induced increase of P_4. Additionally, secondary CL formation as a result eCG acting as LH around day 40-60 also contributes to increases in P_4 production. Prior to chorionic invasion, mechanisms that maintain the CL are undefined. Certain factors are known to maintain early pregnancy in the mare. A mobile conceptus, moving from horn to horn in the mare, is necessary to maintain early pregnancy prior to fixation. However Ginther and First (1971) hysterectomized and hemi-hysterectomized pony mares which resulted in only half the mares returning to estrus. Additionally extreme cases of pyometra, where the endometrial lining was partially destroyed, also inspired a short period of pseudopregnancy (Hughes JP *et al.* 1979). This indicates that equine endometrium has a primary role in the signal of pregnancy recognition. $PGF_2\alpha$ reaches its highest

concentration in uterine vein plasma concentration on day 14 post-ovulation in the mare (Douglas and Ginther 1976). Additionally Vernon *et al.* (1981) concluded that production of $PGF_2\alpha$ reaches its highest concentration and can be produced by the endometrium of pregnant mares in vitro. Results from the prior studies mentioned have shown that $PGF_2\alpha$ production is coming from pregnant endometrium is still capable of production of the hormone. Therefore the presence of the conceptus minimizes or limits $PGF_2\alpha$ production from the endometrium of the mare.

The equine embryo, in addition to secreting PGE_2 also secretes estrogens. Non-pregnant mare endometrium exposure to estradiol leads to an increase in $PGF_2\alpha$ and therefore the conceptus-derived estrogens may be playing a role in the uterine environment of pregnancy. Increased estradiol and estrone concentrations in the uterine lumen coincide with increases in uterine produced proteins from day 10 to 20 analyzed by 2-D gel (McDowell *et al.* 1990). Additionally ovariectomized mares administered progesterone and estradiol compared to just progesterone have increased uterine protein production (McDowell *et al.* 1987). Presence of estrogens in the uterus from the conceptus may be contributing to pregnancy maintenance.

Important in luteolysis in the horse is the relationship between oxytocin and prostaglandin. Unlike ruminants whose large luteal cells produce high concentrations of oxytocin during luteolysis stimulate the positive feedback with $PGF_2\alpha$ (Silvia *et al.* 1991), equine CLs produce only low concentrations of oxytocin that do not appear to change cycle status (Stevenson *et al.* 1991). Luteolysis in the mare is based on a feedback loop established between up-regulation of oxytocin and $PGF_2\alpha$ (Sharp *et al.* 1997) Blood from intercavernous sinus cannulae in mares were used to sample pituitary venous

concentrations of oxytocin and $PGF_2\alpha$. This study identified high magnitude surges of $PGF_2\alpha$ (in this case 13,14-dihydro-15-keto prostaglandin $F_2\alpha$) coincided or were directly followed by high magnitude surges of oxytocin (Vanderwall *et al.* 1998). Behrendt-Adam *et al.* (1999) found oxytocin-neurophysin I mRNA in the endometrium of mares was sequenced and determined to share more than 89% nucleotide and amino acid sequence to that of pituitary gland oxytocin. Oxytocin, therefore, may be produced from the endometrium as well as the posterior pituitary. Levels of endometrial oxytocin mRNA were negatively correlated to serum progesterone and mRNA was significantly down-regulated in non-bred compared to pregnant mares at days 10, 15, and 20 (Starbuck *et al.* 1999). The same study found that oxytocin receptors are up-regulated on day 14 of cycling mares versus day 10 or 18 but not found in pregnant mares. Although the mechanism is not clearly defined yet, it may be that the presence of a conceptus does alter or even uncouple the $PGF_2\alpha$ -oxytocin feedback loop and plays a role in maternal recognition of pregnancy.

Gene expression analysis held promising leads on maternal recognition in the horse. Transcriptional changes in the equine endometrium have been observed by microarray and PCR by Klein *et al.* (2010) and Bruemmer (2010). Klein and Troedsson (2011) recently profiled equine conceptuses by use of microarray and found that equine embryos express fibrinogen subunits. Although this provides insights to possible effectors to the uterine environment during pregnancy, the maternal recognition signal or combination of signals remains enigmatic.

Table 1 depicts the known mechanism of maternal recognition of pregnancy in murine, ovine, bovine, porcine, and human species. Upon entry into the uterus human and murine blastocysts almost immediately invade the uterine surface. Human chorionic

gonadotropin (hCG) from invading trophoblast cells in the forming placenta maintains pregnancy in the woman and serves as a marker for pregnancy. hCG binds to LH receptors on large luteal cells in the CL and causes P_4 production (Channing and Kammerman 1974). P_4 acts in a luteotrophic manner protecting against $PGF_2\alpha$ secretion (Patton and Stouffer 1991). eCG does not appear in horses until after the invasion of the endometrial cups around day 37-40. In rats and mice, cervical stimulation or stimulation of the pelvic nerve releases prolactin (PRL) from the anterior pituitary gland. PRL and subsequent PRL-like luteotropic hormone from the decidua stimulate LH receptors (LH-R) and expression of the estrogen receptors (ER) and resultant protection of the CL as a result of estradiol activity (Frasor and Gibori 2003). In cattle and sheep the signal of maternal recognition of pregnancy is clearly defined. Interferon-τ (IFN-τ) from mononucleate in the elongating embryo on day 12-13 and day 14-16, in sheep and cows respectively, suppresses pulsatile release of $PGF_2\alpha$ protecting the CL. Equine embryos do not produce IFN- τ based on 2-D gel analysis of the trophectoderm (McDowell *et al.* 1990) or based on RNA transcripts (Baker *et al.* 1991). Porcine endometrium produces $PGF_2\alpha$ regardless of pregnancy status. Estrogens from the conceptus sequester $PGF_2\alpha$ into the uterine lumen and prevent a pulsatile release of $PGF_2\alpha$ into the vasculature of the pig and luteolysis (Bazer 1992). Equine conceptuses also produce estrogens (Heap *et al.* 1982) however mares have little $PGF_2\alpha$ in uterine fluids and no evidence has surfaced of any form of pulsatile secretion of uterine $PGF_2\alpha$ in pregnant mares (Sharp *et al.* 1989). Additionally exposing non-pregnant equine endometrium to estradiol actually increased $PGF_2\alpha$ production significantly in a dose-dependent fashion (Vernon *et al.* 1981) Therefore, equine maternal recognition of pregnancy does not appear to match other models and the signal is unknown.

Table 1: Maternal Recognition of Pregnancy in Different Species

Species	***Maternal Recognition Signal***	***Mechanism***	***Source***
Murine	Prolactin (PRL)	Luteostatic	Frasor 2003
Human	Human chorionic gonadotropin (hCG)	Luteotrophic	Ross 1978
Ovine	Interferon-τ (IFN-τ)	Luteostatic	Bazer 1997
Bovine	IFN-τ	Luteotrophic	Bazer 1997
Porcine	Estrogen	Luteotrophic	Bazer 1992

Table 1: Known signals for maternal recognition of pregnancy in different species.

Early Embryonic Loss

Incidence of early embryonic loss in the mare is high and is defined as pregnancies lost prior to 40 days post-ovulation. Ball *et al.* (1988) proposed per cycle conception rates in young fertile mares approaches 90% and beyond, and in subfertile mares (e.g. due to age) high fertilization rates of 81 and 92% were reported. Ball (2005) further suggested that by day 40 of pregnancy, during endometrial cups invasion, 20% and 70% of pregnancies are lost in fertile and subfertile mares respectively. Carnevale *et al.* (2000) corroborates these figures. Ball (2005) additionally determined that embryonic death is at its highest incidence after the embryo enters the uterus and prior to detection by

transrectal ultrasonography (days 6-10), and before maternal recognition of pregnancy (day 12-14). In fact Morris and Allen (2002) demonstrated that 60% of all reproductive loss occurs prior to day 35 and therefore occur in early pregnancy. Early embryonic loss is the a significantly costly part of equine reproduction. When factoring in the cost of reshipping semen, ultrasonography of a mare for multiple cycles, loss of foal production to the industry the cost is staggering considering that the conception rates are high per cycle.

Embryonic loss is multifactorial and often idiopathic. However, there are some embryo losses that are attributable to known causes. In older mares genetic abnormalities are a source of early embryonic loss. This could be either due to morphological abnormalities (Carnevale *et al.* 1999) and aneuploidy (Rambags *et al.* 2005) or a myriad of other factors. Factors such as malnutrition or poor body condition especially in lactating mares also decrease pregnancy rates. Overweight or overfeeding mares has been proven to not affect pregnancy rates (Henneke 1984). Any source of severe or prolonged stress due to illness, disease, or dehydration may also result in embryonic loss in the mare. Uterine cysts in mares may also pose a problem for pregnancy maintenance impeding uterine wide chorion microvilli attachment (Adams *et al.* 1987). Uterine fluids, especially in the presence of infection, will most likely up-regulate $PGF_2\alpha$ and result in early pregnancy loss (Pycock and Newcombe 1996). Most early embryonic losses can be attributed to failure of maternal recognition of pregnancy, primary CL insufficiency, and endometrial irritation resulting in inflammation and $PGF_2\alpha$ induced luteolysis (Ball 2005). In cases of CL regression, perhaps as a result of failure of maternal recognition, the pregnancy can be rescued with exogenous progesterone or progestagen (altrenogest). In some cases however, failure to form a new

CL due to over-suppression of pituitary function from the exogenous progestin can prevent rescue of the pregnancy (Newcombe 2000).

In the mare, maternal recognition of pregnancy must occur in a specific window in order to prevent pregnancy loss. Understanding and harnessing the mechanisms behind maternal recognition in the mare could significantly reduce costs to the equine industry from the vast expenditures as a result of early pregnancy loss. Using conventional approaches has not yet identified the signal of maternal recognition of pregnancy. Therefore, a new approach to identifying possible factors in maternal recognition of pregnancy is necessary.

Exosomes

Exosomes are small vesicles (10-100nm in size) originally characterized as exocytosed vesicles from the plasma membrane originating from the endocytic pathway. Exosomes originate from a variety of cell types and having in an autocrine, paracrine, or endocrine action throughout the body. Exosomes all have a bilipidic membrane, a similar density allowing for purification with the use of a sucrose gradient, similar protein composition, and contain mRNAs and miRNA and can carry tissue or cell specific material (Keller *et al.* 2006).

Trams *et al.* (1981) originally described exosomes as microvesicles derived from specific domains of the plasma membrane released from normal and neoplastic cell culture lines and having enzymatic activity outside the cell. Even at this early stage of discovery it was proposed that these vesicles might play an important role in physiologic functions. Exosomes were then also characterized in ovine maturing reticulocytes (Pan and Johnstone 1983). These reticulocytes lose their transferrin receptor (TfR) to become mature red

blood cells. This was observed through electron microscopic evidence of sorting the TfR into small vesicles contained in endosomes (ILVs in MVBs), fusion of the limiting membrane with the cell surface, and release of the vesicle to the extracellular space (exosomes) (Pan *et al.* 1985). This mechanism was originally thought to shed unnecessary organelles from the reticulocyte cells. The capacity of these vesicles has greatly expanded since their discovery.

More recent discoveries reignited the interest in the extracellular vesicles. Johnstone *et al.* (1991) described both mammalian and avian reticulocytes formed exosomes (a term coined by Johnstone in a paper in 1987) to exocytose the TfR. This proved that mammalian as well as avian cells performed exosome formation but the cells exocytosed their contents however these cells also retained organelles. Raposo *et al.* (1996) first proposed that these exocytosed vesicles may be biologically active from B lymphocytes which secreted antigen-presenting vesicles. Major histocompatibility complexes (MHC) class II packaged in multivesicular bodies (MVBs) fused to the plasma membrane in cells and were released as exosomes. Not only did these exosomes appear to contain these MHC class II complexes but also incited antigen-specific MHC class II–restricted T cell responses. Induction of T cell responses by exosomes *in vitro* gave rise to the idea that exosomes may have a significant physiological role. Since this discovery, exosomes have been identifying originating from a variety of cell types including; dendritic cells (DCs), T cells, and mast cells as well as a variety of tumor cells, mesenchymal stem cells, neural cells, platelets, hepatocytes, and endothelial cells (Ludwig and Giebel 2011). Additionally exosomes have been discovered in a variety of mammalian fluids including; serum, plasma, saliva, breast milk, bronchial lavage fluid, pleural effusions, urine, ocular

fluids, semen, amniotic fluid and synovial fluid. (Simpson *et al.* 2009). It is clear from just the variety of cell types and fluids in which they were identified that exosomes are generated and can travel throughout a given organism.

Many characteristics of exosomes allow purification as well as identification. Biogenesis within the endosomal pathway means that exosomes have a very small amount of proteins associated with other organelles in the cell. Specific proteins appear to be enriched in exosomes. The initial focus of exosomes was on immune cells and thus many exosomes have been characterized with antigen presentation in the form of MHC class I and II protein complexes (Théry *et al.* 2002). These complexes are in exosomes from B cells, DCs, enterocytes, tumor cells, and T cells. Loading antides into MHCs requires chaperone proteins such as heat shock protein 70 and 90 (HSP 70 and HSP 90), which are also enriched in exosomes. Mathivanan *et al.* (2010) found that across 19 studies contained in ExoCarta 89% of proteomic studies reported HSP 70 in exosomes despite the cell origin. HSP 70 is, therefore, a very attractive biomarker for exosomes. Other proteins that are enriched in exosome populations include adhesion molecules including integrins and tetraspanins. These proteins include; CD63, CD9, CD37, CD53, CD81 and CD82 (tetraspanins),α3, α4, αM, αL, β1, β2 (integrins) (Février and Raposo 2004). A study by Laulagnier *et al.* (2005) tagged CD 63 using a single fluorescent label in RBL-2H3 cells MHC-II containing exosomes were enriched in CD 63 but only 47% stained for the positive. This indicates that exosomes that are released are not uniform in content and the protein content can vary greatly based on cell of origin. Additionally tetraspanins associate with other tetraspanins, integrins, growth factor receptors, and MHC Class II proteins in a raft like manner (Boucheix and Rubinstein 2001). Tetraspanins typically play a role in

organization of large molecular complexes and membrane subdomains which may be important for an alternative route for exosome formation without protein ubiquitination.. Small GTPAses, known as Rab proteins, in exosomes play a role in docking, membrane fusion, and vesicle fusion (Mathivanan *et al.* 2010). Additionally annexins aid in membrane trafficking and fusion. Futter and White (2007) propose a role for annexins in clathrin-dependent internalization leading to MVB formation. Some of these annexins have been shown to specifically target the degradation pathway within lysosomes (annexin II and VI) while others have been shown to be specific for inward vesiculation (annexin I) (Futter and White 2007; Ramachandran and Palanisamy 2012). In addition to proteins, exosomes appear to be enriched in lipids such as ceramide and sphingolipids, which promote membrane budding (Trajkovik *et al.* 2008). Sphingolipids are especially enriched in exosomes from the epididymis (Sullivan *et al.* 2005). There is a high level of selection and regulation in the formation, contents, and secretion of exosomes. This suggests specific packaging within exosomes and indicates the importance of exosome biogenesis.

Exosome Biogenesis

All exosomes are derived from the endocytic pathway and associated with particular proteins. Mechanisms of biogenesis may vary across cell types however, there is an accepted generic method for generation of MVBs. The release of exosomes is a highly regulated event (Olver and Vidal 2007). Early endosomes are created via internal budding at the plasma membrane through clathrin-dependent or –independent pathways to internalize receptors, ligands, extracellular fluid and other substances in the extracellular environment (Mellman 1996). These endosomes become MVBs in a pathway that is typically understood as 'inward' or reverse budding which can occur due to ubiquitination,

phosphorylation, glycosylation of proteins or clathrin recruitment (Piper and Katzmann 2007). The inward budding results in ILVs within the MVB that are degraded in the lysosome pathway or fuse to the plasma membrane of the cell and release their contents to the extracellular environment becoming 'exosomes'. The packaging of ILVs within MVBs originally thought to be facilitated by endosomal sorting complexes required for transport (ESCRT), can occur independently as well. ESCRT protein complexes seem to be associated with the ubiquitination pathway of sorting proteins into ILVs (Mathivanan 2010). ESCRT protein complexes -0, -I, -II recognize ubiquitinated proteins and sequester them to the membrane of the endosome while ESCRT-III seems to be responsible for membrane budding (Hurley 2008). The ESCRT complexes are associated with hepatocyte growth factor-regulated kinase substrate (Hrs), apoptosis-linked gene 2 (ALG-2)-interacting protein X (Alix) and tumor susceptibility gene-101 (Tsg101) to function. Hrs functions in sorting ubiquitinated proteins to microdomains in an early endosome that is clathrin-coated (Théry 2002). Tsg101 in the endocytic pathway is important for the transport of membrane proteins where Alix connects ESCRT II to ESCRT III completing the endocytosis of late endosomes (Théry *et al.* 2002). Another pathway independent of the ESCRT machinery for inward budding of endosomes is based on the sphingolipid and ceramide content. Trajkovic *et al.* (2008) tracked the trafficking of proteolipid protein (PLP) and knocked down Hrs, Tsg101, and Alix and showed no influence on inward budding of PLP versus epidermal growth factor receptor (EGF-R), which has been well characterized as using the ESCRT machinery. The findings conclude that independently from the ESCRT machinery, MVB formation can occur and appears to be dependent on raft-based microdomains. Marsh and van Meer (2008) followed up the previous author's suggestion

that ceramide can induce lipid-microdomain accumulation and lead to inward budding. Pathways behind MVB to fusion with the plasma membrane versus fusion with lysosomes for degradation are less clear.

Mechanisms governing lysosome degradation or plasma membrane fusion and release of exosomes is not completely understood. Original hypotheses suggested that soluble NSF-attachment protein (SNAP) and SNAP attachment protein receptor (SNARE) had a function in exosome release because of their role in membrane fusion events in lysosome secretion. However, SNARE proteins still have been relatively uninvestigated in discourse of exosomes. Additionally Rab family guanosine triphosphatases (Rab GTPases) were hypothesized as responsible for membrane fusion due to their importance in membrane trafficking both in endocytic recycling and membrane fusion events (Théry *et al.* 2002). Recent reports from Ostrowski *et al.* (2010) indicated that Rab GTPases are particularly important in MVBs moving to the plasma membrane. In HeLa cells Rab27a silencing by RNA interference (RNAi) increased the size of MVBs whereas Rab27b silencing positioned MVBs towards the perinuclear region. Hsu *et al.* (2010) screened for Rab GTPases in exosomes from oligodendroglial cells via liquid chromatography and mass spectrometry. The most abundant GTPase idenfied was Rab35. The experiments confirmed that Rab35 did not regulate a lysosomal degradation pathway. Moreover Rab35 knockdown resulted in a greater number of intracellular vesicles suggesting that Rab35 has a role in the pathway from the endosomal system to the plasma membrane. Finally experiments from Hsu *et al.* (2010) demonstrated that Rab35 promotes vesicle docking. Other Rab proteins have been identified (4, 5, 7, 10,11, 14) (Olver and Vidal 2007) and implicated in MVB fusion with the plasma membrane (Rab11) (Lakkaraju and Rodriguez-

Boulan 2008) but none have been directly tested. Because Rab proteins have a role in the recycling lysosomal pathway, the presence of these proteins is not sufficient enough for conclusive statements about the MVB to plasma membrane pathway. Figure 3 depicts of exosome formation and release from an individual cell.

