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EXPRESSION OF MICRONRNAS IN THE BOVINE CORPUS LUTEUM AND THEIR IMPACT ON LUTEAL CELL FUNCTION

A Thesis in
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ABSTRACT

The corpus luteum is a temporary endocrine structure that produces progesterone for the maintenance of pregnancy and has many regulatory processes that are involved in its development, function, and regression. MicroRNAs are noncoding RNAs that are 17-25nt in length and function in translational repression by binding to the 3' untranslated region of its target mRNA. Because of this repression, microRNAs are able to regulate the expression of many different genes. Drosha, an RNase III enzyme needed for processing the primary microRNA transcript into the precursor microRNA form, is considered one of the most important components in microRNA biogenesis. Little is known about microRNA expression and regulation pertaining in the corpus luteum. Even less is known about miRNA expression in livestock species.

The studies presented here are the first studies focusing on microRNA expression in the bovine corpus luteum and miRNA regulatory abilities in bovine luteal cells. To investigate the expression of different miRNAs in luteal tissue, corpora lutea were removed from cyclic Holstein cows on days 4, 10, and 18 of the estrous cycle, and on day 18 of pregnancy. Total RNA enriched with small RNAs were isolated from each treatment and were hybridized to Affymetrix microRNA microarrays. Quantitative PCR was used to confirm microarray results. To assess the importance of microRNAs to luteal cell function, luteal cells were collected after the dissociation of midcycle (day 10-11) luteal tissues and luteal cells were transfected with siRNAs against Drosha to inhibit microRNA biogenesis. Transfected cells were treated with or without LH or TNF-α and IFN-γ to assess the effect of decreased microRNA expression on progesterone production and apoptosis, respectively.

Bovine and nonbovine specific microRNAs were found to be differentially expressed among luteal tissue collected on day 4, 10, and 18 of the estrous cycle and on day 18 of pregnancy. Three microRNAs, miR-10a, miR-103, and miR-99a were confirmed to have increased expression in luteal tissue on day 18 of pregnancy. Overall, thirteen nonbovine miRNAs not previously reported as present in bovine tissues were detected only in luteal tissue on day 18 compared to three miRNAs expressed only in luteal tissue on day 18 of pregnancy. Nonbovine microRNAs were also identified in bovine luteal tissue on day 10 and day 4, with 42 miRNAs expressed only on day 10 and thirteen miRNAs expressed only on day 4. The knockdown of Drosha mRNA expression in luteal cells cultured with LH resulted in a significant decrease ($P < 0.05$) in progesterone production compared to luteal cells transfected with Drosha siRNAs cultured with no LH. Also, the percentage of apoptosis for cells transfected with Drosha siRNAs and treated with or without TNF-α and IFN-γ significantly increased ($P < 0.05$) compared to the controls. Overall, these data confirm the presence and differential expression of miRNAs in luteal tissue during the luteal phase and suggest that microRNAs have a role in luteal cell function and survival.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

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DEDICATION

To my Parents, Denise G. and William I. Smith

To my sister, Amanda K. Smith

To my aunt, Sherri E. Barnett

To my significant other, Walter J Alexander

CHAPTER 1

LITERATURE REVIEW

1.1 Luteinization

Hormonal Initiation of Luteinization

In the follicular phase, granulosal and thecal cells are the primary steroidogenic cells that produce estrogen. Thecal cells contain the enzymes for the conversion of cholesterol into androgens for estrogen biosynthesis, but lack the enzymes needed to convert androgens into estradiol (Bao and Gaverick, 1998, reviewed in Niswender et al., 2000, Magoffin, 2005). Granulosal cells contain aromatase and are able to convert the androgens produced by thecal cells into estradiol. As the developing follicle increases in size, production of estrogen increases (Milvae et al., 1991). High estrogen concentrations stimulate neurons in the hypothalamus to produce and secrete gonadotropin-releasing hormone (GnRH), as demonstrated by Chappell and Levine (2000), who showed that ovariectomized mice primed with estrogen had a 3-4 fold increase in GnRH pulse amplitude compared to the control (Chappell and Levine, 2000). GnRH released in surges travels down the hypophysial portal bloodstream at the median eminence and stimulates gondadotrophs in the anterior pituitary, resulting in production of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Chappell and Levine, 2000). Preovulatory LH is released in surges, increasing in concentration, and causes ovulation of the preovulatory follicle, luteinization of the thecal and granulosal cells and a shift from estrogen to progesterone production (reviewed in Niswender, 2000).

Morphological and Cellular Changes

Morphological changes in the thecal and granulosal cells are initiated by the surges of LH. Granulosal and thecal cells begin to luteinize 6-7 hours after the LH surge, starting with the cessation of cell division (Donaldson and Hansel, 1965). For granulosal cells, the ability to aromatize androgens into estradiol is lost (Henderson and Moon, 1979), whereas for the thecal layer, an increase in extravasation of erythrocytes, hyperemia, and edema occurs (Cavendar and Murdoch, 1988; reviewed in Smith et al. 1994). Twenty-four to forty-eight hours after ovulation, the ruptured follicle undergoes dramatic tissue remodeling and cellular migration of granulosa cells and theca cells, facilitated by the breakdown of the basement membrane separating the granulosal cell layer from the thecal cell layer (O'Shea, 1980) which is caused by an increase in collagenase activity due to the LH surge (Curry et al., 1985; reviewed in Curry and Osteen, 2001). Granulosal cells undergo increased hypertrophy (Median et al., 1990), and increase in cytoplasmic to nuclear ratio (O'Shea et al., 1989). The number of smooth endoplasmic reticula, Golgi apparati, and mitochondria also increase (Enders, 1973; reviewed in Smith et al., 1994). Thecal cells undergo more hyperplasia than hypertrophy, which has been demonstrated in bovine thecal cell cultures (Median et al., 1990; reviewed in Smith et al., 1994). Granulosal and thecal cells ultimately develop into large and small luteal cells, respectively (Alila and Hansel, 1984), a process that is mostly completed around day 7 after ovulation (Donaldson and Hansel, 1965).

Steroidogenic Changes

The preovulatory LH surge initiates distinctive changes in expression and regulation of steroidogenic enzymes. The main characteristic of luteal cells is the ability to produce progesterone. The conversion of cholesterol into pregnenolone is considered the rate limiting

step in progesterone biosynthesis, carried out in the mitochondria by cytochrome P450 side chain cleavage (P450scc) enzyme Smith et al., 1994). Cytochrome $P450_{scc}$ mRNA is more highly expressed in large luteal cells compared to small luteal cells (Mamluk et al., 1999), suggesting that large luteal cells produce more progesterone compared to small luteal cells. 3β-hydroxysteroid-dehydrogenase/Δ 5-Δ 4 isomerase (3βHSD), the enzyme required for the conversion of pregnenolone into progesterone, is considered a second key step in progesterone biosynthesis. Within the bovine CL, 3βHSD enzyme increases throughout the estrous cycle, and decreases during luteolysis (Conley et al., 1995), whereas in ovine luteal tissue, 3βHSD mRNA was increased until day 15, the day before luteolysis, with expression confined in the theca interna layer (Hawkins et al., 1993). 3βHSD is abundant in preovulatory follicles of cow, sheep and pig, but is undetectable within later stages of the CL (Conley et al., 1995; reviewed in Smith et al., 1994). Aromatase cytochrome P450 is the key enzyme in estradiol synthesis, converting androgens of thecal origin to estradiol within the granulosal layer (Savard, 1973). This enzyme is greater in the follicles of the cow and other domestic animals, as well as the rat, but mRNA expression and enzymatic activity decreased after ovulation and during the luteal phase (Hickey et al., 1988; Voss and Fortune, 1993).

Corpus Luteum Size Increase

After the onset of the LH surge and ovulation, the rate of CL growth and cell proliferation increase dramatically. Between day 3 and 5, the corpus luteum begins to increase in size, losing its hemorrhagic appearance from the rupturing of the follicle during ovulation. The CL increases in size dramatically from day 4 to day 8 or 9 (Donaldson and Hansel, 1965; Mann, 2009). This growth is the result of increased large luteal cell size and small luteal cell number, including the increase in the number of endothelial cells, fibroblast

cells and other cells important to luteal function (O'Shea et al., 1989; reviewed in Niswender et al., 2000). Plasma progesterone concentrations increased from Day 5 (Donaldson and Hansel, 1965; Mann, 2009) until the onset of luteolysis, strengthening the argument that the CL produces more progesterone as it develops.

Corpus Luteum Angiogenesis

Establishment of a blood supply to the CL is essential for luteal survival. The breakdown of the basement membrane, which occurs due to increases in collagenase activity after the LH surge (Curry et al., 1985), is needed to allow entry of the endothelial cells into developing luteal tissue for vascular development (Cavender and Murdoch, 1988). These cells are abundant in the CL during development (Augustin et al., 1995). After degradation of the basement membrane, capillaries start to grow within 36 hours after the LH surge (Cavender and Murdoch, 1988). Vascularization of the developing CL is very rapid and sprouting of vascular vessels can be identified as early as 1 or 2 days after ovulation (Augustin et al, 1995).

Angiogenesis in the CL is regulated by several endothelial growth factors. Ferrara et al. (1998) has confirmed that one of these factors essential for angiogenesis in the CL is vascular endothelial growth factor (VEGF) (Ferrara et al., 1998). Concentrations of vascular endothelial growth factors are high early in luteal phase at increased concentrations and decreased as the luteal phase progresses (Yamashita et al., 2008; reviewed in Miyamoto et al., 2009). Basic fibroblast growth factors (bFGF), which is needed for cell growth and differentiation, as well as angiogenesis, is also expression at its highest level in early luteal phases (Yamashita et al., 2008; reviewed in Schams and Berisha, 2004; and Miyamoto et al., 2009). bFGF and its receptor have also been shown to increase during luteal regression in

the cow (Schams et al., 1994). This may be to prepare for the development of the next CL following ovulation. Vasoactive factor Angiotensin II (Ang II) regulates biological processes including vascular tone, cellular growth and angiogenesis and may be involved in paracrine and autocrine functions relating to Angiotensin II in the bovine CL (reviewed in Miyamoto et al., 2009).

1.2. Luteotropism

Cellular Content of the Corpus Luteum

As highlighted in the previous section, granulosal cells and thecal cells must go through structural and functional changes, resulting in the change from estrogen production to progesterone production that defines luteal function. Alila and Hansel (1984) were one of the first to show the origin of small and large luteal cells (Alila and Hansel, 1984). Using monoclonal antibodies specific to thecal and granulosal cells of the preovulatory follicle, Alila and Hansel (1984) were able to distinguish luteal cells collected from heifers that expressed specific markers for granulosal and thecal cells through indirect immunofluorescence (Alila and Hansel, 1984). Thecal cell specific antibodies bound to a majority of small luteal cells throughout the bovine estrous cycle, while the granulosal cell specific antibody bound to a majority of large luteal cells, indicating that the fate of the thecal cells was to become small luteal cells and granulosal cells to become large luteal cells (Alila and Hansel, 1984).

The estimated total number of cells within luteal tissue is approximately 1.5×10^9 (O'Shea et al., 1989). Based on morphology, there are at least five distinct types of cells present in the CL: small and large steroidogenic cells, endothelial cells, fibroblasts, and immune cells (O'Shea et al., 1989). Small and large luteal cells have a combined value of

approximately 70% of the volume of the bovine CL. Large luteal cells, ranging from 24 to 38 μm in diameter (O'Shea et al., 1989; Lei et al., 1991), represent 40% of the total luteal tissue volume (O'Shea et al., 1989). However, large luteal cells only represent about 10% of the total cell number (O'Shea et al., 1989). This range in size can vary between 26 and 31 μm in sheep (Farin et al., 1986; O'Shea et al., 1989). These cells have a spherical shape with a spherically shaped nucleus, containing rough and smooth endoplasmic reticula, abundant mitochondria and secretory granules, and a defined basal lamina (reviewed in Wiltbank, 1994). Small steroidogenic cells represent 20-30% of the luteal volume and 25-27% of the total number of cells. The diameter is much smaller, hence the name, at about 16-18 μm (O'Shea et al., 1989). Distinguishing characteristics include abundant mitochondria and smooth endoplasmic reticulum, but less rough endoplasmic reticula and secretory granules (reviewed in Wiltbank et al., 1994). The nuclei of these cells are described as irregular with cytoplasmic inclusions but these cells contain more lipid droplets compared to large luteal cells (O'Shea et al., 1979).

Endothelial cells are also found in luteal tissue and are much smaller in size compared to small and large luteal cells, ranging from 10-11 μm in diameter (O'Shea et al., 1989). Capillary endothelial cells represent about 10% of the luteal volume but can represent about 50% of the total cell number (O'Shea et al., 1989). Other distinguishing characteristics of endothelial cells include an elongated and slender shape, a large nuclear to cytoplasmic ratio, few organelles and a distinct basal lamina (reviewed in Wiltbank, 1994). Functions include vasodilation, vasoconstriction, angiogenesis and immune cell passage into tissue (Michiels, 2003). Fibroblasts, also present in luteal tissue, are slightly larger in diameter compared to endothelial cells, about 10-12 μm, and only represent approximately 6-9% of the

luteal volume and 10-20% of the total number of cells (O'Shea et al., 1989). These cells are important for maintaining the structural framework of the corpus luteum.

Progesterone Production

Progesterone is considered to be the major hormone secreted by the CL of cattle, sheep, pigs and other mammals. Both small and large luteal cells are able to produce progesterone. Luteinizing hormone (LH) is secreted from the anterior pituitary in a pulsatile manner, and acts on small luteal cells, which have been shown to contain receptors specific for LH (Fitz et al., 1982; reviewed in Niswender et al., 2000). Small luteal cells stimulated by LH have been shown to produce more progesterone than those cells cultured without LH (Fitz et al., 1982). The importance of LH was further confirmed through hypothalamic-pituitary stalk disconnection procedures in sheep, which resulted in a decrease in progesterone produced by luteal tissue due to reduced LH stimulation of luteal cells (Niswender et al., 1986).

Luteinizing hormone (LH) has been shown to increase secretion of progesterone in small luteal cells, but much less in large luteal cells (Alila et al., 1988). The binding of LH to its receptor activates adenylate cyclase, leading to increased concentrations of cyclic adenosine monophosphate **(**cAMP), and ultimately leading to protein kinase A (PKA) activation (reviewed in Niswender et al., 2000). Cyclic AMP (cAMP) binds to protein kinase A in small luteal cells, resulting in the release of cholesterol from cholesterol esters (Marsh, 1976; Wiltbank et al. 1993). However, it has also been shown LH-induced that phospholipase C (PLC) activation results in increased luteal cell LH-stimulated production of progesterone (Nishiura et al., 2004).

Cholesterol is essential for the production of progesterone, and is transported into the cell via low density lipoprotein (LDL) and high density lipoprotein (HDL), then transported to the mitochondria for steroid production (reviewed in Niswender et al., 2000). Lipoproteins are serum proteins that circulate in the blood. Luteal cells cultured with LDL and HDL increased progesterone production 2.7 and 3.4-fold, respectively (Pate and Condon, 1982). The essentiality of cholesterol for progesterone production was confirmed when inhibitors of cholesterol synthesis were added to luteal tissues, resulting in the decrease in progesterone production in the presence or absence of LH (Armstrong et al., 1970). In the cow, both LDL and HDL increase progesterone secretion, which was demonstrated in dissociated luteal cells (Pate and Condon, 1982). Cholesterol is contained within LDL, which binds to its receptor and is internalized, dissociating from its receptor to release cholesterol (Brown and Goldstein, 1986).

Cholesterol, now in a usable form in the cell, must be transported to the mitochondria for its first enzymatic conversion in the steroidogenic process. Steroidogenic acute regulatory (StAR) protein, characterized by Clark et al (1994 and 1995) functions to mediate the transport of cholesterol from the outer mitochondrial membrane to the inner membrane where the $P450_{scc}$ enzyme is located. StAR mRNA is expressed highest in luteal tissue at midcycle and throughout pregnancy and is decreased in expression during luteolysis and early luteal development (Hartung et al., 1995). StAR has a cholesterol binding sequence (CBS) that binds to cholesterol for transport. The N-terminus is required for transport of cholesterol to mitochondria transfer from the outer membrane to the inner membrane (reviewed in Niswender et al., 2000; Stocco, 2001). The biosynthesis pathway of steroid hormones is well known. Once in the inner membrane of the mitochondrion, cholesterol is converted by cytochrome $P450_{scc}$ into pregnenolone, which is transported out of the mitochondrion to the

smooth endoplasmic reticulum (SER), where it is then converted into progesterone by 3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase (reviewed in Savard, 1973, reviewed in Niswender et al., 2000). Once made, progesterone is secreted from the cell (Niswender et al., 2000).

The concentration of progesterone produced by luteal cells and released into the blood varies as the luteal phase progresses. Concentrations of progesterone in plasma have been shown to average 2.0-2.3 ng/mL between day 2 and 3 after ovulation, increasing in concentration to about 3.7 ng/mL by Day 6 or 7 after ovulation (Pope et al., 1969; Stanbenfeldt et al., 1969). Average progesterone concentrations at midluteal phase have been shown to range between 5 and 6.6 ng/mL; however, a dramatic decrease in concentration is seen around Day 19 (Pope et al., 1969; Stanbenfeldt et al., 1969). This makes sense given that luteal regression begins around Day 19-20 of the luteal phase.

Progesterone is essential for the establishment and maintenance of pregnancy. The progesterone receptor (PGR) is expressed in the reproductive tissues of cows (Berisha et al., 2002) and primates (reviewed in Stouffer and Duffy, 1995). In the nucleus, binding of progesterone to its receptor, isoform A or B, results in the recruitment of co-activators responsible for initiating chromatin remodeling and, ultimately, transcriptional initiation (reviewed in Conneely et al., 2002). Cytoplasmic PR has also been documented, reported to be expressed more in endometrial tissue during proestrus compared to diestrus (Zelinski et al., 1982). Studies have confirmed the importance of the PGR in other tissues for progesterone-mediated actions. Kimmins and MacLaren (2001) reported that the expression of PRs in the endometrium, and that this expression varies throughout the luteal phase and pregnancy, confirming the reports of Zelinski et al., (1982) (Kimmins and MacLaren, 2001).

Kimmins and MacLaren (2001) not only showed that the PGR mRNA decreases in expression in endometrial tissue as the luteal phase progressed, but also demonstrated that the PR is reduced in ovariectomized mice treated with progesterone (Kimmins and MacLaren, 2001) This could suggest progesterone is able to inhibit the expression of its receptor as part of its function to help establish and maintain pregnancy in the uterus

Progesterone can also suppress further development of follicles and the secretion of estrogen (Johnson et al., 1996). High concentrations of progesterone and low concentrations of estrogen have been shown to prevent LH pulses and reduce the overall amount of LH (Calder et al., 1999). This would result in the preovulatory follicles that are unable to ovulate and would inhibit the return of estrus. However, due to the ability of progesterone to reduce LH pulses, this could reduce of follicular cysts in cows, which would promote the development of normal follicles (Calder et al., 1999).

Other Factors Involved in Progesterone Production

Prolactin (PRL) in circulation dramatically decreases in concentration after the LH surge (Ciereszko and Dusza, 1993); however, when injected into cyclic pigs, PRL was able to simulate progesterone production, increasing the concentration of progesterone in the blood (Ciereszko and Dusza, 1993). Prostaglandins are usually associated with luteal regression. However, it has been suggested that prostacyclin (PGI_2), a member of the prostaglandin family, may have a role in increased luteal cell production of progesterone since its increased biosynthesis occurred during increased progesterone production (Milvae and Hansel, 1980; Milvae and Hansel, 1983). Tumor necrosis factor alpha (TNF-α), which is mostly associated with stimulating $PGF_{2\alpha}$ production for preparation of luteolysis, has also been shown to

increase progesterone production, suggesting a potential luteotropic effect that may depend on time of the cycle that TNF-α is present (Townson et al., 1996).

1.3 Luteolysis

If pregnancy does not occur, it is essential that luteolysis takes place, allowing follicles to grow and the initiation of a new reproductive cycle. Luteolysis is defined as the structural degradation of the corpus luteum, a process that can be divided into two related events. The decrease in progesterone synthesis and secretion is followed by the loss of cells and luteal tissue structure (reviewed in Niswender et al., 1994 and 2000; reviewed in Pate, 1994). Experiments involving hysterectomies have shown that normal luteolysis is dependent on the presence of the uterus in cows, sheep, pigs, and other species (Wiltbank and Casida, 1956; Anderson et al., 1961 and 1966).

Prostaglandin $F_{2\alpha}$

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is considered the primary luteolysin for several domestic animals, including sheep, cows, and pigs (Hafs et al., 1974, Roberts et al., 1975, reviewed in Niswender et al., 2000). This was concluded after years of experiments where uteri as a whole or in portions were removed, resulting in prolonged luteal life (reviewed in Goding, 1974). Both Chamley et al. (1972) and McCracken et al. (1970) demonstrated that infusion of $PGF_{2\alpha}$ result in luteolysis. Chamley et al. (1972) infused doses as low as 10µg/hr, resulting in luteal regression after 4 hours (Chamley et al., 1972; reviewed in Goding, 1974). However, McCracken et al. (1970) demonstrated that infusion of $PGF_{2\alpha}$ resulted in a greater luteolytic response; with 50ug/hr causing slow progesterone decrease over 24 hours and behavioral

estrus after 48 hours. When the concentration of $PGF_{2\alpha}$ infused to the ovary increased to 100µg/hr, this resulted in increased vasoconstriction, leading to decreased blood flow to the ovary, as well as the rapid decrease of progesterone secretion and behavioral estrus at 38 hour after $PGF_{2\alpha}$ was infused (McCracken et al., 1970). Nett et al. (1976) and O'Shea et al. (1977) also observed a decrease in blood flow to the ovary after an injection of $PGF_{2\alpha}$, as well as decreases in capillary numbers in luteal tissue, confirming the findings of McCracken et al. (1970) (Nett et al., 1976; O'Shea et al., 1977).

$PGF_{2\alpha}$ is released from the uterus and travels through the utero-ovarian circulation to the ovary to exert its actions. The release of $PGF_{2\alpha}$ is caused by estradiol from developing preovulatory follicles stimulating oxytocin (OXT) secretion from the hypothalamus and release from the posterior pituitary, and this oxytocin stimulates the increased release of $PGF_{2\alpha}$ from the uterus (Labhsetwar et al., 1964, McCracken et al., 1996; reviewed in Niswender et al., 2000). OXT can also be secreted from the ovarry and can cause increased $PGF_{2\alpha}$ secretion from the uterus as well (Milvae and Hansel, 1980).

Prostaglandin $F_{2\alpha}$ Effects on Luteal Tissue and Function

$PGF_{2\alpha}$ induces many changes in luteal tissue, including decreased progesterone secretion and LH receptor expression (Diekman et al., 1978), and decreased 3β-HSD and StAR mRNA expression (Conley et al., 1995, Pescador et al., 1996). LDL and HDL expression is also decreased in bovine luteal cells when $PGF_{2\alpha}$ is added to luteal cell cultures (Pate and Nephew, 1988). This reduction, along with the reduction of LH receptors, 3β-HSD and StAR mRNA, could explain the decrease in progesterone production during the initiation and process of luteolysis.

Morphological changes after the addition of $PGF_{2\alpha}$ to luteal tissue include reduction of size and number in large and small luteal cells and reduction in capillary number (Nett et al., 1976), cellular shrinkage and luteal disorganization 48 hours after $PGF_{2\alpha}$ infusion (Stacy et al., 1976). Phagocytic activity increases as well (Stacy et al., 1976) indicating an increase in macrophages in the CL during regression (reviewed in Pate and Keyes, 2001). Luteolysis also involves the process of apoptosis. Cytokines are present in luteal tissue and are expressed by luteal cells and immune cell, especially during luteal regression (Fairchild and Pate, 1989; Townson and Pate, 1994; Penny et al., 1999; Petroff et al., 2001). It has also been demonstrated that combined TNF-α with IFN-γ treatment is able to induce luteal cell apoptosis, suggesting that these cytokines may function by inducing cell death during luteolysis in vivo (Petroff et al., 2001).

1.4 The Discovery of MicroRNA

Non-coding RNAs, such as snRNA, participate in a diverse collection of regulatory biological events including chromosomal maintenance and processing (reviewed in Mattick and Makunin, 2006). MicroRNAs (miRNAs) are defined as single stranded non-coding RNAs 17-25 nucleotides in length that function by negatively regulating gene expression (Ambros, 2003; Lai, 2003; Bartel, 2004). MicroRNAs were first discovered in *Caenorhabditis* elegans (C. elegans) with the name of small temporal RNAs (stRNAs) (reviewed in He and Hannon, 2004). Currently these stRNAs, now deemed miRNAs, have been identified in many different species of worms, insects, plants, and animals.

