AN ABSTRACT OF THE THESIS OF

Kristi M. DuBay for the degree of Master of Science in Animal Sciences presented on December 3, 2010.

Title: Differential Gene Expression in the Pregnant Bovine Corpus Luteum During Maternal Recognition of Pregnancy.

Abstract approved:

Alfred R. Menino, Jr.

Interferon tau (IFN-τ) is the pregnancy recognition signal secreted by the trophectodermal cells of the developing bovine embryo that prevents luteolysis and maintains critical progesterone production. Recent evidence has suggested in addition to the mechanism of action identified in the uterus, there may be a direct effect of IFN-τ on the corpus luteum (CL). The objectives of this study were to generate a gene expression profile for the CL during maternal recognition of pregnancy, identify all genes where expression is modulated, and characterize the direction and magnitude of gene expression. To accomplish this two CL were collected from each of five cows on Day 14 of the estrous cycle and pregnancy (Day 0 = onset of estrus). Affymetrix Bovine GeneChip® microarrays were used to identify genes significantly up- or down-regulated in pregnant compared to non-pregnant cows. Microarray analysis detected 29 up-regulated and 6 down-regulated genes with a ≥1.5-fold change ($P < 0.05$). From the differentially

expressed genes, four were selected for validation with real-time RT-PCR. An additional 17 genes related to prostaglandin synthesis, growth hormone, IGF-1, interferon-tau-related genes, and hormone receptors were chosen for investigation with real-time RT-PCR. Analysis of the PCR results identified four genes, three involved in prostaglandin synthesis and the gene encoding the LH receptor, whose expression was ($P < 0.05$) down-regulated in the pregnant CL during maternal recognition of pregnancy. These results suggest that the presence of the embryo on Day 14 of pregnancy cause the CL to become less competent in intraluteal prostaglandin synthesis, thereby contributing to the extension of luteal lifespan.

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Differential Gene Expression in the Pregnant Bovine Corpus Luteum During Maternal Recognition of Pregnancy

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

Presented December 3, 2010
Commencement June 2011

Master of Science thesis of Kristi M. DuBay presented on December 3, 2010.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Kristi M. DuBay, Author

ACKNOWLEDGEMENTS

I would like to acknowledge the contributions of a number of people to the completion of this thesis. First, I would like to thank to my fellow graduate students Adrienne, Leah, and Katherine for their assistance during tissue collection. Next, I would like to acknowledge Dr. Gerd Bobe for his help with the microarray and PCR data analysis. I would also like to thank and acknowledge Dr. Matt Cannon for helping me develop the project and for his mentoring. To Dr. Fred Menino, many thanks for stepping in to be my major professor and helping me to complete my thesis. I would not have been able to finish if it wasn't for all the help you have given. Last, I would like to give a special thanks to my husband, Joseph, and my parents for their unfailing support and encouragement during my graduate school experience.

TABLE OF CONTENTS

Page

1 Literature Review 1

1.1 Corpus Luteum Formation and Function 1

1.2 Corpus Luteum Regression 16

1.3 Maternal Recognition of Pregnancy 22

2 Introduction 30

3 Materials and Methods 33

3.1 Collection of Corpora Lutea 33

3.2 Embryo Recovery 34

3.3 RNA isolation and cDNA synthesis 34

3.4 Microarrays 35

3.5 Real-Time RT-PCR of luteal RNA 36

3.6 Real-Time RT-PCR of embryonic RNA 37

3.7 Progesterone ELISA 37

3.8 Statistics 38

4 Results 39

4.1 Plasma progesterone 39

4.2 Real-time RT-PCR verification of embryonic expression of IFN-τ 40

4.3 Differentially expressed genes identified by microarray 41

TABLE OF CONTENTS (Continued)