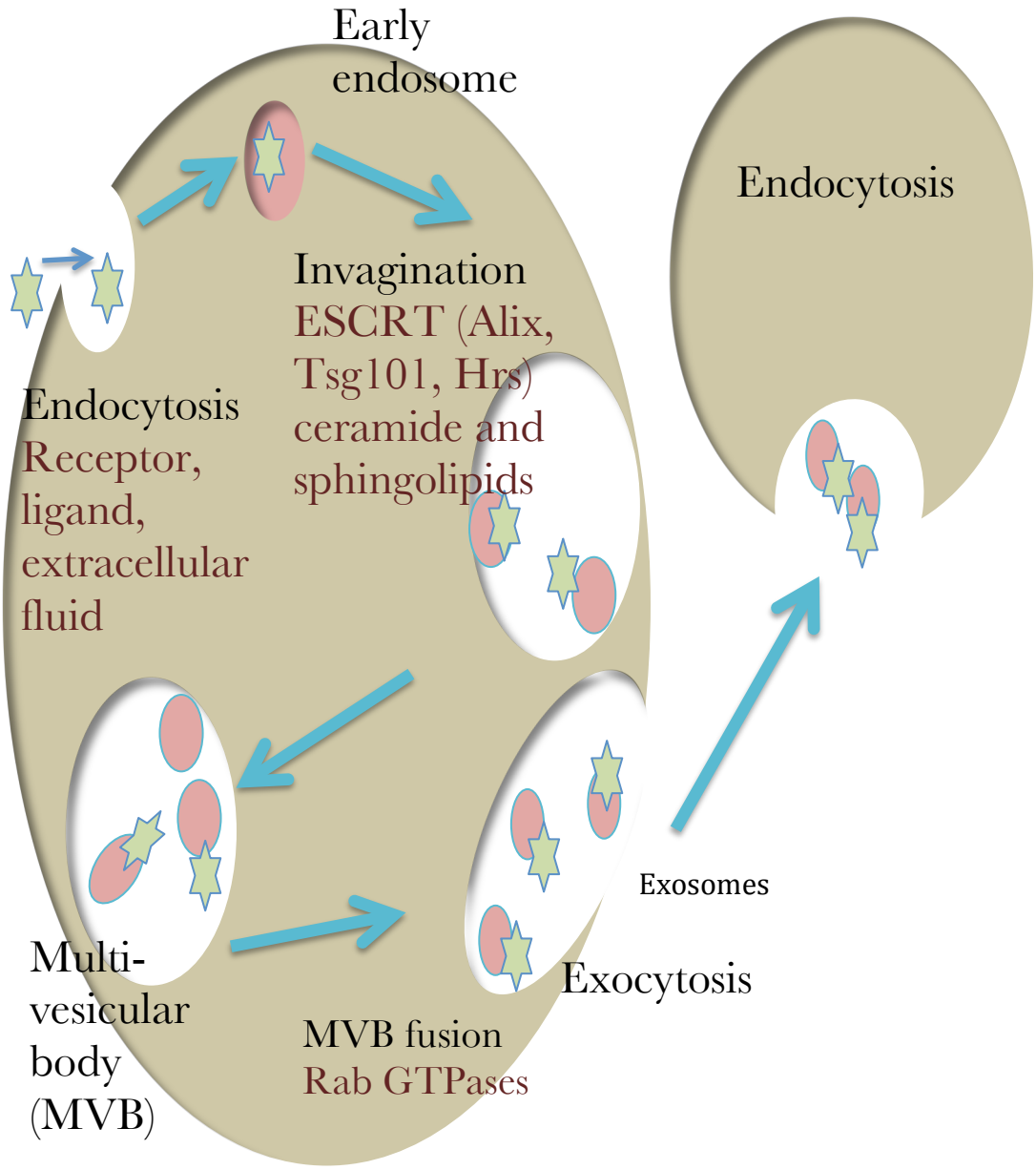

Figure 3: Exosome Biogenesis

Figure 3: Depiction of exosome formation and the important proteins and lipids behind the events of biogenesis of exosomes.

Exosome function in cell-to-cell communication

Once released from the cell of origin, exosomes have a variety of ways of interacting with the extracelluar environment. The exosomal membrane proteins interact with receptors on a target cell to activate intercellular signaling. Membrane proteins cleaved from the exosome bind and activate a target cell surface receptor. Exosomes also fuse with the target cell membrane and release the exosomal cargo to the target cell (Mathivanan *et al.* 2010). Alternatively, the exosome could be internalized into the endocytic pathway obythe target cell (Tian *et al.* 2010; Feng *et al.* 2010).

As early as the Raposo study (1996) where exosome derived from B lymphocytes elicited T-cell responses researchers have examined responses of cells to exosomes. Exosomes have a role in immune response regulation and antigen presentation. The transformation of immune cells just by the presence of exosomes was promising. Exosomes have great potential in increasing the body's defenses (increase in T-cells). Théry *et al.* (2002) showed activation of immature T cells by DC exosomes. Both B cells and DCs have high levels of MHC-I and MHC-II which when in exosomes have potential to activate T cells. *In vivo* the presence of exosomes from tumor-antigen-pulsed DCs have the capacity to elicit antitumor responses (Quah *et al.* 2005). Despite discoveries of increasing immune responses due to the presence of exosomes, other studies have also identified exosomes exhibiting tolerogenic phenotypes. Exosomes containing Fas ligand (FasL) induced T-cell apoptosis in a dose dependent manner (Abusamra *et al.* 2005). Riteau *et al.* (2004) found that melanoma-derived exosomes bore human leucocyte antigen (HLA) class I molecule (HLA-G) which displays immune tolerant properties. Tolerogenic properties have also played a role in increase in survivability in allograft transfers in human patients receiving

exosomes prior to transfer (Peche *et al.* 2003). The experiments suggest that exosomes play a roles immune response augmentation as well as induces tolorogenic phenotype. Exosomes from the epididymides are essential for the maturation of sperm in that they provide important proteins to sperm that play a role in reproduction (Sullivan 2005). Putatively, exosomes are involved a pathway which retroviruses hijack the cell's machinery. This includes notable in diseases like human immunodeficiency virus (HIV) and prion diseases. Exosomes can, therefore, act as a propagation device of the disease (Kramer *et al.* 2005). Most importantly for this study is the exosome content of mRNAs or miRNAs and modification of cell responses at a distance from the cell of origin.

The presence of exosomes elicits a variety of physiological effects. Other applications include the use of exosomes in cancer vaccinations harnessing the power to augment tumor-suppressing capabilities of DC derived exosomes on T-cells (Viaud *et al.* 2010) and suppress the tolerogenic capabilities of some cancer cells. Extracellular communication can be better understood by identifying the cargo that exosomes are delivering to target cells. Exosomes isolated peripherally can be indicative of physiological states within different tissues of the body.

This study aims to identify the role of exosomes in pregnancy. Placental derived (Syncytiotrophoblast) exosomes are found in the circulation of pregnant women with specific miRNA cargo (Luo *et al.* 2009). In humans this is another distinct method of cell-to-cell communication as well as immunomodulation which are important for the maintenance of pregnancy (Mincheva-Nilsson and Baranov 2010). Since placental invasion occurs almost as the embryo enters the uterus in the human, exosomes harvested from serum in women appear to have signature miRNAs associated with trimester or events

during pregnancy. During pregnancy exosomes have an immunomodulatory role specific to T cells (Taylor *et al.* 2006). Modulating the uterus to allow the survival of the embryo is necessary with or without placental formation. The presence of the embryo in the uterus is foreign. Exosomes in the mare may induce the immuno-quiescence of the uterus or act in a cell-to-cell communicative way to signal for maternal recognition of pregnancy. These exosomes harvested peripherally also may serve as a biomarker to indicate pregnancy status, embryonic health, and serve as a possible platform for clinical intervention for compromised pregnancies. Exosomes isolated from mare serum indicated trend for increase (P=0.09) in smaller denser exosomes in serum of pregnant mares at day 12 post-ovulation despite delayed placental formation compared to the human (Hergenreder 2011, Thesis). This slight up-regulation cannot explain the entirety of the maternal recognition signal or the immense changes in the uterus during pregnancy in the mare prior to implantation. Therefore the current study aims to examine the presence of exosomes and their miRNA content.

miRNA

miRNAs lin-4 and let-7 were originally identified as small RNAs silencing mRNAs translation, regulating the timing of larval development in *C. elegans* (Lee *et al.* 1993; Wightman *et al.* 1993; Reinhart *et al.* 2000). Since then miRNAs have been identified in many mammalian species and plants. miRNAs are small (~22nt) non-coding RNAs that post-transcritionally regulate target mRNAs (Cai *et al.* 2009). miRNAs regulate mRNAs through degradation via deadenylation (elimination of the poly[A] tail) of the target mRNA or translational silencing. It is predicted that more than 60% of all protein-coding genes can be controlled by miRNAs (Friedman *et al.* 2009). In fact PicTar computational analysis

suggests that a single miRNA can have up to 200 different targets (Krek *et al.* 2005). Currently there are over 2000 confirmed miRNAs according to mirbase.org in the human. An *in silico* detection model of the horse revealed 407 novel equine miRNAs (Zhou *et al.* 2009).

miRNA biogenesis

Several pathways and means of miRNA biogenesis have been reported however there is one common well established pathway. miRNA genes are transcribed via RNA polymerase II (pol II). Primary miRNAs (pri-miRNAs) exist with a 5'cap and poly(A) tail with one or more hairpin structures (>100bp) (Cai *et al.* 2004) (Figure 4). These hairpin structures that often have unpaired bulges are further processed in the nucleus by a protein complex microprocessor containing both Drosha and RNAse II enzyme and its cofactor DiGeorge syndrome critical region gene 8 (DGCR8) (Kim 2005). The *D. melanogaster* and *C. elegans* homolog of DGCR8; Pasha is commonly referred to as DGCR8/Pasha. At this point miRNAs are referred to as precursor miRNAs (pre-miRNA)

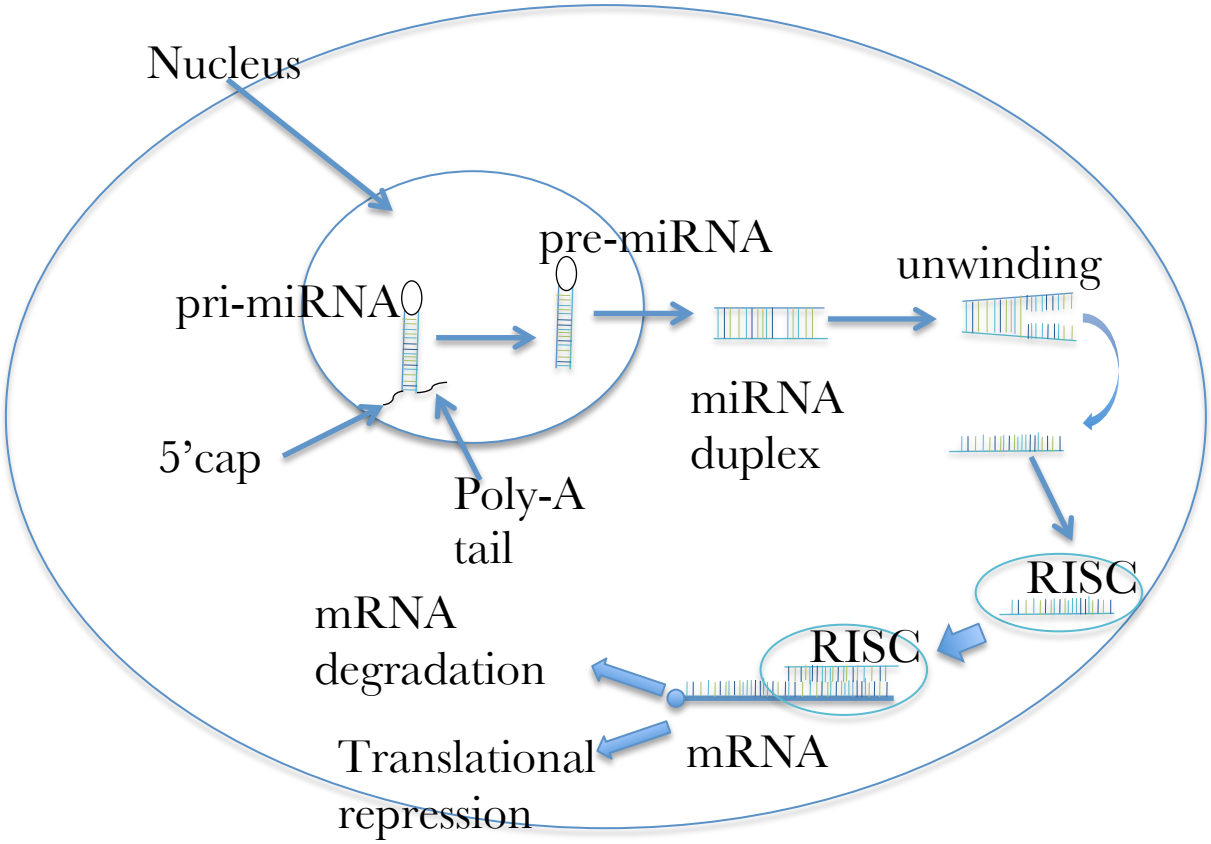

Figure 4: Diagrammatic Description of miRNA Biogenesis

and exist as hairpin intermediates. The pre-miRNA is characterized by ~70nt and a ~2nt 3' overhang. Further processing requires that the pre-miRNAs are moved into the cytoplasm from the nucleus which is executed by exportin-5 (Lund *et al.* 2004). Pre-miRNA couples with multi-domain RNase II enzyme Dicer for further processing. Dicer cleaves the step loop and results in a miRNA duplex. How the double strand is separated is still unknown but analogous small interfering RNA (siRNA) R2D2 enables loading the 5' end of the leading strand into Argonaute in *D. melanogaster* (Liu *et al.* 2006). Many helicases have been implicated in the unwinding of the duplex into the leading strand that will be loaded into the RNA induced silencing complex (RISC) and the degraded passenger strand including; p68 (Salzman *et al.* 2007), RNA helicase A (Robb and Rana 2007), and RCK/p54

(Chu and Rana 2006), and eukaryotic initiation factor 6 (eIF6) (Chendrimada *et al.* 2007). Typically only the leading strand is loaded into the RISC while the other passenger strand is degraded. The single strand loaded into the RISC complex is the strand responsible for mRNA silencing. Unlike pri- and pre-miRNA mature miRNA is rather stable (Winter *et al.* 2009).

miRNAs biological effects

miRNAs mediate their effects through other proteins. AGO proteins of the RISC complex interact with glucine-tryptophan bodies (GW bodies) specifically GW repeat-containing protein of 182 kDa family proteins GW182, in order to target mRNAs for silencing or degradation. Similar to P-bodies, GW-bodies appear to congregate to endosomes and MVBs (Lee *et al.* 2009). Once miRNA are incorporated into the RISC complexes they are often referred to as miRISCs. Of note, both GW182 and AGO2, crucial components of the RISC complex, are found to be enriched in MVB and endosomal compartments (Gibbings *et al.* 2009) indicating a role in packing miRISCs into exosomes for intercellular communication. The AGO and GW-body proteins act to silence translation of the mRNAs and the miRNA acts as a guide for targeting mRNA (Pillai *et al.* 2004). Mammalian miRNAs form imperfect hybrids by base pairing with 3'-untranslated region (UTR) of the mRNA however there are instances of animal miRNAs that may target 5'UTR and coding regions of mRNAs but are less robust. (Orom *et al.* 2008; Rigoutsos 2009). The most important match regions between the mRNA and miRNA are in the so-called 'seed-region' positions 2-8 of the miRNA having the most paring specificity (Doench and Sharp 2004). An imperfect pairing in this region results in greatly impairs translational silencing which prohibits endonucleolytic cleavage of mRNA (Bartel 2009). There must also be

relative complementarity at the 3' end of the miRNA (positions ~13-16) that becomes important when the seed region is not perfectly complementary (Grimson *et al.* 2007).

Understanding mRNA translation is neccessary for appreciating miRNAs role in suppressing translation of mRNAs. Translation consists of three steps; initiation, elongation, and termination. Most translation is cap-dependent requiring certain proteins to bind the 5' cap (Fabian *et al.* 2010). Ribosomes are recruited to mRNA for translation by eukaryote initiation factor 4 (eIF4F) on the 5' cap and poly(A)–binding protein (PABP) of the poly(A) tail. eIF4F is an RNA helicase and unwinds the mRNA while PABP binds to eIF4G functionally circularizing the mRNA for stability and thereby enhancing the rate of translation (Pestova and Hellen 2001). Initiation factors (IF) 1,2, and 3 bind to the small (40S) subunit of the ribosome near the 5' cap and scan the mRNA until it encounters a start codon. The large (60S) ribosomal subunit is then placed in the start site. The 60S ribosomal subunit has two transfer RNA (tRNA) sites. Translation occurs at three sites. The amino acid site (A-site) where the aminoacyl-tRNA anticodon pairs with the mRNA brings in the correct amino acid. The polypeptide site (P-site) is where the amino acid attached to the tRNA is transferred to the polypeptide chain. Finally the exit (E-site) is where the empty tRNA sits before being released back to the cytoplasm to bind another amino acid and bring it back to the ribosome complex to repeat the process. The ribosome complex moves 5'-3' with protein elongation factor G. Peptide bonds form between amino acids and the ribosome complex shifts moving another tRNA into the A-, P-, and E-site for the process to be repeated. When the ribosome complex encounters a stop codon, tRNAs with release factors instead of amino acids bind to the ribosome and cause the release of the

polypeptide (protein) mRNA from the ribosome which are subsequently dissociated. Initiation is the primary target for translational control.

Silencing Translation

There are distinct means in which both pathways in miRNA induced silencing occur. mRNA cap recognition and assembly of the correct proteins appears to be the method in which miRNA inhibits initiation of translation (Mathonnet *et al.* 2007). Lin-4, the first discovered miRNA, inhibited mRNA translation at elongation because levels of mRNA and polysome never shifted but inhibited the translation of lin-14 (Olsen and Ambros 1999). Evidence supports that mRNAs with 3'UTRs are targeted by let-7 or chemokine receptor type 4 (CXCR4) (Humpreys *et al.* 2005). A synthetic RNA duplex, CXCR4 was cotransfected with a plasmid that expresses luciferase mRNA with imperfect biding sites in 3'UTR. CXCR4 acts as a miRNA mimic and is commonly used as a positive control. CXCR4 induces a robust repression of the luciferase protein without the loss of the mRNA (Humphreys *et al.* 2005). The levels of mRNA did not drop but upon testing against polysomal density gradients the mRNAs shifted to a lighter fraction (Pillai *et al.* 2004). This indicates that the correct ribosomal subunits described above are not recruited properly under miRNA-induced repression. These results suggest that miRNAs inhibit initiation of translation specifically rather than elongation. mRNAs with have cap-independent translation do not require assembly of initiation factors and, in some cases, the large ribosomal subunit (Spahn *et al*. 2004). These mRNAs and mRNAs with a mutated 5' cap are unaffected by miRNA or CXCR4 compared to the same sequences with a normal 5'cap (Humphreys *et al.* 2005). Additionally Pillai *et al.* found that binding of initiation factors eIF4E or eIF4G to an intercistronic region in bicistronic mRNAs encouraged translation with or without let-7

target sites in the 3'UTR. While some proposals of the miRNAs inhibiting the recruitment the 60S ribosomal subunit to initiate translation via a disruption of eIF6 showed promise, the idea was inevitably shelved. This was primarily due to results demonstrating no change in miRNA-mediated repression of mRNAs to enhancing miRNA repression in knockdown of eIF6 models (Eulalio *et al.* 2009; Ding *et al.* 2008). The current hypothesis for miRNA induced silencing of translation is that miRNAs disrupt the function of eIF4F (including eIF4E, eIF4G, eIF4A) and PABP. The GW182 of the miRISC complex interacts with the PABP and dissociates if from the initiation factors attached to the 5' cap (Zekri *et al.* 2009). The open conformation inhibits translation and may leave the mRNA more unprotected to decay enzymes (Huntzinger and Izaurralde 2011). This will silence the translation of most mRNAs however there is another mechanism thought to govern the degradation of mRNAs. GW182 recruits Ccr4p associated factor 1 (CAF1)-carbon catabolite repression 4 (CCR4)-negative on TATA-less (NOT) deadenylation complex. Knockdown of CAF1 and NOT1 blocked miRNA-mediated deadenylation (Eulalio *et al.* 2009). PABP can also play a role in deadenylation. GW182 C terminus interacts with PABP in *Drosophila* (Zekri *et al.* 2009) and seems to be evolutionarily conserved in mammals (Fabian et al. 2010) placing the poly(a) tail in close proximity to the deadenylase complex initiating the reaction (Seitz *et al.* 2008). Deadenylation precedes miRNA-mediated decapping via decapping-complex proteins 1 and 2 (DCP1 and DCP2 respectively) disrupting the stability of the mRNA targeting it for degradation (Behm-Ansmant 2006). Either by deadenylation, decapping, destabilization and degradation or inhibiting translation by uncoupling eIF4F from mRNAs, miRNAs have a dramatic silencing effect on mRNA translation.