MicroRNA *lin-4* is the first miRNA discovered (reviewed in He and Hannon, 2004). *lin-4* was identified in C. elegans using genetic screening for defects in the control of post-embryonic development (Ambros and Horvitz, 1987; Lee et al., 1993). C. elegans have four different developmental larval stages, (reviewed in Lee et al., 1993). C. elegans that carry the mutant form of *lin-4*, which results in loss-of-function phenotypes, were unable to develop past the late larval stage, (Ambros and Horvitz, 1987). The lin-4 mutant also mimicked the phenotype of Lin-14 mutant. Thus lin-4 may regulate Lin-14. lin-14 is needed for late stage larval development, suggests a relationship between lin-4 and lin-14 expression (Ambros and Horvitz, 1987). Cloning of lin-4 using chromosomal walking and transformation rescue to determine the mechanism by which lin-4 regulates the level of lin-14 protein expression revealed that lin-4 does not encode a protein product and that lin-4 RNA sequences are complementary to the 3' untranslated region (UTR) of lin-14 (Lee et al., 1993).

The second miRNA discovered, let-7, a 21-nucleotide small RNA, was found to control the developmental transition from L4 stage to adult stage in C. elegans. (Reinhart et al., 2000). Let-7 was also found to bind to the 3' UTR, lin-14 and lin-28 mRNA, resulting in the inhibition of translation (Reinhart et al., 2000). The identification of these two miRNAs gave rise to the possibility of other small RNAs were present in other species. Let-7 was also found to be evolutionarily conserved across different species, with homologs that were readily detected in sea urchins, flies, mice, and human tissues (Pasquinelli et al., 2000). However, lin-4 was shown to be species-specific, helping to establish that some miRNA can be highly conserved across species, whereas others may be species or tissue-specific in their expression (Pasquinelli et al., 2000). This may also indicate that miRNAs may have general roles in developmental regulation and other biological processes.

1.5 MicroRNA Biogenesis

MicroRNA Gene Transcription

MicroRNA genes are located in intergenic regions; however, a small population can reside in the intronic regions of known genes in the sense or antisense form. Some miRNAs are located so closely together in genes, they form clusters on the gene and can be transcribed as a polycistronic unit instead of individually (Lee et al., 2002). RNA polymerase III (RNA pol. III) was initially thought to be the polymerase that transcribes miRNA genes because it transcribes other noncoding RNAs such as tRNAs and snoRNAs (Lee et al., 2004). However, certain miRNA transcripts can be several kilobases in length and contain four or more stops codons, which would result in termination of transcription by pol. III (Lee et al., 2002). Also, the primary miRNA sequence was polyadenylated and spliced, which are characteristics of a gene being transcribed by RNA polymerase II (RNA pol. II) (Lee et al., 2004). Lee et al. (2004) was able to demonstrate that miRNA genes were being transcribed by RNA pol. II. However, it is also hypothesized that miRNA genes containing Alu repeats may be the exception to this transcription rule, since the Alu sequence contains a 75L promoter, which is an RNA pol. III promoter (reviewed in Faller and Guo, 2008).

Nuclear Processing

During transcription, miRNA genes are transcribed in the nucleus as long primary transcripts (pri-miRNA) that are several kilobases in length and contain stem-loop structures (Cai et al., 2004; reviewed in He and Hannon, 2004). Pri-miRNA transcripts are then modified by the addition of a 5’ 7-methyl guanylate cap and a 3’ poly(A) tail (Cai et al., 2004). After transcription, the pri-miRNA transcript is processed by a microprocessor protein complex,

consisting of an RNase III-type protein, Drosha (Han et al., 2004), and a RNA-binding protein known as DiGeorge Syndrome Critical Region 8 (DSCR8) (Wang et al., 2007; Kim et al., 2009). Drosha is an RNase III family enzyme characterized in the human and mouse (Fortin et al., 2002; Han et al., 2004). This enzyme contains proline-rich and arginine-serine domains as well as two canonical C-terminal RNase III domains (Wu et al., 2000). The mechanism by which Drosha establishes specificity for pri-miRNA transcripts is not misunderstood, however, it has been suggested that Drosha may have an affinity for hairpin loops because of pri-miRNA transcript's unique stem-loop structure (Han et al., 2004; reviewed in Murchinson and Hannon, 2004). It has also been suggested that DSCR8 is the component of the microprocessor that functions in recognizes the pri-RNA transcripts for guiding the cleavage by Drosha (Han et al., 2006).

A typical pri-miRNA consists of a stem of approximately 33 base pairs in dsRNA form, a terminal loop, and ssRNA segments flanking the terminal loop. DSCR8 interacts with the ssRNA segments to hold the pri-miRNA transcript while Drosha cleaves between the proximal and distal stem, at the ssRNA and dsRNA junction, about 11 bp away (Zeng and Cullen, 2005; Han et al., 2006; reviewed in Kim et al., 2009). Drosha cleavage results in a ~70nt transcript, now known as the precursor miRNA (pre-miRNA) transcript, consisting of a 5' monophosphate and a 3' 2-nt hydroxyl overhang (Lee et al., 2003). The pre-miRNA transcript is released by DSCR8 and exported out of the nucleus and into the cytoplasm by RanGTP and Exportin-5, a member of the nuclear transport receptor family (Yi et al., 2003). Exportin-5 is the major transporter of pre-miRNAs because of its recognition of the pre-miRNA transcript 3' 2-nt overhang (Lund et al. 2004). Exportin-5, along with RanGTP, binds to the pre-miRNA for transport out of the nucleus as well as stabilization of the transcript as it is being transported (Yi et al., 2003). After a pre-miRNA has been exported out to the

cytoplasm, RanGTP is hydrolyzed to Ran GDP, causing the release of pre-miRNA from Exportin-5 (Yi et al., 2003; Liu et al., 2007). Not all pri-miRNA transcripts are processed by the Drosha-DGCR8 complex. Short introns that have the ability to form hairpin structures, known as mirtrons, can be spliced after transcription into hairpin forms that mimic the end product of Drosha-DGCR8 processing (Berezikov et al., 2007). These mirtons were thought only to be present in invertebrates (Okamura et al., 2007), but also exist in mammalian species (Berezikov et al., 2007)

Cytoplasmic Processing

Dicer is an RNase III enzyme that is predominately located in the cytoplasm and is required for processing pre-miRNA to double stranded miRNA (Lee et al. 2004). There are two forms of Dicer, Dicer1 is required for processing of pre-miRNA, whereas Dicer2 is needed for processing of double stranded precursor siRNA (Lee et al., 2004). However, only Dicer1 gene is found in mammals (Hutvagner et al., 2001). The Dicer complex consists of an RNA helicase, a domain of unknown function (DUF283), a Piwi Argonaute Zwille (PAZ) domain, two RNase III domains and a double-stranded RNA-binding domain (dsRBD) (MacRae et al., 2006; reviewed in Luense et al., 2009). The two RNase domains dimerize to form a single processing center for cleaving the stem of pre-miRNAs or long dsRNAs ~22nt away from their termini, resulting in a 17-25nt double stranded molecule that can be inserted into the RISC complex (Zhang et al., 2004). The PAZ domain may be one of the most important features in the Dicer complex for pre-miRNA processing. The PAZ domain is thought to have the ability to differentiate between long dsRNA, precursors for siRNA, and pre-miRNAs through the recognition the 3’ 2nt overhang added by Drosha/DGCR8

processing (reviewed in Murchinson and Hannon, 2004). Cleavage by Dicer results in a 17-25 nt double stranded miRNA form.

1.6 Assembly of MicroRNAs into Effector Complexes

RNA-induced silencing complex (RISC) is a multiprotein complex essential for gene silencing. After Dicer processing, the miRNA duplex is unwound and the mature miRNA sequence binds to an Argonaute (Ago) protein in a process referred to as miRNA loading (Gregory et al., 2005). Ago proteins are a diverse protein family with PAZ and PIWI domains divided into two main subfamilies, the Ago and Piwi families (Ma et al., 2004). MicroRNAs bind to the Ago proteins and this miRNA-Ago complex forms a core of microRNA ribonucleoprotein complex (miRNP) (Mourelatos et al., 2002; Bartel, 2004). The thermodynamic asymmetry of the duplex is a primary determinant of which of the miRNA duplex strands will be loaded in Ago proteins, with the RNA strand whose 5' end is less stably bound to the opposite strand, being favored (Tomari and Zamore, 2005).The complementary miRNA sequence not bound by Ago protein is degraded (reviewed in He and Hannon, 2004 and Liu et al., 2007).

1.7 MicroRNA Target Recognition

Early studies have demonstrated that the first miRNAs discovered in C. elegans, lin-4 (Lee et al., 2003) and let-7(Reinhart et al., 2000), bound imperfectly to their mRNA targets by complementary matching to sequences within the 3'UTR, resulting in a decrease in mRNA expression (Lee et al., 1993; Reinhart et al., 2000). The level of homology between a particular miRNA and its target varies between target sites and miRNA sequences.

Experimental and bioinformatic approaches have shown that the major determinant of miRNA target recognition is the perfect (or near perfect) complementary binding between the 5'proximal region of the miRNA sequence, known as the seed sequence, and the 3'UTR of the target mRNA sequence (Lewis et al., 2003). The seed sequence is the region of 2–8 nt at the 5' region of the miRNA sequence (Lewis et al., 2005, Friedman et al., 2008). Base pairing between the 3' portion of the miRNA sequence and the 3' UTR of the mRNA target is more rare, but may be essential for enhanced repression if the binding between the 5' proximal region of the miRNA sequence and the 3'UTR of the target mRNA sequence is not sufficient (Brennecke et al.; 2003, Grimson et al., 2007). There are four types of seed pairings between miRNA and the target mRNA. Perfect binding occurs between the 6mer seed sequence of the miRNA and The 6mer is the perfect 6 nt match between the miRNA seed sequence (nt 2-7) and the target (Lewis et al., 2005, Grimson et al., 2007). The 7mer-m8 site contains the seed match including nt 8 on the miRNA seed sequence, whereas the 7mer-A1 site includes the A nt at target position one (Grimson et al., 2007) The 8mer site consists of nt 8 and the A at target position one (Lewis et al., 2005; Brennecke et al., 2005; Grimson et al., 2007). By combining computational and experimental approaches, Grimson et al. (2007) uncovered several features of miRNA target binding that increases site efficacy. Microarrays analysis after miRNA transfection of cells revealed that mRNAs with two sites complementary to miRNA seed sequences were repressed more efficiently than mRNAs with only one site and the closer the sites were to each other on the target, the stronger the repression (Grimson et al., 2007). Increases in AU content in the 5'seed sequence compared to the target seem to result in a decrease in mRNA stabilization as well as protein expression, however, the seed site present in regions of higher local GC content are predicted to have 3'pairing with their

target sequences (Grimson et al., 2007). Location of 3'UTR sites must be at least 15 nt away from the stop codon or binding will not occur (Grimson et al., 2007).

On the basis of the number and position of mismatching nucleotides between the miRNA and its target site, there are three groups of target sites. 5'-dominant extensive target sites show perfect complementarity to the miRNA seed sequence (nucleotides 2–8) and extensive base-pairing to the rest of the miRNA sequence, while 5'-dominant seed-only target sites also have perfect complementarity to the seed sequence, but have limited base-pairing with the remaining miRNA sequence. (Brennecke et al., 2005; Sethupathy et al., 2006). 3'-compensatory target sites have imperfect binding to the seed sequence, but this is compensated by extensive base-pairing with the 3' half of the miRNA (Brennecke et al., 2005). This 3' pairing of miRNA is most efficient when paring is between nt 12-17, especially nt 13-16, on the miRNA sequence (Grimson et al., 2007).

1.8 MicroRNA Function

miRNAs base pair with miRNA recognition elements (MREs) found in their mRNA targets at the 3'untranslated region (3' UTR) and deposit their bound Ago proteins onto the mRNA target resulting in translational repression of the targeted mRNA followed by destabilization or endonucleolytic cleavage (Kiriakidou et al., 2004). This process is dependent upon the extensiveness of complementarity between the miRNA and its target and which Ago protein was deposited onto the target. miRNAs bound to Ago2 protein pairs have extensive complementarity with its mRNA target and initiate cleavage of the mRNA, with the miRNA remaining intact (Hutvagner and Zamore, 2002; Liu et al., 2004). This cleavage produces 5'-phosphate and 3'-hydroxyl terminal products which are degraded by 5'- and 3'-exonucleases (Liu et al.2008). Final steps of degradation may involve P-bodies (GW-

bodies), cellular structures enriched with mRNA-catabolizing enzymes and translational repressors (Parker and Sheth, 2007). P-bodies are involved in the process of post-transcriptional regulation in gene regulation by plant miRNAs (Dugas and Bartel, 2004).

Only one endogenous animal miRNA has been reported to function in causing cleavage (Yekta et al., 2004). Most animal mRNA targets undergo translational repression as well as their destabilization without endonucleolytic cleavage (reviewed in Ambros, 2004). In repression, target mRNAs are not actively degraded, but instead are destabilized by deadenylation and decapping (Wu et al., 2006). Studies have suggested that P-bodies can function as temporary storage sites for repressed mRNAs with recent reports showing Argonaute (AGO) proteins, miRNAs, and mRNAs target enriched in P-bodies for storage, suggesting that P-bodies have a role in miRNA driven repression as well as degradation (Jakymiw et al., 2005; reviewed in Filipowicz et al., 2008). Furthermore, there seems to be a positive correlation between miRNA mediated translational repression and accumulation of mRNAs in visible P-bodies (Liu et al., 2005; Filipowicz et al., 2008).

1.9 MicroRNA Expression in the Ovary

MicroRNAs have been shown to be important for the regulation of many different biological processes in many different tissue types, including cell proliferation, differentiation, and apoptosis (Miska, 2005; Hwang and Mendell, 2006; Jovanovic and Hengartner, 2006). Because of these findings, it is likely that miRNAs also play a vital role in the development and function of the ovary as well. Ovarian regulatory processes include growth and development of follicles, ovulation, and luteal tissue development, maintenance and regression, which can then be divided in subcategories which include cellular growth and differentiation, steroidogenesis, angiogenesis, and apoptosis. Little is known about post-

transcriptional gene regulation that may be critical for ovarian function. MicroRNAs may be the key in understanding post-transcriptional regulation of these molecular processes.

Otsuka et al. (2008) used Dicer1 hypomorph mice (Dicer1$^{d/d}$) to study the effect of global miRNA reduction on fertility in mice, Dicer1$^{d/d}$ 75% reduction of Dicer1 mRNA levels (Otsuka et al., 2008). Mutations resulting in total deletion of Dicer1 mRNA expression has been shown to cause lethality in embryos (Yang et al., 2005), so a Dicer1 knockdown phenotype seems to be a better method for studying effects of global miRNA expression in tissues and organs of interest. Dicer1 hypomorph (Dicer1$^{d/d)}$ in female mice resulted in decreased concentrations of serum progesterone and decreased mRNA expression for LH receptor, cytochrome p450 11A1 (CYP11A1). Furthermore, Dicer reduction results in decreases in number and length of blood vessels in the CL (Otsuka et al., 2008). This indicates that Dicer reduced the expression of miRNAs needed for proper CL function. This was confirmed when a decrease in miR-17-5p and let-7b was also observed in the ovary (Otsuka et al., 2008). Dramatic increases in vasculature are required for proper luteal tissue formation (Augustin et al., 1995). Otsuka et al.(2008) found mRNA expression of anti-angiogenic factors Timp1 and platelet factor 4 increased in Dicer1$^{(d/d)}$ female mice, which are potential target of miR-17-5p and let-7 (Otsuka et al., 2008). When these two miRNAs were injected into the ovarian bursa of Dicer1$^{d/d}$ female mice, this resulted in partial restoration of vasculature in the CL as well as increased progesterone concentrations, while decreasing Timp1 expression, indicating that miR-17-5p and let-7b have a role in the development of luteal vasculature by regulating Timp1 expression (Otsuka et al., 2008).

Ro et al. (2007) used cloning techniques to identify miRNAs expressed in mouse ovaries, at two weeks old and at maturity and found 122 miRNAs expressed in whole ovaries from immature and mature mice. Microarrays were used by Fiedler et al. (2008) on mouse

granulosal cells before and 4 hours after the administration of hCG to test if LH can regulate miRNA expression. One hundred ninety-six miRNAs and 206 miRNAs were detected before and after (4 hours) the administration of hCG, respectively. Thirty-one of these miRNAs showed increased expression, while 117 showed decreased expression between treatments. Three miRNAs were found to be upregulated between 0 hours (before) and 4 hours post-hCG, and 10 miRNAs were found to be downregulated. Overall, miRNAs may have different roles in ovarian function and more research is needed to further understand the targets and exact roles of these miRNAs in ovarian regulation.

The focus of miRNA research has centered more on tissues in the diseased state than in the normal state. Further, miRNA research has focused more on miRNA expression in humans, mice, rats, and other animals for medical research than in livestock. MicroRNA expression has been studied in various types of ovarian cancer. MicroRNAs associated with cancer can be classified into two categories. Oncogenic miRNAs, expressed in cancer tumors, are thought to cause downregulation of tumor suppressor proteins, increase cellular proliferation, low apoptotic effects, de-differentiation, increased angiogenesis and metastasis (Calin and Croce, 2006). Tumor suppressor miRNAs are thought to regulate mRNAs that will ultimately cause the down-regulation of oncogenic proteins, increased apoptotic effects and differentiation, and decreased cellular proliferation, angiogenesis and metastasis (Calin and Croce, 2006). Because of the dramatic difference in miRNA expression in most cancerous tissues compared to normal tissues, miRNAs are now considered, in some cases, to be a molecular marker for the presence of tumors compared to mRNA expression (Iorio et al., 2007, Nam et al., 2008).

MicroRNA-21, miR-125a, miR-125b, miR-100, miR-145, mir-16, and miR-99a were all found to be expressed in serous ovarian carcinoma in more than sixteen out of twenty human cancer patients (Nam et al., 2008). Interestingly, certain miRNAs were found to be correlated with a poor prognosis as well as the survival of the patient after cancer treatment. Increased expression of *miR-200a, miR-200b, miR-200c, miR-141, miR-18a, miR-93*, and *miR-429*, and decreased expression of *ambi-miR-7039, let-7b,* and *miR-199a* were significantly correlated with decreased progression-free survival and overall survival (Nam et al., 2008). Taylor and Taylor (2009) conducted experiments to determine if miRNAs contained in exosomes derived from ovarian cancer could be used to diagnose these cancers in women. With the use of microarrays, Taylor and Taylor (2009) identified 31 miRNAs distinctly upregulated in ovarian cancer compared to 12 miRNAs upregulated only in ovarian tissue (Taylor and Taylor, 2009). In addition, there was a significant increase in the expression of miR-21, miR-203, and miR-205 in cells from epithelial cancer cell lines (OVCAR3) treated with 5-aza-2'-deoxycytidine demethylating treatment, an anticancer treatment (Iorio et al., 2007). The results of these studies and others on miRNA expression in cancerous tissue suggest that miRNAs may offer new biomarkers and therapeutic targets for cancer tumor regulation and treatment.

MicroRNA Expression in Bovine Tissues

The role of miRNAs in animal biology is of significant interest, yet the current literature on livestock animals is limited. Initial research has focused on expanding the current miRNA database (miRBase) to include sequences for livestock species through miRNA expression profiles and homology searches (Coutinho et al., 2007; Gu et al., 2007, reviewed in MacDaneld, 2009). However, only a handful of these studies have focused on miRNA expression in bovine tissues. For the past eight years, the objective for miRNA expression

research in cattle has mainly focused on the identification of conserved miRNAs as well as the discovery of novel miRNAs. Coutinho et al. (2006) used small RNA libraries and miRNA cloning to identify miRNAs expressed across a range of bovine tissues. miR-26a, miR-103, miR-29a, miR125b, and miR-150 were all found to be expressed in all tissues tested, which included tissue samples from the thymus, mesenteric and abomasal lymph nodes, small intestines, and Day 30 embryonic tissue (Coutinho et al., 2006). miR-545-5p and miR-22a-5p were found to be exclusively expressed in bovine thymus tissues, whereas miR-455-3p, miR-21-3p, miR-425-3p and 5p, and miR-127-3p and 5p were expressed only in tissue samples from mesenteric and abomasal lymph nodes, small intestines, and day 30 embryonic tissue, respectively (Coutinho et al, 2006). Glazov et al. (2009) used deep sequencing to identify over 115 miRNAs in bovine kidney cell lines, with miR-30b, miR-193a, miR-345, miR-365, and miR-423 having the highest count of sequence reads from the more thermodynamically stable arm in the precursor sequence (Glazov et al., 2009). Jin et al. (2009) observed varying miRNA expression across eleven different bovine tissues. Using sequencing and bioinformatic analysis, Jin et al. (2009) observed that, of the eleven tissues tested, tissues from the thymus, cerebellum, and kidney had the most miRNA expression, whereas subcutaneous fat had the least (Jin et al., 2009). Nineteen of these miRNAs have not yet been identified in cattle and were found to be conserved across species (Jin et al., 2009).

Currently, there are only two publications focusing on miRNA expression in bovine reproductive tissues. Tesfaye et al. (2009) compared miRNA expression of immature bovine oocytes versus mature oocytes using human, mouse and rat miRNA microarrays. Fifty-nine miRNAs showed differential expression, with 31 miRNAs showing relative preferential expression in immature oocytes. The remaining 28 miRNAs were found to be more abundant in mature bovine oocytes (Tesfaye et al., 2009). Expression profiling of bovine miRNAs using

human, mouse, and rat probes during oocyte maturation resulted in the identification of 32 miRNAs not yet identified and annotated in bovine tissues (Tesfaye et al., 2009). Validation through qPCR analysis revealed that miR-512-5p, miR-25, miR-125a, miR-130b, miR-208, miR-127, and miR-200c were more abundant in immature oocytes, while miR-496, miR-292-3p, miR-206, and miR-145 were found to be higher in expression in mature oocytes (Tesfaye et al., 2009). It was also observed that miR-127 and miR-145 were expressed the most during the 8-cell stage, whereas miR-125a, and miR-496 and miR-208 were expressed more in the 4-cell stage and morula stage, respectively (Tesfaye et al., 2009). miR-25 was found expressed in the oocyte and blastocyst with similar fold change (Tesfaye et al., 2009).

Among all the miRNAs identified in the ovary, 38 miRNAs were found to be new for the cow and were derived from the distinct loci of 43 miRNAs showing characteristic secondary structure (Hossain et al., 2009). Most of these new miRNAs have already been identified as intragenic, intergenic and intronic human miRNAs. Quantitative PCR analysis revealed the detection and expression of five miRNAs that were expressed at a similar level across different tissues, including fetal ovary, oviduct, uterus, placenta, heart, liver and luteal tissue (Hossain et al., 2009). However, these results are from qualitative PCR analysis and not qPCR, which would be a more accurate measurement of the expression of miRNAs as well as the confirmation of miRNA expression change between different tissues.

Despite these discoveries, only 117 miRNAs have been identified and annotated for the cow in the miRBase v.10 and overall, approximately 600 bovine miRNAs have been identified, but not yet annotated. Most of these miRNAs' predicted targets are unknown. Further studies, especially functional studies, are needed to increase our knowledge of the miRNAs and their regulatory abilities in bovine tissues. Because of the high number of mammalian miRNAs that are conserved across species, research involving most model

animals, including the cow could lead to potential targets in not only the species of interest, but in similar species as well, which could increase the current knowledge on post transcriptional gene regulation in reproductive processes in general.

1.10 Statement of the Problem

The number of bovine miRNAs (117) currently identified and annotated in the miRBase 12.0 are inadequate compared to human (695), chimpanzee (595), and mouse (488). Discovery of most of these miRNAs found in the cow have resulted from bioinformatic approaches and small RNA-cDNA libraries from bovine adipose, mammary gland, thymus, small intestine, mesenteric and abomasal lymph nodes,, kidney, ovary, embryo, and oocyte (Coutinho et al., 2006; Gu et al., 2007; Glazov et al., 2009; Hossain et al., 2009; Tesfaye et al., 2009). However, less is known about specific miRNAs expressed in bovine tissues compared to tissues of other species, especially in reproductive tissues. Furthermore, functional studies focusing on miRNA posttranscriptional regulation by translational repression through their mRNA targets are also limited. Currently, only two studies have shown the presence of miRNAs in luteal tissue, one of which focuses on bovine tissues (Otsuka et al., 2008; Hossain et al., 2009). MicroRNAs discovered to be expressed in the bovine corpus luteum may lead to a better understanding of the post-transcriptional regulation of components involved in progesterone production as well as the regulation of the synthesis of molecular products produced by cells within luteal tissue essential for overall luteal function throughout different stages in the estrous cycle.

1.11 Hypothesis and Implications

The hypothesis for this research was that miRNAs are involved in the development, regression, and function of the corpus luteum. The overall goal of the series of experiments presented herein was to examine temporal expression of miRNAs in the CL, determine potential targets for miRNAs, and assess the effect of the reduction of global miRNA expression in luteal cells in progesterone production and induced apoptosis.

CHAPTER 2

EXPRESSION OF MICRORNAS IN THE BOVINE CORPUS LUTEUM

Introduction

The corpus luteum (CL) develops from residual theca and granulosa cells from the ruptured follicle after ovulation, developing rapidly within the first few days after estrus (Donaldson and Hansel, 1965; Mann, 2009). Thecal cells develop into small luteal cells, while granulosal cells develop into large luteal cells, ultimately functioning in the production of progesterone stimulated by LH secretion (Alila and Hansel, 1984).