Page

4.4 Real-time RT-PCR analysis of selected genes 44

5 Discussion 47

6 General Conclusions 51

Bibliography 53

LIST OF FIGURES

Figure Page

1.2.1 Model of luteolysis cascade 21

4.1.1 Plasma progesterone concentrations in pregnant and non-pregnant cows on Day 14. 39

4.2.1 Day 14 embryo recovered from pregnant cow (13.5mm x 0.8mm) 40

4.2.2 INF-τ mRNA in Day 14 embryos collected from pregnant cows. Lane assignments: 1- 1 Kb ladder, 2- embryo 1176, 3- embryo 1277, 4- embryo 3018, 5- embryo 232, 6- embryo 267, 7- Water 40

LIST OF TABLES

Table Page

4.3.1 Genes up-regulated ≥1.5-fold in the pregnant cow corpus luteum.......... 42

4.3.2 Genes down-regulated ≥1.5-fold in the pregnant cow corpus luteum.......... 44

4.4.1 Fold changes in expression for genes selected for analysis by real-time RT-PCR.......... 45

Differential Gene Expression in the Pregnant Bovine Corpus Luteum During Maternal Recognition of Pregnancy

Chapter 1 Literature Review

1.1 Corpus Luteum Formation and Function

The corpus luteum (CL) is an endocrine gland that develops from the ruptured follicle on the mammalian ovary following ovulation. Proper formation of this gland is critical for the successful establishment and maintenance of early pregnancy in mammals. The main function of the CL is to synthesize and secrete the steroid hormone progesterone, which provides support to the developing embryo and prepares the uterus for pregnancy establishment (Mann et al., 1999; Mann and Lamming, 2001; Green et al., 2005). The CL is comprised of transformed theca and granulosa cells that have undergone "luteinization." Luteinization involves two major simultaneous processes; extensive tissue remodeling resulting in CL formation and acquisition of luteal function.

The first steps in the tissue remodeling process are regulation of cell proliferation and hypertrophy. Theca cells from the ovulated follicle luteinize and become rapidly proliferating small luteal cells (Farin et al., 1986). Rapid cell proliferation during CL formation also occurs in other non-steroidogenic cell populations such as endothelial cells during formation of the vascular network of

the CL and other small cells like fibroblasts and parenchymal cells (Alila and Hansel, 1984; Zheng et al., 1994). Conversely, granulosa cells luteinize into large luteal cells that cease proliferating and undergo massive hypertrophy (Alila and Hansel, 1984; Schams and Berisha, 2004). This occurs through the cessation of progression through the cell cycle. Although this process is not well-defined, there is some information regarding cyclins, interactions with cyclin-dependent kinases (CDK), and inhibition of cyclin-CDK complex activity by CDK inhibitors (CKI) (Johnson and Walker, 1999). Dephosphorylation of proteins during luteinization can suppress cyclin D and E activity, thereby suppressing cell passage through “G1” and “G1 to S transition” phases in the cell cycle (Robker and Richards, 1996; Johnson and Walker, 1999). Results in several species have shown that an ovulatory bolus of human chorionic gonadotropin (hCG) induces a transient increase in CKIp27, which resulted in a marked decline in mitotic activity (Robker and Richards, 1996; Chaffin et al., 2001). All of these regulators are likely contributors to the cessation of proliferation characteristic of luteinizing granulosa cells.

Hypertrophy of luteinizing granulosa cells is the major cause of the increased mass of the CL in comparison to the follicle from which it was derived (Enders, 1973). Cellular size is increased through modulation of expression of cytoskeletal components. Multiple studies have shown the capacity of luteinizing granulosa cells to transiently alter tubulin expression and acquire the ability to

express smooth muscle actin, cytokeratin, vimentin, and desmin (Khan-Dawood et al., 1996; Murdoch, 1996).

Formation of CL structure also requires modification of the extra-cellular matrix (ECM) and cell-ECM interactions. Luck and Zhao (1993) demonstrated a change in collagen type during CL formation. Type IV collagen found in follicular basement membrane is replaced by fibrillar (type I) collagen, which becomes the main component of the luteal ECM. ECM-degrading enzyme expression and activity levels are elevated during the extensive remodeling associated with luteinization. A number of proteases have been indicated to play important roles in luteal remodeling including: the plasminogen-plasmin system, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), matrix metalloproteinases (MMP) and their inhibitors, tissue inhibitors of MMP (TIMP) (Liu et al., 1997; Curry and Osteen, 2003; Nothnick, 2003; Young et al., 2004).