Exosomes containing miRNAs are diverse enactors of intercellular communication. Exosomal miRNAs have been implicated in transfer of miRNA loaded exosomes from T-cells to APCs (Mittelbrunn *et al.* 2011), viruses (Pegtel *et al.* 2010), in the equine ovarian follicle (da Silveira *et al.* 2012) as well as important biomarkers for diagnosis and prognosis in a variety of neoplasms (Rabinowits *et al.* 2009; Taylor and Gerçel-Taylor 2008; Kosaka *et al.* 2010) and pregnancy (Luo *et al.* 2009; Mincheva-Nilsson and Baranov 2010). Profiles of exosomal miRNAs are indicative of the physiological state of different cell types. Exosomal miRNAs are valuable as peripheral biomarkers as well as may be informative of the differential expression of mRNAs associated with specific physiological processes.

Conclusion

Early pregnancy and recognition in the mare is a physiological process that has not been intricately defined or identified. The precise signal of maternal recognition to protect the CL around day 14 post-ovulation remains mysterious. A smaller, denser population of exosomes appear to trend toward significant up-regulation in pregnant mares on day 12 post-ovulation. A profile of the miRNAs associated with these exosomes may be informative as unique biomarkers, mRNA targets, and physiological ramifications of the presence of miRNAs. The goal of this study is to determine differential expression of miRNAs from serum exosomes between samples from pregnant and non-pregnant mares.

CHAPTER II: PROFILING miRNAs AFTER MATERNAL RECOGNITION OF PREGNANCY (DAY 16)

Introduction

The equine maternal-embryo interface must exchange a variety of factors to signal that she is pregnant. Conventional approaches to understanding this intricate physiological process have not identified a single candidate responsible for maternal recognition of pregnancy in the mare, but rather a multitude. To aid in understanding which pathways of primary importance during this delicate time of pregnancy, another approach is necessary. Exosomes, which can communicate in an intercellular manner, may aid in current understanding maternal recognition of pregnancy. Exosomes often contain miRNA that are responsible for regulating the mRNA translation (Valadi *et al.* 2007). miRNAs have been indicated in human pregnancy. The presence or lack of miRNAs from exosomes in pregnant versus non-pregnant animals may indicate mRNAs regulated during pregnancy in the mare. This experiment profiled 380 human miRNAs by quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR). Exosome miRNA profiles at Day 16 may be indicative of mRNAs are of interest within the window for maternal recognition or may serve as peripheral biomarkers for pregnancy in the mare.

Materials and Methods:

Horse Care

Colorado State University Institutional Animal Care and Use Committee approved all horse use. Mares (n=3) were utilized in a simple cross-over design where mares had pregnant and non-pregnant cycles. Mares were monitored by transrectal ultrasonography

for ovarian follicle development every other day. Mares were monitored every other day with a follicle size of 35 or greater until ovulation. Mares were bred when they had a follicle size of 35 or greater and bred every other day until ovulation was detected. Mares were inseminated with $500x10^6$ progressively motile sperm cells every other day with from stallions of known fertility. Mares were monitored using transrectal ultrasonography starting on day 10 to confirm pregnancy. At day 18 embryos were collected via trans-cervical lavage.

Serum Collection

Blood samples were drawn daily by jugular venipuncture from mares 12-18 post ovulation. Blood samples were allowed to clot and serum was removed from the top of the sample and placed in -80° C until further processing.

Exosome Isolation

Exosomes were isolated according to manufacturer's protocol using Exoquick (System Biosciences, Inc. (SBI), Mountain View, CA). Exoquick is a polymer based reagent used to enrich samples for exosomes. Briefly, 400 µL of serum samples were incubated with 100 µL Exoquick for 12 hours at 4° C followed by centrifugation at 1500 x g for 30 minutes to pellet the population of exosomes. The pellet was re-suspended in 250 µL phosphate buffered saline (PBS) (pH 7.4).

RNA Isolation

Total RNA was isolated according to manufacturer's protocols using TRI Reagent BD-RNA/DNA/Protein Isolation Reagent for Blood Derivatives (Molecular Research Center, Cincinnati, OH). Samples were lysed and separated by chloroform into RNA and protein

layers. RNA is precipitated with isopropanol and washed with 75% ethanol. All samples were treated with DNA-free DNase Treatment and Removal Reagent (Invitrogen/Life Sciences, Grand Island, NY) to remove any DNA contamination. RNA purity values were assessed using the Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE). Typically 260/280 values of above 1.8 are considered acceptable for PCR. For this RNA isolation using cholorform caused samples to have lower 260/280 values and therefore values of 1.7 were accepted for PCR. Samples were stored at -80° C until further qRT-PCR analysis.

Reverse Transcription

500 ng of RNA generated cDNA using QuantiMir RT Kit (SBI) per the manufacture's protocol. Poly(A) tail was added using 5 µL of total RNA, 2 µL of 5X PolyA Buffer, 1 µL 25mM $MnCl_2$, 1.5 µL 5mM ATP and 0.5 µL PolyA Polymerase and incubated for 30 minutes at 37°C. Samples then had the dT adaptor annealed by adding 0.5 µL Oligo dT Adaptor and heated for 5 minutes at 60°C and thencooled to room temperature for 2 minutes. cDNA synthesis and reverse transcription reaction (4 µL 5x RT Buffer, 2 µL dNTP mix, 1.5 µL 0.1M DTT, 1.5 µL RNase-free H_2O, 1 µL Reverse Transcriptase) was incubated for 60 minutes at 42°C then heated to 95°C for 10 minutes. cDNA reaction was then immediately added to qRT-PCR master mix for PCR reaction.

qRT-PCR

Human miRNA primers were used from human miRNome Profiler plates (SBI). Primers were 380 mature human miRNA sequences (forward primers), and three endogenous controls; U6 snRNA, RNU43 snoRNA and U1 snRNA. An additional well

without a primer served as a negative control. qRT-PCR was performed on 384 well plates. Each well containing 6 µL of total reaction including 2X SYBR Green I master mix (Roche Applied Science, Indianapolis, IN) 10 µM Universal reverse primer, and aforementioned miRNA specific forward primer (SBI) and 0.1 µL of cDNA. qRT-PCR was performed using the LightCycler480 PCR system (Roche). Cycle conditions ran at 95°C for 5 minutes, followed by 45 cycles of 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 15 seconds, ending in a melt curve analysis to confirm single cDNA amplification.

Statistical Analysis

To test differences between pregnant and non-pregnant mares on Day 16 exosomal miRNA differences in raw Cp values were normalized to the geometric mean of RNU43 snoRNA and U1 snRNA. These noramlizers were utilized due to their presence at less than 37 cycles of PCR and less than 1 standard deviation across all samples. Using a paired, two-tailed students t-test within miRNAs between samples from pregnant and non-pregnant samples. Statistical differences were assessed and significance placed at $P<0.05$.

Results

Exosomal miRNAs were considered to be present when number of cycles to detect fluorescence signal above background was ≤37. 378 miRNAs were present in either samples from pregnant or non-pregnant mares based on raw Cp values. Amplification curves and melt peaks were examined to confirm single product amplification. All raw Cp values were normalized to the geometric mean of two control genes: RNU43 snoRNA and U1 snRNA based on standard deviations less than one across all samples and Cp values of less than 37. qRT-PCR analysis revealed six differentially expressed miRNAs associated

with pregnancy status. Two miRNAs were present only in samples from pregnant mares (miR-26b and miR-183). Two additional miRNAs were present only in samples from non-pregnant mares (mir-146b-5p and miR-424) Finally two miRNAs present in both samples from pregnant and non-pregnant mares but significantly higher in non-pregnant mares (miR-205 and miR-145) (Figure 5). miR-183 was present only in non pregnant mares however was only expressed in two animals. Results from the Diana mirPath analysis for pregnancy-related miRNAs(miR-26b and miR-183) indicated pathways involved in various cancers but also included pathways in focal adhesion and Wnt signaling. Pathway analysis of top 10 pathways are listed in tables 4 and 5. Samples from non-pregnant mares included miRNAs expressed higher in non-pregnant samples as well as those expressed only in non-pregnant samples (mir-146b-5p, miR-424, miR-205, and miR-145) (Table 3) While non-pregnant-related miRNA pathways also included focal adhesion the top ranked pathways were adherens junction and Wnt signanling pathway.

Table 2: Day 16 Comparison of Differentially Expressed miRNAs

miRNA	Expressed in
hsa-miR-26b	Non-pregnant only
hsa-miR-145	Higher in NP
hsa-miR-146b-5p	Pregnant only
hsa-miR-183	Non-pregnant only
hsa-miR-205	Higher in NP
has-miR-424	Pregnant only

Table 2 demonstrates the presence of different human mi-RNAs in pregnant, non-pregnant, or up-regulated in non-pregnant samples.

Table 3: P-values and Fold Changes of Significantly Different miRNAs

miRNA	p-value	Fold Change
miR-205	0.02	1.04
miR-145	0.01	1.01

Table 3 demonstrates the p-values associated with the student's T-Test performed on the $2^{-\Delta CT}$ transformation of and the fold change associated with the $2^{\Delta CT}$ values

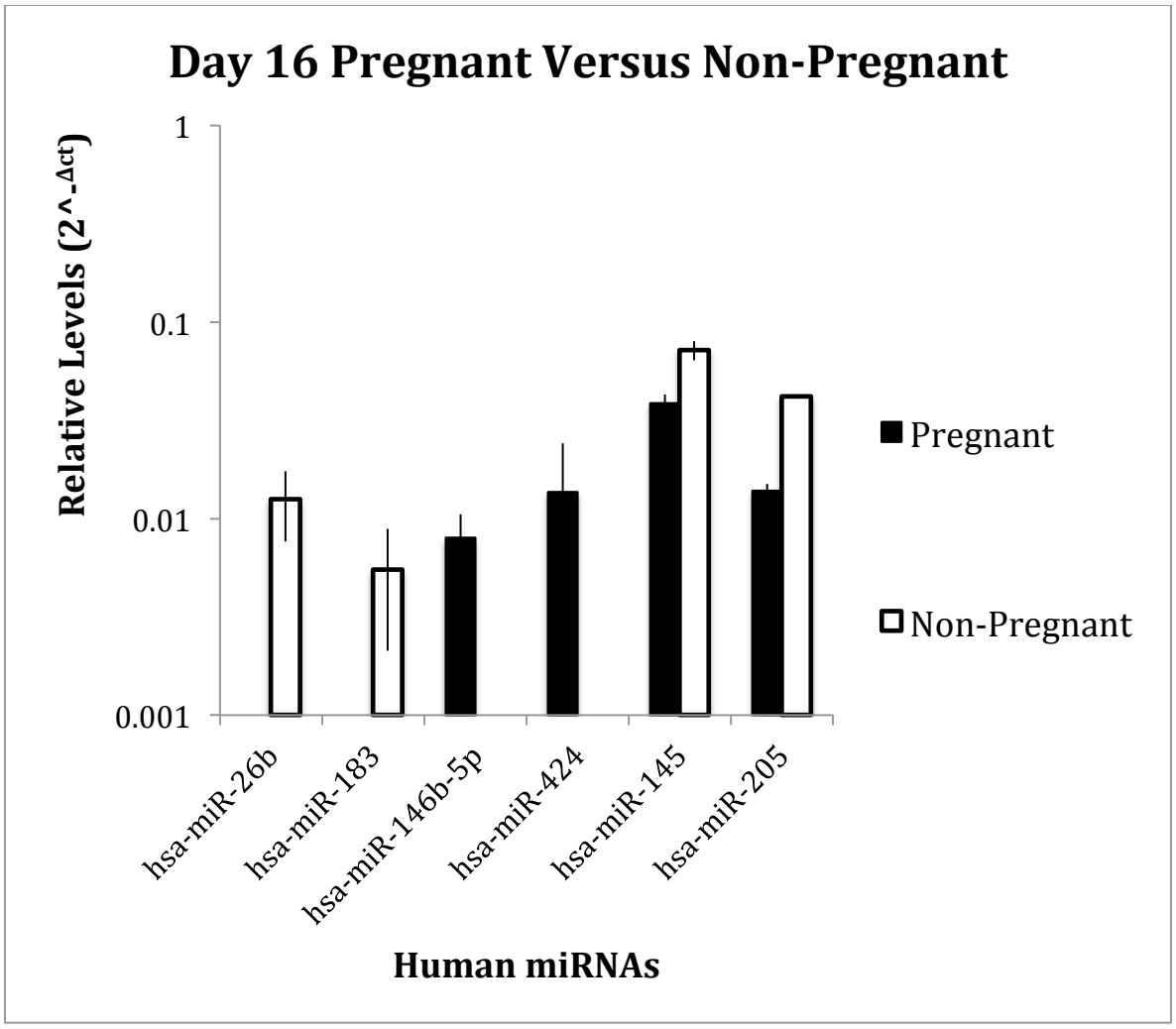

Figure 5: Day 16 Pregnant versus Non-Pregnant Significant miRNA

Figure 5: Relative levels of significantly different miRNAs on day 16 post-ovulation. All values are expressed in $2^{-\Delta ct}$ and in logarithmic scale. Error bars represent SEM values

Table 4: Pathways of miRNAs Present in Samples from Pregnant Mares

Rank	Pathway	Number of Genes Targeted	-ln(p-value)
1	Pancreatic cancer	19	19.41
2	Glioma	17	18
3	Prostate cancer	20	16.09
4	Non-small cell lung cancer	14	13.86
5	Acute myeloid leukemia	14	13.44
6	Chronic myeloid leukemia	17	13.33
7	Melanoma	16	12.42
8	Focal adhesion	30	11.64
9	Wnt signaling	24	10.48
10	Axon guidance	21	9.79

Table 4: Displays the top ten results in pathway analysis of miRNAs at higher levels in samples from pregnant animals using Diana mirPath software.

Table 5: Pathways of miRNAs Expressed Higher in Samples from Non-Pregnant Mares

Rank	Pathway	Number of Genes Targeted	-ln(p-value)
1	Adherens junction	24	21.51
2	Wnt signaling	35	16.91
3	TGF-beta signaling	25	16.15
4	MAPK signaling pathway	49	14.28
5	Long-term potentiation	18	12.18
6	Tight Junctions	29	10.91
7	Focal adhesion	36	9.28
8	Axon Guidance	25	7.92
9	Colorectal cancer	18	6.23
10	Ubiquitin mediated proteolysis	24	5.92

Table 5: Displays the top ten results in pathway analysis of miRNAs at higher levels in samples from non-pregnant animals using Diana mirPath software.

Discussion

Experiment I profiled a list of miRNAs expressed at different levels in samples from pregnant and non-pregnant mares following maternal recognition of pregnancy. Of the 380 human miRNAs profiled by qRT-PCR; 6 miRNAs were present at significantly different levels in regards to pregnancy status. Of significant note in this experiment is the small sample size. Power is added to this study by using a paired T-Test with mares acting as their own controls. miRNAs hsa-miR-26b and hsa-miR-183 were present only in samples from non-pregnant mares however hsa-miR-183 was expressed only two of three animals. hsa-miR-183 may not serve as a marker for pregnancy in the mare due to this further complication of the small sample size. Additionally miRNAs hsa-miR-146-5p and hsa-miR-424 were present only in samples from pregnant mares. Finally miRNAs hsa-miR-145 and hsa-miR-205 were present at significantly higher levels in non-pregnant mares.

Pathway analysis using Diana mirPath software was employed to indicate physiological components potentially affected by these miRNAs. Pathways related to miRNAs present higher in pregnant mares resulted in a variety of cancer targets with focal adhesion molecules and Wnt signaling ranked lower. These pathways are not particularly indicative of any physiological processes in regards to pregnancy of the mare. However pathways related to significantly higher levels of miRNAs from non-pregnant mares may be more indicative of physiological changes in pregnancy. Wnt signanling ranked number 2 according to pathway analysis is implicated in a variety of embryogenesis processes. TGF-beta signaling controls cell proliferation and differentiation. The first ranked target on the list for day 16 non-pregnant miRNAs was adherens junctions (AJs). AJs are multi-protein

complexes that mediate cell adhesion between adjacent cells (Rudini and Dejana, 2008). These junctions are important in embryonic development as well as intercellular signaling to transfer signals and inhibit cell growth, regulate cell shape, and increase resistance to apoptosis. Higher relative levels of miRNAs in non-pregnant samples target mRNAs for silencing and degradation. Therefore, classical of miRNA function mRNA transcripts are translated in pregnant mares but are suppressed in non-pregnant mares. As discussed in the review of literature embryo fixation occurs on day 16 in mares in the base of one horn or another. This fixation would logically lead to direct signaling from the embryo to the endometrium via adherens junctions. The targeted supression of this pathway in non-pregnant animals may be indicative of the lack of an embryo. This data must be confirmed with more samples in order to further elucidate miRNAs role in equine pregnancy.

There are at least six differentially expressed exosomal miRNAs on day 16 post-ovulation between pregnant and non-pregnant animals. Pathway analysis of non-pregnant samples indicate a target down-regulating adherens junctions. Allowing these transcripts to be expressed in pregnant mares may play a role in cell-cell signaling between the embryo and the endometrium of the mare through adherens junctions once the embryo is fixed in the uterus.

CHAPTER III: PROFILING miRNAs BEFORE AND DURING MATERNAL RECOGNITION OF PREGNANCY (Days 9,11, and 13)

Introduction

The presence of the embryo in the uterus moving from horn to horn is playing a dynamic role signaling pregnancy to the mare. Exosomes carrying miRNA can convey signals and release cargo outside the cell of origin. Therefore exosomes can signal to other cells the presence of the conceptus or change mRNA translation due to miRNA content. Coupled with miRNAs differentially expressed after maternal recognition signal in Experiment I examined miRNA expression before and during maternal recognition of pregnancy. Exosomal miRNAs have been implicated in a variety of cell-cell signaling. The signal of maternal recognition must be communicated in order to maintain pregnancy. Therefore day 11 post-ovulation serum samples profiled 346 equine miRNAs and 6 human miRNAs significantly expressed at higher levels from Experiment I results. In addition the 6 human miRNAs significantly expressed at higher levels identified in the previous experiment were simultaneously profiled. Of the 346 equine miRNAs; 83 were expressed in pregnant, non-pregnant or both. Therefore these 83 miRNAs were used to investigate profiles day 9 and day 13 post-ovulation to match the time period of before and during maternal recognition of pregnancy. To further validate enrichment of exosomes from isolation protocols, Western Blot analysis for HSP 70 and Cytochrome C (CYT C) was performed as positive and negative controls respectively.

Materials and Methods:

Horse Care

Colorado State University Institutional Animal Care and Use Committee approved all horse use. Mares (n=8) were utilized in a simple cross-over design where mares had pregnant and non-pregnant cycles. Mares were monitored by transrectal ultrasonography for ovarian follicle development every other day. Mares were monitored every other day with a follicle size of 35 or greater until ovulation. Mares were bred when they had a follicle size of 35 or greater and bred every other day until ovulation was detected. Mares were inseminated with $500x10^6$ progressively motile sperm cells every other day with from stallions of known fertility. Mares were monitored using transrectal ultrasonography starting on day 10 to confirm pregnancy. At days 11, 13, or 15 embryos were collected via trans-cervical lavage.

Serum Collection

Blood samples were drawn daily by jugular venipuncture from mares days 0-15 post ovulation depending of time of embryo collection (Day 9 n=8 Day 11 n=8 Day 13 n=5). Pregnancy was confirmed by ultrasound evaluation and embryo collection on day 11, 13 or 15. Blood samples were allowed to clot and serum was removed from the top of the sample and placed in 4°C until differential centrifugation. Samples were spun at 300 x g for 10 min and 2000 x g for 10 min to remove residual cells and debris, at 10,000 x g for 30 min to remove microparticles according to Théry et al. (2006).

Exosome Isolation

Exosomes were isolated according to manufacture's protocol using Exoquick™ (System Biosciences, Inc. (SBI), Mountain View, CA). Exoquick is a polymer based reagent used to enrich samples for exosomes. Briefly, 400 µL of serum samples were incubated with 100 µL Exoquick for 12 hours at 4° C followed by centrifugation at 1500xg for 30 minutes to pellet and purify the population of exosomes. The pellet was re-suspended in 250 µL phosphate buffered saline (PBS) (pH 7.4).