Cattle have a 21-day estrous cycle. Development of luteal tissue begins after the onset of the LH surge and ovulation, and rapid development occurs between day 2-8. Between day 10 to 16, the CL is fully developed and produces progesterone at maximal concentrations. LH directly stimulates progesterone production and secretion from small luteal cells through the activation of the protein kinase A (Fuller and Hansel, 1970; McCracken, 1971; Wiltbank, 1994). Large luteal cells contain $PGF_{2\alpha}$ receptors, which mediate luteolytic actions in the CL (Stacy et al., 1976; Diekman et al., 1978). Pregnancy results in continued secretion of progesterone required to help ensure embryonic survival. Absence of pregnancy results in regression of the CL initiated by $PGF_{2\alpha}$, allowing a new reproductive cycle to begin. Because of the rapid development and changes in luteal tissue throughout the estrous cycle, optimal regulation of mRNA and its protein products during this process is required. A considerable amount of information has been uncovered on transcriptional gene regulation and cell signaling pathways in reproductive tissues, including the ovary. However, much less is known about the post-

transcriptional gene regulation of the CL, which would be important for regulation of cell differentiation and proliferation, as well as protein synthesis.

MicroRNAs are small noncoding RNAs approximately 17 to 25nt in length that function as post-translational regulators of gene expression in plants and animals (Ambros, 2004). Post-transcriptional regulation consists of miRNAs interacting with their mRNA targets by binding to the 3' UTRs with near perfect or mismatch complementarity. This binding results in translational repression for most miRNAs found in animal species (Kim and Nam, 2006), however, some animal miRNAs have also been found to cause mRNA degradation (Yetka et al., 2004; Wu et al., 2006). Most miRNAs have been initially characterized as conserved across species (Pasquinelli et al., 2000), however, it has also been shown that miRNA expression can be development (Ason et al., 2006; Plasterk, 2006).

The miRNA expression profiling of bovine miRNAs is limited, especially in reproductive tissues. Tissues already analyzed for the presence miRNAs in the cow include adipose tissue, mammary gland, thymus, lymph node, small intestines, and kidney (Coutinho et al., 2006; Gu et al., 2007; Glazov et al., 2009; Jin et al., 2009). Recently, an abundance of miRNAs have been found in embryonic, ovarian, placental, and uterine tissues as well as in oocytes (Hossain et al., 2009; Tesfaye et al., 2009). However, no extensive miRNA expression profiling has been performed on the corpus luteum. Given the emerging roles of miRNAs in development and regulation, identification of differentially expressed miRNAs in luteal tissue during different stages in the estrous cycle is essential to begin investigating the function of miRNAs in the CL. In the present study, differential miRNA expression was determined in luteal tissue during different stages of the estrous cycle using microarray technology and quantitative PCR.

Potential targets of miRNAs found present in luteal tissue during different stages of estrus were also determined using various miRNA target prediction databases.

Materials and Methods

Luteal Tissue Collection

Estrus was synchronized in 12 dairy cattle using controlled internal drug release (CIDRs) devices for progesterone. CIDRs were inserted vaginally for a period of 7 days. An injection of prostaglandin $F_{2\alpha}$ (PGF_{2a}) was administered on day 6 of this period to induce luteal regression. Three cows designated for collection of luteal tissue on day 4 after the estrous cycle were injected with gonadotropin releasing hormone (GnRH) to synchronize ovulation of the dominant follicle and slaughtered 4 days after the onset of estrus to collect luteal tissue. Animals were observed to determine the onset of estrus after the removal of CIDRs for cows designated for the collection of luteal tissue on day 10 and day 18 after estrus and day 18 of pregnancy. Cows designated for day 18 of pregnancy were bred within 12 hours after estrus and uterine flushes were performed on day 18 to confirm pregnancy by presence of embryonic fragments in uterine flush. Corpora lutea from these groups were collected transvaginally. All corpora lutea were frozen in liquid nitrogen immediately after collection and stored in the -80^{o}C freezer until processing.

MicroRNA Labeling and Microarray Analysis

MicroRNAs were isolated from luteal tissue using the MirVana miRNA Isolation kit (Ambion Inc., Austin, TX) and miRNA quality was assessed on an Agilent Bioanalyzer. Samples enriched with miRNA were poly (A)-tailed and biotinylated using the Genisphere

FlashTag Biotin RNA Labeling kit (Genisphere Inc., Hatfield, PA). Genechip miRNA microarray chips (Affymetrix, Santa Clara, CA) were used for miRNA analysis. For the comparison of miRNA expression in luteal tissue of pregnant versus nonpregnant animals, RNA isolated from luteal tissue on day 18 of pregnancy and day 18 of the estrous cycle were compared and for the comparison of miRNA expression in developing luteal tissue versus fully developed luteal tissue, luteal tissue on day 4 and day 10 of the estrous cycle were compared. Labeling controls, with reagents provided by Genisphere, were used to assess successful labeling of RNA present in sample. Hybridization control probes were used to assess hybridization efficiency of RNA sample to the microarray. Microarray Dot files were converted into Cel files and analyzed using the Affymetrix miRNA QC Tools software. Microarrays were normalized between each microarray chip for each treatment using the Affymetrix miRNA QC Tools software.

Confirmation of MicroRNA Expression using Quantitative PCR

Total RNA was isolated from bovine luteal tissue using the MirVana miRNA Isolation kit (Ambion Inc., Austin, TX) to conserve small RNA during the isolation process. DNase treatment was performed using RQ1 RNase-Free DNase (Promega, Madison, WI) and single stranded cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription kit (Ambion Inc., Austin, TX). Specific loop primers for each miRNA of interest were designed by Ambion Inc. based on microRNA sequences. MicroRNA sequences used to design RT and qPCR primers are as follows:

MicroRNA	Sequence
Let-7a	5'-UGAGGUAGUAGGUUGUAUAGUU-3'
miR-10a	5'-UACCCUGUAGAUCCGAAUUUGUG-3'
miR-103	5'-AGCAGCAUUGUACAGGGCUAUGA-3'
miR-107	5'-AGCAGCAUUGUACAGGGCUAUCA-3'
miR-138	5'-AGCUGGUGUUGUGAAUC-3'

miR-142-star	5'-UGUAGUGUUUCCUACUUUAUGGA-3'
miR-150	5'-UCUCCCAACCCUUGUACCAGUG-3'
miR-25	5'-CAUUGCACUUGUCUCGGUCUGA-3'
miR-34a	5'-UGGCAGUGUCUUAGCUGGUUGU-3'
miR-99a	5'-AACCCGUAGAUCCGAUCUUGUG-3'

Stem-looped primers used to generate cDNA with extension of the 17-25nt sequence of miRNA as preparation for qPCR assay. The PCR product was amplified from cDNA using the TaqMan MicroRNA Assay for qPCR and the TaqMan Universal PCR Master Mix (Ambion Inc., Austin TX). Data were analyzed using the Student's T-test and compared to microarray results for confirmation of differential miRNA expression between two treatments.

MicroRNA Target Prediction

Potential targets for miRNAs were predicted using miRNA target prediction databases including miRanda, miRDB, and TargetScan (http://microrna.sanger.ac.uk/, http://mirdb.org/miRDB/, http://www.targetscan.org/), which are linked to miRBase, the microRNA database (http://www.mirbase.org/). Potential mRNA targets to the corresponding miRNAs were then BLASTed on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to confirm sequence alignment between the miRNA of interest and the potential mRNA target.

Statistical Analyses

Data from microarray analysis and qPCR are presented as mean ± SEM for three biological replicates. Data were analyzed using the miRNA QC Tools microarray software. miRNA QC Tools software uses Wilcoxon Rank-Sum test for replicate probe sets. MicroRNAs were considered expressed if probe hybridization resulted in $P \leq 0.06$. P-value cut offs, alpha 1 (α_1) and alpha 2 (α_2), provide boundaries for defining microRNAs that are present, marginal, or

absent. Any miRNA probes with a p-value falling below $\alpha_1 = 0.04$ was considered present and any p-value falling between α_1 and α_2 ($\alpha_2 = 0.06$) was considered marginally expressed. All bovine-specific miRNAs had an average P-value ≤ 0.04. Microarray data were also analyzed using Student's T-test to compare expression of miRNA between day 18 and day 18 pregnancy treatments and day 4 and day 10 treatments. For confirmation of day 18 and day 18 of pregnancy miRNA expression using q-PCR, cycle threshold values were analyzed using Student's T-test and compared to miRNA expression patterns detected using microarray analyses. Data were considered significantly different at P ≤ 0.05. Fold change was calculated from delta delta CT values for each miRNA. Fold change was determined relative to the reference standard in luteal tissue at day 18 of pregnancy (Figure 3B). miR-34a did not change in expression between treatments and was used as a reference standard.

Results

Bovine-specific microRNAs vary in expression in luteal tissue during different stages of the estrous cycle

Heterologous arrays containing microRNA capture probes that were a match for various miRNAs considered to be conserved across species or specie-specific were used to analyze conserved miRNAs in bovine luteal tissue at different stages of the estrous cycle. MicroRNA sequences represented on the microarrays have been registered and annotated in the Sanger miRNA Database VII. MicroRNAs present in bovine luteal tissue on day 4, day 10, and day 18 of the estrous cycle as well as day 18 of pregnancy were analyzed using heterologous miRNA microarrays that contained probes specific for miRNA genes expressed in over 70 different organisms. Over 1300 miRNAs were detected in luteal tissue on day 4 of the estrous cycle,

whereas 1941 miRNAs were detected on day 10. For luteal tissue collected on day 18 of pregnancy, 1943 miRNAs were detected and 2041 miRNAs were detected in luteal tissue on day 18 of the estrous cycle. Differential expression of bovine-specific miRNAs were observed in luteal tissue collected on day 4 compared to day10 of the estrous cycle and in luteal tissue collected from pregnant compared to cyclic animals on day 18 of the estrous cycle. Of the 125 bovine-specific miRNAs represented on the array, 79 microRNAs were expressed in luteal tissue from cyclic cows, whereas 78 were expressed in luteal tissue from pregnant cows (Figure 1). Sixty-seven bovine-specific microRNAs were expressed in luteal tissue on day 10 of the estrous cycle, but only 25 bovine-specific microRNAs were expressed on day 4 (Figure 1). Ten of the bovine-specific microRNAs were differentially expressed (P-value ≤ 0.05) between day 18 of pregnancy and day 18 of the estrous cycle. MicroRNA let-7a, miR-142-star, miR-150, miR-25 and miR-34a were greater in CL of nonpregnant cows, while miR-103, miR-107, miR-10a, miR-138, and miR-99a were greater in CL of pregnant cows (Figure 2). Sixty-six bovine-specific microRNAs were differentially expressed (P-value ≤ 0.05) in day 4 compared to day 10 luteal tissue. Of these, 63 microRNAs were more abundant in luteal tissue on day 10, whereas the remaining 3 miRNAs were slightly more abundant in luteal tissue on day 4 (Table 1).

Analysis of binding to heterologous capture probes

Expression profiling of luteal microRNAs using miRNA probes led to the identification of 18 miRNAs present in day 18 luteal tissue that have not been previously described in the cow (Table 2). Five of miRNAs (dre-miR-10d, hsa-miR-543, rno-miR-346, rno-miR-370, hsa-miR-223) were conserved in different species. Probes on the microarray representing the same miRNA for different species hybridized to miRNAs present in bovine luteal tissue, confirming

this conservation. MicroRNA probes representing three miRNAs (hsa-miR-296-3p, hsa-miR-299-5p, and hsa-miR-939) also detected miRNA sequences in the bovine luteal samples, but did not display the same conservation across specie-specific probes. Eleven of these miRNAs have only been identified in a species (Table 2). In day 4 and day 10 CL, 74 miRNAs were detected that have not been previously described in the cow (Table 3). Twenty-nine of these miRNAs were conserved while 21 miRNAs displayed limited conservation. Overall, 24 of these miRNAs have only been identified in a single species (Table 3).

The *miRBase* miRNA database contains the most complete list of discovered and annotated miRNAs for a variety of species. Currently, 665 miRNAs have been identified in the cow and annotations of these are limited. Comparatively, 940 miRNAs have been identified in the human and 590 miRNAs have been identified in the mouse, with more annotated miRNAs in these species compared to the cow. The miRBase was used to compare miRNAs expressed in bovine luteal tissue to miRNAs already identified in the cow. Thirteen miRNAs not previously reported as present in bovine tissues were detected only in luteal tissue on day 18 when compared to miRNA expression in luteal tissue on day 18 of pregnancy (Table 4). Three miRNAs were only expressed in luteal tissue on day 18 of pregnancy compared to day 18 of the estrous cycle (Table 5). Most of these microRNAs are conserved, except for hsa-miR-379-star, mmu-miR-1198, mmu-miR-1195, and mmu-miR-290-3p, mml-miR-1230, and mmu-miR-700, which have only been identified in a single species (Table 4).Nonbovine microRNAs were also identified in bovine luteal tissue on day 10 and day 4, with 42 miRNAs expressed only on day 10 (Table 6) and thirteen miRNAs expressed only on day 4 (Table 7).

Comparison of miRNAs detected in day 18 bovine luteal tissue

Human, mouse, rat, and rhesus monkey have the most extensive miRBase sequence entries for identified and annotated miRNAs. Overall, 20 human-specific miRNAs, fourteen mouse-specific miRNAs, seven rhesus monkey-specific miRNAs, and one rat-specific miRNA were identified in bovine luteal tissue on day 18 of pregnancy or the estrous cycle using heterologous probes . These miRNAs have not previously been identified in the cow and were found not to be conserved across species. In total, only 6 miRNAs were conserved across species (Figure 4).

qPCR Analysis

The 10 bovine-specific miRNAs that were differentially expressed in the day 18 CL of cyclic compared to pregnant cattle were further analyzed using qPCR. miR-138 was determined undetectable by qPCR analysis. Three miRNAs were found to be statistically significant compared to all 10 miRNAs and show similar expression patterns compared to the microarray analyses (Figure 3A). Fold change was calculated from delta delta CT values for each miRNA. Three miRNAs (miR-103, miR-10a, and miR-99a) were confirmed to be differentially expressed, each of them having a 2.3 fold increase or higher relative to the reference standard in luteal tissue at day 18 of pregnancy (Figure 3B). miR-34a did not change in expression between treatments and was used as a reference standard. MicroRNAs let-7a, miR-107, miR-142-star, miR-150, miR-25, and miR-34a had opposite expression patterns in qPCR analysis compared to the microarray analyses and statistical analysis confirmed no differences in miRNA expression between treatments (Figure 3A). miR-138 was found expressed more in luteal tissue during pregnancy compared to day 18 of estrous. However,

the difference in hybridization values between the treatments was minimal. qPCR analysis was unable to determine expression of miR-138 within the treatments.

Comparison of microRNA expression in luteal tissue on day 10 versus day 4 of the estrous cycle

Bovine-specific microRNAs expressed on day 4 and 10 of the estrous cycle were analyzed using the Student's T-test to assess differential expression. Sixty-three microRNAs were preferentially expressed in luteal tissue on day 10; whereas the remaining 3 were more abundant in luteal tissue on day 4 (Table 1). Although the three miRNAs were greater in day 4 luteal tissue, the difference in average hybridization between day 10 and day 4 is small. Nine miRNAs, which include let-7a, let-7b, let-7c, let-7d, miR-145, miR-23b, miR-24, miR-320, and miR-342, all had a hybridization of 10 or higher on day 10 of the estrous cycle (Table 1). Let-7b had the highest hybridization value of 13.1 (Table 1).

MicroRNA target prediction

Messenger RNA targets were predicted using the miRNA prediction databases, including miRanda, TargetScan, and miRDB. Some of these miRNAs have not yet been identified in the cow and bovine target mRNA searches were not possible. These targets were selected base on their potential function pertaining to reproductive processes.Therefore, targets for these miRNAs were predicted from their human homologs. Targets for confirmed bovine-specific miRNAs include the estrogen receptor (*ESR1)* and insulin like growth factor receptor 1 (IGFR1) (Table 7). miRNAs expressed in Day 18 CL of cyclic or pregnant cows include miR-543, miR-939, and miR-379/miR-379-star. Potential mRNA targets for these miRNAs include progesterone receptor (*PGR*), targeted by miR-543, *CD4*, targeted by miR-939, and oxytocin receptor (*OXTR)* targeted by miR-379/379-star. When comparing the

microarray analyses for miRNA expression in luteal tissue on day 10 versus day 4, miR-296-3p was consistently expressed only in day 10 CL, whereas miR-296-5p was consistently expressed in day 4 luteal tissue. These two miRNAs also have very different targets. Targets for 296-3p include cell adhesion molecule L1CAM (CHL1), whereas the targets for 296-5p include cell adhesion molecule 3 (CADM3), genes that code for cell adhesion molecules. MicroRNA-378-star, expressed in day 10 CL, is predicted to be a regulator of insulin like growth factor 1 receptor (IGF1R), and microRNA-331-3p, also expressed only in day 10 CL, has a predicted mRNA target of tumor necrosis factor (TNF). All of these mRNA targets were predicted based on the criteria for miRNA binding to its target and will need to be confirmed through other scientific methods. Target mRNAs predicted by one or by a combination of target prediction databases increase the probability of the mRNA truly being a target for a particular miRNA (Table 11). Target mRNAs predicted using two prediction databases will be more likely to be a possible target to miRNA than target mRNAs predicted using one prediction database since mRNAs predicted by two databases have been filtered by more criteria.

Discussion

The microRNAs present in bovine luteal tissue on day 4, 10, and 18 of the estrous cycle and day 18 of pregnancy were analyzed using heterologous miRNA microarrays. These arrays contain probes specific for miRNA genes expressed in over 70 different organisms. Fluorescent signaling in microarrays, including the Affymetrix microarray chip, is due to the detection of mature miRNA sequences, for the precursor sequences (Barad et al., 2004). This is an important feature of the miRNA microarray design, since hybridization between the microarray probes and miRNAs present in the sample indicate mature miRNAs present in the

sample. This would help in assessing miRNA function since the microarray is only considering mature miRNA present or absent in the tissue of interest. MicroRNAs differ in the first three letters of their identifying name, indicating the same miRNA in different species (Griffiths-Jones et al., 2006). Most miRNAs among organisms have similar nucleotide sequences that differ in single nucleotide additions or deletions. miRNAs present in bovine luteal tissue that hybridized to probes specific for a certain miRNA usually bound to miRNA probes for the same miRNA represented in a different species also on the microarray chip. The hybridization of miRNAs present in bovine luteal tissue to the heterologous microarray chip can not only be used to confirm the presence of bovine specific miRNAs, but also identify the presence of miRNAs known in other species, but not yet reported in bovine tissues.

When luteal tissue from day 18 of the estrous cycle and day 18 of pregnancy were compared, 10 miRNAs were found to be differentially expressed, with let-7a, miR-142-star, miR-150, miR-25 and miR-34a more highly expressed in luteal tissue from day 18 of the estrous cycle and miR-103, miR-107, miR-10a, miR-138, and miR-99a more highly expressed in day 18 of pregnancy. The fold change calculated from the qPCR result for miR-10a, miR-103, and miR-99a showed that these three miRNAs had similar expression patterns compared to the microarray results confirming that these miRNAs were differentially expressed in luteal tissue during day 18 of pregnancy when compared to luteal tissue from day 18 of the estrous cycle. However, the remaining seven bovine specific miRNAs were found not be expressed differentially between day 18 of the estrous cycle and day 18 of pregnancy when assessed with qPCR. This may be due to sensitivity differences between the two assays. Comparisons of microarray, qPCR, and deep sequencing analyses for measuring differential miRNA expression have shown that there can be discrepancies between microarray and qPCR results, which may suggest that using qPCR for miRNA confirmation of microarray analyses

may not be flawless (Git et al., 2010). When assessing miRNA expression in the microarray, thousands of probes are being compared to the background probes for confirming expression in a particular tissue. This many comparisons being made, although it would give information on the expression of thousands of miRNAs, would increase chances of committing type one error. Using qPCR for confirming differential expression of the ten bovine specific miRNAs identified to be differentially expressed by the microarray would decrease the chances of false positive results since the comparison is between less miRNAs and the detection in differences may be more accurate.

Experimentally validated miRNA targets are limited and the rules of target prediction are just now becoming clear. Recent studies have suggested several criteria that should be considered when determining the mRNA targets of miRNAs. Perfect seed sequence matching between nt 2-8 on the miRNA and its target is a popular criterion that many target prediction databases use in their designed algorithms (Lewis et al., 2005).Target mRNAs bind perfectly to the miRNA seed sequence, even if the binding between the mRNA target and the miRNA is not extensive past this point. However, validated targets can have imperfect binding to the miRNA seed sequence (Johnson et al., 2005), which could increase issues in target detection if prediction database algorithms are too strict. The miRNA can usually correct for this with 3' compensatory binding, where the 3' end of the miRNA binds to the 5'UTR of the target mRNA to enhance weak binding (reviewed in Maziere and Enright, 2007). Using cross-species conservation of seed sequences as a parameter for target prediction could be a better option, since most miRNA seed sequences are conserved across species (Pasquinelli et al., 2000; Lewis et al., 2003; Robins et al., 2005). However, there is a risk of overlooking valid mRNA targets due to incomplete genome annotations of non-human species (Robins et al., 2005). Using the concept of miRNA conservation and sequence similarity may decrease this risk,

since similar miRNAs expressed in similar tissues across species may have similar targets. Certain parameters can also be weighted based on the importance of the particular parameter to miRNA that would increase the chances of binding to its potential mRNA target. For example, MiRTarget miRNA target database uses the combination of algorithm filters for scoring computation, which increases the number of correct mRNA targets to a certain miRNA. Seed pairing conservation filter focusing on seed pairing at the 5' end of the miRNA would have a higher weight for scoring than 3'-end paring and miRNAs conserved between six different species would have increased scores compared to miRNAs conserved in only two species (Wang and Wang, 2006). Overall, miRNA target prediction is important for the identification of potential miRNA targets for further study, leading to better understanding of miRNA post-transcriptional regulation.

The presence of miR-10a, miR-103, and miR-99a in bovine luteal tissue at day 18 of pregnancy suggests a possible role of microRNAs during early pregnancy. MicroRNA-10a has been reported to interact with the 5'UTR region of mRNAs encoding for ribosomal proteins, resulting in the enhancement of their translation (Orom et al., 2008). This is interesting since miRNAs bind to the 3' UTR of their mRNA target to cause translational repression or mRNA degradation. Orom et al. (2008) showed that miR-10a can bind immediately downstream of the regulatory 5'TOP (terminal oligopyrimidine tract) motif, which is found in mRNAs encoding proteins involved in ribosomal biogenesis (Meyuhas, 2000). miR-10a transfection into NIH 3T3/RAS-V12 cells resulted in an increase of almost 40% in total protein synthesis (Orom et al., 2008). This demonstrates that miRNAs may be able to enhance the translation of mRNAs important to cellular function. However, this is only one study compared to the many studies published on miRNA's translational repression abilities. The fact that miR-10a is expressed in luteal tissue in both day 18 of the estrous cycle and day 18 of pregnancy, but is greater in day

18 of pregnancy, may suggest that miR-10a may increase protein synthesis during pregnancy. Many proteins are associated with progesterone production and luteal cell signaling. Further research will be needed to determine if this is the true function of miR-10a in bovine luteal tissues during these stages.

Macrophages have been shown to increase during luteolysis (Hume et al., 1984) and it is thought that luteolysis is delayed when an animal becomes pregnant due to the secretion of interferon-τ from the developing trophoblast to the uterine lumen, ultimately causing the inhibition of an estrogen-induced increase in endometrial estrogen and oxytocin receptors in ewes (Spencer et al., 1995). One of the predicted targets for miR-10a expressed in bovine luteal tissue is *BCL6* mRNA. BCL6 (B-cell Lymphoma 6) protein has been shown to negatively affect macrophage-specific chemokines, MCP-1, MCP-3, and MRP-1 (Toney et al., 2000). This relationship suggests that miR-10a may cause the inhibition of macrophages from entering luteal tissue during pregnancy by regulating the expression of BCL6.

One of the predicted targets for miR-103 includes estrogen receptor 1 (ESR1), also known as estrogen receptor alpha (ERα). It has been well established that the corpus luteum in multiple species, including rabbits (Lee et al., 1971), rats (Telleria et al., 1998), cows (Kimball and Hansel, 1974), and rhesus monkeys (Duffy et al., 2000), contains the estrogen receptor and that its expression varies throughout the estrous cycle. Estradiol-17β, the most biologically active estrogen, has also been shown to be present in the bovine corpus luteum (Okuda et al., 2001) as well as the corpus luteum of human (Shutt et al., 1975), rat (Elbaum and Keyes, 1976), and pig (Einspainer et al., 1991). Further, estradiol-17β has been shown to stimulate $PGF_{2\alpha}$ secretion in cultured bovine luteal cells, while having no effect on progesterone or oxytocin secretion, suggesting that luteal estradiol enhances $PGF_{2\alpha}$

production (Okuda et al., 2001) and that this effect may be controlled by estrogen receptors (Kimball and Hansel, 1974). The presence of miR-103 in bovine luteal tissue on day 18 of pregnancy suggests a role in the regulation of estrogen receptor expression. MicroRNA-103 may function in luteal tissue by inhibiting the translation of ERα, which would inhibit the binding of estradiol-17β to ERα, ultimately leading to decreased stimulation of $PGF_{2\alpha}$ production. This could be one of the mechanisms occurring in luteal tissue to ensure inhibiting of luteal regression.