One of the most important processes in CL formation is development of the microvasculature. In the follicle, a basement membrane lies between the theca interna and the stratum granulosa, preventing penetration by any capillaries. When ovulation occurs, this basement membrane is broken down, allowing microvascular cells to invade the luteinizing granulosa cells. Following CL formation, every steroidogenic luteal cell is adjacent to a microvascular element and in the mature CL, 50% of the total cell population is microvasculature-associated cells (Christenson and Stouffer, 1996).

The precise mechanisms through which the microvasculature of the CL is formed are not well defined. Rather, a number of factors that affect angiogenesis within the CL have been identified, giving rise to a general idea of possible mechanisms involved. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) have been identified in the developing CL and are hypothesized to be important regulators of angiogenesis in luteal tissue (Schams et al., 1994; Berisha et al., 2000). These two growth factors promote vascular endothelial cell proliferation, migration, tube formation and vessel permeability (Hazzard and Stouffer, 2000). Yamashita and co-workers (2008) demonstrated when an antibody to VEGF and bFGF was introduced locally, immediately following ovulation, the bovine CL that developed was smaller and secreted significantly less progesterone than a normal CL (Yamashita et al., 2008). High levels of VEGF promoted destabilization of blood vessels and formation of new vascular networks, as is found in the developing CL (Hanahan, 1997). This is further evidence of the critical role these angiogenic factors play in CL formation and function (Yamashita et al., 2008). Other factors that play an important role in angiogenesis are angiopoietins 1 (ANPT-1) and 2 (ANPT-2). In general, ANPT-1 promotes vessel stability, while ANPT-2 works as an agonist to promote remodeling of the vasculature (Yancopoulos et al., 2000). The balance of angiogenic factors appears to play a crucial role in development of vascular system of the CL, which is critical for acquisition of luteal cell function.

Preparation of the follicle for luteinization begins prior to ovulation, as a result of the pre-ovulatory surge luteinizing hormone (LH) . Within the follicular cells, nuclear chromatin disperses and the nucleolus forms concomitant with an increase in the number of polyribosomes (Enders, 1973; McClellan et al., 1975). Gap junctions between granulosa cells also begin to disappear. The amount of smooth endoplasmic reticulum in granulosa cells dramatically increases, mitochondria become more rounded, and mitochondrial cristae transform from lamellar to primarily tubular morphology (McClellan et al., 1975; Smith et al., 1994).

The pre-ovulatory surge of LH also induces changes in the activity and concentrations of steroidogenic enzymes in preovulatory follicular cells resulting in the loss of their ability to produce estrogen. The changes that take place in these luteinizing follicular cells after the LH surge are both quantitative and qualitative. Gene expression is altered from estrogen to progesterone synthesis. This switch is also quantitative because the amount of progesterone produced by luteal cells is far greater than the amount of estrogen by follicular cells. By 15-20 hours after the LH surge, mRNA concentrations of aromatase, the key enzyme for estrogen production, decline dramatically (Voss and Fortune, 1993a). Consequently, these steroidogenic cells begin to produce substantial amounts of progesterone. Interestingly, immediately following the LH surge there is no detectable increase in mRNA for enzymes involved in progesterone production,

suggesting that the periovulatory increase in progesterone production is transient. However, by 72 hours post-LH surge mRNA for enzymes cytochrome p450 cholesterol side chain cleavage enzyme (P450scc) and 3β-hydroxysteriod dehydrogenase (3βHSD) significantly increases, thereby increasing progesterone synthesis and secretion (Voss and Fortune, 1993b).