RNA Isolation

Total RNA was isolated according to manufacture's protocols using TRI Reagent BD-RNA/DNA/Protein Isolation Reagent for Blood Derivatives (Molecular Research Center, Cincinnati, OH). Samples were lysed and separated by chloroform into RNA and protein layers (*Note samples should not contain any DNA). RNA is precipitated with isopropanol and washed with 75% ethanol. All samples were treated with DNA-free DNase Treatment and Removal Reagent (Invitrogen/Life Sciences, Grand Island, NY) to remove any DNA contamination. RNA purity values were assessed using the Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE). Typically 260/280 values of above 1.8 are considered acceptable for RNA quantification by PCR. For this RNA isolation using cholorform causes the Nanodrop reading to have lower 260/280 values and therefore values of 1.7 were accepted for PCR. Samples were stored at -80° C until further qRT-PCR analysis.

Protein Isolation and Quantification

Protein isolation of serum samples was completed per manufacture's protocols with the use of M-PER (Themo Scientific, Rockford, IL). Exosomes were repelleted using centrifugation at 12,000xg for 30 minutes. 1mL of M-Per reagent was used for cell lysis. Samples were mixed for 10 minutes and then debris was pelleted by centrifugation and 14,000xg for 15 minutes. Supernatant was then frozen in a new tube at -80°C until Western Blot analysis. For control samples protein isolation of equine epididymal and endometrial samples was completed per manufacture's protocols with the use of TRI Reagent BD (MRC) due to presence of proteins of interest in most tissues and cells. From the RNA isolation solution after taking off the aqueous phase (RNA layer) protein was precipitated with isopropanol, washed with a series of centrifugation steps adding 500 µL 0.3M guanidine hydrochloride and 95% ethanol, storing at room temperature for 10 minutes and centrifuging at 12,000xg for 5 minutes. These centrifugation steps were repeated until the resulting pellet looked white or beige in color indicating the full removal of the TRI Reagent. Samples were solubilized in 8M urea Tris-HCl (pH8.0). Samples were stored in -80°C until Bradford assays. Exosomal and tissue protein concentrations were determined using Bradford Assay technique from purified protein. Standard curve of protein concentration was achieved by adding 0.1µg/µL of bovine serum albumin (BSA) with 8M urea in Tris-HCl to cuvettes in increasing volumes (20, 40, 100, 150, and 200µL). Cuvettes containing 10µL of sample were prepared and 2mL 4:1 Protein Assay Dye Reagent Concentrate Solution (Bio-Rad, Hercules, CA) was added to each cuvette. Analysis via spectrophotometer at 595nm ascertained protein concentration based on the standard curve.

Reverse Transcription

cDNA was generated using miScript (Qiagen, Valencia, CA) per the manufacture's protocol. 4µL 5xmiScript RT Buffer, 1µL reverse transcriptase mix and 1µg of RNA and water were used in a 20µL solution and incubated for 60 minutes at 37°C and then 5 minutes at 95°C. cDNA reaction was then immediately added to qRT-PCR master mix for PCR analysis.

qRT-PCR

Equine miRNA forward primers were used and sequences were obtained by an in *silico* detection model (Zhou et al. 2009). Of the 340 equine miRNAs (Appendix B) 250 were conserved human miRNAs and 75 equine miRNAs (Appendix C) had different nucleotide sequences than those profiled by Experiment I. The remaining 15 miRNAs have no apparent human homologue (Appendix D). The same three endogenous controls were used as in Experiment I; RNU43 snoRNA, U1 snRNA. An additional well without a primer served as a negative control. qRT-PCR was performed in 384 well plates. Each well containing 6 µL of total reaction including 2X QuantiTect SYBR Green PCR Master Mix, 10X miScript Universal reverse primer (Qiagen), and miRNA specific forward primer and 0.1 µL of cDNA. qRT-PCR was performed using the LightCycler480 PCR system (Roche). Cycle conditions ran at 95°C for 15 minutes, followed by 45 cycles of 94°C for 15 seconds for denaturation, annealing at 55°C for 30 seconds, and extension at 70°C for 30 seconds, ending in a melt curve analysis to confirm single cDNA amplification. Day 11 samples profiled 340 equine specific miRNAs while day 9 and day 13 samples analyzed a smaller subset of 83 equine miRNAs as well as 6 miRNAs found in Experiment I (Appendix E). The 83 miRNAs analyzed using samples from mares on day 9 and 13 were found to be in all

samples regardless of pregnancy status on day 11. miRNAs were considered present at a Cp value of less than 37. Amplicons were confirmed with analysis of appropriate amplification curves and singular melt peaks.

Western Blot Analysis

Western blot analysis aimed to identify the presence of HSP 70 in serum exosomes and controls due to the prevalence of that protein in exosomes across multiple studies (Mathivanan *et al.* 2010). Organelle-related proteins are not found in exosomes due to their biogenesis within the endocytic pathway (Lotvall and Valadi 2007). Therefore cytochrome C was chosen as a negative control due to its presence in on the external portion of mitochondria. For Western blot analysis of HSP 70, 25µg of protein was loaded into each well of 12% SDS-PAGE poly acrylamide gels. The gels were made with 2.0mL 1M Tris (pH 8.8), 4.0mL acrylamide (30:0.8), 100µL 10% SDS, 5µL TEMED, and 100µL 10% APS. Cytochrome C western blot analysis were performed using Novex® 4-20% Tris-Glycine 1.0 mm 10 well gels to allow for better separation between bands of small (15kDa) size. Samples were incubated with buffer and DTTmix and incubated at 90°C for 10 minutes. Samples were transferred into the wells and run at 30mA for 30 minutes and transferred to Protran nitrocellulose membranes (Fisher Scientific, Pittsburg, PA). Gels are transferred at 100 V for 60 minutes. Membranes were incubated in 5% blocking buffer for 1 hour at room temperature (5% non-fat dried milk in TBST). Membranes were exposed to primary antibody rabbit HSP 70 (1:200, ab79852, Abcam, San Francisco, CA) or rabbit Cytochrome C (1:200, sc-13156, Santa Cruz Biotechnology, Santa Cruz, CA). After thrice washing the membrane in 1x TBST for five minutes membranes were incubated with horseradish peroxidase conjugated anti-rabbit secondary antibody (1:2000, sc-2004, Santa Cruz

Biotechnology Inc., Santa Cruz, CA). Membranes were again washed thrice in 1X TBST for five minutes each and then subsequently developed using ECL Plus Western Blotting Detection System (Amersham, Buckinghamshire, UK).

Statistical Analysis

To identify differences between pregnant and non-pregnant mares exosomal miRNA differences raw Cp values were normalized to the geometric mean four consistently and invariably expressed miRNAs across all time points; miRNAs 99b, 127, 129a-5p, 323-5p. Only miRNAs that were present in all samples were compared using a paired, two-tailed student's t-test statistical differences were assessed at $P<0.05$.

Pathway analysis

Targets of differentially expressed miRNAs were identified using Diana Labs mirPath which is used to identify the pathways of multiple miRNAs. Top pathways are based on the negative natural log of an enrichment p-value calculated from a modified Fisher's Exact Test.

Results

qRT-PCR for day 11 samples from pregnant and non-pregnant mares indicated 90 miRNAs that were present in either pregnant, non-pregnant, or both based on amplification curves and single melt peaks. These 90 miRNAs were used to profile time points during maternal recognition of pregnancy (day 13) and prior to maternal recognition of pregnancy (day 9).

On each day there were exosomal miRNAs that were significantly higher according to pregnancy status. Day 9 results exhibited a higher level of eca-miR-433 in pregnant animals (Figure 6). This represents the only miRNA that was higher in pregnant animals in this experiment. Results from serum exosomes isolated from samples on day 11 post-ovulation found four miRNAs that were higher in non-pregnant animals: eca-let-7c, eca-miR-25, eca-miR-195, and eca-miR-451 (Figure 7). Seven miRNAs were differentially expressed and up-regulated in non-pregnant animals on day 13 including eca-miR-130b, eca-miR-140-3p, eca-miR-195, eca-miR-433, eca-miR-508-3p, eca-miR-767-5p, and eca-miR-770 (Figure 8). Interestingly, eca-miR-433 was lower in non-pregnant animals on day 9 post ovulation, the same on day 11, and higher in non-pregnant animals on day 13 (Table 6). Fold changes of all miRNA are in Table 6.

Statistically significant miRNAs from Experiment I did not have the same profile in earlier days post-ovulation. Hsa-miR-26b, hsa-miR-145 and hsa-miR-205 were expressed in both pregnant and non-pregnant samples across all days. Hsa-miR-146-5p was not consistently expressed in either pregnant or non-pregnant samples across time points. Hsa-miR-183 was not expressed in day 9 or day 11 samples but was expressed in both pregnant and non-pregnant samples on day 13. Finally hsa-miR-424 was present in only

some samples and varied across time points. No miRNAs were expressed in only pregnant or non-pregnant mares (Table 7).

Diana Labs mirPath for pathway analysis was performed on day 11 and day 13 samples for specific targets for the differentially expressed miRNAs. Day 11 and day 13 pathway analyses were similar and are displayed in tables 8 and 9 respectively. Differential expression of miRNAs were higher in non-pregnant samples only and therefore these pathways reflect potential targets of the non-pregnant miRNAs. The top target for both day 11 and day 13 results were focal adhesion molecules. Focal adhesion pathway is exhibited in Figure 9 describing the pathway for both day 11 and day 13 results. The top five gene targets for each day is given in Table 10. B-cell lymphoma 2 (BCL2) controls apoptotic regulation is the top predicted gene target in both day 11 and day 13 analysis of the top target within focal adhesion molecules.

Western blot analysis confirmed an enrichment of exosome population. Exosome isolation from serum stained positive for HSP 70 as well as just endometrium staining positive for Cytochrome C (Figure10).

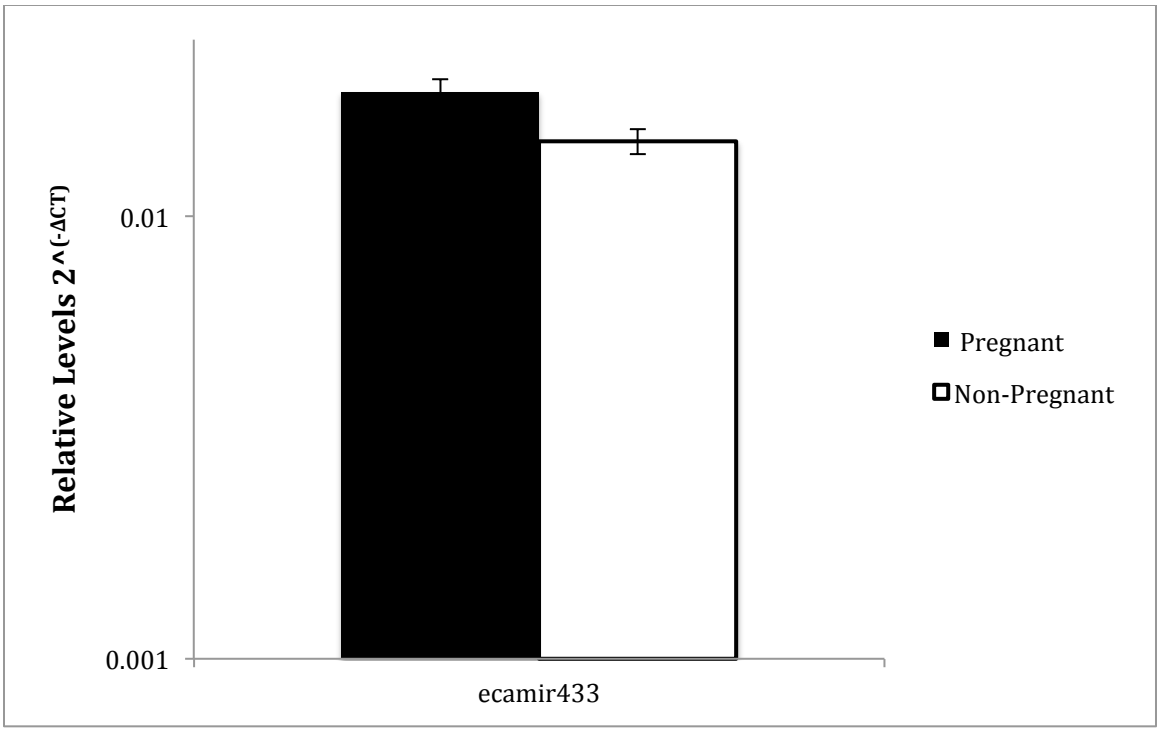

Figure 6: Exosomal miRNAs Significantly Higher in Samples from Pregnant Mares on Day 9

Figure 6: This graph demonstrates eca-miR-433 expressed higher in pregnant animals on day 9 post ovulation. The graph is depicted in logarithmic scale. Error bars represent SEM values. $P<0.05$

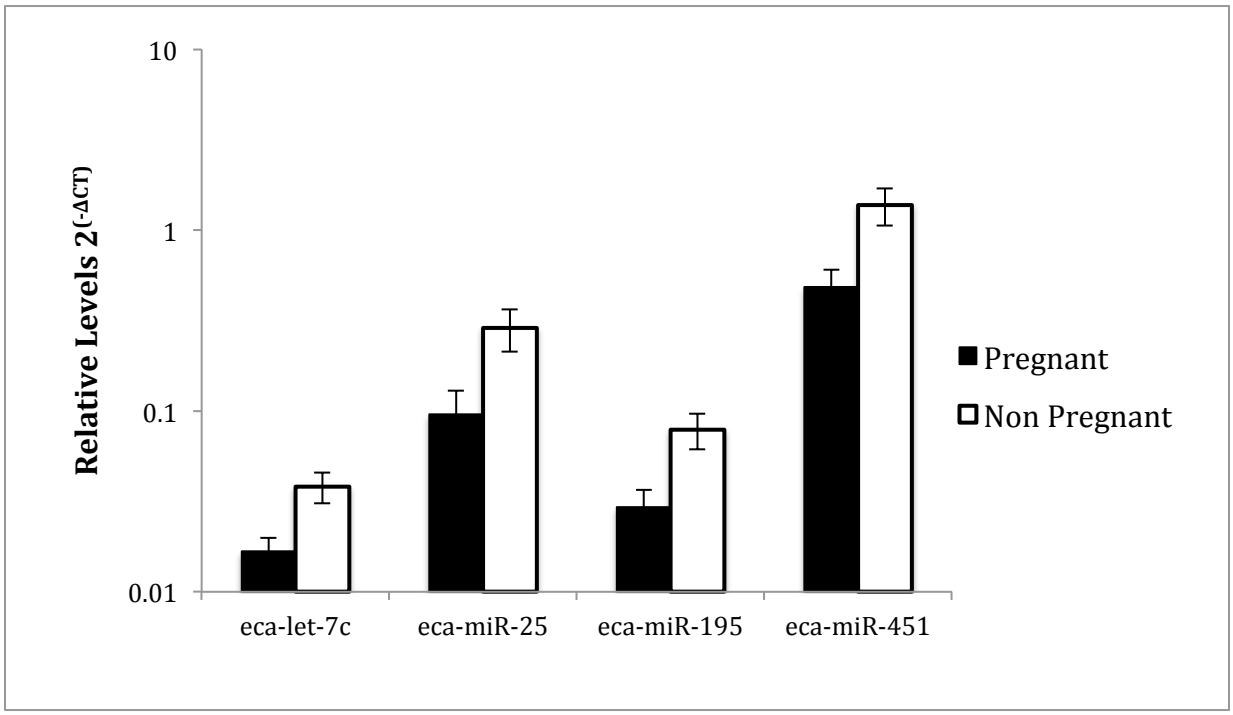

Figure 7: Exosomal miRNAs Significantly Higher in Samples from Non-Pregnant Mares on Day 11

Figure 7: Demonstrates up-regulation of the four miRNAs in samples from non-pregnant mares. Graph is depicted in logarithmic scale. Error bars represent SEM values. $P<0.04$

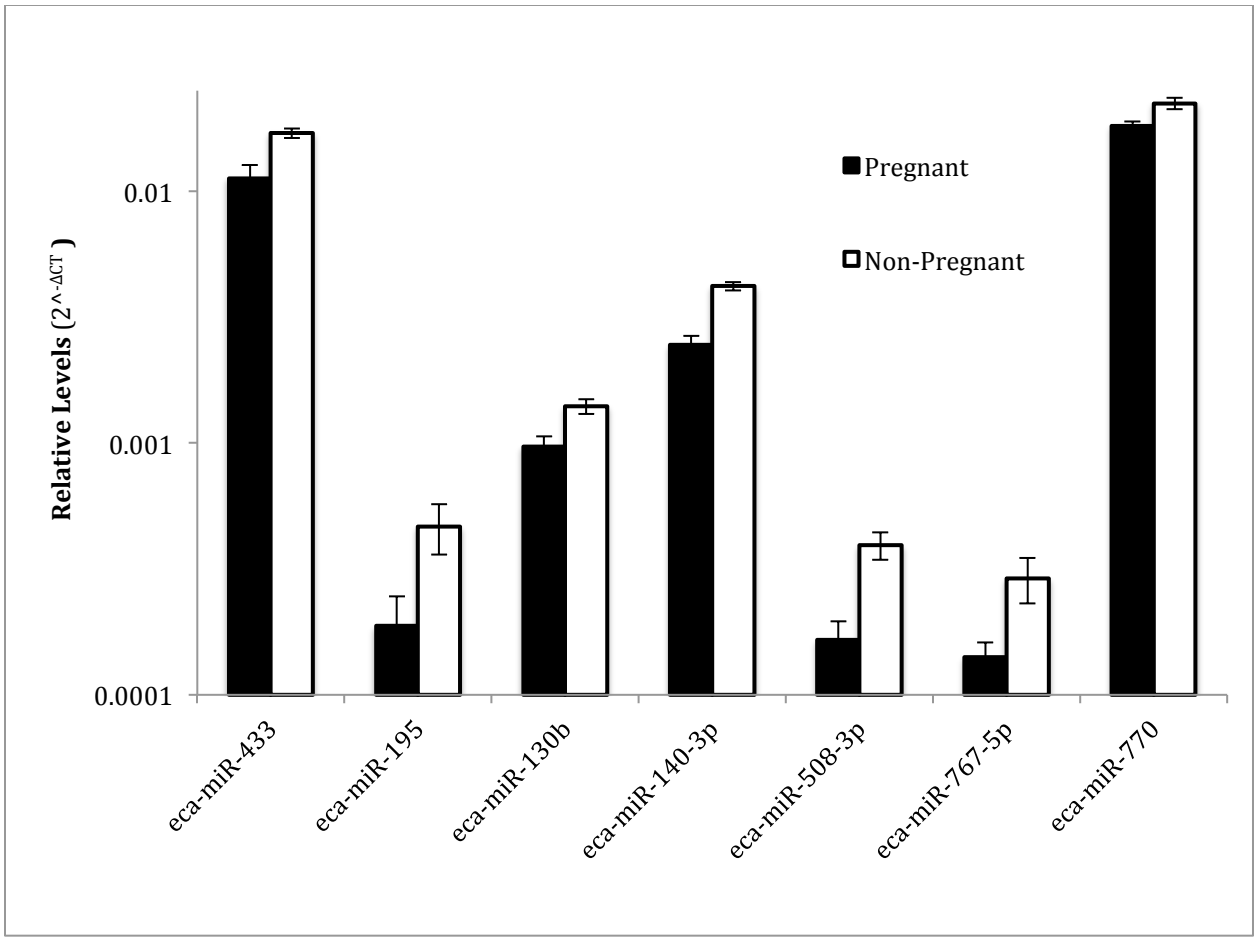

Figure 8: Exosomal miRNAs Significantly Higher in Samples from Non-Pregnant Mares on Day 13

Figure 8: Demonstrates up-regulation of seven miRNAs in samples from non-pregnant mares. Graph is depicted in logarithmic scale. Error bars represent SEM values. $P<0.04$

Table 6: Exosomal miRNAs Across Time and Associated Fold Changes

Day 9		
Higher in P+	eca-miR-433	1.31
Day 11		
Higher in NP	eca-let-7c	2.24
Higher in NP	eca-miR-25	3.64
Higher in NP	eca-miR-195	3.01
Higher in NP	eca-miR-451	2.75
Day 13		
Higher in NP	eca-miR-130b	1.45
Higher in NP	eca-miR-140-3p	1.73
Higher in NP	eca-miR-195	2.56
Higher in NP	eca-miR-433	1.56
Higher in NP	eca-miR-508-3p	2.44
Higher in NP	eca-miR-767-5p	2.00
Higher in NP	eca-miR-770	1.22

Table 6: Fold change between samples from pregnant and non-pregnant mares based on $2^{\wedge\Delta CT}$ values.