MicroRNA-99a has moderate to high expression in a variety of bovine tissues, including brain, kidney, liver, spleen, thymus, medulla, abdominal and back subcutaneous fat, hypothalamus and medulla (Jin et al, 2009) as well as in mature bovine oocytes (Tesfaye et al., 2007). Predicted targets are sometimes shared with miR-99b, which only varies from miR-99a due to a difference in one or two nucleotides in its sequence (Griffiths-Jones et al, 2006). One of the predicted targets of miR-99a that may be of relevance to the CL is insulin-like growth factor receptor 1 (IGFR1) mRNA. During follicular growth, insulin-like growth factors (IGFs) function in cooperation with FSH and LH to regulate proliferation and differentiation of granulosal and thecal cells (Guiterrez et al., 1997; Campbell et al., 1998). IGF-I and IGF–II are expressed in the bovine CL (Einspanier et al., 1990) and increase progesterone production in bovine luteal cells(McArdle and Holtorf (1989) Sauerwein et al. (1992) further established that IGF-I, -II, and insulin could stimulate the release of progesterone and oxytocin throughout the luteal phase. IGFR1 is a cell surface receptor expressed in ovarian stroma, ovarian follicles (Perks et al., 1999) and during the luteal phase (Einspanier et al., 1990). MicroRNA-99a may regulate IGFR1 expression for the indirect control of progesterone and oxytocin production. Oxytocin also has an effect on bovine luteal cells during early pregnancy, enhancing progesterone production at concentrations of 4 to 40 mi.u./mL after 3 hour of incubation, but

also reducing progesterone production of luteal cells at concentrations of 800 mi.u./mL (Tan et al., 1982). However, the role of oxytocin in bovine luteal tissue is still debated. miR-99a may have a role in regulating oxytocin secretion through translational repression of IGFR1 mRNA, which may result in decreased oxytocin secretion, promoting the stimulation of progesterone production in luteal tissue during early pregnancy.

Progesterone receptors (PGR), one of the mRNA targets of miR-543, are expressed as two isoforms, PR-A and PR-B, and are found in a number of vertebrate species, including mice (Park-Sarge et al., 1995), rats (Shyamala et al., 1990; Natraj et al., 1993), humans (Misao et al., 1998) and bovine (Berisha et al., 2002). Berisha et al. (2002) showed that progesterone receptors are expressed in large and small luteal cells, theca interna and granulosal cells, as well as endometrial tissues. Progesterone receptors may also have the ability to aid in the inhibition of apoptosis by interacting with progesterone produced by luteal cells (Rueda et al., 2002). The expression of progesterone receptors on bovine luteal cells and our microarray data showing the expression of miR-543 in luteal tissue during Day 18 of the estrous cycle may suggest that miR-543 is present during this stage in the luteal phase for the translational repression of progesterone receptors. This repression would lead to the inability of PR to interact with progesterone, and may ultimately aid in the preparation for luteal regression.

As mentioned before, oxytocin receptor gene (OXTR) is a target of miR-379/miR-379-star, which was expressed in luteal tissue during day 18 of the estrous cycle, but not during day 18 of pregnancy. Our lab has previously shown that T lymphocytes express OXTRs and that culturing midcycle luteal cells with T lymphocytes decreased OXTR mRNA expression compared to T lymphocytes cultured alone (Ndiaye et al., 2008). Since this miR-379/miR-379-star is present in luteal tissue and its target is predicted to be OXTR, there may be regulation

of the interaction between T lymphocytes and luteal cells to cause this decrease in OXTR expression. OXTR is also expressed in luteal cells (Okuda et al., 1992). Decreasing OXTR expression may also affect the process of luteal regression where (oxytocin) OXT is released to stimulate $PGF_{2\alpha}$ from the uterus. The involvement of OXT in luteal regression and luteal function is still being debated; however, miR-543 could be an important regulator of OXT in luteal function and regression.

Only three miRNAs were expressed in luteal tissue on day 18 of pregnancy, not present on day 18, and not previously identified in bovine tissues. miR-939 is considered low- or poorly-conserved miRNA and our data confirms its low conservation through hybridization of miRNAs present in luteal tissue only to probes representing miR-939 for human and rhesus monkey. Currently, miR-939 expression has only been studied in cancerous tissues, research which involves the use of microarrays to monitor changes in miRNA expression to aid in the identification of tumors (Lui et al., 2007). Our data is the first to show miR-939 expression in bovine luteal tissue and this may be the first evidence of miR-939 in agricultural animal species. MicroRNA-939 is expressed in cervical cancer tissue and may be used to differentiate between cancerous tissues and normal tissues (Lui et al., 2007). Potential targets of miR-939 include tumor necrosis factor (TNF); however the subtype is not specified. These cytokines are associated with the ability to induce cytolysis or cell death (Benyo and Pate, 1992, Petroff et al., 2001), and binding to its receptor may lead to triggering the caspase pathway since the TNF receptor may be associated with caspase adaptor proteins (Nagata, 1997). TNF-α, produced by macrophages, mast cells and natural killer (NK) cells to induce cell death and aid in cytokine expression, is expressed in bovine luteal cells, remaining consistent in expression throughout the estrous cycle (Petroff et al., 1998), causing acute stimulation of progesterone secretion in luteal cells *in vitro* (Townson and Pate, 1996), and is able to

suppress LH-stimulated secretion (Benyo and Pate, 1992). TNF-α is also cytotoxic to luteal cells when combined with IFN-γ (Benyo and Pate, 1992, Petroff et al., 2001). The expression of miR-939 in luteal tissue during day 18 of pregnancy and its target of TNF may suggest function on regulation of TNF-α during this phase in luteal tissue to inhibit apoptosis and promote CL survival for the maintenance of pregnancy. Further study on this miRNA is needed to determine the true regulatory function.

Mammals have been shown to have lower levels of mirtrons compared to flies and worms (Lim and Burge, 2001). MicroRNA -1230 is also categorized by the miRBase database as a poorly-conserved miRNA, expressed only in rhesus monkey. mir-1230 is classified as a mirton, microRNAs processed from short introns with hairpin potential that are able to bypass Drosha processing (Okamura et al., 2007; Ruby et al., 2007). Our microarray data may be the first to show miR-1230 expression in species other than rhesus monkey. Due to its poor conservation and being a mirtron, the targets of miR-1230 are currently unknown. However, given that this miRNA has so far only been shown to be expressed in the tissues of rhesus monkey, our work showing that it is expressed in bovine luteal tissues can be considered an accomplishment in providing the first evidence of its conservation across species. We have shown that miR-1230 is expressed in luteal tissue during pregnancy, and its unknown targets leave many questions about what these targets may be in the CL. miR-700 has been shown mainly to be expressed in various mouse tissues (Mineno et al., 2006; Hackler et al., 2009; Ahn et al., 2010) however, recent studies have shown its expression in goat and sheep skin, indicating a role in hair growth regulation (Wenguang et al., 2007). Targets for this miRNA are limited.

The same comparative approach was done for the microarray results of microRNA expression in luteal tissue on day 10 and day 4. Results from this comparative approach showed that 42 miRNAs were abundant at higher hybridization levels in luteal tissue only during day 10 and13 miRNAs were found only present during day 4 when compared to miRNA expression during day 10. These miRNAs have also not yet been identified in bovine tissues. The increased amount of miRNAs expressed only during day 10 compared to day 4 resulted in an increased number of potential targets. Some of the most interesting miRNAs are miR-518b and miR-378-star, both expressed during day 10, which have DiGeorge Syndrome Critical Region 8 (DGCR8) and exportin-5 (XPO5) as potential targets. This could suggest that these miRNAs are able to regulate their own processing and expression. By repressing the components needed for biogenesis, microRNAs may cause negative feedback regulation on their own expression.

MicroRNA-331-3p is a miRNA derived from the 3'end of the precursor sequence, expressed only in luteal tissue during day 10 of the estrous cycle. Similar to miR-939, one of the predicted targets for miR-331-3p is tumor necrosis factor (TNF), indicating that the CL contains different miRNAs with similar targets that may need to regulate the same targets at different stages in the luteal phase. Comparing overall miRNA expression between the four stages, day 4 has the least amount of miRNA expression. During the RNA labeling process, RNA samples designated day 4 of the estrous cycle would not initially label properly. After several attempts, RNA from the day 4 treatments were able to be label and labeling controls fall within the range to indicate that labeling was successful. However, because of these labeling issues, the microarray results for microRNA expressed on day 4 may need to be repeated. During day 4 of the estrous cycle, theca interna and granulosal cell layers luteinize, the cavity from ovulation becomes obliterated and the CL overall increases in size and ability

to produce (Donaldson and Hansel, 1965). Enzymes and other proteins would be needed during this process, and microRNAs for the induction of translational repression would not be needed in similar levels compared to day 10 and day 18 of the estrous cycle and day 18 of pregnancy. However, a few miRNAs, both bovine-specific and non-bovine-specific, are present during this luteal stage. miR-296-5p was found only to be expressed in day 4 luteal tissue, however, miR-296-3p, which is derived from the same precursor sequence as miR-296-5p, was found to be only expressed during day 10 in luteal tissue. Both miRNAs have different cell adhesion molecules as potential mRNA targets, including CADM3 and CHL1, which is possible given that the seed sequence in both miRNAs is the same. Previous studies have demonstrated that miRNAs sharing the same seed sequence usually targets similar genes (Lewis et al., 2005; Linsley et al., 2007). Thermodynamic stability of the miRNA sequences determines which miRNA strand will be loaded into the RISC complex and the mechanism behind this selection is unknown.

CHAPTER 3

THE EFFECT OF GLOBAL MICRORNA REDUCTION ON LUTEAL CELL FUNCTION

Introduction

Drosha and Dicer are RNase III enzymes involved in miRNA biogenesis and processing. Drosha is one of three RNase III family enzymes characterized in the human and mouse (Fortin et al., 2002) and contains proline-rich and arginine-serine domains as well as two canonical C-terminal RNase III domains (Wu et al., 2000). The mechanism by which Drosha establishes specificity for pri-miRNA transcripts is not yet understood, however, it has been suggested that Drosha may have an affinity for the hairpin loop because of the unique stem-loop structure of the pre-miRNA transcript (Murchinson and Hannon, 2004). Drosha associates with DiGeorge Syndrome Critical Region 8 (DSCR8), an RNA binding protein that interacts with the ssRNA segments of primary miRNA (pri-miRNA) transcript, holding the transcript while Drosha cleaves at the proximal and distal stem (Zeng and Cullen, 2005; Han et al., 2006; Kim et al., 2009). Cleavage by Drosha generates a ~70 nt transcript known as the precursor miRNA (pre-miRNA) transcript, which consists of a 5' monophosphate and a 3' 2-nt hydroxyl overhang (Lee et al., 2003). The pre-miRNA transcript is released by DSCR8 and exported out of the nucleus and into the cytoplasm by RanGTP and Exportin-5 (Bohnsack et al., 2004; Kim, 2004).

Entry into the cytoplasm allows pre-miRNAs to come into contact with Dicer, an RNase III enzyme predominately located in the cytoplasm. There are two forms of Dicer, Dicer1 being required for processing of pre-miRNA, whereas Dicer2 is needed for processing of double

stranded precursor siRNA (Lee et al., 2004), however, mammalian cells seem to only encode for the Dicer1 enzyme (Hutvagner et al., 2001). Dicer consists of an RNA helicase, a domain of unknown function (DUF283), a Piwi Argonaute Zwille (PAZ) domain, two RNase III domains and a double-stranded RNA-binding domain (dsRBD) (MacRae et al., 2006; Luense et al., 2009). The PAZ domain has particular specificity for the 3' 2nt overhang on pre-miRNAs that have been processed by Drosha, thus functioning to select only pre-miRNAs (Murchinson and Hannon, 2004; Luense et al, 2009). The two RNase III domains dimerize to form a single processing center and function by cleaving the stem of the pre-miRNA ~22nt away from its termini, resulting in a 17-25 nt double stranded miRNA with the ability to be inserted into the RISC complex (Luense et al., 2009).

Bernstein et al. (2003) and Yang et al. (2005) have demonstrated that the loss of the Dicer gene resulted in embryonic lethality in mice. While demonstrating the necessity of Dicer for normal mouse development, the result of embryonic lethality may make it difficult to study global reduction miRNA expression in vivo (Berstein et al., 2003; Yang et al., 2005). The knockdown of the components of miRNA biogenesis and maturation may be a better method to study the global reduction of miRNA expression. Knockdown of Dicer expression in human endothelial cells using siRNAs resulted in reduced global miRNA expression compared to nontransfected endothelial cells (Kuehbacher et al., 2007; Suarez et al., 2007). Kuehbacher et al. (2007) also demonstrated that the knockdown of Drosha resulted in the increase of thrombospondin-1 mRNA expression, an antiangiogenic protein. This suggests that thrombospondin-1 is a potential target of miRNAs present in endothelial cells (Kuehbacher et al., 2007).

Few studies have been done assessing the effect of decreased Dicer and Drosha expression in reproductive tissues. Otsuka et al. (2008) demonstrated that a decrease in Dicer expression in the ovaries of mice resulted in decreased serum progesterone concentrations decreased expression of genes of components associated with progesterone production, and the inability to carry pregnancy to full term. Decreases in Dicer expression also cause reduced development of oviducts and uteri (Hong et al., 2008). Dicer knockdown has also been shown to induce cyst formation at the isthmus of the oviduct and result in the inhibition of embryonic development (Gonzalez and Behringer, 2009). To date, there are no studies focusing on the effects of decreased Drosha in reproductive tissues. The effect of the reduction of global miRNA expression in luteal cells has not yet been determined. In the present study, siRNAs specific for Dicer and Drosha genes were transfected into luteal cells to assess the effect of decreased Dicer and Drosha mRNA and protein expression, global miRNA expression, and its affect on luteal cell function in vitro.

Materials and Methods

Luteal Cell Isolation

Luteal tissue was collected transvaginally from cyclic Holstein cows (n=5) during midluteal phase (day 10-11; day 0 = day of estrus) of the estrous cycle. Handling of animals and surgical procedures were conducted according to protocols approved by the Institutional Laboratory Animal Care and Use Committee (IACUC) of Pennsylvania State University. Dissociation of luteal tissues was performed according to the procedure described previously (Pate, 1993). Luteal tissues were minced and placed in 24 mM Hepes-buffered Ham's F-12 culture medium (Invitrogen Corp., Carlsbad, CA) containing 0.5% BSA (Sigma Aldrich, St.

Louis, MO), 20 µg/mL gentamycin (Invitrogen Corp., Carlsbad, CA), and 2000 U/g tissue collagenase type I (Worthington Biochemical Corp., Lakewood, NJ). Centrifugation washes were performed at 90xg to prepare cells for transfection. The number of viable cells was determined using the Guava ViaCount program in the Guava EasyCyte system.

RNA Interference and Luteal Cell Transfection

For siRNA-mediated gene knockdown, two different siRNA duplexes for Dicer and Drosha were used for transfection. Sequences for Dicer and Drosha siRNAs are as follows:

Dicer I	5'-GAGUAAUGCUGAAACUGCAACUGAC-3'
	5'-GUCAGUUGCAGUUUCAGCAUUACUC-3'

Dicer II	5'-GAAUGAUGGUCAGAGAGCUGCAGAA-3'
	5'-UUCUGCAGCUCUCUGACCAUCAUUC-3'

Drosha I	5'-CAUGGAUCAGGUGGGAGAUUCUACA-3'
	5'-UGUAGAAUCUCCCACCUGAUCCAUG-3'

Drosha II	5'-CAGUGAAUCCGAGUGUGAGUCUGAU-3'
	5'-AUCAGACUCACACUCGGAUUCACUG-3'

Two siRNAs were designed for each gene using the siRNAs design programs and purchased from Invitrogen Corp (Carlsbad, CA). Sequences were BLASTed on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to confirm homology to the targets. A nonsilencing scrambled siRNA was used as a control (Invitrogen Corp., Carlsbad, CA). For cell transfection, luteal cells were isolated as described above and transfected using the Basic Mammalian Epithelial Cell kit (LONZA Inc., Williamsport, PA) according to the protocol of the manufacturer. To determine appropriate cell number and

transfection program used for culture of luteal cells transfected with Dicer and Drosha siRNAs, increasing numbers of luteal cells (250,000; 300,000; and 400,000 cells/well) were transfected with GFP plasmids (20ug; LONZA, Inc.) using several different Nucleofector transfection settings and cultured in 24 well plates for 24 hours in a humidified atmosphere of 5% CO_2 in air at 37^{o}C. The number of viable cells was determined using the Guava ViaCount program in the Guava EasyCyte. Transfection efficiency was evaluated by visual appraisal in fluorescence microscope and flow cytometry. Based on this preliminary experiment, 300,000 cells/well transfected with GFP plasmids using transfection program S-24 was determined to be appropriate based on transfection efficiency (85%) and cell viability (70-80%).

To determine siRNA concentrations and time in culture for Dicer and Drosha knockdown experiments, 300,000 luteal cells were transfected with siRNAs at concentrations of 25, 50, 75, and 100nM siRNAs using the S-24 Nucleofector setting and cultured for 24 and 48 hours. Luteal cells transfected with 100nM of Dicer or Drosha siRNAs and cultured for 48 hours resulted in the greatest decrease in Dicer and Drosha mRNA expression.

For Drosha knockdown experiments, Drosha (100 nM) or nonsilencing siRNA (75 nM) were mixed with Nucleofector solution (LONZA Inc., Williamsport, PA) and added to 300,000 luteal cells. Cells were transfected using Nucleofector program S-24.

Luteal Cell Culture

After transfection, luteal cells (3 X 10^5) were plated in 24-well plates and cultured in serum free Ham's F-12 media with gentamicin (20 µg/mL), L-gluamine (200nM), insulin-transferrin-selenium (ITS; 5 µg/mL insulin and transferring, 5ng/mL selenium) for 12 hours in a humidified atmosphere of 5% CO_2 in air at 37^{o}C. Ham's F-12 culture media was replaced with fresh culture media as describe above after 12 hours. Nontransfected and transfected luteal

cells with scrambled or Drosha siRNAs were cultured in three sets. For the first set, cells were cultured without treatments for 48 hours, collected and stored at -20^{o}C for RNA and protein extraction. For the second set, cells were treated with or without bovine TNF-α (50 ng/mL, R & D Systems Inc., Minneapolis, MN) and IFN-γ (500 ng/mL, R & D Systems Inc., Minneapolis, MN) for 48 hours. Cells cultured without TNF-α and IFN-γ served as the control. Cells treated with or without TNF-α and IFN-γ were also cultured with LH (10ng/mL). Cells were collected and processed for apoptosis assay. For the third set, cells were treated with or without LH (10 ng/mL) for 48 hours and cell culture media was collected and stored at

-20^{o}C until ELISA for progesterone. Cells cultured without LH served as the control.

Total RNA Extraction and Quantitative PCR

Total RNA was extracted from nontransfected and transfected luteal cells with scrambled or Drosha siRNAs with RNAqueous RNA Isolation kit (Ambion, Austin, TX) according to the manufacturer's protocol. DNase treatment was performed using RQ1 RNase-Free DNase kit (Promega, Madison, WI) and cDNA was synthesized using DyNAmo cDNA Synthesis Kit (Woburn, MA) following manufacturer's instructions. Quantitative PCR was performed in triplicate using SenisMixPlus SYBR kit (Taunton, MA) on the 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). RPL19 was used as the house keeping gene. Primer sets used for Dicer were 5'-TCCGGCTTCACGTTATCTCT-3' AND 5'-TCAATGTCCAAAGTGCTGGA-3' and for Drosha were 5'-GGAGATTGCCAATATGCTTCA-3' and 5'-GGACAGAGCTTGGTTTCGTC-3'.

Western Blot Analysis of Dicer and Drosha Protein Expression

Nontransfected and transfected luteal cells with scrambled or Drosha siRNAs

Transfected and nontransfected from the first set with no treatments were trypsinized and proteins were extracted using the CellLytic MT Cell Lysis Reagent (Sigma Aldrich) in the presence of the protease inhibitor cocktail (Sigma-Aldrich) following manufacturer instructions. Protein samples (100ng) were subjected to 8% SDS-polyacrylamide gel and the separated proteins were blotted onto polyvinylidene difluoride membranes (PVDF) (Hybond-P, Amersham Pharmacia Biotech). Western analyses were performed using polyclonal rabbit anti-Dicer and polyclonal rabbit anti-Drosha antibody (catalog #5818 and #12286, AbCam, Cambridge, MA). Beta-actin was used as a loading control. Membranes were incubated with blocking buffer (20nM Tris [pH 7.4], 150 mM NaCl, 5% BSA, and 0.05% Tween-20) for 30 minutes at room temperature followed by an overnight (12-16 hr) incubation at 4^{o}C with anti-Dicer at 4µg/mL or anti-Drosha at 1:250 dilution. Concentrations of Dicer and Drosha antibodies were determined through preliminary experiments to determine the concentrations that result in the most efficient detection of Dicer and Drosha proteins. Membranes were washed four times with TBS-Tween (20nM Tris [pH 7.4], 150 mM NaCl, and 0.05% Tween-20) and incubated with horseradish peroxidase-labeled anti-rabbit secondary antibody (Amersham Biosciences, GE Healthcare Life Sciences, Piscataway, NJ) at a dilution of 1:20000. Blots were washed four times with TBS-Tween and the antigen-antibody complex was visualized using the enhanced chemiluminescence system (ECL Western Blotting Analysis System; Amersham Biosciences) following the manufacturer's protocol. Membranes were exposed using Basic Quantity One program for the Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, CA).

Flow Cytometric Analysis of Induced Apoptosis

Nontransfected and transfected luteal cells with scrambled or Drosha siRNAs treated with or without bovine specific TNF-α (50 ng/mL) and IFN-γ (500 ng/mL), but also cultured in the presence of LH (10ng/mL) were trypsinized and processed 48 hours after treatment. Concentrations of TNF-α and IFN-γ were determined through preliminary experiments to determine the concentrations that result in the most efficient induction of apoptosis. Cell number was adjusted to approximately 4×10^5 cells/mL and 200 µL of this suspended cell concentration added to each well of a 96-well plate for the MitoPotential assay to assess apoptosis. MitoPotential dyes JC-1 and 7-amino actinomycin D (7-AAD) were used to assess changes in the mitochondrial membrane potential and cell membrane permeability, respectively. The 100x JC-1 dye was mixed with the 7-AAD dye in a 1:1 ratio to make a 50x staining solution and 4µL of this mixture was added to each well of cells for a final concentration of 1X staining dye per well of cells. The percentage of depolarized and apoptotic cells for each treatment was determined using the MitoPotential Assay on the Guava EasyCyte.

ELISA

After culture, cell culture media from nontransfected and transfected luteal cells with scrambled or Drosha siRNAs was collected and stored at -20^{o}C until analysis. Samples were thawed and diluted to 1:100 using Ham's F-12 cell culture media. Progesterone concentrations in culture samples were analyzed using the Correlate EIA progesterone enzyme immunoassay kit from Assay Designs (Ann Arbor, MI). Ninty-six-well precoated ELISA plates with goat anti-mouse secondary antibody coating buffer. Primary antibody (monoclonal mouse anti-progesterone) in 50 Lµ was and 100 µL cell culture media samples were added to

corresponding wells. Progesterone concentrations for the standard curve ranged from 25 to 1000 ng/mL. Standards (100µL) were added to corresponding wells on coated plates and plates were placed on a shaker and incubated at room temperature (25^{o}C) for two hours. Plates were washed three times and 200uL of p-nitrophenyl phosphate (pNpp) substrate was added to each well and incubated at room temperature for 45 minutes. Stop solution (50µL) was added to wells and progesterone concentrations were analyzed on a microtiter plate reader with test filter set at 405nM and reference filter set at 600nM.

Results

Confirmation of knockdown of Dicer and Drosha mRNA and protein in transfected luteal cells.