Many factors affect progesterone production within the CL. The first factor is the gonadotropin LH, acting through the LH receptor (LHR). LH principally stimulates progesterone synthesis and secretion by the small luteal cells, as these cells are where most of the LHR are located (Niswender and Nett, 1988). The LHR is a seven trans-membrane domain G-protein coupled receptor. LH binds to the LHR, stimulating formation of cAMP, leading to activation of protein kinase A and subsequently increasing progesterone production (Scham and Berisha, 2004). A study where heifers were treated with an antiserum against LH led to reduced circulating progesterone concentrations, demonstrating the critical role of LH in progesterone synthesis. Juengel and co-workers (1997) also found an additional role of LH may be to maintain the concentration of mRNA encoding growth hormone receptor (GHR) in luteal cells. In hypophysectomized ewes a 60% decrease in GHR in luteal tissue was observed. Treatment of hypophysectomized ewes with LH restored concentrations of GHR to values of pituitary-intact ewes. Thus, LH may

ultimately regulate the responsiveness of luteal tissue to growth hormone (GH) (Juengel et al., 1997).

GH is another factor that appears to play an important role in luteal function. Somatotrophs located in the adenohypophysis secrete this single-chain, polypeptide hormone. After synthesis, it is secreted into the bloodstream and acts on its target tissues which include: the liver, adipose tissue, and the reproductive organs. Growth hormone receptors have been identified in luteal tissue and were localized to large luteal cells (Koelle et al., 1998). This is a significant observation as large luteal cells produce the majority of progesterone secreted by the CL (Milvae et al., 1991). Investigations have uncovered a role for GH in regulating CL function in several species, including the cow. A study of GHR-deficient cows demonstrated that these animals also had a partial progesterone deficiency (Chase et al., 1998). Additionally, administration of exogenous recombinant bovine somatotrophin (rBST) to dairy cattle resulted in an observed increase in plasma progesterone concentrations (Schemm et al., 1990). Furthermore, treatment of cows with rBST resulted in increased weight of the CL compared to control animals (Lucy et al., 1992). Because many of the earlier studies on GH interaction with the CL were performed on dispersed luteal cells in culture, researchers were skeptical of the effects of GH on the CL *in vivo*. However, in an experiment performed by Liebermann and Schams (1994), administering GH to a microdialysed bovine CL resulted in a significant increase

in progesterone secretion during the early-luteal (day 5 to 7) and mid-luteal (day 8-12) phases, validating the results of the cultured luteal cell studies. Progesterone synthesis was also increased in this experiment in early gestational CL (day 60-120) indicating that GH may play a role in progesterone production during this period, as well (Liebermann and Schams, 1994). These results led to the conclusion that GH affects progesterone production from large luteal cells.

Soon thereafter, Juengel and co-workers (1997) conducted an *in vivo* experiment investigating the effects of administration of GH on plasma progesterone, GHR, and insulin-like growth factor 1 (IGF-1) concentrations in luteal tissue of hypophysectomized ewes. Treatment with GH increased concentrations of IGF-1 in luteal tissue, suggesting that GH's effects may be mediated through the actions of IGF-1 within the CL (Juengel et al., 1997).

IGF-1 is one of two ligands in the IGF superfamily (Spicer, 2004). It is so named for its structural similarity to insulin and growth promoting effects (Velazquez et al., 2009). The IGF superfamily also includes IGF-2, two receptors, six high-affinity insulin-like growth factor binding proteins (IGFBP), and binding protein proteases (Giudice, 1995; Hwa et al., 1999; Spicer, 2004). IGFBP play a pivotal role in the availability of IGF-1 for actions within the ovary and other reproductive tissues (Schams et al., 2002). IGFBP can inhibit the effects of IGF-1 by sequestering extracellular IGF-1 and limiting its availability for binding to cell surface receptors. Conversely, IGFBP can also potentiate IGF-1 actions by

protecting it from degradation, acting as a reservoir to sustain controlled delivery to target cells, and facilitating transport from the peripheral circulation to target tissues (Clemmons, 1998; Baxter, 2000; Firth and Baxter, 2002). Of the IGFBP, IGF-1 is predominately bound to IGFBP3. The complexity of the IGF system is further illustrated by the production of IGFBP proteases, which alter the bioavailability of IGF-1 by degradation of its binding protein (Velazquez et al., 2009).