Table 7: Day 16 Significant miRNAs on Days 9, 11, 13

Human miRNA from Day 16	**Status on Day 16**	**Status on Day 9, 11, and 13**
hsa-miR-26b	NP Only	Both P+ and NP
hsa-miR-145	Up-regulated in NP	Both P+ and NP
hsa-miR-146b-5p	P+ Only	None
hsa-miR-183	NP Only	None
hsa-miR-205	Up-regulated in NP	Both P+ and NP
hsa-miR-424	P+ Only	Bath P+ and NP

Table 7: miRNAs differentially expressed on day 16 examined at days 9, 11, and 13.

Table 8: Pathway Analysis for Exosomal miRNAs Expressed Higher in Samples from Non-Pregnant Mares on Day 11

Rank	Pathway	Number of Genes Targeted	-ln(p-value)
1	Focal adhesion	43	15.46
2	MAPK signaling pathway	51	14.17
3	Prostate cancer	23	11.38
4	p53 signaling pathway	19	10.98
5	Melanoma	19	9.7
6	Amyotrophic lateral sclerosis	8	9.33
7	Glioma	17	8.87
8	Wnt signaling pathway	30	8.57
9	TGF-beta signaling pathway	21	8.21
10	Colorectal cancer	20	7.79

Table 8: Displays the top ten targets in the pathway analysis of the miRNAs present at higher levels on day 11 post ovulation using Diana mirPath software.

Table 9: Pathway Analysis of Exosomal miRNAs Higher in Samples from Non-Pregnant Mares on Day 13

Rank	Pathway	Number of Genes	-ln(p-value)
1	Wnt signaling pathway	36	16.57
2	TGF-beta signaling pathway	26	16.47
3	Adherens junction	21	13.37
4	Focal adhesion	41	13.23
5	Prostate cancer	24	13.2
6	Colorectal cancer	23	12.46
7	Melanoma	20	11.54
8	Renal cell carcinoma	19	10.79
9	Pancreatic cancer	18	10.71
10	p53 signaling pathway	19	9.55

Table 9: Displays the top ten targets in the pathway analysis of the miRNAs present at higher levels on day 13 post ovulation using Diana mirPath software.

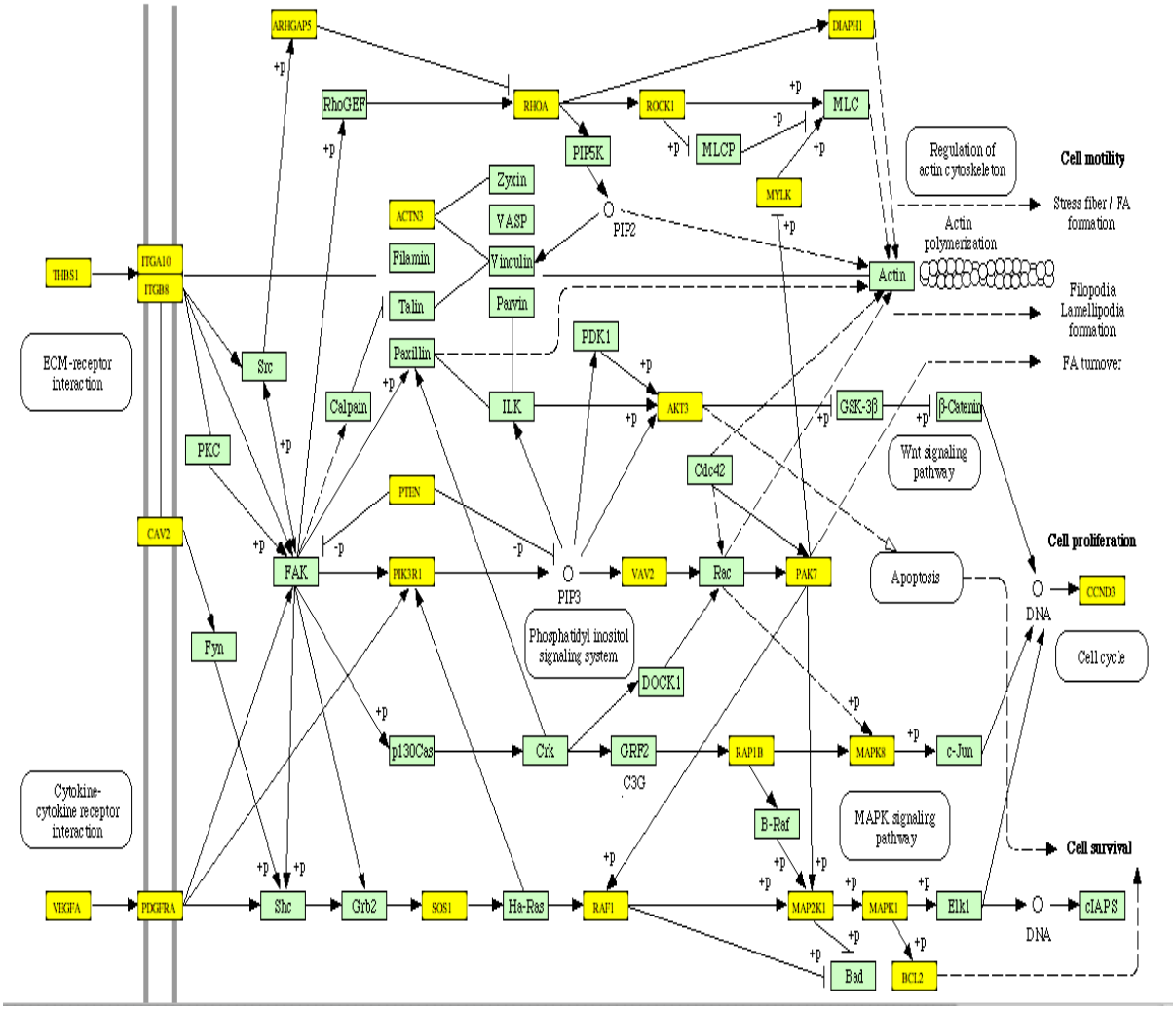

Figure 9: Schematic of Focal Adhesion Pathway

Figure 9: The figure above is a schematic of the focal adhesion pathway which the miRNAs of non-pregnant samples on day 11 and day 13 target

Table 10: Top Gene Targets of miRNAs Up-Regulated on Day 11

Rank	Day 11	Day 13
1	B-cell CLL/lymphoma 2 (BCL2)	BCL2
2	Mitogen-activated protein kinase 8 (MAPK8)	Collagen, type VI, alpha 3 (COL6A3)
3	Protein kinase C, alpha (PRKCA)	Phosphoinositide-2-kinase, catalytic, alpha polypeptide (PIK3CA)
4	Collagen, type IV, alpha 6 (COL4A6)	MAPK8
5	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12) (ITGB1)	ITGB1

Table 10: The top five gene targets of the miRNAs at higher levels in day 11 and day 13. B-cell cLL/lymphoma 2 an apoptotic gene is number one predicted target in both time points.

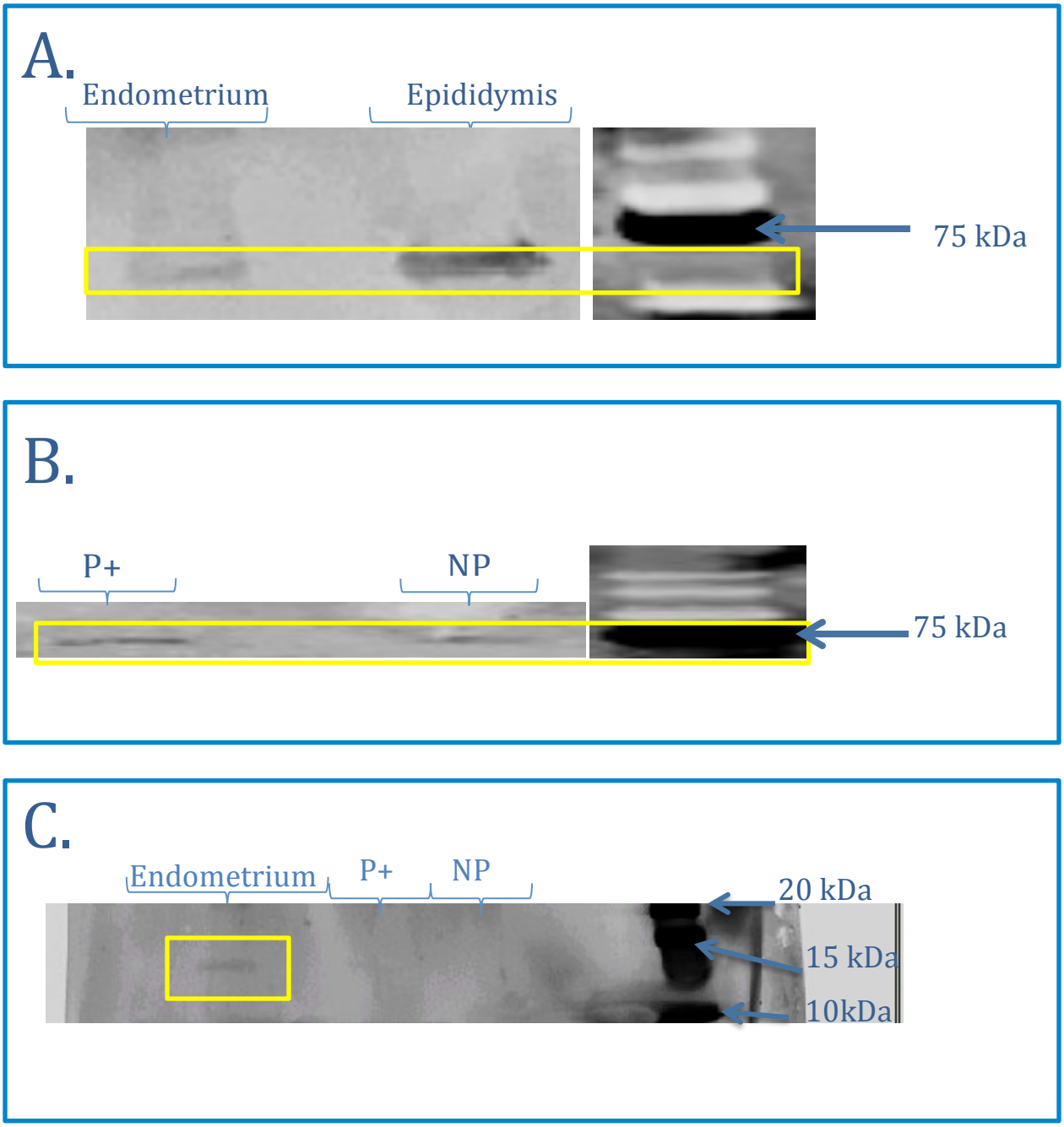

Figure 10: Western Blot Analysis of HSP 70 and Cytochrome C in Serum Exosome Isolations and Endometrium and Epididymis Controls

Figure 10: Panel A demonstrates Western blot analysis of HSP 70 (70 kDa protein) in equine endometrium and epididymis. Panel B demonstrates western blot analysis of HSP 70 from serum exosome isolations using ExoQuick™. Panel C demonstrates Cytochrome C (15 kDa protein) in equine endometrium but not in serum exosome isolations from pregnant and non-pregnant mares.

Discussion

Western blot analysis identified the presence of HSP 70 in endometrium, epididymis, and exosome isolations from serum using ExoQuick™. Additionally we identified cytochrome C, which is tightly associated with mitochondria and therefore separate from the endocytic pathway, in endometrium but not in serum samples by Western blot analysis. Tightly regulated packaging of exosomes within cells, discussed in the review of literature, indicates that the miRNAs that are in higher levels in serum samples from non-pregnant mares are also tightly regulated.

qRT-PCR analysis identified 12 miRNAs that are differentially expressed according to pregnancy status across days 9, 11, and 13 post ovulation. Day 9 results demonstrated eca-miR-433 in higher levels in pregnant mare serum exosomes, the only miRNA that was at a higher level in pregnant animals throughout Experiment II. Day 11 non-pregnant mare serum exosomes had significantly higher levels of miRNAs eca-let-7c, eca-miR-125, eca-miR-195, and eca-miR-451. Day 13 Non-pregnant serum exosomes had significantly higher levels of miRNAs eca-miR-433, eca-miR-195, eca-miR-130b, eca-miR-140-3p, eca-miR-508-3p, eca-miR-767-5p, and eca-miR-770. Interestingly there were higher levels of eca-miR-433 in in samples from pregnant mares preceding maternal recognition of pregnancy but higher levels of the same miRNA in non-pregnant mares during maternal recognition of pregnancy. qRT-PCR analysis of more exosome samples are necessary to confirm these findings. It is possible that the strict regulation of this miRNA may have a role in maternal recognition of pregnancy in the mare. Additionally eca-miR-195 levels are higher in non-pregnant mares on days 11 and 13 indicating possible importance of this miRNA in the mare during maternal recognition of pregnancy.

Pathway analysis of these miRNAs with Diana mirPath analysis software is based on human miRNAs with human targets. Day 11 results are rather straightforward and Table 8 demonstrates the top 10 pathways that are targeted by the miRNAs in higher levels in samples from non-pregnant mares. The top ranked pathway for both day 11 and day 13 analysis is focal adhesion molecules (FAMs). FAMs are macromolecular complexes usually comprised of heterodimeric Transmembrane integrin receptors regulate effects in the extracellular matrix (Burghardt et al. 2009). Vogel and Sheetz (2006) demonstrated that focal adhesions sense and transduce mechanical forces. The transmission of force at cell adhesion sites can regulate pathways regulating growth, development, and proliferation. We found FAMs targeted by non-pregnancy associated exosomal miRNAs which indicates that this pathway is not translationally silenced in pregnant animals. Burghardt et al. (2009) found a variety of differentially organized FAMs in endometrium, myometrium, subepithelial stroma, and maternal-conceptus interface in ovine pregnancy. These FAMs appeared as a result of sensation of transduction of mechanical force from the presence of the elongating conceptus in the peri-implantation period. As stated in the review of the literature maintenance of pregnancy in the mare is dependent upon the embryo moving from one uterine horn to the other many times per day reaching the peak amount of times during day 10. From this understanding of the embryo interacting with the endometrium as well as the target of non-pregnant miRNAs we can propose that the embryo movement is acting as a signal through FAMs that may maintaining the CL in a luteotrophic or luteostatic manner. Wnt signaling is the second ranked pathway analysis of day 13 samples and was ranked number 9 in the day 11 results. Wnt signaling is characteristic in cell-cell signaling in embryogenesis (Logan et al. 2004). Our understanding of what the embryo does when it

interacts with the endometrium is limited but the pathway analysis contributes a number of genes that the serum exosomal miRNAs higher would target in non-pregnant mares. Further analysis of mRNA in the endometrium of the genes targeted by the miRNAs expressed higher serum exosomes from pregnant or non-pregnant mares at the time of maternal recognition of pregnancy may ultimately unveil the enigmatic signal. Additionally these miRNAs may serve as a biomarker for pregnancy or maternal recognition of pregnancy in the mare with further analysis and understanding of the underlying mechanisms.

Significant miRNAs identified on day 16 in Experiment I were not significant on days 9, 11, or 13. hsa-miR-26b does not yet have a homologue identified in the equine genome. However the other 5 significant miRNAs have identical sequences to identified equine miRNAs. Therefore significantly different miRNAs from the first experiment may not be ideal markers for pregnancy across time, however miRNAs may markers for pregnancy at day 16 post-ovulation.

Pathway analysis of miRNAs at significantly different levels from an enriched population of exosomes in pregnant and non-pregnant serum may serve as biomarkers for pregnancy related to uterine changes and maintenance of the CL as well as indicated a pathway that may be related to the signal for maternal recognition of pregnancy in the mare.

CHAPTER IV: DISCUSSION

Results from Experiment I indicate that pregnancy status modifies expression of exosomal miRNAs. Experiment II examined 340 equine miRNAs 75 have different nucleotide sequences. Currently, no homologous human sequence to 15 miRNAs has been identified similar to equine sequences used in Experiment II. The remaining miRNAs have the same nucleotide sequence and similar nomenclature. Our study is the first to our knowledge to identify serum exosomal miRNAs associated with pregnancy in the mare. Exosomal miRNAs can be indicative of physiological processes or disease states. Exosomal miRNAs isolated from serum can serve as peripheral, non-invasive biomarkers. Our aim was to identify exosomal miRNAs differentially expressed between pregnancy and non-pregnant mares before during and after maternal recognition of pregnancy that may serve as biomarkers of pregnancy.

Six miRNAs were found to be at higher levels on day 16 post ovulation; 2 in non-pregnant mares only, 2 in pregnant mares only, and 2 expressed significantly higher in non-pregnant mares. One miRNA, hsa-miR-26b, seems particularly promising as a possible biomarker for pregnancy. This miRNA, up-regulated in exosomes from non-pregnant mares only, may play a role in targeting adherens junctions which has a physiological role in the embryo-endometrial interface once the embryo fixes during pregnancy. Interestingly this miRNA has not been identified in horses prior to this study. Data from day 16 was not identical to samples on day 9, 11, and 13 serum samples of the significantly different human miRNAs. It is possible that there is direct regulation of these miRNAs at specific time-point of pregnancy.

Analysis of day 9, 11, and 13 samples revealed 12 miRNAs present at different levels. Day 9 samples had a higher levels of a miRNA in serum exosomes from pregnant samples. All other miRNAs at higher levels were in non-pregnant serum exosomes. Pathway analysis of day 11 and day 13, before and during maternal recognition of pregnancy in the mare respectively, demonstrated FAMs as the number one target pathway. miRNA targeting FAMs in non-pregnant mares indicates that the pathway is expressed in pregnant mares and it may play a large role in maintaining pregnancy. Due to the significance of a mobile embryo within the mare's uterus to maintain pregnancy FAMs may be conducting a signal through mechanical force and may be a key in answering the unknown signal for maternal recognition of pregnancy.

The cell-of-origin was not identified in the current study. Literature suggests that exosomes can come from many cell types. Exosomes, could therefore, could originate from the endometrium, the embryo, or the CL. These exosomes, isolated from specific cell types during maternal recognition of pregnancy could be much more informative to intercellular communication and inclusion of FAMs during the time maternal recognition of pregnancy in the mare. This study indicates the need for research into the relationship between the targets of the miRNAs at different levels and may lead to the identification of the signal for maternal recognition of pregnancy in the mare.

While our current study was unsuccessful in identifying a biomarker of pregnancy in the mare, it has given more insight into mechanism that may be underlying the signal of maternal recognition of pregnancy. The exosomal miRNAs isolated from serum indicated 18 total miRNAs differentially regulated before, during, and after maternal recognition of pregnancy in the mare. Understanding and harnessing this signal could lead to answering

many problems that currently face the equine industry. Identifying the maternal recognition of pregnancy signal could abrogate early embryonic loss, induce pseudopregnancy in horse herds with over-population problems, and perhaps increase the ability to sustain embryo life *in vitro* all of which are currently impossible. Our data indicate pathways, specifically FAMs, which match to physiological events in mares' pregnancy, namely embryo mobility. These exosomal miRNAs may be indicative of processes very important in pregnancy maintenance and maternal recognition of pregnancy.