To investigate the role of endogenous Dicer and Drosha on luteal cell functions, we developed siRNAs to reduce expression of Dicer and Drosha in luteal cells. Luteal cells were transfected with either Dicer (100nM) or Drosha (100nM) siRNAs. Luteal cells were also transfected with nonspecific scrambled siRNAs to serve as a negative control and to assess specificity of the siRNAs for Dicer and Drosha. Non-transfected luteal cells also served as a control. PCR revealed that transfection of bovine luteal cells with Dicer or Drosha siRNA duplexes (100nM) resulted in reduced expression of Dicer and Drosha mRNA (Figure 6B). RPL19 was used as a house keeping gene and to compare specificity of siRNAs designed for Dicer and Drosha (Figure 6A). Western blotting of cell extracts confirmed the reduction of Dicer and Drosha expression (Figure 6C-E). β-actin was used as a housekeeping protein and to compare specificity of the siRNAs designed for Dicer and Drosha knockdown (Figure 6C). The knockdown of Drosha using Drosha-specific siRNAs resulted in a more copious decrease in Drosha mRNA and protein expression compared to the knockdown of Dicer using Dicer-

specific siRNAs (Figure 6B and 6D-E). Therefore, the knockdown of Drosha was studied further for effects on luteal cell function.

*Effect of Drosha knockdown on luteal cell progesterone production***.**

To determine whether the reduction of Drosha expression, and ultimately the reduction of miRNAs, affected the production of progesterone, cell culture media from transfected and nontransfected luteal cells cultured with or without LH was assessed using ELISA. As shown in Figure 7, there was no significant difference in progesterone concentration between nontransfected luteal cells and luteal cells transfected with scrambled or Drosha siRNAs cultured without LH (Figure 7). However, progesterone production in Drosha knockdown luteal cell cultures with added LH significantly decreased ($P < 0.05$), indicating that decreased Drosha expression in luteal cells has a negative effect on LH-stimulated progesterone production.

Effect of Drosha knockdown on luteal cells during TNF-α and IFN-γ induced apoptosis.

The addition of TNF-α and IFN-γ to luteal cells in culture can result in the induction of apoptosis (Benyo and Pate, 1992). To determine whether miRNAs may be involved in the induction of apoptosis, nontransfected and transfected luteal cells with scrambled or Drosha siRNAs were cultured with or without TNF-α (50ng/mL) and IFN-γ (500ng/mL) and assessed for percentage of mitochondrial depolarization and apoptosis. Mitochondrial depolarization is considered an early sign of apoptosis (Zamzami et al.,1996). For nontransfected luteal cells and luteal cells transfected with scrambled siRNAs, no significant difference was observed in mitochondrial depolarization (Figure 8A). However, a significant increase in mitochondrial depolarization ($P < 0.05$) was seen in luteal cells transfected with Drosha siRNAs compared to nontransfected luteal cells or luteal cells transfected with scrambled siRNAs (Figure 8A). For

cells treated with TNF-α (50 ng/mL) and IFN-γ (500 ng/mL), mitochondrial depolarization was significantly increased in transfected and nontransfected cells ($P < 0.05$) compared to the control (Figure 8A). However, there was no difference in apoptosis observed between nontransfected and transfected luteal cells when treated with TNF-α and IFN-γ (Figure 8A).

Transfection of luteal cells with Drosha siRNAs resulted in a significant increase ($P < 0.05$) in the percentage of apoptosis compared to non-transfected luteal cells and luteal cells transfected with scrambled siRNAs s (Figure 8B). There was no difference observed in apoptosis between non-transfected luteal cells and luteal cells transfected with scrambled siRNAs . When TNF-α (50 ng/mL) and IFN-γ (500 ng/mL) were added to transfected and nontransfected luteal cell cultures, a significant increase was seen in the percentage of apoptotic cells in these treated cells compared to the control cells (Figure 8B). Apoptosis of luteal cells was significantly increased in Drosha reduced luteal cells treated with TNF-α and IFN-γ compared to nontransfected and non-specific siRNA transfected cells (Figure 8B). Cell culture media from non transfected luteal cells and luteal cells transfected with scrambled siRNAs or Drosha siRNAs treated with or without TNF-α and IFN-γ was assessed for progesterone concentrations. Progesterone concentrations were significantly decreased in transfected and nontransfected luteal cells treated with TNF-α and IFN-γ compared to transfected and nontransfected luteal cells without the treatment of TNF-α and IFN-γ (Figure 8C).

Discussion

RNase III enzymes play a key role in microRNA biogenesis and processing. Drosha is responsible for miRNA processing to ensure appropriate length and export out of the nucleus

for further processing, while Dicer is needed to cleave the pre-cursor sequence into a 17-25nt dsRNA sequence in preparation for RISC complex loading. Without these two RNase III enzymes, miRNA biogenesis and processing may not occur, leading to the global reduction of miRNA expression and function.

Dicer knockdown and knockout experiments are common when assessing role of Dicer and miRNAs (Yang et al., 2005; Suarez et al., 2007; Otsuka et al., 2008; Gonzalez and Behringer, 2009). Dicer knockouts result in impaired development, usually leading to a lethal outcome (Berstein et al., 2003; Yang et al., 2005). Because of this, Dicer knockdown experiments using siRNAs have been used to study the global reduction of miRNAs on cell function (Suarez et al., 2007; Otsuka et al., 2008; Gonzalez and Behringer, 2009). However, Dicer knockdowns to study the global reduction of miRNA expression may not be the best approach due to the involvement of Dicer in processing precursors for miRNAs and siRNAs (Lee et al., 2004). Invertebrates, such as Drosophila , have homologues for two Dicer proteins, Dicer-1 and Dicer-2, which are involved in the processing of precursors for mature miRNA and siRNA sequences (Lee et al., 2004) However, mammals express only Dicer1, which processes both miRNAs and siRNAs (Hutvagner et al., 2001).

Research on the global reduction of miRNA expression and its effect on the function of primary cells is very limited. Endothelial cells were transfected with Drosha and Dicer specific siRNAs to study the role of these enzymes and miRNAs in angiogenic functions (Kuehbacher et al., 2007). The knockdown of Drosha in endothelial cells resulted in decreased cumulative sprout vessel length, tube formation and miRNA expression (Kuehbacher et al., 2007). However, knockdown of Dicer resulted in a greater reduction of cumulative sprout vessel length and tube formation, but a similar result in miRNA expression compared to Drosha

knockdown (Kuehbacher et al., 2007). This suggests that the pronounced decreased in sprout vessel length and tube formation are caused by the global reduction of miRNAs and siRNAs due to the knockdown of Dicer. Therefore, the knockdown of Drosha in specific cells or tissues for studying the effects of the global reduction in miRNA expression may be a more beneficial approach (Dalzell et al., 2010). The transfection of cells with different siRNA sequences can have varying effects on the mRNA target, resulting in comparable differences in decreased mRNA expression (Aagaard et al., 2007). This could be a reason that Dicer-specific siRNAs did not result in a similar decrease in mRNA expression seen in our Drosha knockdowns. To date, this is the first successful transfection of luteal cells to decrease Drosha expression.

The main function of luteal cells is to produce progesterone, a process enhanced by the addition of LH (Pate and Condon, 1982; Harrison et al., 1987). LH binds to its receptor located on small luteal cells, leading to protein kinase A (PKA) activation and the increase in progesterone production (Rekawiecki et al., 2008). Several functions associated with progesterone include the inhibition of Fas-ligand activated apoptosis (Okuda et al., 2004) and the establishment and maintenance of pregnancy (reviewed in Spencer and Bazer, 2002). In vivo, Dicer knockdown decreases progesterone concentrations, threatening the survival of established pregnancies in mice (Otsuka et al., 2008). However, the effect of Drosha knockdown on progesterone concentrations, especially in luteal cell cultures, had not been determined. A significant decrease in progesterone production was seen in LH treated luteal cells transfected with siRNAs compared to the control. This suggests that the reduction of miRNAs in luteal cells has an effect on LH-stimulated progesterone production.

Transfection of cells with mature miRNAs usually results in immediate mRNA repression within the first 24 hours (Parker and Wen, 2009). However, miRNA and siRNA turnover is fairly rapid, with mature miRNAs and siRNAs being degraded 48-72 hours after binding to their target (reviewed in Winter et al., 2009). This suggests the importance of analyzing the effects of Drosha siRNA-induced knockdowns before 72 hours. LH is unable to stimulate progesterone production in luteal cells between day 1 and 3 in culture (Pate and Condon, 1982; Fairchild and Pate, 1991); however, because of the potential for siRNA repression to be relieved past 72 hours after transfection, cells were removed from culture after 48 hours. This may be the reason that we did not observe an increase in progesterone production in LH-stimulated luteal cells. Although LH is unable to stimulate progesterone production during this time in culture, data suggests miRNAs are important for the continued progesterone production of luteal cells stimulated with LH.

The relationship between reproductive hormones and miRNAs expression and regulation has recently been recognized. Several miRNAs are associated with the increase of estrogen receptor α (ERα), estrogen receptor β (ERβ) and progesterone receptor (PR or PGR) expression in tumors. (Mattie et al., 2005, reviewed in Klinge, 2009). miR-206, miR-221, and miR-222 can inhibit the expression of ERα in ERα negative tumors (Mattie et al., 2005), whereas the decrease in estrogen and progesterone inhibits miR-21 expression (Hu et al., 2008). miR-21 is an apoptotic inhibitor (Park et al., 2009), which overall suggests that estrogen and progesterone can regulate miRNAs to ensure the survival of the dominant follicle or the survival of the CL, respectively. In the case of progesterone production, the effect of LH on miRNA expression is not known.

Tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) are cytokines that function in the communication between immune cells. TNF-α can act on luteal cells to increase prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production, as well as MHC class I expression (Benyo and Pate, 1992). Increasing concentrations of $PGF_{2\alpha}$ from luteal cells may stimulate oxytocin production by neighboring luteal cells and signal to the uterus to produce more $PGF_{2\alpha}$ during luteolysis (Milvae and Hansel, 1980). By increasing the expression of MHC class I molecules (Benyo and Pate, 1992), which are needed for fragment presentation of luteal cells to T lymphocytes, TNF-α may play a role in immune cell signaling for luteolysis. IFN-γ added to luteal cell cultures affects basal and LH-stimulated progesterone production past day 5 of culture (Fairchild and Pate, 1991), and increases MHC class II molecule expression on luteal cells (Fairchild and Pate, 1989). IFN-γ also decreases the number of luteal cells in culture (Benyo and Pate, 1992); however, the ability of this cytokine to induce cell death greatly increases when combined with TNF-α (Benyo and Pate, 1992). This indicates that TNF-α and IFN-γ are act synergistically to induce apoptosis (Benyo and Pate, 1992; Petroff et al., 2001; Okuda et al., 2004).

Several miRNAs are involved in either the induction or inhibition of apoptosis by regulating the expression of certain proteins in the apoptotic pathway. BCL-2 (B cell lymphoma 2) functions to inhibit cell death by inhibiting the BAX protein, a member of the BCL-2 family that binds to the voltage-dependent anion channel in the outer mitochondrial membrane. This binding causes the mitochondria to depolarize through the loss of its membrane potential (Wolter et al., 1997; reviewed in Cory and Adams, 2002 and in Cimmino et al., 2005). miR-15 and miR-16 are inversely expressed with BCL-2 in chronic lymphocytic leukemia cells and when transfected into leukemia cells, miR-15 and miR-16 can cause the reduction of BCL-2 protein and the induction of apoptosis (Cimmino et al., 2005). Anti-apoptotic miRNAs are

abundant in normal as well as cancerous tissues. Transfection of anti-miR-21 in pancreatic cancer cell lines can lead to increased apoptosis as well as decreased proliferation (Park et al., 2009). This suggests that miR-21 functions to repress certain components of the apoptosis pathway and repress inhibitors of proliferation. Our microarray data has detected the presence of miR-21, miR-145, and miR-221in bovine luteal tissues, which were all expressed on day 10 and all considered to be anti-apoptotic miRNAs studied thoroughly in cancerous tissues (Subramanian and Steer, 2010). Drosha knockdown could lead to the decreased expression of miR-21, miR-145, and miR-221 and this could explain why increased apoptosis was observed in luteal cells transfected with Drosha siRNAs compared to the controls. A decrease in these anti-apoptotic miRNAs would lead to an increase in apoptotic activity. The addition of TNF-α and IFN-γ to luteal cells with decreased Drosha expression and decreased miRNA expression would enhance these apoptotic effects. A few other microRNAs known to be pro-apoptotic in nature (Subramanian and Steer, 2010) were also present in Day 10 luteal tissue; however, using microarrays, we detected greater hybridization of miRNAs known to have anti-apoptotic functions compared to pro-apoptotic miRNAs in midcycle luteal tissue.

There was no difference in concentrations of progesterone secreted by nontransfected and transfected luteal cells, but a dramatic decrease in progesterone concentration occurred in apoptotic luteal cells. This was expected, since TNF-α and IFN-γ are considered potent inducers of apoptosis (Benyo and Pate, 1992; Petroff et al., 2001; Okuda et al., 2004), which would result in decreases in progesterone concentrations. In conclusion, our data show that microRNAs are involved in luteal cell function and are important for luteal cell survival.

FINAL CONCLUSIONS

MicroRNAs are fascinating in that they are produced based on cell type and developmental situation. They function through translational repression and mRNA degradation and are degraded soon after, demonstrating that they can regulate processes as well as be regulated. Many components are involved in their biogenesis and processing but the most important components are considered to be Dicer and Drosha. Without Dicer or Drosha, miRNA expression would be significantly reduced. MicroRNA expression and function in the corpus luteum has not been fully assessed. Determining which miRNAs are expressed in luteal tissues and their function in luteal cells could lead to further understanding of the regulation of processes essential to luteal function and survival. Overall, our data suggests that miRNAs are expressed differentially in luteal tissue as the luteal phase progresses, and that miRNAs may play an important role in the survival and maintenance of luteal cells. This is the first work in microRNA expression and function in the CL.

There are several different possibilities when further assessing miRNA function in luteal cells. A potential way to further assess miRNA targets that are truly affected by Drosha suppression would be through a cDNA microarray of different genes associated with reproduction or to design a microarray with probes for genes expressed specifically in the CL. However, microarrays have limited probe space for gene expression analysis. Gene sequencing is another option, and would assess how all the genes in the cow genome differ in expression when miRNAs are reduced in luteal cells. This would probably be a better approach so that no reproductive genes are missed. Expression of certain genes associated with luteal cells, such as PGR and OXTR, could be assessed using qPCR for Drosha knockdown luteal cells and would confirm if these potential miRNA targets were reduced due

to global miRNA reduction. The knockdown of other components in miRNA Biogenesis and processing, such as Exportin-5, may be another option for further study in global miRNA reduction. Studies focusing on the knockdown of Exportin-5 are limited in cell lines, and almost nonexistent in primary cells, but would result in the decrease in mature miRNA due to the inability of precursor miRNA to be transported out of the nucleus. These are only a few suggestions on how to further assess the affect of global reduction of miRNAs on luteal cells.

FIGURES

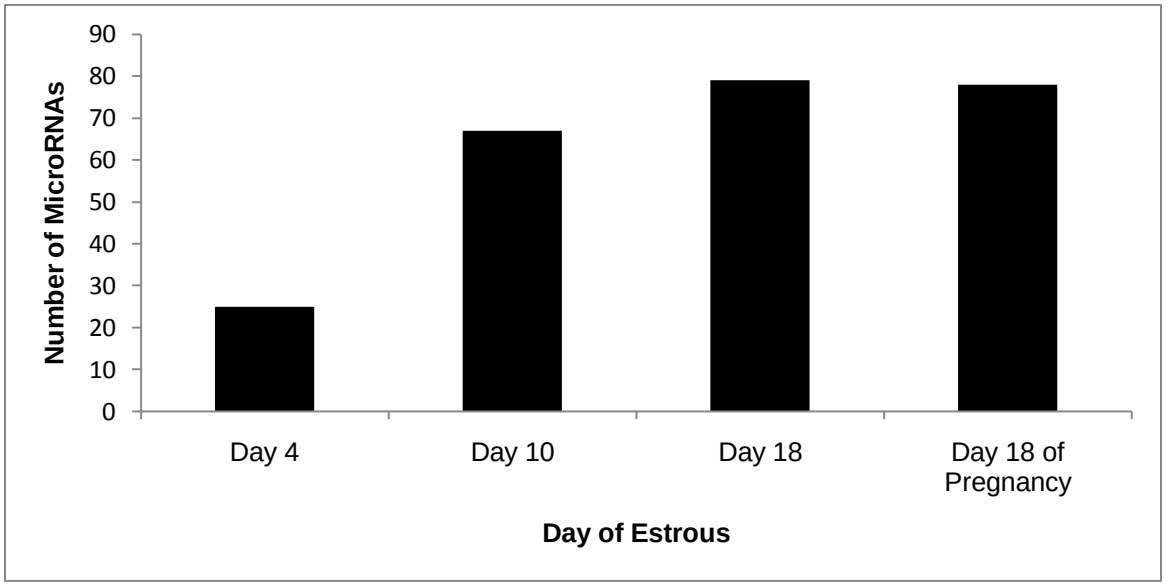


Figure 1: Examination of bovine-specific microRNAs present in luteal tissue. n=3; P ≤ 0.04 represents confident expression, whereas 0.04 ≤ P ≤ 0.06 represent marginal expression. Positive hybridization was indicated by a P≤ 0.04.

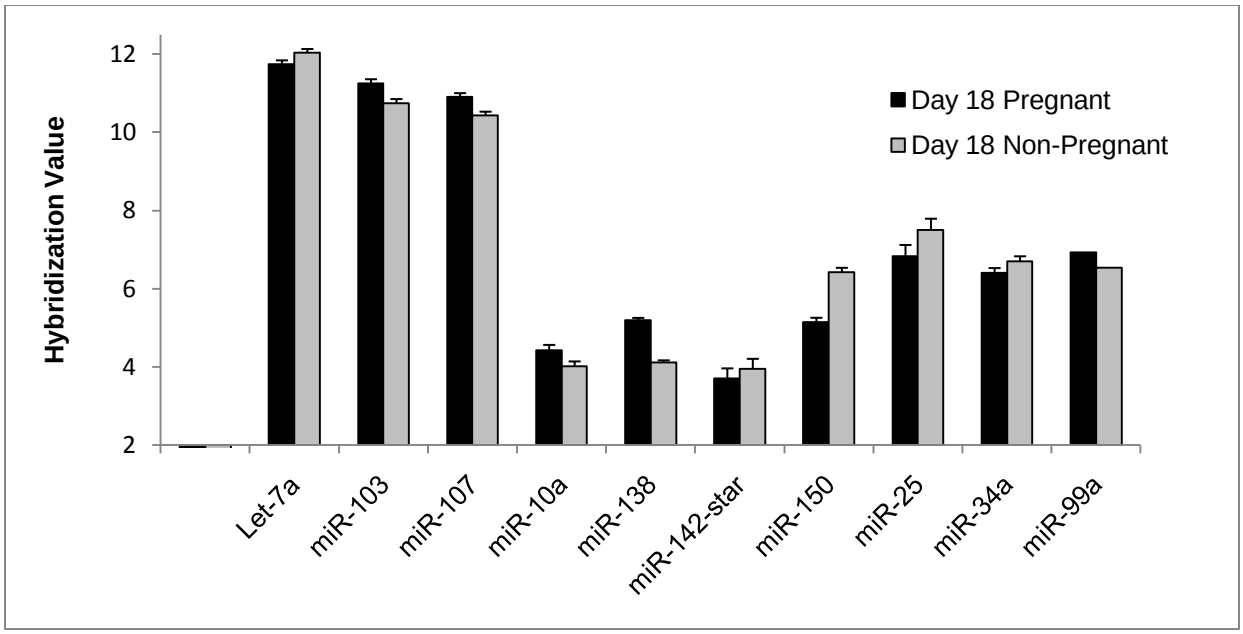
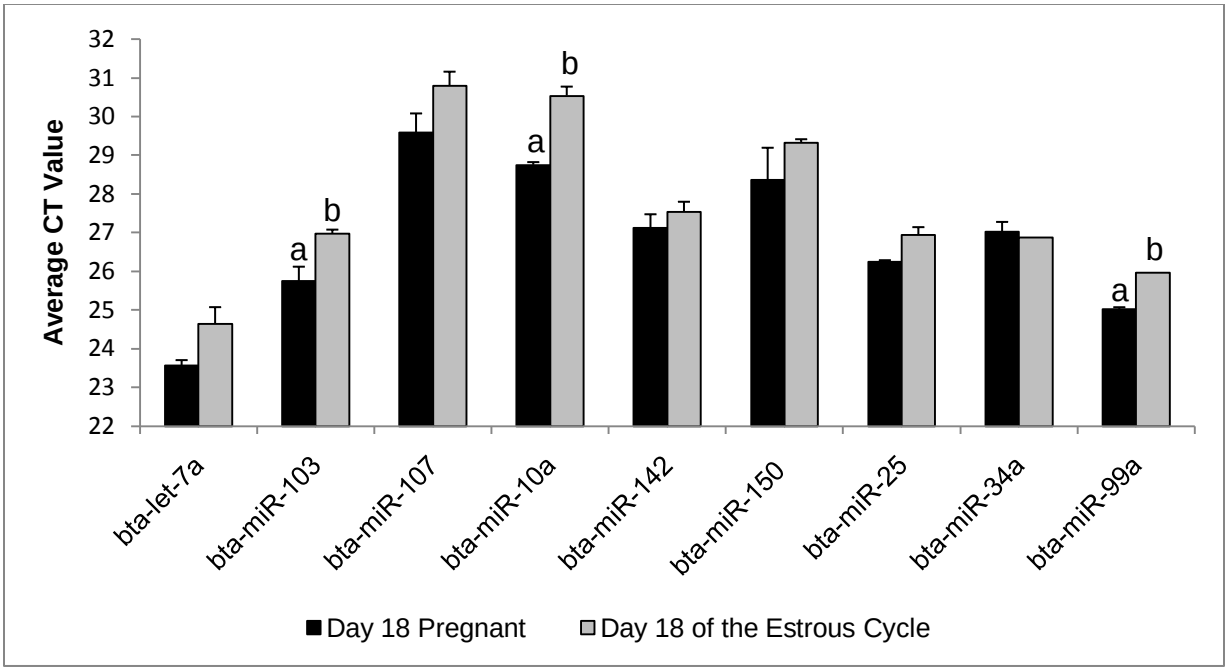


Figure 2: Bovine-specific microRNAs differentially expressed in luteal tissue from day 18 cyclic versus pregnant cattle. Comparison of hybridization values revealed treatment differences in 10 miRNAs. Bars indicate mean ± SEM, n = 3 animals per treatment. P<0.05.

A



B

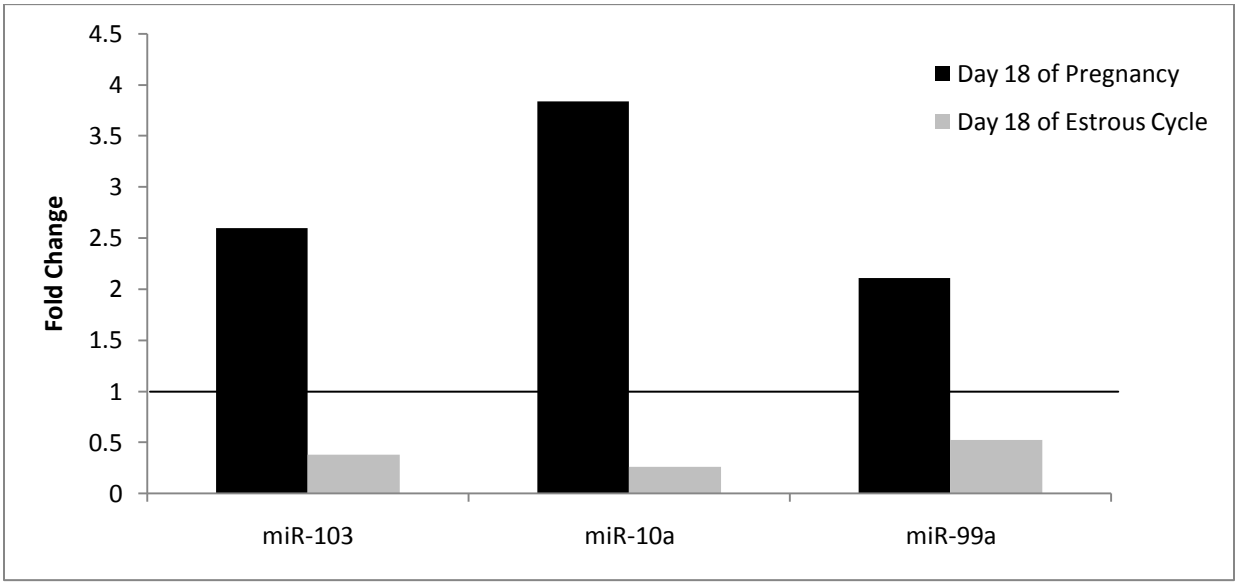


Figure 3: Verification of differential expression of bovine-specific microRNA at day 18 of pregnancy and day 18 of the estrous cycle using quantitative PCR analysis. A) Ct values are inversely proportional to the steady state concentration of miRNAs. Bars indicate mean ± SEM, n = 3 animals per treatment. $P < 0.05$. miR-138 was undetected. B) Fold change of miRNAs

differentially expressed at day 18 of pregnancy. Fold change was relative to the reference standard.

Table 1: Bovine-specific microRNAs differentially expressed in luteal tissue from day 10 cyclic versus day 4 cyclic cattle.