The role of IGF-1 in luteal function has been heavily investigated, especially its effects on regulating steroidogenesis. Early studies demonstrated the presence of a functional IGF-1 tyrosine kinase receptor cascade in bovine luteal cells and established the relationship between IGF-1 exposure and increased progesterone production (Sauerwein et al., 1992; Chakravorty, 1993). Furthermore, receptors for IGF-1 are present in the CL throughout all stages of the estrous cycle. If luteal tissue is exposed to IGF-1 at any time during the cycle, progesterone production is stimulated. The greatest stimulation by IGF-1 occurs during the late luteal phase, with the peak of progesterone release related directly to peptide infusion (Sauerwein et al., 1992). The mechanism through which luteal IGF-1 causes progesterone release has also been studied. Denner and co-workers (2010) found that IGF-1 appears to activate the ERK pathway, leading to activation of the transcription factor Sp1, which increases expression of cytochrome P450scc, a critical steroidogenic enzyme. However, there is

evidence that this pathway is also under the control of other factors. Administration of the luteolytic hormone prostaglandin $F_{2\alpha}$ (PGF) also results in an increase in ERK signaling, but does not increase progesterone production. Instead, PGF decreases the capacity of IGF-1 to stimulate regulators of Sp1 (Arvisais et al., 2010). Although the mechanism of action of IGF-1 is still not fully elucidated, its importance in luteal function has been well established.

During the lifespan of the CL, this tissue's main function is to secrete progesterone. The key role progesterone plays in the establishment and maintenance of pregnancy has been demonstrated in numerous studies (Graham and Clark, 1997; Mann et al., 1999; Mann and Lamming, 2001; Green et al., 2005). Increased progesterone is also associated with an increased survival rate of embryos (Lamming et al., 1989; Starbuck et al., 1999). However, the mechanisms through which progesterone acts to establish and maintain pregnancy are poorly understood. A number of more recent studies have demonstrated the effects of progesterone on gene expression within the uterus. The results have indicated that much of progesterone's beneficial effects on pregnancy are likely mediated downstream of progesterone-induced changes in gene expression (Bauersachs et al., 2006; Gray et al., 2006; Satterfield et al., 2006). Forde and co-workers (2009) used a cDNA microarray to identify genes differentially regulated in early pregnancy by progesterone between groups of high progesterone and normal progesterone-producing cattle. A number of differentially expressed genes at

different stages in early pregnancy, many of which involved in protein metabolic processes and transport, were identified. Of particular note was the increased expression of diacylglycerol O-acyltransferase-2 (DGAT2), an enzyme that catalyzes the final step in the formation of triglyceride to acylcoenzyme A. Triglycerides are a potential energy source for the developing conceptus up to the blastocyst stage in cattle. This regulation of genes associated with energy production indicates one mechanism by which progesterone supports the establishment and maintenance of pregnancy (Forde et al., 2009).

Progesterone also acts in a paracrine/autocrine manner as well. Recently, progesterone receptors have been identified in the nuclei of large and small luteal cells, as well as vascular endothelial cells (Sakumoto et al., 2010). Within the CL, progesterone appears to play a largely luteotropic role. In the mid-cycle CL, treatment of luteal cells with a progesterone antagonist inhibited oxytocin and stimulated PGF secretion, indicating that progesterone inhibits mid-cycle PGF secretion (Pate, 1988; Skarzynski and Okuda, 1999). Progesterone can also stimulate synthesis of LH receptors in luteal cells and repress the onset of apoptosis by a progesterone receptor-dependent mechanism within the bovine CL further demonstrating progesterone's luteotropic role (Jones et al., 1992; Rueda et al., 2000).