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APPENDICES

Appendix A: 380 Human miRNA Mature Sequences

Mature miRNA ID	*Target miRNA Mature Sequence*
hsa-let-7a	UGAGGUAGUAGGUUGUAUAGUU
hsa-let-7b	UGAGGUAGUAGGUUGUGUGGUU
hsa-let-7c	UGAGGUAGUAGGUUGUAUGGUU
hsa-let-7d	AGAGGUAGUAGGUUGCAUAGUU
hsa-let-7e	UGAGGUAGGAGGUUGUAUAGUU
hsa-let-7f	UGAGGUAGUAGAUUGUAUAGUU
hsa-let-7g	UGAGGUAGUAGUUUGUACAGUU
hsa-let-7i	UGAGGUAGUAGUUUGUGCUGUU
hsa-miR-1	UGGAAUGUAAAGAAGUAUGUAU
hsa-miR-7	UGGAAGACUAGUGAUUUUGUUGU
hsa-miR-9	UCUUUGGUUAUCUAGCUGUAUGA
hsa-miR-10a	UACCCUGUAGAUCCGAAUUUGUG
hsa-miR-10b	UACCCUGUAGAACCGAAUUUGUG
hsa-miR-15a	UAGCAGCACAUAAUGGUUUGUG
hsa-miR-15b	UAGCAGCACAUCAUGGUUUACA
hsa-miR-16	UAGCAGCACGUAAAUAUUGGCG
hsa-miR-17	CAAAGUGCUUACAGUGCAGGUAG
hsa-miR-18a	UAAGGUGCAUCUAGUGCAGAUAG
hsa-miR-18b	UAAGGUGCAUCUAGUGCAGUUAG
hsa-miR-19a	UGUGCAAAUCUAUGCAAAACUGA
hsa-miR-19b	UGUGCAAAUCCAUGCAAAACUGA
hsa-miR-20a	UAAAGUGCUUAUAGUGCAGGUAG
hsa-miR-20b	CAAAGUGCUCAUAGUGCAGGUAG
hsa-miR-21	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-22	AAGCUGCCAGUUGAAGAACUGU
hsa-miR-23a	AUCACAUUGCCAGGGAUUUCC
hsa-miR-23b	AUCACAUUGCCAGGGAUUACC
hsa-miR-24	UGGCUCAGUUCAGCAGGAACAG
hsa-miR-25	CAUUGCACUUGUCUCGGUCUGA
hsa-miR-26a	UUCAAGUAAUCCAGGAUAGGCU
hsa-miR-26b	UUCAAGUAAUUCAGGAUAGGU
hsa-miR-27a	UUCACAGUGGCUAAGUUCCGC
hsa-miR-27b	UUCACAGUGGCUAAGUUCUGC
hsa-miR-28-3p	CACUAGAUUGUGAGCUCCUGGA
hsa-miR-28-5p	AAGGAGCUCACAGUCUAUUGAG
hsa-miR-29a	UAGCACCAUCUGAAAUCGGUUA

hsa-miR-29b	UAGCACCAUUUGAAAUCAGUGUU
hsa-miR-29c	UAGCACCAUUUGAAAUCGGUUA
hsa-miR-30a	UGUAAACAUCCUCGACUGGAAG
hsa-miR-30b	UGUAAACAUCCUACACUCAGCU
hsa-miR-30c	UGUAAACAUCCUACACUCUCAGC
hsa-miR-30d	UGUAAACAUCCCCGACUGGAAG
hsa-miR-30e	UGUAAACAUCCUUGACUGGAAG
hsa-miR-31	AGGCAAGAUGCUGGCAUAGCU
hsa-miR-32	UAUUGCACAUUACUAAGUUGCA
hsa-miR-33a	GUGCAUUGUAGUUGCAUUGCA
hsa-miR-33b	GUGCAUUGCUGUUGCAUUGC
hsa-miR-34a	UGGCAGUGUCUUAGCUGGUUGU
hsa-miR-34b	CAAUCACUAACUCCACUGCCAU
hsa-miR-34c-3p	AAUCACUAACCACACGGCCAGG
hsa-miR-34c-5p	AGGCAGUGUAGUUAGCUGAUUGC
hsa-miR-92a	UAUUGCACUUGUCCCGGCCUGU
hsa-miR-92b	UAUUGCACUCGUCCCGGCCUCC
hsa-miR-93	CAAAGUGCUGUUCGUGCAGGUAG
hsa-miR-95	UUCAACGGGUAUUUAUUGAGCA
hsa-miR-96	UUUGGCACUAGCACAUUUUUGCU
hsa-miR-98	UGAGGUAGUAAGUUGUAUUGUU
hsa-miR-99a	AACCCGUAGAUCCGAUCUUGUG
hsa-miR-99b	CACCCGUAGAACCGACCUUGCG
hsa-miR-100	AACCCGUAGAUCCGAACUUGUG
hsa-miR-101	UACAGUACUGUGAUAACUGAA
hsa-miR-103	AGCAGCAUUGUACAGGGCUAUGA
hsa-miR-103-as	UCAUAGCCCUGUACAAUGCUGCU
hsa-miR-105	UCAAAUGCUCAGACUCCUGUGGU
hsa-miR-106a	AAAAGUGCUUACAGUGCAGGUAG
hsa-miR-106b	UAAAGUGCUGACAGUGCAGAU
hsa-miR-107	AGCAGCAUUGUACAGGGCUAUCA
hsa-miR-122	UGGAGUGUGACAAUGGUGUUUG
hsa-miR-124	UAAGGCACGCGGUGAAUGCC
hsa-miR-125a-3p	ACAGGUGAGGUUCUUGGGAGCC
hsa-miR-125a-5p	UCCCUGAGACCCUUUAACCUGUGA
hsa-miR-125b	UCCCUGAGACCCUAACUUGUGA
hsa-miR-126	UCGUACCGUGAGUAAUAAUGCG
hsa-miR-127-3p	UCGGAUCCGUCUGAGCUUGGCU
hsa-miR-127-5p	CUGAAGCUCAGAGGGCUCUGAU
hsa-miR-128	UCACAGUGAACCGGUCUCUUU
hsa-miR-129-3p	AAGCCCUUACCCCAAAAAGCAU

hsa-miR-129-5p	CUUUUUGCGGUCUGGGCUUGC
hsa-miR-130a	CAGUGCAAUGUUAAAAGGGCAU
hsa-miR-130b	CAGUGCAAUGAUGAAAGGGCAU
hsa-miR-132	UAACAGUCUACAGCCAUGGUCG
hsa-miR-133a	UUUGGUCCCCUUCAACCAGCUG
hsa-miR-133b	UUUGGUCCCCUUCAACCAGCUA
hsa-miR-134	UGUGACUGGUUGACCAGAGGGG
hsa-miR-135a	UAUGGCUUUUUAUUCCUAUGUGA
hsa-miR-135b	UAUGGCUUUUCAUUCCUAUGUGA
hsa-miR-136	ACUCCAUUUGUUUUGAUGAUGGA
hsa-miR-137	UUAUUGCUUAAGAAUACGCGUAG
hsa-miR-138	AGCUGGUGUUGUGAAUCAGGCCG
hsa-miR-139-3p	GGAGACGCGGCCCUGUUGGAGU
hsa-miR-139-5p	UCUACAGUGCACGUGUCUCCAG
hsa-miR-140-3p	UACCACAGGGUAGAACCACGG
hsa-miR-140-5p	CAGUGGUUUUACCCUAUGGUAG
hsa-miR-141	UAACACUGUCUGGUAAAGAUGG
hsa-miR-142-3p	UGUAGUGUUUCCUACUUUAUGGA
hsa-miR-142-5p	CAUAAAGUAGAAAGCACUACU
hsa-miR-143	UGAGAUGAAGCACUGUAGCUC
hsa-miR-144	UACAGUAUAGAUGAUGUACU
hsa-miR-145	GUCCAGUUUUCCCAGGAAUCCCU
hsa-miR-146a	UGAGAACUGAAUUCCAUGGGUU
hsa-miR-146b-3p	UGCCCUGUGGACUCAGUUCUGG
hsa-miR-146b-5p	UGAGAACUGAAUUCCAUAGGCU
hsa-miR-147	GUGUGUGGAAAUGCUUCUGC
hsa-miR-147b	GUGUGCGGAAAUGCUUCUGCUA
hsa-miR-148a	UCAGUGCACUACAGAACUUUGU
hsa-miR-148b	UCAGUGCAUCACAGAACUUUGU
hsa-miR-149	UCUGGCUCCGUGUCUUCACUCCC
hsa-miR-150	UCUCCCAACCCUUGUACCAGUG
hsa-miR-151-3p	CUAGACUGAAGCUCCUUGAGG
hsa-miR-151-5p	UCGAGGAGCUCACAGUCUAGU
hsa-miR-152	UCAGUGCAUGACAGAACUUGG
hsa-miR-153	UUGCAUAGUCACAAAAGUGAUC
hsa-miR-154	UAGGUUAUCCGUGUUGCCUUCG
hsa-miR-155	UUAAUGCUAAUCGUGAUAGGGGU
hsa-miR-181a	AACAUUCAACGCUGUCGGUGAGU
hsa-miR-181b	AACAUUCAUUGCUGUCGGUGGGU
hsa-miR-181c	AACAUUCAACCUGUCGGUGAGU
hsa-miR-181d	AACAUUCAUUGUUGUCGGUGGGU

hsa-miR-182	UUUGGCAAUGGUAGAACUCACACU
hsa-miR-183	UAUGGCACUGGUAGAAUUCACU
hsa-miR-184	UGGACGGAGAACUGAUAAGGGU
hsa-miR-185	UGGAGAGAAAGGCAGUUCCUGA
hsa-miR-186	CAAAGAAUUCUCCUUUUGGGCU
hsa-miR-187	UCGUGUCUUGUGUUGCAGCCGG
hsa-miR-188-3p	CUCCCACAUGCAGGGUUUGCA
hsa-miR-188-5p	CAUCCCUUGCAUGGUGGAGGG
hsa-miR-190	UGAUAUGUUUGAUAUAUUAGGU
hsa-miR-190b	UGAUAUGUUUGAUAUUGGGUU
hsa-miR-191	CAACGGAAUCCCAAAAGCAGCUG
hsa-miR-192	CUGACCUAUGAAUUGACAGCC
hsa-miR-193a-3p	AACUGGCCUACAAAGUCCCAGU
hsa-miR-193a-5p	UGGGUCUUUGCGGGCGAGAUGA
hsa-miR-193b	AACUGGCCCUCAAAGUCCCGCU
hsa-miR-194	UGUAACAGCAACUCCAUGUGGA
hsa-miR-195	UAGCAGCACAGAAAUAUUGGC
hsa-miR-196a	UAGGUAGUUUCAUGUUGUUGGG
hsa-miR-196b	UAGGUAGUUUCCUGUUGUUGGG
hsa-miR-197	UUCACCACCUUCUCCACCCAGC
hsa-miR-198	GGUCCAGAGGGGAGAUAGGUUC
hsa-miR-199a-3p	ACAGUAGUCUGCACAUUGGUUA
hsa-miR-199a-5p	CCCAGUGUUCAGACUACCUGUUC
hsa-miR-199b-3p	ACAGUAGUCUGCACAUUGGUUA
hsa-miR-199b-5p	CCCAGUGUUUAGACUAUCUGUUC
hsa-miR-200a	UAACACUGUCUGGUAACGAUGU
hsa-miR-200b	UAAUACUGCCUGGUAAUGAUGA
hsa-miR-200c	UAAUACUGCCGGGUAAUGAUGGA
hsa-miR-202	AGAGGUAUAGGGCAUGGGAA
hsa-miR-203	GUGAAAUGUUUAGGACCACUAG
hsa-miR-204	UUCCCUUUGUCAUCCUAUGCCU
hsa-miR-205	UCCUUCAUUCCACCGGAGUCUG
hsa-miR-206	UGGAAUGUAAGGAAGUGUGUGG
hsa-miR-208a	AUAAGACGAGCAAAAAGCUUGU
hsa-miR-208b	AUAAGACGAACAAAAGGUUUGU
hsa-miR-210	CUGUGCGUGUGACAGCGGCUGA
hsa-miR-211	UUCCCUUUGUCAUCCUUCGCCU
hsa-miR-212	UAACAGUCUCCAGUCACGGCC
hsa-miR-214	ACAGCAGGCACAGACAGGCAGU
hsa-miR-215	AUGACCUAUGAAUUGACAGAC
hsa-miR-216a	UAAUCUCAGCUGGCAACUGUGA

hsa-miR-216b	AAAUCUCUGCAGGCAAAUGUGA
hsa-miR-217	UACUGCAUCAGGAACUGAUUGGA
hsa-miR-218	UUGUGCUUGAUCUAACCAUGU
hsa-miR-219-1-3p	AGAGUUGAGUCUGGACGUCCCG
hsa-miR-219-2-3p	AGAAUUGUGGCUGGACAUCUGU
hsa-miR-219-5p	UGAUUGUCCAAACGCAAUUCU
hsa-miR-220a	CCACACCGUAUCUGACACUUU
hsa-miR-220b	CCACCACCGUGUCUGACACUU
hsa-miR-220c	ACACAGGGCUGUUGUGAAGACU
hsa-miR-221	AGCUACAUUGUCUGCUGGGUUUC
hsa-miR-222	AGCUACAUCUGGCUACUGGGU
hsa-miR-223	UGUCAGUUUGUCAAAUACCCCA
hsa-miR-224	CAAGUCACUAGUGGUUCCGUU
hsa-miR-296-3p	GAGGGUUGGGUGGAGGCUCUCC
hsa-miR-296-5p	AGGGCCCCCCCUCAAUCCUGU
hsa-miR-297	AUGUAUGUGUGCAUGUGCAUG
hsa-miR-298	AGCAGAAGCAGGGAGGUUCUCCCA
hsa-miR-299-3p	UAUGUGGGAUGGUAAACCGCUU
hsa-miR-299-5p	UGGUUUACCGUCCCACAUACAU
hsa-miR-300	UAUACAAGGGCAGACUCUCUCU
hsa-miR-301a	CAGUGCAAUAGUAUUGUCAAAGC
hsa-miR-301b	CAGUGCAAUGAUAUUGUCAAAGC
hsa-miR-302a	UAAGUGCUUCCAUGUUUUGGUGA
hsa-miR-302b	UAAGUGCUUCCAUGUUUUAGUAG
hsa-miR-302c	UAAGUGCUUCCAUGUUUCAGUGG
hsa-miR-302d	UAAGUGCUUCCAUGUUUGAGUGU
hsa-miR-302e	UAAGUGCUUCCAUGCUU
hsa-miR-302f	UAAUUGCUUCCAUGUUU
hsa-miR-320a	AAAAGCUGGGUUGAGAGGGCGA
hsa-miR-320b	AAAAGCUGGGUUGAGAGGGCAA
hsa-miR-320c	AAAAGCUGGGUUGAGAGGGU
hsa-miR-320d	AAAAGCUGGGUUGAGAGGA
hsa-miR-323-3p	CACAUUACACGGUCGACCUCU
hsa-miR-323-5p	AGGUGGUCCGUGGCGCGUUCGC
hsa-miR-324-3p	ACUGCCCCAGGUGCUGCUGG
hsa-miR-324-5p	CGCAUCCCCUAGGGCAUUGGUGU
hsa-miR-325	CCUAGUAGGUGUCCAGUAAGUGU
hsa-miR-326	CCUCUGGGCCCUUCCUCCAG
hsa-miR-328	CUGGCCCUCUCUGCCCUUCCGU
hsa-miR-329	AACACACCUGGUUAACCUCUUU
hsa-miR-330-3p	GCAAAGCACACGGCCUGCAGAGA

hsa-miR-330-5p	UCUCUGGGCCUGUGUCUUAGGC
hsa-miR-331-3p	GCCCCUGGGCCUAUCCUAGAA
hsa-miR-331-5p	CUAGGUAUGGUCCCAGGGAUCC
hsa-miR-335	UCAAGAGCAAUAACGAAAAAUGU
hsa-miR-337-3p	CUCCUAUAUGAUGCCUUUCUUC
hsa-miR-337-5p	GAACGGCUUCAUACAGGAGUU
hsa-miR-338-3p	UCCAGCAUCAGUGAUUUUGUUG
hsa-miR-338-5p	AACAAUAUCCUGGUGCUGAGUG
hsa-miR-339-3p	UGAGCGCCUCGACGACAGAGCCG
hsa-miR-339-5p	UCCCUGUCCUCCAGGAGCUCACG
hsa-miR-340	UUAUAAAGCAAUGAGACUGAUU
hsa-miR-342-3p	UCUCACACAGAAAUCGCACCCGU
hsa-miR-342-5p	AGGGGUGCUAUCUGUGAUUGA
hsa-miR-345	GCUGACUCCUAGUCCAGGGCUC
hsa-miR-346	UGUCUGCCCGCAUGCCUGCCUCU
hsa-miR-361-3p	UCCCCCAGGUGUGAUUCUGAUUU
hsa-miR-361-5p	UUAUCAGAAUCUCCAGGGGUAC
hsa-miR-362-3p	AACACACCUAUUCAAGGAUUCA
hsa-miR-362-5p	AAUCCUUGGAACCUAGGUGUGAGU
hsa-miR-363	AAUUGCACGGUAUCCAUCUGUA
hsa-miR-365	UAAUGCCCCUAAAAAUCCUUAU
hsa-miR-367	AAUUGCACUUUAGCAAUGGUGA
hsa-miR-369-3p	AAUAAUACAUGGUUGAUCUUU
hsa-miR-369-5p	AGAUCGACCGUGUUAUAUUCGC
hsa-miR-370	GCCUGCUGGGGUGGAACCUGGU
hsa-miR-371-3p	AAGUGCCGCCAUCUUUUGAGUGU
hsa-miR-371-5p	ACUCAAACUGUGGGGGCACU
hsa-miR-372	AAAGUGCUGCGACAUUUGAGCGU
hsa-miR-373	GAAGUGCUUCGAUUUUGGGGUGU
hsa-miR-374a	UUAUAAUACAACCUGAUAAGUG
hsa-miR-374b	AUAUAAUACAACCUGCUAAGUG
hsa-miR-375	UUUGUUCGUUCGGCUCGCGUGA
hsa-miR-376a	AUCAUAGAGGAAAAUCCACGU
hsa-miR-376b	AUCAUAGAGGAAAAUCCAUGUU
hsa-miR-376c	AACAUAGAGGAAAUUCCACGU
hsa-miR-377	AUCACACAAAGGCAACUUUUGU
hsa-miR-378	ACUGGACUUGGAGUCAGAAGG
hsa-miR-379	UGGUAGACUAUGGAACGUAGG
hsa-miR-380	UAUGUAAUAUGGUCCACAUCUU
hsa-miR-381	UAUACAAGGGCAAGCUCUCUGU
hsa-miR-382	GAAGUUGUUCGUGGUGGAUUCG

hsa-miR-383	AGAUCAGAAGGUGAUUGUGGCU
hsa-miR-384	AUUCCUAGAAAUUGUUCAUA
hsa-miR-409-3p	GAAUGUUGCUCGGUGAACCCCU
hsa-miR-409-5p	AGGUUACCCGAGCAACUUUGCAU
hsa-miR-410	AAUAUAACACAGAUGGCCUGU
hsa-miR-411	UAGUAGACCGUAUAGCGUACG
hsa-miR-412	ACUUCACCUGGUCCACUAGCCGU
hsa-miR-421	AUCAACAGACAUUAAUUGGGCGC
hsa-miR-422a	ACUGGACUUAGGGUCAGAAGGC
hsa-miR-423-3p	AGCUCGGUCUGAGGCCCCUCAGU
hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU
hsa-miR-424	CAGCAGCAAUUCAUGUUUUGAA
hsa-miR-425	AAUGACACGAUCACUCCCGUUGA
hsa-miR-429	UAAUACUGUCUGGUAAAACCGU
hsa-miR-431	UGUCUUGCAGGCCGUCAUGCA
hsa-miR-432	UCUUGGAGUAGGUCAUUGGGUGG
hsa-miR-433	AUCAUGAUGGGCUCCUCGGUGU
hsa-miR-448	UUGCAUAUGUAGGAUGUCCCAU
hsa-miR-449a	UGGCAGUGUAUUGUUAGCUGGU
hsa-miR-449b	AGGCAGUGUAUUGUUAGCUGGC
hsa-miR-450a	UUUUGCGAUGUGUUCCUAAUAU
hsa-miR-450b-3p	UUGGGAUCAUUUUGCAUCCAUA
hsa-miR-450b-5p	UUUUGCAAUAUGUUCCUGAAUA
hsa-miR-451	AAACCGUUACCAUUACUGAGUU
hsa-miR-452	AACUGUUUGCAGAGGAAACUGA
hsa-miR-453	AGGUUGUCCGUGGUGAGUUCGCA
hsa-miR-454	UAGUGCAAUAUUGCUUAUAGGGU
hsa-miR-455-3p	GCAGUCCAUGGGCAUAUACAC
hsa-miR-455-5p	UAUGUGCCUUUGGACUACAUCG
hsa-miR-483-3p	UCACUCCUCUCCUCCCGUCUU
hsa-miR-483-5p	AAGACGGGAGGAAAGAAGGGAG
hsa-miR-484	UCAGGCUCAGUCCCCUCCCGAU
hsa-miR-485-3p	GUCAUACACGGCUCUCCUCUCU
hsa-miR-485-5p	AGAGGCUGGCCGUGAUGAAUUC
hsa-miR-486-3p	CGGGGCAGCUCAGUACAGGAU
hsa-miR-486-5p	UCCUGUACUGAGCUGCCCCGAG
hsa-miR-487a	AAUCAUACAGGGACAUCCAGUU
hsa-miR-487b	AAUCGUACAGGGUCAUCCACUU
hsa-miR-488	UUGAAAGGCUAUUUCUUGGUC
hsa-miR-489	GUGACAUCACAUAUACGGCAGC
hsa-miR-490-3p	CAACCUGGAGGACUCCAUGCUG