MicroRNA	Stage of Estrus	Hybridization Value	Standard Error	P-Value
Let-7a	Day 10	10.2631	0.3064	0.0012
	Day 4	6.693		
Let-7b	Day 10	13.1044	0.5356	0.0118
	Day 4	9.7844		
Let-7c	Day 10	11.2034	0.3317	0.0021
	Day 4	7.86		
Let-7d	Day 10	10.09	0.2027	0.0002
	Day 4	6.33		
Let-7e	Day 10	9.87	0.3198	0.0022
	Day 4	6.7		
Let-7g	Day 10	5.53	0.1108	0.0006
	Day 4	4		
Let-7i	Day 10	7.6	0.1003	0.0001
	Day 4	4.56		
miR103	Day 10	9.93	0.5686	0.0056
	Day 4	5.57		
miR107	Day 10	9.44	0.3291	0.0006
	Day 4	4.89		
miR10b	Day 10	5.26	0.0588	0.0003
	Day 4	4.26		
miR125a	Day 10	8.01	0.1416	0.0001
	Day 4	4.54		
miR125b	Day 10	9.07	0.2827	0.0026
	Day 4	6.4		
miR126	Day 10	7.08	0.114	0.0001
	Day 4	4.05		
miR127	Day 10	7.69	0.3928	0.0043
	Day 4	4.45		
miR138	Day 10	4.04	0.01553	0.0341
	Day 4	4.11		
miR139	Day 10	6.39	0.2119	0.0009

	Day 4	3.96		
miR140	Day 10	6.59	0.21	0.0014
	Day 4	4.26		
miR145	Day 10	9.91	0.4929	0.0041
	Day 4	5.8		
miR151	Day 10	5.57	0.0733	0.0002
	Day 4	4.16		
miR151star	Day 10	9.03	0.1676	0.0001
	Day 4	5.07		
miR15b	Day 10	6.65	0.09443	0.0001
	Day 4	4.14		
miR16	Day 10	5.36	0.1037	0.0009
	Day 4	4.06		
miR017-5p	Day 10	7.03	0.07103	0.0001
	Day 4	4.69		
miR181a	Day 10	8.41	0.1169	0.0001
	Day 4	4.25		
miR181b	Day 10	8.11	0.6208	0.0067
	Day 4	4.36		
miR181c	Day 10	4.56	0.1195	0.032
	Day 4	4.01		
miR186	Day 10	3.96	0.01643	0.0012
	Day 4	4.15		
miR191	Day 10	8.82	0.2869	0.0006
	Day 4	4.81		
miR193a	Day 10	4.3	0.014776	0.0001
	Day 4	3.97		
miR193a-star	Day 10	6.53	0.4643	0.0363
	Day 4	4.49		
miR195	Day 10	7.06	0.1402	0.0001
	Day 4	4.177		
miR199a-3p	Day 10	6.37	0.09879	0.0001
	Day 4	4.36		
miR199a-5p	Day 10	5.188	0.1237	0.0025
	Day 4	4		
miR19b	Day 10	4.48	0.08258	0.0145
	Day 4	4		
miR20a	Day 10	6.3	0.08121	0.0001
	Day 4	4.14		
miR21	Day 10	6.74	0.2261	0.0032
	Day 4	4.72		

miR221	Day 10	6.57	0.0546	0.0001
	Day 4	4.17		
miR222	Day 10	6.75	0.1117	0.0001
	Day 4	4.13		
miR22_5p	Day 10	4.306	0.05022	0.0063
	Day 4	3.9347		
miR23a	Day 10	8.88	0.1724	0.0001
	Day 4	4.815		
miR23b	Day 10	10.7297	0.508	0.0052
	Day 4	6.7424		
miR24	Day 10	10.3304	0.4961	0.0045
	Day 4	6.284		
miR25	Day 10	5.6929	0.2285	0.005
	Day 4	3.8862		
miR26a	Day 10	9.4856	0.2057	0.0002
	Day 4	5.4566		
miR27a	Day 10	4.4342	0.0784	0.0073
	Day 4	3.8763		
miR27b	Day 10	6.5095	0.113	0.0001
	Day 4	4.0486		
miR29b	Day 10	4.9439	0.06864	0.0009
	Day 4	4.0802		
miR30a-5p	Day 10	6.6141	0.167	0.0008
	Day 4	4.4396		
miR30d	Day 10	7.2289	0.08934	0.0001
	Day 4	4.7158		
miR30e-5p	Day 10	5.0226	0.1354	0.0222
	Day 4	4.3273		
miR31	Day 10	7.9277	0.3826	0.0053
	Day 4	4.9535		
miR320	Day 10	10.3847	0.9682	0.0702
	Day 4	7.0252		
miR342	Day 10	10.0074	0.2137	0.0009
	Day 4	7.337		
miR34a	Day 10	4.995	0.04198	0.0001
	Day 4	3.9313		
miR361	Day 10	8.3262	0.199	0.0002
	Day 4	4.6979		
miR423	Day 10	7.1461	0.3577	0.0103
	Day 4	4.8373		
miR425-5p	Day 10	4.9959	0.1182	0.008
	Day 4	4.1741		

miR455-star	Day 10	8.1113	0.2923	0.0012
	Day 4	4.7252		
miR497	Day 10	5.1582	0.05549	0.0001
	Day 4	4.0336		
miR532	Day 10	6.217	0.1502	0.0005
	Day 4	4.0754		
miR660	Day 10	5.1606	0.1433	0.005
	Day 4	4.0273		
miR7	Day 10	3.773	0.01964	0.0005
	Day 4	4.0642		
miR92	Day 10	8.8526	0.5111	0.0104
	Day 4	5.5637		
miR93	Day 10	6.4167	0.1171	0.0001
	Day 4	4.025		
miR99a	Day 10	5.2447	0.05952	0.0003
	Day 4	4.2319		
miR99b	Day 10	8.1985	0.3024	0.0018
	Day 4	5.02		

Hybridization values indicate mean ± SEM, n = 3 animals per treatment. $P < 0.05$.

Table 2: MicroRNAs present in day 18 bovine luteal tissue detected with heterologous probes.

MicroRNA	Species	Sequence	Chromosomal Location	Start[1]	End[2]	Human Chromosome Assignment[3]	Conservation[4]	Species with Similar miRNA Expression	P or NP
cfa-miR-1306	Canine familiaris	CCACCUCCCCUGCAAACGUCC	26	32322258	32322315	22	H		NP
dre-miR-10d	Danio rerio	UACCCUGUAGAACCGAAUGUGUG	6	9885911	9885911	n/a	P (2-two types of pufferfish)	P (2-two types of pufferfish)	NP
hsa-miR-296-3p	Homo Sapien	GAGGGUUGGGUGGAGGCUCUCC	20	56826065	56826144	n/a	Rh, M	Rh	NP
hsa-miR-299-5p	Homo Sapien	UGGUUUACCGUCCCACAUACAU	14	100559884	100559946	n/a	Rh	Rh	NP
hsa-miR-543	Homo sapiens	AAACAUUCGCGGUGCACUUCUU	14	100568077	100568154	n/a	C, M, R	M, R	NP
hsa-miR-552	Homo sapiens	AACAGGUGACUGGUUAGACAA	1	34907787	34907882	n/a	Rh		NP
mml-miR-1240	Macaca mulatta	UCACCAUGACCCUGAUCCCACU	12	83077563	83077645	n/a	n/a	n/a	NP
rno-miR-346	Rattus norvegicus	UGUCUGCCUGAGUGCCUGCCUCU	16	10553485	10553582	10	H, M, Rh	H, M, Rh	NP
rno-miR-370	Rattus norvegicus	GCCUGCUGGGGUGGAACCUGGUU	6	134210194	134210272	14	H, M, Rh	H, M, Rh	NP
hsa-miR-379-star	Homo sapiens	UAUGUAACAUGGUCCACUAACU	14	100558156	100558222	n/a	n/a	n/a	NP
mml-miR-1230	Macaca mulatta	GUGGGUGGGGGCAUCUCGGA	?	28162	28234	n/a	n/a	n/a	P

mml-miR-939	Macaca mulatta	UGGGGAGCUGAGGCUCUGGGGGUG	8	147020034	147020116	8	H	H	P
mmu-miR-1198	Mus musculus	UAUGUGUUCCUGGCUGGCUUGG	X	7384227	7384347	n/a	n/a	n/a	NP
mmu-miR-1195	Mus musculus	UGAGUUCGAGGCCAGCCUGCUCA	17	71209818	71209940	n/a	n/a	n/a	NP
mmu-miR-700	Mus musculus	CACGCGGGAACCGAGUCCACC	4	134972470	134972548	n/a	n/a	n/a	P
dre-miR-727-star	Danio rerio	UCAGUCUUCAAUUCCUCCCAGC	24	24847536	24847626	n/a	n/a	n/a	NP
hsa-miR-223	Homo sapiens	UGUCAGUUUGUCAAAUACCCCA	X	65155437	65155546	n/a	Bo, C, Ch, F, G, M, O, Or, P (2), R, Rh, T, Z	Bo, C, Ch, F, R, Rh, T, Z	NP
hsa-miR-483-3p	Homo sapiens	UCACUCCUCUCCUCCCGUCUU	11	2111940	2112015	n/a	n/a	n/a	NP
mmu-miR-290-3p	Mus musculus	AAAGUGCCGCCUAGUUUUAAGCCC	7	3218627	3218709	n/a	n/a	n/a	NP

[1]Position on the species-specific genome where microRNA sequence starts. [2]Position on the species-specific genome where microRNA sequence ends.[3]Chromosome assignment on the Human genome. [4]Conservation of the microRNA in Human (H), Bovine (B), Zebrafish (Z), Gorilla (G), Opposum (O), Rhesus Monkey (Rh), Mouse (M), Orangutan (Or), Rat (R), Chicken (C), Tamarind Monkey (T), Pufferfish (P), Chimpanzee (Ch), Pig (Pi), Macaque Monkey (Ma), Bobono Monkey (Bo), Dog (D) and Frog (F). P indicates miRNA expressed in pregnant luteal tissue. NP indicates miRNA expressed in day 18 cyclic luteal tissue.

Table 3: MicroRNAs in day 10 and day 4 bovine luteal tissue detected with heterologous probes.

MicroRNA	Species	Sequence	Chromosomal Location	Start[1]	End[2]	Human Chromosome Assignment[3]	Conservation[4]	Species with Similar miRNA Expression	Day 4 or Day 10
cfa-miR-497	Canis familiaris	CAGCAGCACACUGUGGUUUGU	5	35042178	35042244	17	H, M, R, Rh	H, M, R, Rh	Day 10
cfa-miR-652	Canis familiaris	AAUGGCGCCACUAGGGUUGUGC	X	86340979	86341041	X	M, R, H, Rh	M, R, H, Rh	Day 10
dre-miR-126-star	Danio rerio	CAUUAUUACUUUUGGUACGCG	27	688926	689026	5	R, F, B, Z, C, H	n/a	Day 10
fru-miR-429	Fugu rubripes	UAAUACUGUCUGGUAAUGCCGU	23	22588863	22588944	1	Z, C, H, Rh, M, R, P (2), D	P (2)	Day 10
hsa-miR-106b-star	Homo sapiens	CCGCACUGUGGGUACUUGCUGC	7	99529552	99529633		M, R	n/a	Day 10
hsa-miR-1228	Homo sapiens	UCACACCUGCCUCGCCCCCC	12	55874554	55874626		n/a	n/a	Day 10
hsa-miR-193b-star	Homo sapiens	CGGGGUUUUGAGGGCGAGAUGA	16	14305325	14305407		Rh	Rh	Day 10
hsa-miR-199a-3p	Homo sapiens	ACAGUAGUCUGCACAUUGGUUA	1	170380298	170380407		W, Rh, M, O, Ch, Ma, R, F, G	W, Rh, M, O, Ch, Ma, R, F, G	Day 10
			19	10789102	10789172				
hsa-miR-21-star	Homo sapiens	CAACACCAGUCGAUGGGCUGU	17	55273409	55273432		M, R, B	M	Day 10
hsa-miR-28-5p	Homo sapiens	AAGGAGCUCACAGUCUAUUGAG	3	189889263	189889348		n/a	n/a	Day 10

hsa-miR-296-3p	Homo sapiens	GAGGGUUGGGUGGAGGCUCUCC	20	56826065	56826144		n/a	n/a	Day 10
hsa-miR-328	Homo sapiens	CUGGCCCUCUCUGCCCUUCCGU	16	65793725	65793799		M, R, D	M, R, D	Day 10
hsa-miR-331-3p	Homo sapiens	GCCCCUGGGCCUAUCCUAGAA	12	94226327	94226420		Rh, M	Rh, M	Day 10
hsa-miR-342-5p	Homo sapiens	AGGGGUGCUAUCUGUGAUUGA	14	99645745	99645843		M, R, Rh	M, R, Rh	Day 10
hsa-miR-362-5p	Homo sapiens	AAUCCUUGGAACCUAGGUGUGAGU	X	49660312	49660376		Rh, M	Rh, M	Day 10
hsa-miR-378-star	Homo sapiens	CUCCUGACUCCAGGUCCUGUGU	5	149092581	149092646		M, R	M, R	Day 10
hsa-miR-422a	Homo sapiens	ACUGGACUUAGGGUCAGAAGGC	15	61950182	61950271		Rh	Rh	Day 10
hsa-miR-423-5p	Homo sapiens	UGAGGGGCAGAGAGCGAGACUUU	17	25468223	25468316		M, Rh	M, Rh	Day 10
hsa-miR-425-star	Homo sapiens	AUCGGGAAUGUCGUGUCCGCCC	3	49032585	49032671		M	M	Day 10
hsa-miR-500-star	Homo sapiens	AUGCACCUGGGCAAGGAUUCUG	X	49659779	9659862		n/a	n/a	Day 10
hsa-miR-502-3p	Homo sapiens	AAUGCACCUGGGCAAGGAUUCA	X	49665946	49666031		Rh	Rh	Day 10
hsa-miR-503	Homo sapiens	UAGCAGCGGGAACAGUUCUGCAG	X	133508024	133508094		Rh	Rh	Day 10
hsa-miR-505-star	Homo sapiens	GGGAGCCAGGAAGUAUUGAUGU	X	138833973	138834056		n/a	n/a	Day 10
hsa-miR-532-3p	Homo sapiens	CCUCCCACACCCAAGGCUUGCA	X	49654494	49654584		Rh, M, R	Rh, M, R	Day 10

hsa-miR-542-5p	Homo sapiens	UCGGGGAUCAUCAUGUCACGAGA	X	133503037	133503133		Rh, M, R	Rh, M, R	Day 10
hsa-miR-635	Homo sapiens	ACUUGGGCACUGAAACAAUGUCC	17	63932187	63932284		n/a	n/a	Day 10
hsa-miR-642	Homo sapiens	GUCCCUCUCCAAAUGUGUCUUG	19	50870026	50870122		Rh	n/a	Day 10
hsa-miR-652	Homo sapiens	AAUGGCGCCACUAGGGUUGUG	X	109185213	109185310		n/a	**n/a**	Day 10
hsa-miR-663	Homo sapiens	AGGCGGGGCGCCGCGGGACCGC	2	132731009	132731123		Rh	Rh	Day 10
hsa-miR-92b-star	Homo sapiens	AGGGACGGGACGCGGUGCAGUG	1	153431592	153431687		n/a	n/a	Day 10
hsa-miR-939	Homo sapiens	UGGGGAGCUGAGGCUCUGGGGGUG	8	145590172	145590253		Rh	n/a	Day 10
hsa-miR-93-star	Homo sapiens	ACUGCUGAGCUAGCACUUCCCG	7	99529327	99529406		M	n/a	Day 10
mdo-miR-152	Monodelphis domestica	UCAGUGCAUGACAGAACUUGGG	2	200362981	200363053	17	O, M, R, P, D Z, H,	R, M, P, H, D	Day 10
mdo-miR-181b	Monodelphis domestica	AACAUUCAUUGCUGUCGGUGGG	2	99872867	99872953		Rh, M, Ma, Ch, T, R, Pi, P, F	Rh, M, Ma, Ch, T, R, Pi, P, F	Day 10
mdo-miR-193	Monodelphis domestica	AACUGGCCUACAAAGUCCCAG	2	504311646	504311711	17	F, D, Z, P, C, H	F, D, Z, P, C, H	Day 10
mdo-miR-221	Monodelphis domestica	AGCUACAUUGUCUGCUGGGUUUC	4	17427280	17427353	17	H, Rh, M, R, P (2), F, D, Z, C, G	H, Rh, M, R, P (2), F, D, Z, C, G	Day 10
mml-miR-1227	Macaca mulatta	GUGGGGCCAGGCGGUGGUGG	19	2034269	2034356	17	H	H	Day 10
mml-miR-125a-	Macaca	ACAGGUGAGGUUCUUGGGAGCC	19	57751589	57751589		M, R, P, F, D	M, R, P, F,	Day 10

3p	mulatta							D	
mml-miR-129-3p	Macaca mulatta	AAGCCCUUACCCCAAAAAGUAU	3	165758238	165758309		H, M	M	Day 10
mml-miR-139-3p	Macaca mulatta	GGAGACGCGGCCCUGUUGGAGU	14	70701137	70701204		M, R	n/a	Day 10
mml-miR-142-3p	Macaca mulatta	UGUAGUGUUUCCUACUUUAUGGA	16	42574680	42574766		M, R, F, C	C	Day 10
mml-miR-149	Macaca mulatta	UCUGGCUCCGUGUCUUCACUCCC	12	104321121	104321209	2	H,M	H	Day 10
mml-miR-151-3p	Macaca mulatta	CUAGACUGAAGCUCCUUGAGG	8	143221103	143221192	8	H, M	H, M	Day 10
mml-miR-151-5p	Macaca mulatta	UCGAGGAGCUCACAGUCUAGU	8	143221103	143221192	8	H, M	H, M	Day 10
mml-miR-152	Macaca mulatta	UCAGUGCAUGACAGAACUUGG	16	32253411	32253497	17	O, M, R, P, D Z, H,	R, M, P, H, D	Day 10
mml-miR-193b	Macaca mulatta	AACUGGCCCUCAAAGUCCCGCU	20	14416035	14416117	16	H	H	Day 10
mml-miR-199a-5p	Macaca mulatta	CCCAGUGUUCAGACUACCUGUUC	19	10626280	10626350		Rh, M, O, Ch, Ma, R, F, G	Rh, M, O, Ch, Ma, R, F, G	Day 10
mml-miR-210	Macaca mulatta	CUGUGCGUGUGACAGCGGCUGA	14	372934	373043	4	H, M, R, P, F	H, M, R, P, F	Day 10
mml-miR-362-5p	Macaca mulatta	AAUCCUUGGAACCUAGGUGUGAGU	X	47644032	47644096	X	M, H	M, H	Day 10
mml-miR-432	Macaca mulatta	UCUUGGAGUAGGUCAUUGGGUGG	7	164168430	164168523	14	H	H	Day 10
mml-miR-455-3p	Macaca mulatta	GCAGUCCAUGGGCAUAUACAC	15	21918824	21918919	9	H	H	Day 10

mmu-miR-532-3p	Mus musculus	CCUCCCACACCCAAGGCUUGCA	X	47638245	47638322		n/a	n/a	Day 10
mmu-miR-532-5p	Mus musculus	CAUGCCUUGAGUGUAGGACCGU	X	47638245	47638322			n/a	Day 10
mmu-miR-542-5p	Mus musculus	CUCGGGGAUCAUCAUGUCACGA	X	132766718	132766814	4	R, C, H	H	Day 10
mmu-miR-574-5p	Mus musculus	UGAGUGUGUGUGUGUGAGUGUGU	5	65361557	65361634	4	H	H, M	Day 10
mmu-miR-720	Mus musculus	AUCUCGCUGGGGCCUCCA	3	88920531	88920594	3	H	M, H	Day 10
mne-miR-181a-star	Macaca nemestrina	ACCAUCGACCGUUGAUUGUACC	14	115443073	115443168		Ch	Ch	Day 10
mne-miR-211	Macaca nemestrina	UUCCCUUUGUCAUCCUUCGCCU	7	71350692	71350797		C, H, M, Ma	M, Ma	Day 10
rno-miR-139-5p	Rattus norvegicus	UCUACAGUGCACGUGUCUCCAG	1	158976576	158976643	11	H	H	Day 10
dre-miR-735	Danio rerio	CUCUCCCACCGCUAAACUUGAC	24	33517840	33517902		Z	Z	Day 4
hsa-miR-129-3p	Homo sapiens	AAGCCCUUACCCCAAAAAGCAU	11	43559520	43559609	11	M	M	Day 4
hsa-miR-191-star	Homo sapiens	GCUGCGCUUGGAUUUCGUCCCC	3	49033055	49033146		M	M	Day 4
hsa-miR-454-star	Homo sapiens	ACCCUAUCAAUAUUGUCUCUGC	17	54569901	54570015		Z	n/a	Day 4
hsa-miR-483-3p	Homo sapiens	UCACUCCUCUCCUCCCGUCUU	11	2111940	2112015		H	n/a	Day 4
hsa-miR-498	Homo sapiens	UUUCAAGCCAGGGGGCGUUUUUC	19	58869263	58869386	11	M	M	Day 4
hsa-miR-589-	Homo	UCAGAACAAAUGCCGGUUCCCAGA	7	5501976	5502074		n/a	n/a	Day 4

star	sapiens								
hsa-miR-767-3p	Homo sapiens	UCUGCUCAUACCCCAUGGUUUCU	X	151312549	151312657	17	Rh	n/a	Day 4
hsa-miR-877	Homo sapiens	GUAGAGGAGAUGGCGCAGGG	6	30660088	30660173		Rh, M, R	Rh, M, R	Day 4
hsa-miR-877-star	Homo sapiens	UCCUCUUCUCCCUCCUCCCAG	6	30660088	30660173		Rh, M, R	Rh, M, R	Day 4
mml-miR-296-5p	Macaca mulatta	AGGGCCCCCCCUCAAUCCUGU	10	5712878	5712957		n/a	n/a	Day 4
mml-miR-518b	Macaca mulatta	CAAAGCGCUCCCCUUUAGAGG	19	59804018	59804100	3	H	H	Day 4
mml-miR-615-3p	Macaca mulatta	UCCGAGCCUGGGUCUCCCUCUU	11	51132754	51132849		M	M	Day 4
mml-miR-657	Macaca mulatta	GGCAGGUCCUCACCCUCUCUAGG	16	76575354	76575354		H	n/a	Day 4
mmu-miR-467a	Mus musculus	UAAGUGCCUGCAUGUAUAUGCG	2	10397973	10398045		n/a	n/a	Day 4

[1]Position on the species-specific genome where microRNA sequence starts. [2]Position on the species-specific genome where microRNA sequence ends.[3]Chromosome assignment on the Human genome. [4]Conservation of the microRNA in Human (H), Bovine (B), Zebrafish (Z), Gorilla (G), Opposum (O), Rhesus Monkey (Rh), Mouse (M), Orangutan (Or), Rat (R), Chicken (C), Tamarind Monkey (T), Pufferfish (P), Chimpanzee (Ch), Pig (Pi), Macaque Monkey (Ma), Bobono Monkey (Bo), Dog (D) and Frog (F). Day 10 and Day 4 indicate miRNA expressed in luteal tissue on day 10 and 4 of the estrous cycle, respectively.

Table 4: MicroRNAs in luteal tissue of day 18 cyclic cattle previously reported in human, mouse, rat, or rhesus monkey.

Probe ID	Human	Mouse	Rat	Rhesus Monkey
miR-296-3p	hsa-miR-296-3p	not expressed	no known rno target	mml-miR-296-3p
miR-299-5p	hsa-miR-299-5p	no known mmu target	no known rno target	mml-miR-299-5p
miR-543	hsa-miR-543	mmu-miR-543	no known rno target	mml-miR-543
miR-552	hsa-miR-552	no known mmu target	no known rno target	not expressed
miR-1240	no known hsa target	no known mmu target	no known rno target	mml-miR-1240
miR-346	hsa-miR-346	mmu-miR-346	rno-miR-346	no known mml target
miR-370	hsa-miR-370	mmu-miR-370	rno-miR-370	no known mml target
miR-379-star	hsa-miR-379-star	no known mmu target	no known rno target	no known mml target
miR-1198	no known hsa target	mmu-miR-1198	no known rno target	no known mml target
miR-1195	no known hsa target	mmu-miR-1195	no known rno target	no known mml target
miR-223	hsa-miR-223	not expressed	rno-miR-223	mml-miR-223
miR-483-3p	hsa-miR-483-3p	no known mmu target	no known rno target	no known mml target
miR-290-3p	no known hsa target	mmu-miR-290-3p	no known rno target	no known mml target

Table 5: MicroRNAs in luteal tissue of day 18 pregnant cattle previously reported in human, mouse, rat or rhesus monkey.

Probe ID	Human	Mouse	Rat	Rhesus Monkey
miR-1230	no known hsa target	no known mmu target	no known rno target	mml-miR-1230
miR-939	hsa-miR-939	no known mmu target	no known rno target	mml-miR-939
miR-700	no known hsa target	mmu-miR-700	no known rno target	no known mml target

Table 6: MicroRNAs in luteal tissue day 10 cyclic cattle previously reported in human, mouse, rat and rhesus monkey.