As stated previously, the main function of the CL is to produce progesterone. Progesterone synthesis and secretion by the CL is a complex

process involving many enzymes. The first step is acquisition of cholesterol, which can either be derived from the diet or synthesized *de novo* (Rekawiecki and Kotwica, 2007). Cholesterol is transported to the ovaries by lipoproteins (high-density lipoproteins and low-density lipoproteins) and taken into the cells by endocytosis. Cholesterol esters are converted to free cholesterol via hydrolysis in the cytoplasm (Niswender et al., 2000). Cholesterol is transported across the double mitochondrial membrane by Steroidogenic Acute Regulatory Protein (StAR) and this process is the rate-limiting step in progesterone synthesis (Stocco, 2001; Rekawiecki et al., 2008). Once cholesterol has been moved to the inner mitochondrial membrane, the enzyme p450scc catalyzes the conversion of cholesterol to pregnenolone (Diaz et al., 2002). Pregnenolone possesses two hydrophilic residues that allows it to diffuse out of the mitochondria and move to the smooth endoplasmic reticulum. In the smooth endoplasmic reticulum, the enzyme 3βHSD converts pregnenolone to progesterone (Niswender, 2002). Progesterone then diffuses out of the cell and into the bloodstream to be carried to its target tissues.

Progesterone mediates its actions on target cells via the progesterone receptors. The nuclear progesterone receptor (PR) belongs to a superfamily of nuclear receptors that contain a similar basic structure. These receptors consist of a C-terminal ligand-binding domain, a highly conserved DNA-binding domain near the center of the receptor, and an N-terminal domain that varies in length

(Stormshak and Bishop, 2008). Within the domains of the progesterone receptor are two transcription activation subdomains, AF-1 and AF-2. AF-1 is located in the N-terminal domain, and AF-2 is in the ligand-binding domain (Edwards, 2005). Progesterone receptors are activated when they dissociate from their chaperone molecules, dimerize, and the receptor complex binds a progesterone response element in the promoter region of a gene (Kumar and Thompson, 2003). Coactivators or corepressors are also recruited to one or both of the transription activation subdomains of the progesterone-receptor complex to alter gene transcription. The resulting association of coactivators or corepressors with the ligand-bound receptor facilitates the assembly of the RNA polymerase II complex which then promotes gene transcription (McKenna et al., 1999; Stormshak and Bishop, 2008).

Within the uterus, progesterone receptor concentrations are at the highest between days 4 to 10 of pregnancy in both the endometrial glands and sub-epithelial stroma (Robinson et al. 2001). Studies examining changes in PR expression in the CL during the estrous cycle have yielded conflicting results. Berisha and co-workers (2002) found no changes in PR concentrations, while a more recent study by Sakumoto and co-workers (2010) found the quantity of progesterone receptors highest in the early CL (day 2-4 post-ovulation) and a steady decline thereafter. The differences in these two studies may be due to a variety of factors such as differences in methods of classifying CL or different

primer/probe sequences. Regardless, PR have been proven to be at detectable levels in the bovine CL during all stages of the estrous cycle.

Estrogen also affects luteal function via its receptors; estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Several studies have described expression patterns of ERα and ERβ mRNA and proteins during the estrous cycle and pregnancy in the bovine CL (Berisha et al., 2002; Shibaya et al., 2007). Greatest expression of ERα is in the early luteal phase (Day 1-5), with expression decreasing significantly during the mid (Day 8-12) and late (Day 13-16) luteal phases. In contrast, ERβ mRNA is relatively high during the early luteal phase, decreases during the mid-luteal phase, and significantly increases again during the late luteal phase and after luteal regression (Berisha et al., 2002). These results suggest that actions perpetuated by ERα may be important for the establishment and regulation of early CL function, while estrogen may play a different role via ERβ during and after regression of the CL. This hypothesis is further supported by Shibaya and co-workers (2007), who investigated the expression pattern of ERα and ERβ protein in the bovine CL. Shibaya and co-workers (2007) demonstrated that ERα protein was highest at the early luteal stages and decreased throughout the estrous cycle. However, ERβ protein increased from the early to mid stages and then decreased thereafter. Interestingly, the ratio of ERβ to ERα was much higher in the regressed CL than in other stages, again indicating a probable role for ERβ in CL regression.