hsa-miR-490-5p	CCAUGGAUCUCCAGGUGGGU
hsa-miR-491-3p	CUUAUGCAAGAUUCCCUUCUAC
hsa-miR-491-5p	AGUGGGGAACCCUUCCAUGAGG
hsa-miR-492	AGGACCUGCGGGACAAGAUUCUU
hsa-miR-493	UGAAGGUCUACUGUGUGCCAGG
hsa-miR-494	UGAAACAUACACGGGAAACCUC
hsa-miR-495	AAACAAACAUGGUGCACUUCUU
hsa-miR-496	UGAGUAUUACAUGGCCAAUCUC
hsa-miR-497	CAGCAGCACACUGUGGUUUGU
hsa-miR-498	UUUCAAGCCAGGGGGCGUUUUUC
hsa-miR-499-3p	AACAUCACAGCAAGUCUGUGCU
hsa-miR-499-5p	UUAAGACUUGCAGUGAUGUUU
hsa-miR-500	UAAUCCUUGCUACCUGGGUGAGA
hsa-miR-501-3p	AAUGCACCCGGGCAAGGAUUCU
hsa-miR-501-5p	AAUCCUUUGUCCCUGGGUGAGA
hsa-miR-502-3p	AAUGCACCUGGGCAAGGAUUCA
hsa-miR-502-5p	AUCCUUGCUAUCUGGGUGCUA
hsa-miR-503	UAGCAGCGGGAACAGUUCUGCAG
hsa-miR-504	AGACCCUGGUCUGCACUCUAUC
hsa-miR-505	CGUCAACACUUGCUGGUUUCCU
hsa-miR-506	UAAGGCACCCUUCUGAGUAGA
hsa-miR-507	UUUUGCACCUUUUGGAGUGAA
hsa-miR-508-3p	UGAUUGUAGCCUUUUGGAGUAGA
hsa-miR-508-5p	UACUCCAGAGGGCGUCACUCAUG
hsa-miR-509-3-5p	UACUGCAGACGUGGCAAUCAUG
hsa-miR-509-3p	UGAUUGGUACGUCUGUGGGUAG
hsa-miR-509-5p	UACUGCAGACAGUGGCAAUCA
hsa-miR-510	UACUCAGGAGAGUGGCAAUCAC
hsa-miR-511	GUGUCUUUUGCUCUGCAGUCA
hsa-miR-512-3p	AAGUGCUGUCAUAGCUGAGGUC
hsa-miR-512-5p	CACUCAGCCUUGAGGGCACUUUC
hsa-miR-513a-3p	UAAAUUUCACCUUUCUGAGAAGG
hsa-miR-513a-5p	UUCACAGGGAGGUGUCAU
hsa-miR-513b	UUCACAAGGAGGUGUCAUUUAU
hsa-miR-513c	UUCUCAAGGAGGUGUCGUUUAU
hsa-miR-514	AUUGACACUUCUGUGAGUAGA
hsa-miR-515-3p	GAGUGCCUUCUUUUGGAGCGUU
hsa-miR-515-5p	UUCUCCAAAAGAAAGCACUUUCUG
hsa-miR-516a-3p	UGCUUCCUUUCAGAGGGU
hsa-miR-516a-5p	UUCUCGAGGAAAGAAGCACUUUC
hsa-miR-516b	AUCUGGAGGUAAGAAGCACUUU

hsa-miR-517a	AUCGUGCAUCCCUUUAGAGUGU
hsa-miR-517b	UCGUGCAUCCCUUUAGAGUGUU
hsa-miR-517c	AUCGUGCAUCCUUUUAGAGUGU
hsa-miR-518a-3p	GAAAGCGCUUCCCUUUGCUGGA
hsa-miR-518a-5p	CUGCAAAGGGAAGCCCUUUC
hsa-miR-518b	CAAAGCGCUCCCCUUUAGAGGU
hsa-miR-518c	CAAAGCGCUUCUCUUUAGAGUGU
hsa-miR-518d-3p	CAAAGCGCUUCCCUUUGGAGC
hsa-miR-518d-5p	CUCUAGAGGGAAGCACUUUCUG
hsa-miR-518e	AAAGCGCUUCCCUUCAGAGUG
hsa-miR-518f	GAAAGCGCUUCUCUUUAGAGG
hsa-miR-519a	AAAGUGCAUCCUUUUAGAGUGU
hsa-miR-519b-3p	AAAGUGCAUCCUUUUAGAGGUU
hsa-miR-519b-5p	CUCUAGAGGGAAGCGCUUUCUG
hsa-miR-519c-3p	AAAGUGCAUCUUUUUAGAGGAU
hsa-miR-519c-5p	CUCUAGAGGGAAGCGCUUUCUG
hsa-miR-519d	CAAAGUGCCUCCCUUUAGAGUG
hsa-miR-519e	AAGUGCCUCCUUUUAGAGUGUU
hsa-miR-520a-3p	AAAGUGCUUCCCUUUGGACUGU
hsa-miR-520a-5p	CUCCAGAGGGAAGUACUUUCU
hsa-miR-520b	AAAGUGCUUCCUUUUAGAGGG
hsa-miR-520c-3p	AAAGUGCUUCCUUUUAGAGGGU
hsa-miR-520c-5p	CUCUAGAGGGAAGCACUUUCUG
hsa-miR-520d-3p	AAAGUGCUUCUCUUUGGUGGGU
hsa-miR-520d-5p	CUACAAAGGGAAGCCCUUUC
hsa-miR-520e	AAAGUGCUUCCUUUUUGAGGG
hsa-miR-520f	AAGUGCUUCCUUUUAGAGGGUU
hsa-miR-520g	ACAAAGUGCUUCCCUUUAGAGUGU
hsa-miR-520h	ACAAAGUGCUUCCCUUUAGAGU
hsa-miR-521	AACGCACUUCCCUUUAGAGUGU
hsa-miR-522	AAAAUGGUUCCCUUUAGAGUGU
hsa-miR-523	GAACGCGCUUCCCUAUAGAGGGU
hsa-miR-524-3p	GAAGGCGCUUCCCUUUGGAGU
hsa-miR-524-5p	CUACAAAGGGAAGCACUUUCUC
hsa-miR-525-3p	GAAGGCGCUUCCCUUUAGAGCG
hsa-miR-525-5p	CUCCAGAGGGAUGCACUUUCU
hsa-miR-526a	CUCUAGAGGGAAGCACUUUCUG
hsa-miR-526b	CUCUUGAGGGAAGCACUUUCUGU
hsa-miR-527	CUGCAAAGGGAAGCCCUUUC
hsa-miR-532-3p	CCUCCCACACCCAAGGCUUGCA
hsa-miR-532-5p	CAUGCCUUGAGUGUAGGACCGU

hsa-miR-539	GGAGAAAUUAUCCUUGGUGUGU
hsa-miR-541	UGGUGGGCACAGAAUCUGGACU
hsa-miR-542-3p	UGUGACAGAUUGAUAACUGAAA
hsa-miR-542-5p	UCGGGGAUCAUCAUGUCACGAGA
hsa-miR-543	AAACAUUCGCGGUGCACUUCUU
hsa-miR-544	AUUCUGCAUUUUUAGCAAGUUC
hsa-miR-545	UCAGCAAACAUUUAUUGUGUGC
hsa-miR-548a-3p	CAAAACUGGCAAUUACUUUUGC
hsa-miR-548a-5p	AAAAGUAAUUGCGAGUUUUACC
hsa-miR-548b-3p	CAAGAACCUCAGUUGCUUUUGU
hsa-miR-548b-5p	AAAAGUAAUUGUGGUUUUGGCC
hsa-miR-548c-3p	CAAAAAUCUCAAUUACUUUUGC
hsa-miR-548c-5p	AAAAGUAAUUGCGGUUUUUGCC
hsa-miR-548d-3p	CAAAAACCACAGUUUCUUUUGC
hsa-miR-548d-5p	AAAAGUAAUUGUGGUUUUUGCC
hsa-miR-548e	AAAAACUGAGACUACUUUUGCA
HUman U6 snRNA	CGCAAGGAUGACACGCAAAUUC
RNU43 snoRNA	CUUAUUGACGGGCGGACAGAAAC
Hm/Ms/RU U1 snRNA	CGACUGCAUAAUUUGUGGUAGUGG

Appendix B: 340 Equine Mature Sequence miRNAs

Mature miRNA ID	*Target miRNA Mature Sequence*
eca-let-7a	ugagguaguagguuguauaguu
eca-let-7c	ugagguaguagguuguaugguu
eca-let-7d	agagguaguagguugcauaguu
eca-let-7e	ugagguaggagguuguauaguu
eca-let-7f	ugagguaguagauuguauaguu
eca-let-7g	ugagguaguaguuuguacaguu
eca-miR-1	uggaauguaaagaaguauguau
eca-miR-7	uggaagacuagugauuuuguugu
eca-miR-9a	ucuuugguuaucuagcuguauga
eca-miR-10a	uacccuguagauccgaauuugug
eca-miR-10b	uacccuguagaaccgaauuugug
eca-miR-15a	uagcagcacauaaugguuugug
eca-miR-15b	uagcagcacaucaugguuuaca
eca-miR-16	uagcagcacguaaauauuggcg
eca-miR-17	caaagugcuuacagugcagguag
eca-miR-18a	uaaggugcaucuagugcagauag
eca-miR-18b	uaaggugcaucuagugcaguuag
eca-miR-19a	ugugcaaaucuaugcaaaacuga
eca-miR-19b	ugugcaaaucuaugcaaaacuga
eca-miR-20a	uaaagugcuuauagugcagguag
eca-miR-20b	caaagugcucauagugcagguag
eca-miR-21	uagcuuaucagacugauguuga
eca-miR-22	aagcugccaguugaagaacugu
eca-miR-23a	aucacauugccagggauuucc
eca-miR-23b	aucacauugccagggauuacc
eca-miR-24	uggcucaguucagcaggaacag
eca-miR-25	cauugcacuugucucggucuga
eca-miR-26a	uucaaguaauccaggauaggcu
eca-miR-27a	uucacaguggcuaaguuccgc
eca-miR-27b	uucacaguggcuaaguucugc
eca-miR-28-3p	cacuagauugugagcuccugga
eca-miR-28-5p	aaggagcucacagucuauugag
eca-miR-29a	uagcaccaucugaaaucgguua
eca-miR-29b	uagcaccauuugaaaucaguguu
eca-miR-29c	uagcaccauuugaaaucgguua
eca-miR-30b	uguaaacauccuacacucagcu
eca-miR-30c	uguaaacauccuacacucucagc

eca-miR-30d	uguaaacaucccgacuggaag
eca-miR-30e	uguaaacauccuugacuggaag
eca-miR-31	aggcaagaugcuggcauagcu
eca-miR-32	uauugcacauuacuaaguugca
eca-miR-33a	gugcauuguaguugcauugca
eca-miR-33b	gugcauugcuguugcauugc
eca-miR-34	uggcagugucuuagcugguugu
eca-miR-92a	uauugcacuugucccggccugu
eca-miR-92b	uauugcacucgucccggccucc
eca-miR-93	caaagugcuguucgugcagguag
eca-miR-95	uucaacgggucuuuauugagca
eca-miR-96	uuuggcacuagcacauuuuugcu
eca-miR-98	ugagguaguaaguuguauuguu
eca-miR-99a	aacccguagauccgaucuugug
eca-miR-99b	cacccguagaaccgaccuugcg
eca-miR-100	aacccguagauccgaacuugug
eca-miR-101	uacaguacugugauaacugaa
eca-miR-103	agcagcauuguacagggcuauga
eca-miR-105	ucaaaugcucagacuccuguggu
eca-miR-106a	caaagugcuuacagugcagguag
eca-miR-106b	uaaagugcugacagugcagau
eca-miR-107b	agcagcauuguacagggcuauca
eca-miR-122	uggagugugacaaugguguuug
eca-miR-124	uaaggcacgcggugaaugcc
eca-miR-125a-3p	acaggugagguucuugggagcc
eca-miR-125a-5p	ucccugagacccuuuaaccuguga
eca-miR-125b	ucccugagacccuaacuuguga
eca-miR-126-3p	ucguaccgugaguaauaaugcg
eca-miR-127	ucggauccgucugagcuuggcu
eca-miR-128	ucacagugaaccggucucuuu
eca-miR-129a-3p	aagcccuuaccccaaaaaguau
eca-miR-129a-5p	cuuuuugcggucugggcuugc
eca-miR-130a	cagugcaauguuaaaagggcau
eca-miR-130b	cagugcaaugaugaaagggcau
eca-miR-132	uaacagucuacagccauggucg
eca-miR-133a	uuuggucccccuucaaccagcug
eca-miR-133b	uuuggucccccuucaaccagcua
eca-miR-134	ugugacugguugaccagagggg
eca-miR-135a	uauggcuuuuuauuccuauguga
eca-miR-135b	uauggcuuuucauuccuauguga
eca-miR-136	acuccauuuguuuugaugaugg

eca-miR-137	uuauugcuuaagaauacgcguag
eca-miR-138	agcugguguugugaaucaggccg
eca-miR-139-3p	ggagacgcggcccuguuggagu
eca-miR-139-5p	ucuacagugcacgugucuccag
eca-miR-140-3p	uaccacaggguagaaccacgg
eca-miR-140-5p	cagugguuuuacccuaugguag
eca-miR-141	uaacacugucugguaaagaugg
eca-miR-142-3p	uguaguguuuccuacuuuaugga
eca-miR-142-5p	cauaaaguagaaagcacuacu
eca-miR-143	ugagaugaagcacuguagcuc
eca-miR-144	uacaguauagaugauguacu
eca-miR-145	guccaguuuucccaggaaucccu
eca-miR-146a	ugagaacugaauuccauggguu
eca-miR-146b-3p	ugcccuagggacucaguucugg
eca-miR-146b-5p	ugagaacugaauuccauaggcu
eca-miR-147b	gugugccgaaaugcuucugcua
eca-miR-148a	ucagugcacuacagaacuuugu
eca-miR-148b-3p	ucagugcaucacagaacuuugu
eca-miR-149	ucuggcuccgugucuucacuccc
eca-miR-150	ucucccaacccuuguaccagug
eca-miR-151-5p	ucgaggagcucacagucuagu
eca-miR-153	uugcauagucacaaaagugauc
eca-miR-154	uagguuauccguguugccuucg
eca-miR-155	uuaaugcuaaucgugauaggggu
eca-miR-181a	aacauucaacgcugucggugagu
eca-miR-181b	aacauucauugcugucgguggguu
eca-miR-182	uuuggcaaugguagaacucacacug
eca-miR-183	uauggcacugguagaauucacu
eca-miR-184	uggacggagaacugauaagggu
eca-miR-186	caaagaauucuccuuuugggcu
eca-miR-187	ucgugucuuguguugcagccgg
eca-miR-188-3p	cucccacaugcaggguuugca
eca-miR-188-5p	caucccuugcaugguggaggg
eca-miR-190	ugauauguuugauauauuaggu
eca-miR-190b	ugauauguuugauauuggguu
eca-miR-191	caacggaaucccaaaagcagcug
eca-miR-192	cugaccuaugaauugacagcc
eca-miR-193a-3p	aacuggccuacaaagucccagu
eca-miR-193a-5p	uggguuuugcgggcgagauga
eca-miR-193b	aacuggcccacaaagucccgcu
eca-miR-194	uguaacagcaacuccaugugga

eca-miR-195	uagcagcacagaaauauuggc
eca-miR-196a	uagguaguuucauguuguuggg
eca-miR-196b	uagguaguuuccuguuguuggg
eca-miR-197	uucaccaccuucuccacccagc
eca-miR-199a-3p	acaguagucugcacauugguag
eca-miR-199a-5p	cccaguguucagacuaccuguuc
eca-miR-199b-3p	acaguagucugcacauugguua
eca-miR-199b-5p	cccaguguuuagacuaucuguuc
eca-miR-200a	uaacacugucugguaacgaugu
eca-miR-200b	uaauacugccugguaaugauga
eca-miR-200c	uaauacugccggguaaugaugga
eca-miR-204b	uucccuuugucauccuaugccu
eca-miR-205	uccuucauuccaccggagucug
eca-miR-206	uggaauguaaggaagugugugg
eca-miR-208a	auaagacgagcaaaaagcuugu
eca-miR-208b	auaagacgaacaaaagguuugu
eca-miR-211	uucccuuugucauccuuugccu
eca-miR-212	uaacagucuccagucacggcc
eca-miR-214	acagcaggcacagacaggcagu
eca-miR-215	augaccuaugaauugacagac
eca-miR-216a	uaaucucagcuggcaacuguga
eca-miR-216b	aaaucucugcaggcaaauguga
eca-miR-217	uacugcaucaggaacugauugga
eca-miR-218	uugugcuugaucuaaccaugu
eca-miR-219-5p	ugauuguccaaacgcaauucu
eca-miR-221	agcuacauugucugcuggguuuc
eca-miR-222	agcuacaucuggcuacugggu
eca-miR-223	ugucaguuugucaaauacccca
eca-miR-224	caagucacuagugguuccguu
eca-miR-296	gaggguuggguggaggcuuucc
eca-miR-299	uaugugggaugguaaaccgcuu
eca-miR-301a	cagugcaauaguauugucaaagc
eca-miR-301b-3p	cagugcaaugauauugucaaagc
eca-miR-302a	uaagugcuuccauguuuuaguga
eca-miR-302b	uaagugcuuccauguuuuaguag
eca-miR-302c	uaagugcuuccauguuucagugg
eca-miR-302d	uaagugcuuccauguuuuagugu
eca-miR-323-3p	cacauuacacggucgaccucu
eca-miR-323-5p	aggugguccguggcgcguucgc
eca-miR-324-3p	ccacugccccaggugcugcugg
eca-miR-324-5p	cgcauccccuagggcauuggugu

eca-miR-326	ccucugggcccuuccuccagc
eca-miR-328	cuggcccucucugcccuuccgu
eca-miR-329	aacacaccuaguuaaccucuuu
eca-miR-330	ucucugggccugugucuuaggc
eca-miR-331	gccccugggccuauccuagaa
eca-miR-335	ucaagagcaauaacgaaaaaugu
eca-miR-337-3p	cuccuaugagaugccuuuccuc
eca-miR-337-5p	gaacggcuucauacaggagcu
eca-miR-338-3p	uccagcaucagugauuuuguug
eca-miR-338-5p	aacaauauccuggugcugagug
eca-miR-340-5p	uuauaaagcaaugagacugauu
eca-miR-342-3p	ucucacacagaaaucgcacccgu
eca-miR-342-5p	aggggugcuaucugugauugag
eca-miR-345-5p	gcugacuccuaguccagugcuc
eca-miR-346	ugucugcccgcaugccugccucu
eca-miR-361-3p	uccccaggcgugauucugauuu
eca-miR-361-5p	uuaucagaaucuccaggguac
eca-miR-3623-p	aacacaccuauucaaggauuca
eca-miR-362-5p	aauccuuggaaccuaggugugagu
eca-miR-363	aauugcacgguauccaucugua
eca-miR-365	uaaugccccuaaaaauccuuau
eca-miR-367	aauugcacuuuagcaaugguga
eca-miR-369-3p	aauaauacaugguugaucuuu
eca-miR-369-5p	agaucgaccgugucauauucgc
eca-miR-370	gccugcuggguggaaccuggu
eca-miR-371-3p	aagugccgccauuuuuugagugu
eca-miR-371-5p	acucaaacuguggggcacu
eca-miR-374a	uuauaauacaaccugauaagug
eca-miR-374b	auauaauacaaccugcuaagug
eca-miR-376a	aucauagaggaaaauccacgu
eca-miR-376b	aucauagaggaaaauccaugu
eca-miR-376c	aacauagaggaaauuccacgu
eca-miR-377	aucacacaaaggcaacuuuugu
eca-miR-378	acuggacuuggagucagaagg
eca-miR-379	ugguagacuauggaacguagg
eca-miR-380	uauguaauaugguccacgucuu
eca-miR-381	uauacaagggcaagcucucugu
eca-miR-382	gaaguuguucgugguggauucg
eca-miR-383	agaucagaaggugauugguggcu
eca-miR-384	auuccuagaaauuguucaca
eca-miR-409-3p	gaauguugcucggugaacccu