Probe ID	Human	Mouse	Rat	Rhesus Monkey
miR-652	hsa-miR-652	mmu-miR-652	rno-miR-652	mml-miR-652
miR-429	hsa-miR-429	mmu-miR-429	rno-miR-429	mml-miR-429
miR-106b-star	hsa-miR-106b-star	not expressed	not expressed	no known mml target
miR-1228	hsa-miR-1228	no known mmu target	no known rno target	no known mml target
miR-193b-star	hsa-miR-193b-star	no known mmu target	no known rno target	mml-miR-193b-star
miR-328	hsa-miR-328	mmu-miR-328	not expressed	no known mml target

miR-331-3p	hsa-miR-331-3p	mmu-miR-331-3p	no known rno target	mml-miR-331-3p
miR-342-5p	hsa-miR-342-5p	mmu-miR-342-5p	rno-miR-342-5p	mml-miR-342-5p
miR-362-5p	hsa-miR-362-5p	mmu-miR-362-5p	no known rno target	mml-miR-362-5p
miR-378-star	hsa-miR-378-star	mmu-miR-378-star	rno-miR-378-star	no known mml target
miR-422a	hsa-miR-422a	no known mmu target	no known rno target	mml-miR-422a
miR-423-5p	hsa-miR-423-5p	mmu-miR-423-5p	no known rno target	mml-miR-423-5p
miR-425-star	hsa-miR-425-star	mmu-miR-425-star	no known rno target	no known mml target
miR-500-star	hsa-miR-500-star	no known mmu target	no known rno target	no known mml target
miR-502-3p	hsa-miR-502-3p	no known mmu target	no known rno target	mml-miR-502-3p
miR-503	hsa-miR-503	no known mmu target	no known rno target	mml-miR-503
miR-505-star	hsa-miR-505-star	no known mmu target	no known rno target	no known mml target
miR-532-3p	hsa-miR-532-5p	mmu-miR-532-5p	rno-miR-532-5p	mml-miR-532-5p
mir-542-5p	hsa-miR-542-5p	mmu-miR-542-5p	rno-miR-542-5p	mml-miR-542-5p
miR-635	hsa-miR-635	no known mmu target	no known rno target	no known mml target
miR-642	hsa-miR-642	no known mmu target	no known rno target	mml-miR-642
miR-652	hsa-miR-652	no known mmu target	no known rno target	no known mml target
miR-663	hsa-miR-663	no known mmu target	no known rno target	mml-miR-663
miR-92b-star	hsa-miR-92b-star	no known mmu target	no known rno target	no known mml target
miR-939	hsa-miR-939	no known mmu target	no known rno target	not expressed
miR-93-star	hsa-miR-93-star	not expressed	no known rno target	no known mml target
miR-152	hsa-miR-152	mmu-miR-152	rno-miR-152	no known mml target
miR-181b	no known hsa target	mmu-miR-181b	rno-miR-181b	mml-miR-181b
miR-193	hsa-miR-193	no known mmu target	no known rno target	no known mml target

miR-1227	hsa-miR-1227	no known mmu target	no known rno target	mml-miR-1227
miR-129-5p	hsa-miR-129-5p	mmu-miR-129-5p	no known rno target	mml-miR-129-5p
miR-139-3p	no known hsa target	mmu-miR-139-3p	rno-miR-139-3p	mml-miR-139-3p
miR-142-3p	no known hsa target	mmu-miR-142-3p	rno-miR-142-3p	mml-miR-142-3p
miR-149	hsa-miR-149	mmu-miR-149	no known rno target	mml-miR-149
miR-151-5p	hsa-miR-151-5p	mmu-miR-151-5p	no known rno target	mml-miR-151-5p
miR-193b	hsa-miR-193b	no known mmu target	no known rno target	mml-miR-193b
miR-432	hsa-miR-432	no known mmu target	no known rno target	mml-miR-432
miR-455-5p	hsa-miR-455-3p	no known mmu target	no known rno target	mml-miR-455-5p
miR-574-5p	hsa-miR-574-5p	mmu-miR-574-5p	no known rno target	no known mml target
miR-720	hsa-miR-720	mmu-miR-720	no known rno target	no known mml target
miR-211	hsa-miR-211	mmu-miR-211	no known rno target	no known mml target
miR-139-5p	hsa-miR-139-5p	no known mmu target	rno-miR-139-5p	no known mml target
miR-296-3p	hsa-miR-296-3p_st	no known mmu target	no known rno target	no known mml target

Table 7: MicroRNAs in luteal tissue day 4 cyclic cattle previously reported in human, mouse, rat and rhesus monkey.

Probe ID	Human	Mouse	Rat	Rhesus Monkey
miR-483	hsa-miR-483	no known mmu target	no known rno target	no known mml target
miR-498	hsa-miR-498	mmu-miR-498	no known rno target	no known mml target
miR-589-star	hsa-miR-589-star	no known mmu target	no known rno target	no known mml target
miR-767-3p	hsa-miR-767-3p	no known mmu target	no known rno target	mml-miR-767-3p
miR-877	hsa-miR-877	mmu-miR-877	rno-miR-877	mml-miR-877
miR-877-star	hsa-miR-877-star	mmu-miR-877-star	rno-miR-877-star	mml-miR-877-star
miR-296-5p	no known hsa target	no known mmu target	no known rno target	mml-miR-296-5p
miR-518b	hsa-miR-518b	no known mmu target	no known rno target	mml-miR-518b
miR-615-3p	no known hsa target	mmu-miR-615-3p	no known rno target	mml-miR-615-3p
miR-657	hsa-miR-657	no known mmu target	no known rno target	mml-miR-657
miR-467	no known hsa target	mmu-miR-467	no known rno target	no known mml target
miR-129-5p	hsa-miR-129-5p	mmu-miR-129-5p	no known rno target	no known mml target
miR-191-star	hsa-miR-191-star	mmu-miR-191-star	no known rno target	no known mml target

Table 8: Targets for bovine specific microRNAs expressed in luteal tissue on day 18 of pregnancy.

MicroRNA	Species	Conservation of Expression Across Species[1]	Predicted Gene Target(s)[2]
bta-miR-10a	Bovine	mml, ppy	BCL2L11, BCL6, HOXD10, INHBB
bta-miR-103	Bovine	fru, gga, ggo, hsa, ppa,ppy, ptr, cfa,dre,lla, mdo,mml, mmu, mne, rno, ssc, tni, xtr, age	ESR1, TGFBR3, NKTR, LTBP2
bta-miR-99a	Bovine	ggo, hsa, lla, mml, mmu, mne, ppa, ppy, ptr, rno, cfa, gga	THAP2, IGFR1, CYP26B1

[1]Conservation of miRNA expression across species. Species names are represented by the 3-letter prefixes that is used to identify miRNA expressed in a particular species (Griffiths-Jones et al., 2006). [2]Predicted gene targets of miRNAs generated from miRanda, miRDB, and TargetScan miRNA prediction databases.

Table 9: Selected targets for orthologous miRNAs expressed in day 18 luteal tissue.

MicroRNA	Species	Conservation of Expression Across Species[1]	Predicted Gene Target(s)[2]
cfa-miR-1306	Canine familiaris	H	LEP, TPM2, AK2, MAPK6, GOLT1B, KCND3,
cfa-miR-200c	Canine familiaris	B, H, G, M, O, Or, R, Rh, Z	GIT2, RAP1B, RAP2C, ACAD8, SOX2, SMAD2, STARD7
cfa-miR-204	Canine familiaris	B, Bo, C, F, G, H, M, Ma, O, Or, P (2), Pi, R, Rh, T, Z	EBF2, IL7R, RPL17, TGFBR2, CDH2, SOCS6, BCL2, DNASE1L1, DDX6, ACOX3, EBF1
dre-miR-10d	Danio rerio	P (2-two types of pufferfish)	n/a
gga-miR-365	Gallus gallus	B, D, F, H, M, O, P, R, Rh, Z	BCL2, BCL11B, IGF1,
hsa-miR-296-3p	Homo Sapien	Rh, M	COL4A4, CX3CL1, CHL1, PRK3

hsa-miR-299-5p	Homo Sapien	Rh	CYP20A1, THAP6, CD83, ESR1, IL1RAP, H3F3B, CCNG1,
hsa-miR-543	Homo sapiens	C, M, R	PGR, SMAD5, ANGPT2, MAP7, PAK7, DDX52, MAPK1, IL18R1, TP63, MSR1, CDK6, TNFSF11, DDX3X, IL1A, CYP26B1
hsa-miR-552	Homo sapiens	Rh	SMAD3, SMAD4, VLDLR, API5, INSR, CYP4A11, CYP7B1, CYP24A1, IGF1R, CYP20A1,
mml-miR-1240	Macaca mulatta	n/a	n/a
rno-miR-346	Rattus norvegicus	H, M, Rh	SMAD3, SMAD4, COL2A1, EBF2, PECAM1, TNFRSF12A,
rno-miR-370	Rattus norvegicus	H, M, Rh	CCL21, IL25, TGFBR2, DDX3Y, DDX3X, CD9, IGFBP4, PDCD11, STARD13, IL13RA2
hsa-miR-379-star_st	Homo sapiens	n/a	EDN1, COL4A2, CYP2U1, OXTR, G0S2, DDX52, INSR, IGF1R
mml-miR-1230	Macaca mulatta	n/a	n/a
mml-miR-939_st	Macaca mulatta	H	RARA, CD4, ELN,INHBB, CDH5, MUC13, MAP4, TNF, CADM3, GDF6, CCL22,
mmu-miR-1198	Mus musculus	n/a	IL7, NOX4, CYP2C55, RAP2C, CYP1B1, CD53, IL16, IGF1R, CYP11B1, IL22RA2,
mmu-miR-1195	Mus musculus	n/a	ICOS, MTSS1, CYP2S1
mmu-miR-700	Mus musculus	n/a	ID1
dre-miR-727-star	Danio rerio	n/a	BCMOL, BIK, CDON
hsa-miR-223	Homo sapiens	Bo, C, Ch, F, G, M, O, Or, P (2), R, Rh, T, Z	INHBB, RASSF6, MSR1, IGF1R, EBF3,
hsa-miR-483-3p	Homo sapiens	n/a	IGF1, SMAD4
mmu-miR-290-3p	Mus musculus	n/a	IRF1, CASP2, CXCR4, CADM1, IL25,

			BCOR, TP63, DDX6

[1]Conservation of the microRNA in Human (H), Bovine (B), Zebrafish (Z), Gorilla (G), Opposum (O), Rhesus Monkey (Rh), Mouse (M), Orangutan (Or), Rat (R), Chicken (C), Tamarind Monkey (T), Pufferfish (P), Chimpanzee (Ch), Pig (Pi), Macaque Monkey (Ma), Bobono Monkey (Bo), Dog (D) and Frog (F). [2]Predicted gene targets of miRNAs generated from miRanda, miRDB, and TargetScan miRNA prediction databases.

Table 10: Selected targets for orthologous miRNAs expressed in day 10 and day 4 luteal tissue.

MicroRNA	Species	Conservation	Predicted Gene Target(s)
cfa-miR-497	Canis familiaris	H, M, R, B, Rh	BCL2L2, VEGFA, GHR, XPO7, TGFBR3
cfa-miR-652	Canis familiaris	M, R, H, Rh	ACVR2B
dre-miR-126-star	Danio rerio	R, F, B, Z, C, H	THAP6
fru-miR-429	Fugu rubripes	Z, C, H, Rh, M, R, P (2), D	THAP1, VEGFA, XIAP
hsa-miR-106b-star	Homo sapiens	M, R	TNFRSF21, BCL11B, CD69, IL8, IGF2BP1, CXCL14, IL25
hsa-miR-1228	Homo sapiens	n/a	BCL2
hsa-miR-193b-star	Homo sapiens	Rh	IL10RB
hsa-miR-199a-3p	Homo sapiens	W, Rh, M, O, Ch, Ma, R, F, B, G	API5, IGF1
hsa-miR-21-star	Homo sapiens	M, R, B	IL12A, PDCD4, TIMP3, SOCS6
hsa-miR-28-5p	Homo sapiens	n/a	MTCP1, MTSS1
hsa-miR-296-3p	Homo sapiens	n/a	CX3CL1, CHL1, PRK3

hsa-miR-328	Homo sapiens	M, R, D	EBF3, IGF1R
hsa-miR-331-3p	Homo sapiens	Rh, M	TNF, TGFBR1
hsa-miR-342-5p	Homo sapiens	M, R, Rh	IGFBP5
hsa-miR-362-5p	Homo sapiens	Rh, M	IL17RD, LRP6
hsa-miR-378-star	Homo sapiens	M, R	IGF1R, XPO5
hsa-miR-422a	Homo sapiens	Rh	IGF1R, XPO5
hsa-miR-423-5p	Homo sapiens	M, Rh	CASP2, CYP4F22
hsa-miR-425-star	Homo sapiens	M	IGF1, PDCD10, TIMP2
hsa-miR-500-star	Homo sapiens	n/a	XPO1, RAI14
hsa-miR-502-3p	Homo sapiens	Rh	XPO1, RAI14
hsa-miR-503	Homo sapiens	Rh	GADD45G, PERP
hsa-miR-505-star	Homo sapiens	n/a	CCR6, IL11, IFNG, PGR, FAS
hsa-miR-532-3p	Homo sapiens	Rh, M, R	GDF6, IL6R, IL17RD, IL18R1, IGFBP4, FGF5
hsa-miR-542-5p	Homo sapiens	Rh, M, R	IL28RA
hsa-miR-635	Homo sapiens	n/a	IL61, CCL20, CASP10, IGFBP4
hsa-miR-642	Homo sapiens	Rh	RASSF6, IGF2BP1, IL2RA,
hsa-miR-652	Homo sapiens	n/a	ATP9A, PTGRF
hsa-miR-663	Homo sapiens	Rh	LSP1, FGFRL1, MUC21
hsa-miR-92b-star	Homo sapiens	n/a	BTLA, MOAP1, GHR, SOCS5
hsa-miR-939	Homo sapiens	Rh	RARA, CD4, ELN,INHBB, CDH5, MUC13, MAP4, TNF, CADM3, GDF6, CCL22,

hsa-miR-93-star	Homo sapiens	M	IRAK4, LDLR, VLDLR, BCL11B
mdo-miR-152	Monodelphis domestica	O, M, R, P, D Z, H,	ING2, DDX58
mdo-miR-181b	Monodelphis domestica	Rh, M, Ma, Ch, T, R, Pi, P, F	TNPO1, TNFSF4, MUC7
mdo-miR-193	Monodelphis domestica	F, D, Z, P, C, H	CXCL2, CADM1, ING1, GAS1
mdo-miR-221	Monodelphis domestica	H, Rh, M, R, P (2), F, B, D, Z, C, G	IRF2, GDF9, CYP7A1
mml-miR-1227	Macaca mulatta	H	IRF2, CRX
mml-miR-125a-3p	Macaca mulatta	M, R, P, F, B, D	CYP20A1, TNFRSF19, SMAD4
mml-miR-129-3p	Macaca mulatta	H, M	MEMO1, SMAD3, KL
mml-miR-139-3p	Macaca mulatta	M, R	RELT, GCDH, AP1S3
mml-miR-142-3p	Macaca mulatta	M, R, F, C	TGFBR1, FGF23, TBRG1
mml-miR-149	Macaca mulatta	H,M	THAP6, FGFBP1, TCL6, CASP2
mml-miR-151-3p	Macaca mulatta	H, M	IL26, SOCS5
mml-miR-151-5p	Macaca mulatta	H, M	GRM3, BTLA, SMARCAD1
mml-miR-152	Macaca mulatta	O, M, R, P, D Z, H,	TGFA, LDLR
mml-miR-193b	Macaca mulatta	H	IL10RB
mml-miR-199a-5p	Macaca mulatta	W, Rh, M, O, Ch, Ma, R, F, B, G	IGFBP1, DDX3X
mml-miR-210	Macaca mulatta	H, M, R, P, F, B	FGFRL1, AIFM3, IGF2
mml-miR-362-5p	Macaca mulatta	M,H	IL17RD, PDCD2, XOP7
mml-miR-432	Macaca mulatta	H	COL4A5, P53AIP1

mml-miR-455-3p	Macaca mulatta	H	IGSF11, SMAD2, NKTR
mmu-miR-532-5p	Mus musculus	n/a	IL6T, FRS2, IL1A, TNFRSF1B
mmu-miR-574-5p	Mus musculus	H	CD96, RFX4
mmu-miR-720	Mus musculus	H	TUSC1, CYP4F11
mne-miR-181a-star	Macaca nemestrina	Ch	TNPO1, DDX3X, IPO8, MUC7
mne-miR-211	Macaca nemestrina	C, H, M, Ma	COX5A, SOX11,
rno-miR-139-5p	Rattus norvegicus	H	EBF1, TNPO1, TGIF1
dre-miR-735	Danio rerio	Z	n/a
hsa-miR-191-star	Homo sapiens	M	DDHD1, TAF5
hsa-miR-454-star	Homo sapiens	Z	IGF1, PLAA, KCTD16
hsa-miR-483-3p	Homo sapiens	H	SMC2, RASSF6, BCAP29
hsa-miR-498	Homo sapiens	M	CD93, CCPG1, SOCS3, GPR171
hsa-miR-589-star	Homo sapiens	n/a	FAS, FGFBP3, BAG1
hsa-miR-767-3p	Homo sapiens	Rh	IPO9, ALCAM
hsa-miR-877	Homo sapiens	Rh, M, R	CD302, CDC40, ING3
hsa-miR-877-star	Homo sapiens	Rh, M, R	CD302, CDC40, ING3, CDH19
mml-miR-296-5p	Macaca mulatta	n/a	BMF, TNFSF15, BOK
mml-miR-518b	Macaca mulatta	H	DGCR8, CD5
mml-miR-615-3p	Macaca mulatta	M	LCOR, FADS1
mml-miR-657	Macaca mulatta	H	ABCD3, FCRL2

mmu-miR-467a	Mus musculus	n/a	LCOR, IPO7

Table 11: Selected predicted targets for microRNAs present in bovine luteal tissue.

MicroRNAs Present in day 18 Luteal Tissue

		Predicted Databases		
MicroRNAs	**Predicted Targets**	**miRanda**	**miRDB**	**TargetScan**
cfa-miR-1306	LEP, TPM2, AK2, MAPK6, GOLT1B, KCND3,	n/a	LEP, TPM2, AK2, MAPK6, GOLT1B, KCND3	LEP, TPM2, AK2, MAPK6, GOLT1B, KCND3
cfa-miR-200c	GIT2, RAP1B, RAP2C, ACAD8, SOX2, SMAD2, STARD7	n/a	RAP1B, RAP2C, ACAD8, SOX2, SMAD2, STARD7	GIT2, RAP1B, SOX2, SMAD2, STARD7
cfa-miR-204	EBF2, IL7R, RPL17, TGFBR2, CDH2, SOCS6, BCL2, DNASE1L1, DDX6, ACOX3, EBF1	n/a	EBF2, IL7R, RPL17, TGFBR2, CDH2, SOCS6, BCL2, DNASE1L1, DDX6, ACOX3, EBF1	EBF2, RPL17, TGFBR2, CDH2, SOCS6, BCL2, DDX6, ACOX3, EBF1
dre-miR-10d	n/a	n/a	n/a	n/a
gga-miR-365	BCL2, BCL11B, IGF1,	n/a	BCL2, BCL11B, IGF1,	BCL2, BCL11B, IGF1,
hsa-miR-296-3p	COL4A4, CX3CL1, CHL1, PRK3	COL4A4, CX3CL1, CHL1, PRK3	COL4A4, CX3CL1, CHL1, PRK3	COL4A4, CX3CL1, CHL1

hsa-miR-299-5p	CYP20A1, THAP6, CD83, ESR1, IL1RAP, H3F3B, CCNG1,	CYP20A1, THAP6, CD83, ESR1, IL1RAP, H3F3B, CCNG1,	CYP20A1, THAP6, CD83, ESR1, IL1RAP, H3F3B, CCNG1,	n/a
hsa-miR-543	PGR, SMAD5, ANGPT2, MAP7, PAK7, DDX52, MAPK1, IL18R1, TP63, MSR1, CDK6, TNFSF11, DDX3X, IL1A, CYP26B1	PGR, SMAD5, ANGPT2, MAP7, PAK7, DDX52, MAPK1, IL18R1, TP63, MSR1, CDK6, TNFSF11, DDX3X, IL1A, CYP26B1	PGR, SMAD5, ANGPT2, MAP7, PAK7, DDX52, MAPK1, IL18R1, TP63, MSR1, CDK6, TNFSF11, DDX3X, IL1A, CYP26B1	PGR, SMAD5, MAP7, DDX52, MAPK1, IL18R1, TP63, MSR1, CDK6, TNFSF11, DDX3X, IL1A, CYP26B1
hsa-miR-552	SMAD3, SMAD4, VLDLR, API5, INSR, CYP4A11, CYP7B1, CYP24A1, IGF1R, CYP20A1,	SMAD3, SMAD4, VLDLR, API5, INSR, CYP4A11, CYP7B1, CYP24A1, IGF1R, CYP20A1,	SMAD3, SMAD4, VLDLR, API5, INSR, CYP4A11, CYP7B1, CYP24A1, IGF1R,	SMAD3, SMAD4, VLDLR, API5, INSR, CYP4A11, CYP24A1, IGF1R, CYP20A1,
mml-miR-1240	n/a	n/a	n/a	n/a
rno-miR-346	SMAD3, SMAD4, COL2A1, EBF2, PECAM1, TNFRSF12A,	SMAD3, SMAD4, COL2A1, EBF2, PECAM1, TNFRSF12A,	SMAD3, SMAD4, COL2A1, EBF2, PECAM1	SMAD3, SMAD4, EBF2, PECAM1
rno-miR-370	CCL21, IL25, TGFBR2, DDX3Y, DDX3X, CD9, IGFBP4, PDCD11, STARD13, IL13RA2	CCL21, IL25, TGFBR2, DDX3Y, DDX3X, CD9, IGFBP4, PDCD11, STARD13, IL13RA2	CCL21, IL25, TGFBR2, DDX3Y, DDX3X, CD9, IGFBP4, PDCD11, IL13RA2	CCL21, IL25, TGFBR2, DDX3Y, DDX3X, CD9, IGFBP4
hsa-miR-379-star_st	EDN1, COL4A2, CYP2U1, OXTR, G0S2, DDX52, INSR, IGF1R	EDN1, COL4A2, CYP2U1, OXTR, G0S2, DDX52, INSR, IGF1R	COL4A2, CYP2U1, OXTR, G0S2, DDX52, INSR, IGF1R	COL4A2, CYP2U1, OXTR, G0S2, DDX52, INSR, IGF1R
mml-miR-1230	n/a	n/a	n/a	n/a
mml-miR-939_st	RARA, CD4, ELN,INHBB, CDH5, MUC13, MAP4, TNF, CADM3, GDF6, CCL22,	n/a	n/a	RARA, CD4, ELN,INHBB, CDH5, MUC13, MAP4, TNF, CADM3, GDF6, CCL22,

mmu-miR-1198	IL7, NOX4, CYP2C55, RAP2C, CYP1B1, CD53, IL16, IGF1R, CYP11B1, IL22RA2,	IL7, NOX4, CYP2C55, RAP2C, CYP1B1, CD53, IL16, IGF1R, CYP11B1, IL22RA2,	IL7, NOX4, CYP2C55, RAP2C, CYP1B1, CD53, IL16, IGF1R, CYP11B1, IL22RA2,	IL7, CYP2C55, RAP2C, CYP1B1, CD53, IL16, IGF1R, CYP11B1,
mmu-miR-1195	ICOS, MTSS1, CYP2S1	ICOS, MTSS1, CYP2S1	ICOS, MTSS1, CYP2S1	n/a
mmu-miR-700	ID1	ID1	n/a	n/a
dre-miR-727-star	BCMOL, BIK, CDON	n/a	n/a	BCMOL, BIK, CDON
hsa-miR-223	INHBB, RASSF6, MSR1, IGF1R, EBF3,	INHBB, RASSF6, MSR1, IGF1R, EBF3,	INHBB, RASSF6, IGF1R	IGF1R
hsa-miR-483-3p	IGF1, SMAD4	IGF1, SMAD4	IGF1, SMAD4	IGF1
mmu-miR-290-3p	IRF1, CASP2, CXCR4, CADM1, IL25, BCOR, TP63, DDX6	IRF1, CASP2, CXCR4, CADM1, IL25, BCOR, TP63, DDX6	IRF1, CASP2, CXCR4, CADM1, IL25, BCOR, TP63, DDX6	IRF1, CASP2, CXCR4, CADM1, IL25, BCOR, TP63, DDX6
bta-miR-10a	BCL2L11, BCL6, HOXD10, INHBB	n/a	n/a	BCL2L11, BCL6, HOXD10, INHBB
bta-miR-103	ESR1, TGFBR3, NKTR, LTBP2	n/a	n/a	ESR1, TGFBR3, NKTR, LTBP2