eca-miR-409-5p	agguuacccgagcaacuuugcau
eca-miR-410	aauauaacacagauggccugu
eca-miR-411	uaguagaccguauagcguacg
eca-miR-412	uucaccugguccacuagccg
eca-miR-421	ggccucauuaaauguuuguug
eca-miR-423-3p	agcucggucugaggccccucagu
eca-miR-423-5p	ugaggggcagagagcgagacuuu
eca-miR-424	cagcagcaauucauguuuugaa
eca-miR-429	uaauacugucugguaaugccg
eca-miR-431	ugucuugcaggccgucaugcagg
eca-miR-432	ucuuggaguaggucauugggugg
eca-miR-433	aucaugaugggcuccucggugu
eca-miR-448	uugcauauguaggaugucccau
eca-miR-449a	uggcaguguauuguuagcuggu
eca-miR-450a	uuuugcgauguguuccuaauau
eca-miR-450b-3p	uugggaacauuuugcauccaua
eca-miR-450b-5p	uuuugcaauauguuccugaaua
eca-miR-451	aaaccguuaccauuacuguguu
eca-miR-454	uagugcaauauugcuuauagggu
eca-miR-485-3p	gucauacacggcucuccucucu
eca-miR-485-5p	agaggcuggccgugaugaauuc
eca-miR-486-3p	cggggcagcucaguacaggau
eca-miR-486-5p	uccuguacugagcugccccgag
eca-miR-487a	aaucauacagggacauccaguu
eca-miR-487b	aaucguacagggucauccacuu
eca-miR-488	uugaaaggcuauuucuugguc
eca-miR-489	gugacaucacauauacggcggc
eca-miR-490-3p	caaccuggaggacuccaugcug
eca-miR-490-5p	ccauggaucuccaggugggu
eca-miR-491-3p	cuuaugcaagauucccuucuac
eca-miR-491-5p	aguggggaacccuuccaugagg
eca-miR-492	aggagcugcgggacaagauucuu
eca-miR-493b	ugaaggucuuccgugugccagg
eca-miR-494	ugaaacauacacgggaaaccuc
eca-miR-495	aaacaaacauggugcacuucuu
eca-miR-496	ugaguauuacauggccaaucuc
eca-miR-497	cagcagcacacugugguuugu
eca-miR-499-3p	aacaucacagcaagucugugcu
eca-miR-499-5p	uuaagacuugcagugauguuu
eca-miR-500	uaauccuugcuaccugggugaga
eca-miR-501	auccuucgucccugggugaga

eca-miR-502-3p	aaugcaccugggcaaggauuca
eca-miR-502-5p	auccuugcuaucugggugcua
eca-miR-503	uagcagcgggaacaguacugcag
eca-miR-504	agacccuggucugcacucuauc
eca-miR-505	cgucaacacuugcugguuuccu
eca-miR-507	auuggcaccucuuagagugaa
eca-miR-508-3p	ugauugucaccuuuuggaguaga
eca-miR-508-5p	uacuccagagggugucauucaca
eca-miR-509-5p	uacugcagacaguggcaauca
eca-miR-514	auugacaccucugugagugga
eca-miR-532-3p	ccucccacacccaaggcuugca
eca-miR-532-5p	caugccuugaguguaggaccgu
eca-miR-539	ggagaaauuauccuugcugugu
eca-miR-541	ugggugggcacagaauccagucu
eca-miR-542-3p	ugugacagauugauaacugaaa
eca-miR-542-5p	cucggggaucaucaugucacga
eca-miR-543	aaacauucgcggugcacuucuu
eca-miR-544b	auucugcauuuuuuaacaaguuc
eca-miR-545	ucaacaaacauuuauugugugc
eca-miR-551a	gcgacccacucuugguuucca
eca-miR-551b	gcgacccauacuugguuucag
eca-miR-568	auguauaaauguauacacac
eca-miR-582-5p	uuacaguuguucaaccaguuacu
eca-miR-582-3p	uaaccgguugaacaacugaacc
eca-miR-590-5p	gagcuuauucauaaaaguacag
eca-miR-590-3p	uaauuuuauguauaagcuagu
eca-miR-592	uugugucaauaugcgaugaugu
eca-miR-598	uacgucaucguugucaucguca
eca-miR-615-5p	gggggucccggugcucggauc
eca-miR-615-3p	uccgagccuggucucccucuc
eca-miR-628a	augcugacauauuuacuagagg
eca-miR-632	gugccuguuuccuguggga
eca-miR-652	aauggcgccacuaggguugug
eca-miR-653	guguugaaacaaucucugcug
eca-miR-655	auaauacaugguuaaccucuuu
eca-miR-656	aauauuauacagucaaccucu
eca-miR-660	uacccauugcauaucggaguug
eca-miR-664	uauucauuuaucuccuagccuaca
eca-miR-670	gucccugaguguauguggugaa
eca-miR-671-5p	aggaagcccuggaggggcuggag
eca-miR-671-3p	uccgguucucagggcuccacc

eca-miR-672	ugagguugguguacugugugga
eca-miR-674-5p	ggugcucacuuguccuccu
eca-miR-674-3p	aggaggccauaguggcaacugu
eca-miR-675	uggcgcggagagggcccacagug
eca-miR-684	aguuuucccuucaauucag
eca-miR-703	aaaaccuucagaaggaaagga
eca-miR-708	aaggagcuuacaaucuagcuggg
eca-miR-711	gggacccagggagagacguaag
eca-miR-758	uuugugaccugguccacuaacc
eca-miR-761	gcagcagggugaaacugacaca
eca-miR-763	ccagcugggaggaaccaguggc
eca-miR-767-5p	ugcaccaugguugucugagcaug
eca-miR-767-3p	ucugcucauacuccaugguuccu
eca-miR-769-5p	ggagaccucuggguucugagcu
eca-miR-769-3p	cugggaucucgggggucuugguu
eca-miR-769b	ggaaaccucuggguucugagcu
eca-miR-770	agcaccacgugucugggccaug
eca-miR-802	caguaacaaagauucauccuugu
eca-miR-872	aagguuacuuguuaguucagg
eca-miR-873	gcaggaacuugugagucuccu
eca-miR-874	cugcccuggcccgagggaccga
eca-miR-876-5p	uggauuucuuugugaaucacca
eca-miR-876-3p	ugguggugguuuacaaaguaauuca
eca-miR-885-5p	uccauuacacuacccugccucu
eca-miR-885-3p	aggcagcgggguguaguggaua
eca-miR-889	uuaauaucggacaaccauugu
eca-miR-1179	aagcauucuuucauugguugg
eca-miR-1180	uuuccggcucgaguggguguu
eca-miR-1185	agaggauacccuuuguauguu
eca-miR-1193	uaggucacccguuugacuauc
eca-miR-1197	uaggacacauggucuacuucu
eca-miR-1204	ucguggccuggucccacuau
eca-miR-1244	gagugguugguuuguaugagaugguu
eca-miR-1248	uccuucuuguauaagcacugugcuaaa
eca-miR-1255b	cggauaagcaaagaaaguggguu
eca-miR-1261	guggauuaggcuuuggcuu
eca-miR-1264	caagucuuauuugagcaccuguu
eca-miR-1271	cuuggcaccucguaagcacuca
eca-miR-1282	agugguugguuuguaugagaugguu
eca-miR-1289	uggaguccaggaaucugcauuuu
eca-miR-1291a	uggcccugacugaagaccagcagu

eca-miR-1291b	aggcccugaaucaagaccagcagu
eca-miR-1296	uuagggcccuggcuccaucucc
eca-miR-1298	uucauucggcuguccagaugua
eca-miR-1301	uugcagcugccugggagugauuuc
eca-miR-13021	uugggacauacuuauacuaaa
eca-miR-1302b2	uugggacauacuuauacuaga
eca-miR-1302d4	uugggacauacuuaugcuaaa
eca-miR-1302e6	uugggauauacuuauacuaaa
eca-miR-1302e7	uugggauauacuuauacuaaa
eca-miR-1302c5	uugcgacauacuuauacuaaa
eca-miR-1461	aucucuacggguaaguguguga
eca-miR-1468	cuccguuugccuguuuugcug
eca-miR-1597	ugaggagcucugcgagcaugua
eca-miR-1839	aagguagauagaacaggucuug
eca-miR-1842	uggcucugugaggucggcuca
eca-miR-1892	auuuggggugggggaugggga
eca-miR-1898	aggucaagguucacaggggauc
eca-miR-1902	agaggugcaguaggcaugacuu
eca-miR-1905a	caccacgagcccuaccacgcgguag
eca-miR-1905b	caccagccccacuacgcgguag
eca-miR-1905c	caccaccagccccaccacgcgguag
eca-miR-1912	uacccagagcgugcagugugaa

Appendix C: 75 Equine miRNAs with Different Nucleotide Sequences

*Note: Locations of sequence differences between human and equine miRNAs are underlined

Mature miRNA ID	*Target miRNA Mature Sequence*
eca-mir-19b	ugugcaaauc***u***augcaaaacuga
eca-mir-95	uucaacgggu***c***uuuauugagca
eca-mir-106a	***c***aaagugcuuacagugcagguag
eca-mir-129a-3p	aagcccuuaccccaaaaag***u***au
eca-mir-136	acuccauuuguuuugaugaugg_
eca-mir-146b-3p	ugcccu***ag***ggacucaguucugg
eca-mir-147b	gugugc***c***gaaaugcuucugcua
eca-mir-182	uuuggcaaugguagaacucacacu***g***
eca-mir-193b	aacuggccc***a***caaagucccgcu
eca-mir-199a-3p	acaguagucugcacauuggu***ag***
eca-mir-211	uucccuuugucauccuu***u***gccu
eca-mir-296	gaggguuggguggaggcu***u***ucc
eca-mir-302a	uaagugcuuccauguuuu***a***guga
eca-mir-302d	uaagugcuuccauguuu***u***agugu
eca-mir-324-3p	***cc***acugccccaggugcugcugg
eca-mir-329	aacacaccu***a***guuaaccucuuu
eca-mir-337-3p	cuccuau***ga***gaugccuuuc***c***uc
eca-mir-337-5p	gaacggcuucauacaggag***c***u
eca-mir-342-5p	aggggugcuaucugugauuga***g***
eca-mir-345-5p	gcugacuccuaguccag***u***gcuc
eca-mir-361-3p	ucccccagg***c***gugauucugauuu
eca-mir-369-5p	agaucgaccgugu***c***auauucgc
eca-mir-371-3p	aagugccgccau***u***uuuugagugu
eca-mir-376b	aucauagaggaaaauccaugu_
eca-mir-380	uauguaauaugguccac***g***ucuu
eca-mir-384	auuccuagaaauuguuca***c***a
eca-mir-412	__uucaccugguccacuagccg_
eca-mir-421	***gg***c***cu***ca***uuua***a***a***u***gu***uug***uug***
eca-mir-429	uaauacugucugguaa***ug***ccg_
eca-mir-431	ugucuugcaggccgucaugca***gg***
eca-mir-450b-3p	uuggga***a***cauuuugcauccaua
eca-mir-451	aaaccguuaccauuacug***u***guu
eca-mir-489	gugacaucacauauacggc***g***gc
eca-mir-492	agga***g***cugcgggacaagauucuu
eca-mir-493b	ugaaggucu***u***c***c***gugugccagg
eca-mir-501	_auccuu***c***gucccugggugaga

eca-mir-503	uagcagcgggaacagu***a***cugcag
eca-mir-507	***a***uu***g***gcaccu***c***uu***a***gagugaa
eca-mir-508-3p	ugauugu***ca***ccuuuuggaguaga
eca-mir-508-5p	uacuccagaggg***u***guca***u***uca***ca***
eca-mir-514	auugacac***c***ucugugagu***g***ga
eca-mir-539	ggagaaauuauccuug***c***ugugu
eca-mir-541	uggugggcacagaauc***ca***g***u***cu
eca-mir-542-5p	***c***ucggggaucaucaugucacga__
eca-mir-544b	auucugcauuuuua***a***caaguuc
eca-mir-545	uca***a***caaacauuuauugugugc
eca-mir-582-3p	uaac***c***gguugaacaacugaacc
eca-mir-590-5p	gagcuuauucauaaaagu***a***cag
eca-mir-615-3p	uccgagccugggucucccucu***c***
eca-mir-632	gug***c***cug***u***uuccuguggga
eca-mir-653	guguugaaacaaucucu***g***cug
eca-mir-664	uauucauuuauc***u***cc***u***agccuaca
eca-mir-675	ugg***c***gcggagagggcccacagug
eca-mir-767-3p	ucugcucauac***u***ccaugguu***c***cu
eca-mir-769-5p	***g***gagaccucuggguucugagcu
eca-mir-769-3p	cugggaucuc***g***ggggucuugguu
eca-mir-769b	***g***ga***a***accucuggguucugagcu
eca-mir-770	***ag***ca***cc***ac***g***uguc***u***gggcca***ug***
eca-mir-1180	uuuccggcucg**a**gugggugugu
eca-mir-1204	ucguggccugguc***c***cca***c***uau
eca-mir-1244	***g***agu***g***guugguuuguaugagaugguu
eca-mir-1248	***u***ccuucuuguauaagcacugugcuaaa
eca-mir-1255b	cggau***a***agcaaagaaaguggguu
eca-mir-1261	***g***uggau***u***aggcuuuggcuu
eca-mir-1271	cuuggcaccu***c***g***u***aagcacuca
eca-mir-1282	_agu***g***guugguuuguaugagaugguu
eca-mir-1291b	***a***ggcccuga***a***u***c***aagaccagcagu
eca-mir-1301	uugcagcugccugggaguga***u***uuc
eca-mir-1302-1	uugggacauacuuau***a***cuaaa
eca-mir-1302b-2	uugggacauacuuau***a***cua***g***a
eca-mir-1302e-6	uuggga***u***auacuuau***a***cuaaa
eca-mir-1302e-7	uuggga***u***auacuuau***a***cuaaa
eca-mir-1302c-5	uug***c***gacauacuuau***a***cuaaa
eca-mir-1468	cuccguuugccuguuu***u***gcug
eca-mir-1912	uacccagagc***g***ugcagugugaa

Appendix D: 15 miRNAs Without a Human Homologue

Mature miRNA ID	*Target miRNA Mature Sequence*
eca-miR-674-3p	aggaggccauaguggcaacugu
eca-miR-684	aguuuucccuucaauucag
eca-miR-703	aaaaccuucagaaggaaagga
eca-miR-763	ccagcugggaggaaccaguggc
eca-miR-872	aagguuacuuguuaguucagg
eca-miR-1193	uaggucacccguuugacuauc
eca-miR-1461	aucucuacggguaaguguguga
eca-miR-1597	ugaggagcucugcgagcaugua
eca-miR-1839	aagguagauagaacaggucuug
eca-miR-1842	uggcucugugaggucggcuca
eca-miR-1892	auuuggggugggggaugggga
eca-miR-1898	aggucaagguucacaggggauc
eca-miR-1902	agaggugcaguaggcaugacuu
eca-miR-1905a	caccacgagcccuaccacgcgguag
eca-miR-1905b	caccagccccacuacgcgguag
eca-miR-1905c	caccaccagccccaccacgcgguag

Appendix E: Subset of 83 miRNAs for sample Days 9 and 13 in Experiment II

mature miRNA ID	Target microRNA Mature Sequence
eca-let-7a	ugagguaguagguuguauaguu
eca-let-7c	ugagguaguagguuguaugguu
eca-let-7d	agagguaguagguugcauaguu
eca-let-7e	ugagguaggagguuguauaguu
eca-let-7f	ugagguaguagauuguauaguu
eca-let-7g	ugagguaguaguuuguacaguu
eca-miR-15a	uagcagcacauaaugguuugug
eca-miR-15b	uagcagcacaucaugguuuaca
eca-miR-16	uagcagcacguaaauauuggcg
eca-miR-19a	ugugcaaaucuaugcaaaacuga
eca-miR-19b	ugugcaaaucuaugcaaaacuga
eca-miR-20a	uaaagugcuuauagugcagguag
eca-miR-20b	caaagugcucauagugcagguag
eca-miR-21	uagcuuaucagacugauguuga
eca-miR-22	aagcugccaguugaagaacugu
eca-miR-23a	aucacauugccagggauuucc
eca-miR-23b	aucacauugccagggauuacc
eca-miR-24	uggcucaguucagcaggaacag
eca-miR-25	cauugcacuugucucggucuga
eca-miR-26a	uucaaguaauccaggauaggcu
eca-miR-27a	uucacaguggcuaaguuccgc
eca-miR-27b	uucacaguggcuaaguucugc
eca-miR-29a	uagcaccaucugaaaucgguua
eca-miR-29c	uagcaccauuugaaaucgguua
eca-miR-30c	uguaaacauccuacacucucagc
eca-miR-30e	uguaaacauccuugacuggaag
eca-miR-92b	uauugcacucgucccggccucc
eca-miR-99b	cacccguagaaccgaccuugcg
eca-miR-101	uacaguacugugauaacugaa
eca-miR-106a	caaagugcuuacagugcagguag
eca-miR-106b	uaaagugcugacagugcagau
eca-miR-127	ucggauccgucugagcuuggcu
eca-miR-129a-5p	cuuuuugcggucugggcuugc
eca-miR-130-b	cagugcaaugaugaaagggcau
eca-miR-139-3p	ggagacgcggcccuguuggagu
eca-miR-140-3p	uaccacaggguagaaccacgg
eca-miR-142-3p	uguaguguuuccuacuuuaugga
eca-miR-143	ugagaugaagcacuguagcuc
eca-miR-145	guccaguuuucccaggaaucccu

eca-miR-148-a	ucagugcacuacagaacuuugu
eca-miR-148-b-3p	ucagugcaucacagaacuuugu
eca-miR-150	ucucccaacccuuguaccagug
eca-miR-151-5p	ucgaggagcucacagucuagu
eca-miR-181-b	aacauucauugcugucgguggguu
eca-miR-188-3p	cucccacaugcaggguuugca
eca-miR-188-5p	caucccuugcaugguggaggg
eca-miR-191	caacggaaucccaaaagcagcug
eca-miR-192	cugaccuaugaauugacagcc
eca-miR-195	uagcagcacagaaauauuggc
eca-miR-214	acagcaggcacagacaggcagu
eca-miR-215	augaccuaugaauugacagac
eca-miR-221	agcuacauugucugcuggguuuc
eca-miR-222	agcuacaucuggcuacugggu
eca-miR-223	ugucaguuugucaaauacccca
eca-miR-323-3p	cacauuacacggucgaccucu
eca-miR-378	acuggacuuggagucagaagg
eca-miR-382	gaaguuguucgugguggauucg
eca-miR-409-5p	agguuacccgagcaacuuugcau
eca-miR-423-3p	agcucggucugaggccccucagu
eca-miR-423-5p	ugaggggcagagagcgagacuuu
eca-miR-433	aucaugaugggcuccucggugu
eca-miR-451	aaaccguuaccauuacuguguu
eca-miR-485-3p	gucauacacggcucuccucucu
eca-miR-486-5p	uccuguacugagcugccccgag
eca-miR-487-b	aaucguacagggucauccacuu
eca-miR-489	gugacaucacauauacggcggc
eca-miR-490-5p	ccauggaucuccaggugggu
eca-miR-494	ugaaacauacacgggaaaccuc
eca-miR-500	uaauccuugcuaccugggugaga
eca-miR-502-3p	aaugcaccugggcaaggauuca
eca-miR-502-5p	auccuugcuaucugggugcua
eca-miR-508-3p	ugauugucaccuuuuggaguaga
eca-miR-541	ugguggggcacagaauccagucu
eca-miR-615-5p	gggggucccggugcucggauc
eca-miR-652	aauggcgccacuaggguugug
eca-miR-653	guguugaaacaaucucugcug
eca-miR-671-3p	uccgguucucagggcuccacc
eca-miR-767-5p	ugcaccaugguugucugagcaug
eca-miR-769-5p	ggagaccucuggguucugagcu
eca-miR-769-3p	cugggaucucgggggucuugguu

eca-miR-770	agcaccacgugucugggccaug
eca-miR-874	cugcccuggcccgagggaccga
eca-miR-1842	uggcucugugaggucggcuca