bta-miR-99a	THAP2, IGFR1, CYP26B1	n/a	n/a	THAP2, IGFR1, CYP26B1

MicroRNAs Present in day 10 and 4 Luteal Tissue

		Predicted Databases		
MicroRNAs	**Predicted Targets**	**miRanda**	**miRDB**	**TargetScan**
cfa-miR-497	BCL2L2, VEGFA, GHR, XPO7, TGFBR3	n/a	BCL2L2, VEGFA, GHR, XPO7, TGFBR3	BCL2L2, VEGFA, GHR, XPO7, TGFBR3
cfa-miR-652	ACVR2B	n/a	ACVR2B	ACVR2B
dre-miR-126-star	THAP6	n/a	n/a	THAP6
fru-miR-429	THAP1, VEGFA, XIAP	n/a	n/a	THAP1, VEGFA, XIAP
hsa-miR-106b-star	TNFRSF21, BCL11B, CD69, IL8, IGF2BP1, CXCL14, IL25	TNFRSF21, BCL11B, CD69, IL8, IGF2BP1, CXCL14, IL25	TNFRSF21, BCL11B, CD69, IL8, IGF2BP1, CXCL14, IL25	CD69, IL8, IGF2BP1, CXCL14
hsa-miR-1228	BCL2	BCL2	BCL2	BCL2
hsa-miR-193b-star	IL10RB	IL10RB	IL10RB	IL10RB
hsa-miR-199a-3p	API5, IGF1	API5, IGF1	API5, IGF1	API5, IGF1

hsa-miR-21-star	IL12A, PDCD4, TIMP3, SOCS6	IL12A, PDCD4, TIMP3, SOCS6	IL12A, PDCD4, TIMP3, SOCS6	IL12A, TIMP3, SOCS6
hsa-miR-28-5p	MTCP1, MTSS1	MTCP1, MTSS1	MTCP1, MTSS1	n/a
hsa-miR-296-3p	CX3CL1, CHL1, PRK3	CX3CL1, CHL1, PRK3	CX3CL1, CHL1, PRK3	CHL1
hsa-miR-328	EBF3, IGF1R	EBF3, IGF1R	EBF3, IGF1R	EBF3, IGF1R
hsa-miR-331-3p	TNF, TGFBR1	TNF, TGFBR1	TNF, TGFBR1	n/a
hsa-miR-342-5p	IGFBP5	IGFBP5	n/a	n/a
hsa-miR-362-5p	IL17RD, LRP6	IL17RD, LRP6	n/a	n/a
hsa-miR-378-star	IGF1R, XPO5	IGF1R, XPO5	IGF1R, XPO5	IGF1R, XPO5
hsa-miR-422a	IGF1R, XPO5	IGF1R, XPO5	IGF1R, XPO5	IGF1R, XPO5
hsa-miR-423-5p	CASP2, CYP4F22	CASP2, CYP4F22	CASP2, CYP4F22	CASP2, CYP4F22
hsa-miR-425-star	IGF1, PDCD10, TIMP2	IGF1, PDCD10, TIMP2	IGF1, PDCD10, TIMP2	IGF1, PDCD10, TIMP2

hsa-miR-500-star	XPO1, RAI14	XPO1, RAI14	XPO1, RAI14	XPO1
hsa-miR-502-3p	XPO1, RAI14	XPO1, RAI14	XPO1, RAI14	XPO1
hsa-miR-503	GADD45G, PERP	GADD45G, PERP	GADD45G, PERP	n/a
hsa-miR-505-star	CCR6, IL11, IFNG, PGR, FAS	CCR6, IL11, IFNG, PGR, FAS	CCR6, IL11, IFNG, PGR, FAS	CCR6, IL11, IFNG, PGR, FAS
hsa-miR-532-3p	GDF6, IL6R, IL17RD, IL18R1, IGFBP4, FGF5	GDF6, IL6R, IL17RD, IL18R1, IGFBP4, FGF5	GDF6, IL6R, IL17RD, IL18R1, IGFBP4, FGF5	GDF6, IL6R, IL17RD, IL18R1, IGFBP4, FGF5
hsa-miR-542-5p	IL28RA	IL28RA	n/a	n/a
hsa-miR-635	IL61, CCL20, CASP10, IGFBP4	IL61, CCL20, CASP10, IGFBP4	IL61, CASP10, IGFBP4	IL61,CASP10, IGFBP4
hsa-miR-642	RASSF6, IGF2BP1, IL2RA,	RASSF6, IGF2BP1, IL2RA,	RASSF6, IGF2BP1, IL2RA,	RASSF6, IGF2BP1, IL2RA,
hsa-miR-652	ATP9A, PTGRF	ATP9A, PTGRF	ATP9A, PTGRF	ATP9A, PTGRF
hsa-miR-663	LSP1, FGFRL1, MUC21	LSP1, FGFRL1, MUC21	LSP1, FGFRL1, MUC21	LSP1, MUC21
hsa-miR-92b-star	BTLA, MOAP1, GHR, SOCS5	BTLA, GHR, SOCS5	BTLA, MOAP1, GHR, SOCS5	BTLA, MOAP1, GHR, SOCS5

hsa-miR-939	RARA, CD4, ELN,INHBB, CDH5, MUC13, MAP4, TNF, CADM3, GDF6, CCL22,	RARA, CD4, ELN,INHBB, CDH5, MUC13, MAP4, TNF, CADM3, GDF6, CCL22,	RARA, CD4, ELN,INHBB, CDH5, MUC13, MAP4, TNF, CADM3, GDF6, CCL22,	RARA, CD4, ELN, INHBB, CDH5, MUC13, MAP4, TNF, CADM3, GDF6
hsa-miR-93-star	IRAK4, LDLR, VLDLR, BCL11B	IRAK4, LDLR, VLDLR, BCL11B	IRAK4, LDLR, VLDLR, BCL11B	IRAK4, LDLR, VLDLR, BCL11B
mdo-miR-152	ING2, DDX58	n/a	n/a	ING2, DDX58
mdo-miR-181b	TNPO1, TNFSF4, MUC7	n/a	n/a	TNPO1, TNFSF4, MUC7
mdo-miR-193	CXCL2, CADM1, ING1, GAS1	n/a	n/a	CXCL2, CADM1, ING1, GAS1
mdo-miR-221	IRF2, GDF9, CYP7A1	n/a	n/a	IRF2, GDF9, CYP7A1
mml-miR-1227	IRF2, CRX	n/a	n/a	IRF2, CRX
mml-miR-125a-3p	CYP20A1, TNFRSF19, SMAD4	n/a	n/a	CYP20A1, TNFRSF19, SMAD4
mml-miR-129-3p	MEMO1, SMAD3, KL	n/a	n/a	MEMO1, SMAD3, KL
mml-miR-139-3p	RELT, GCDH, AP1S3	n/a	n/a	RELT, GCDH, AP1S3
mml-miR-142-3p	TGFBR1, FGF23, TBRG1	n/a	n/a	TGFBR1, FGF23, TBRG1

mml-miR-149	THAP6, FGFBP1, TCL6, CASP2	n/a	n/a	THAP6, FGFBP1, TCL6, CASP2
mml-miR-151-3p	IL26, SOCS5	n/a	n/a	IL26, SOCS5
mml-miR-151-5p	GRM3, BTLA, SMARCAD1	n/a	n/a	GRM3, BTLA, SMARCAD1
mml-miR-152	TGFA, LDLR	n/a	n/a	TGFA, LDLR
mml-miR-193b	IL10RB	n/a	n/a	IL10RB
mml-miR-199a-5p	IGFBP1, DDX3X	n/a	n/a	IGFBP1, DDX3X
mml-miR-210	FGFRL1, AIFM3, IGF2	n/a	n/a	FGFRL1, AIFM3, IGF2
mml-miR-362-5p	IL17RD, PDCD2, XOP7	n/a	n/a	IL17RD, PDCD2, XOP7
mml-miR-432	COL4A5, P53AIP1	n/a	n/a	COL4A5, P53AIP1
mml-miR-455-3p	IGSF11, SMAD2, NKTR	n/a	n/a	IGSF11, SMAD2, NKTR
mmu-miR-532-5p	IL6T, FRS2, IL1A, TNFRSF1B	IL6T, FRS2, IL1A, TNFRSF1B	IL6T, FRS2, IL1A, TNFRSF1B	IL6T, FRS2, IL1A, TNFRSF1B

mmu-miR-574-5p	CD96, RFX4	CD96, RFX4	CD96, RFX4	CD96, RFX4
mmu-miR-720	TUSC1, CYP4F11	TUSC1, CYP4F11	TUSC1, CYP4F11	TUSC1, CYP4F11
mne-miR-181a-star	TNPO1, DDX3X, IPO8, MUC7	n/a	n/a	TNPO1, DDX3X, IPO8, MUC7
mne-miR-211	COX5A, SOX11,	n/a	n/a	COX5A, SOX11,
rno-miR-139-5p	EBF1, TNPO1, TGIF1	EBF1, TNPO1, TGIF1	EBF1, TNPO1, TGIF1	EBF1, TNPO1, TGIF1
dre-miR-735	n/a	n/a	n/a	n/a
hsa-miR-191-star	DDHD1, TAF5	DDHD1, TAF5	TAF5	DDHD1, TAF5
hsa-miR-454-star	IGF1, PLAA, KCTD16	IGF1, PLAA, KCTD16	IGF1, PLAA	IGF1, PLAA, KCTD16
hsa-miR-483-3p	SMC2, RASSF6, BCAP29	RASSF6, BCAP29	SMC2, RASSF6, BCAP29	RASSF6, BCAP29
hsa-miR-498	CD93, CCPG1, SOCS3, GPR171	CD93, CCPG1, SOCS3, GPR171	CD93, CCPG1, SOCS3, GPR171	CD93, CCPG1, SOCS3
hsa-miR-589-star	FAS, FGFBP3, BAG1	FAS, FGFBP3, BAG1	FAS, FGFBP3	FAS

hsa-miR-767-3p	IPO9, ALCAM	IPO9, ALCAM	IPO9, ALCAM	IPO9, ALCAM
hsa-miR-877	CD302, CDC40, ING3	CD302, CDC40, ING3	CD302, CDC40, ING3	CD302, CDC40
hsa-miR-877-star	CD302, CDC40, ING3, CDH19	CD302, CDC40, ING3, CDH19	CD302, CDC40, ING3, CDH19	CD302, CDC40, CDH19
mml-miR-296-5p	BMF, TNFSF15, BOK	n/a	n/a	BMF, TNFSF15, BOK
mml-miR-518b	DGCR8, CD5	n/a	n/a	DGCR8, CD5
mml-miR-615-3p	LCOR, FADS1	n/a	n/a	LCOR, FADS1
mml-miR-657	ABCD3, FCRL2	n/a	n/a	ABCD3, FCRL2
mmu-miR-467a	LCOR, IPO7	LCOR, IPO7	LCOR, IPO7	LCOR, IPO7
mml-miR-467b	FADS2, IPO4	n/a	n/a	FADS2, IPO4
mne-miR-572	API2, IPO1	n/a	n/a	API2, IPO1

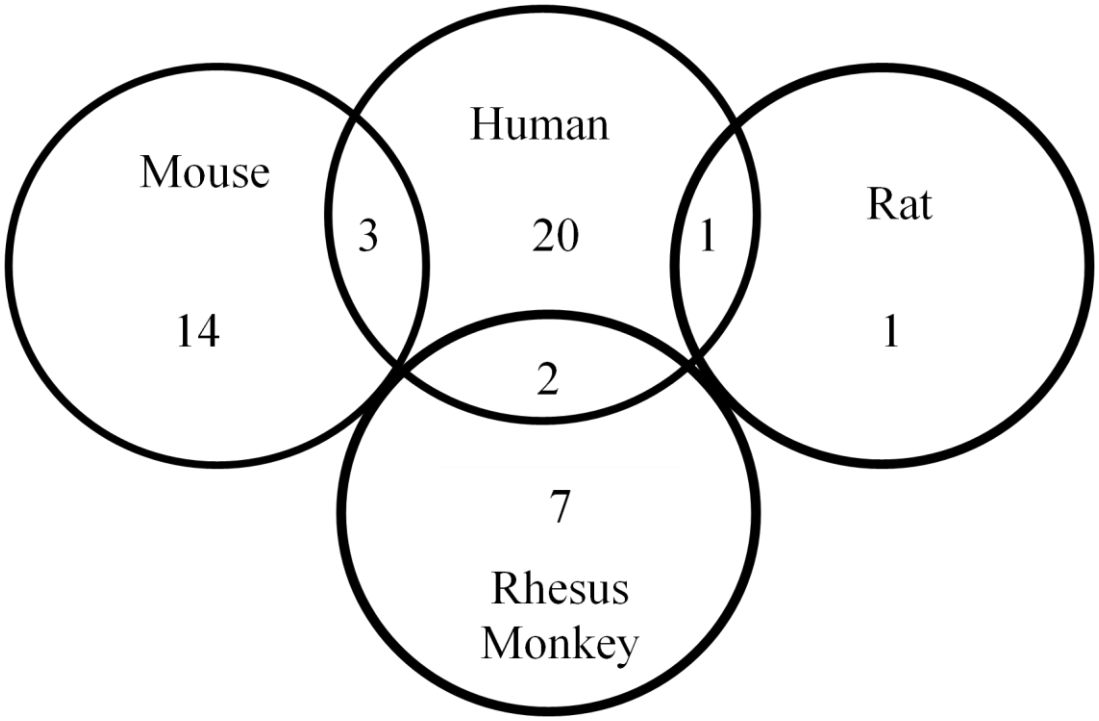
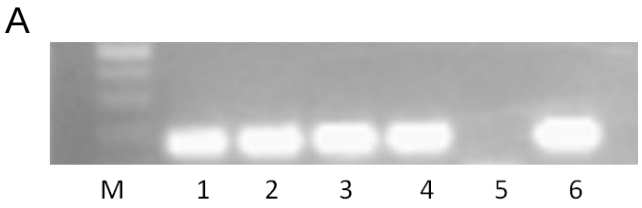
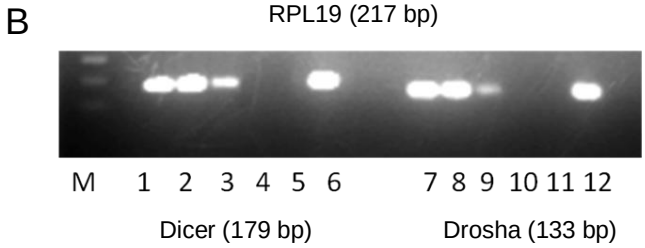


Figure 4: Distribution of species-specific microRNA capture probes resulting in the detection of luteal microRNAs that have not been reported in the cow.

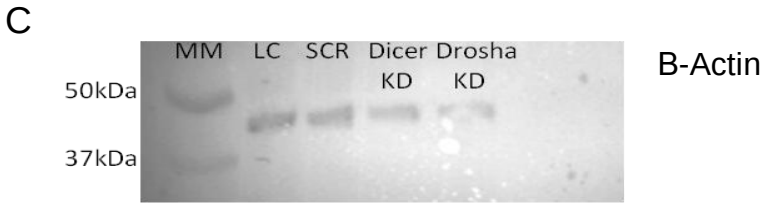




RLP19	
1	Luteal Cells
2	Scrambled (SCR)
3	Dicer KD (100nM)
4	Drosha KD (100nM)
5	No RT
6	Endothelial Cells

Dicer	
1	Luteal Cells
2	Scrambled (SCR)
3	Dicer KD (100nM)
4	No RT
5	H2O Only
6	Endothelial Cells

Drosha	
7	Luteal Cells
8	Scrambled (SCR)
9	Drosha KD (100nM)
10	No RT
11	H2O Only
12	Endothelial Cells



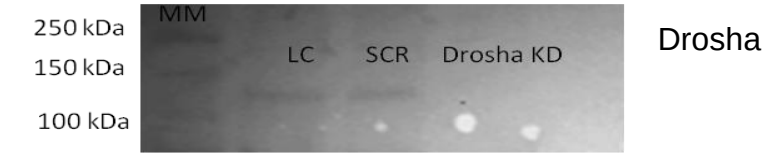


Figure 5: Dicer and Drosha expression and siRNA mediated Dicer and Drosha knockdown in luteal cells. A-B) Representative images of PCR products for Dicer and Drosha gene expression in nontransfected and

transfected luteal cells. A) Expression of endogenous control RPL19 transcript (217 bp) in 1- nontransfected luteal cells, 2 - luteal cells transfected with non-specific scrambled sequence, 3 - luteal cells transfected with Dicer siRNA (100nM) or 4- Drosha siRNA (100nM). 5 - No RT negative controls .6- Endothelial cell positive control. B) Expression of Dicer (179 bp) and Drosha (133 bp) transcripts in 1and 7- nontransfected luteal cells, 2 and 8- luteal transfected with non-specific scrambled sequences, luteal cells transfected with 3- Dicer siRNA (100nM) and 9- Drosha siRNA (100nM). 4 and 10- No RT and 5 and 11- H_2O only negative controls. 6 and 12- Endothelial cell positive control. C-E) Representative images of western blot analysis for (C) β- actin (43kDa), (D) Drosha (179kDa) and (E) Dicer (217kDa).

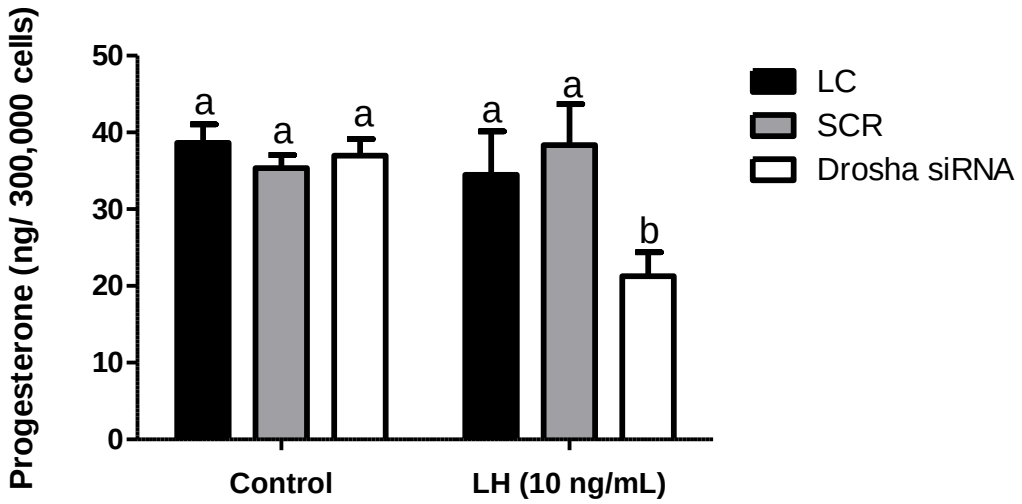
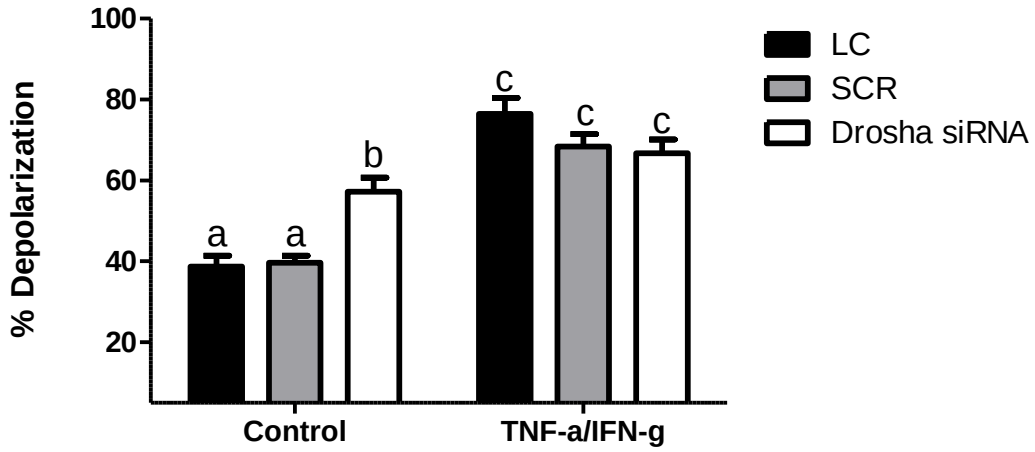
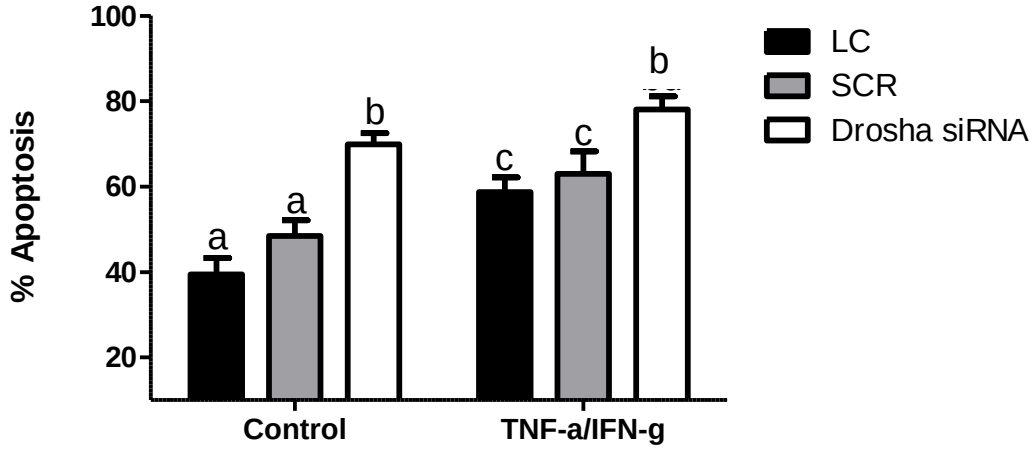


Figure 6: Progesterone concentration by Drosha-depleted knockdown luteal cells. Concentration of progesterone produced by plated nontransfected luteal cells (LC) and LC transfected with scrambled siRNA (SCR; 75nM) and Drosha siRNA (100nM). Bars indicate mean ± SEM; n= 5 separate CL (animals). ^{a,b} indicate differences; $P < 0.05$.

A


% Depolarization
100
80
60
40
20
a
a
b
c
c
c
Control
TNF-a/IFN-g
LC
SCR
Drosha siRNA

B


% Apoptosis
100
80
60
40
20
a
a
b
c
c
b
Control
TNF-a/IFN-g
LC
SCR
Drosha siRNA

C

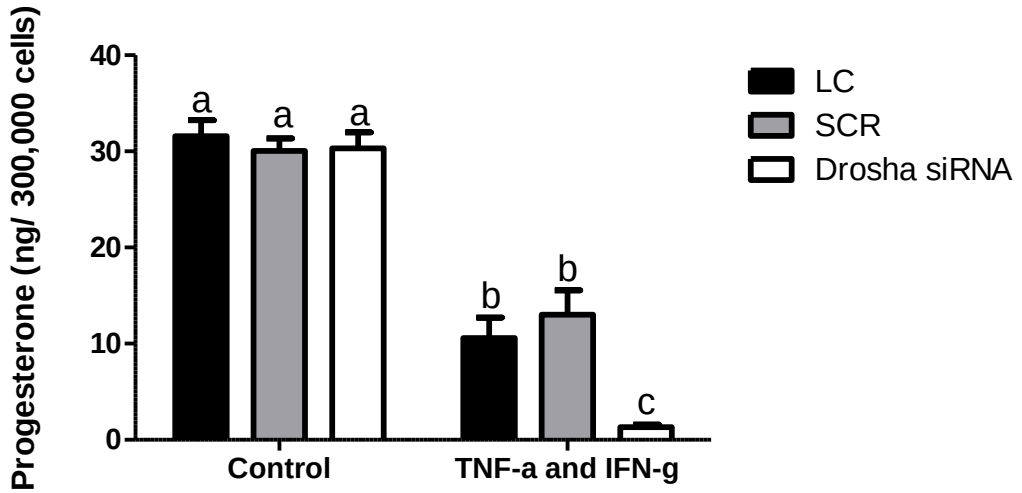

Progesterone (ng/ 300,000 cells)
40
30
20
10
0
a
a
a
b
b
c
Control
TNF-a and IFN-g
LC
SCR
Drosha siRNA

Figure 7: Effect of Drosha depletion on cytokine-stimulated apoptosis and progesterone production A) Depolarization of mitochondria in nontransfected luteal cells (LC), luteal cells transfected with non-specific scrambled sequence (SCR), luteal cells transfected with Drosha siRNA (100nM). B) Apoptosis of nontransfected luteal cells (LC), luteal transfected with non-specific scrambled sequence (SCR), luteal cells transfected with Drosha siRNA (100nM). C) Progesterone concentration in Drosha- depleted LC treated with or without TNF-α and IFN-γ. Bars indicate mean ± SEM; n = 4 CL. ^{a,b} indicate differences; $P < 0.05$.

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