Furthermore, administration of PGF to cultured luteal cells resulted in a dose-dependent decrease of both ERβ and ERα mRNA (Shibaya et al., 2007). An injection of estrogen during the late luteal phase of the estrous cycle can induce luteolysis (Salfen et al., 1999). Therefore, it is possible that in the late luteal phase estrogen may act collaboratively with PGF to induce luteal regression.

1.2 Corpus Luteum Regression

In cattle in the absence of an embryo, the CL will begin to regress, a process termed luteolysis, between days 17-19 of the estrous cycle (McCracken et al., 1999). Luteolysis is characterized by both the cessation of progesterone production in the CL, or functional regression, and the elimination of luteal tissue, termed structural regression.

Preparation of the uterus for luteolysis begins prior to the release of the luteolytic signal, PGF. Progesterone priming of the uterus is required for this organ to be responsive to estradiol and oxytocin, two important hormones in the luteolytic cascade (Okuda et al., 2002). Exposure of the uterus to high concentrations of estradiol causes up-regulation of oxytocin receptors within the endometrium. Additionally, high estradiol concentrations stimulate the release of oxytocin from the posterior pituitary (Hixon and Flint, 1987; Asselin et al., 1996; McCracken et al., 1996). Oxytocin binds to its receptor in the uterus

activating the protein kinase C pathway, resulting in up-regulation of genes involved in PGF synthesis, including phospholipase A2 (PLA2) (Okuda et al., 2002). Upon activation, PLA2 liberates arachidonic acid from membrane phospholipids. Conversion of arachidonic acid to the intermediate compounds PGG2 and then PGH2 is catalyzed by the enzymes Prostaglandin G/H Synthase 1 (PGHS1) and Prostaglandin G/H Synthase 2 (PGHS2), also known as COX-1 and COX-2, respectively, because of their cyclooxygenase activity (Wlodawer et al., 1976). PGH2 is converted to PGF by the enzyme PGF synthase. Alternatively, PGH2 can be converted into prostaglandin E_2 and then converted to PGF by the enzyme PGE_2-9-keto reductase (Okuda et al., 2002). Once PGF has been synthesized it is immediately released into the bloodstream. PGF participates in a countercurrent exchange mechanism between the uterine vein and the ovarian artery to reach its target, the CL (Niswender et al., 2000).

Within the CL, PGF modulates expression of a number of factors to promote both functional and structural regression. PGF inhibits transport of cholesterol from the outer mitochondrial membrane to the inner membrane by decreasing StAR mRNA and protein. In studies done with cattle, administration of PGF quickly resulted in a significant decrease of StAR mRNA within luteal cells, followed closely by a decline in StAR production (Pescador et al., 1996). In support of these results, studies on the effects of PGF on the enzymes P450scc and 3β-HSD and the LH receptor have shown that it is unlikely PGF mediates

luteolysis through these factors (Niswender et al., 2000). Therefore PGF causes cessation of progesterone synthesis mainly through down-regulation of StAR mRNA and protein.

PGF also stimulates production of luteal prostaglandins. Studies have suggested that endometrial PGF stimulates production of intraluteal PGF to complete the luteolytic process (Tsai and Wiltbank, 1997; Hayashi et al., 2003). This is supported by Arosh and co-workers (2004a), as expression of prostaglandin F synthase is up-regulated withing the CL during luteal regression. It has been further suggested that locally produced PGF may establish a positive feedback loop with luteolytic factors endothelin-1 and angiotensin II, as each has been shown to stimulate release of the others (Shirasuna et al., 2004).

Endothelin-1 (EDN1) and angiotensin II (Ang II), both potent vasoconstrictors, are upregulated in the CL under the influence of PGF (Miyamoto et al., 2009). This has been shown using cultured luteal cells, as well as microdialyzed CL both *in vivo* and *in vitro*. An intraluteal injection of EDN1 or Ang II following a sub-luteolytic dose of PGF results in a reduction of plasma progesterone concentrations and the animal's subsequent return to estrus (Hayashi et al., 2002; Miyamoto et al., 2005). EDN1 acts to inhibit progesterone secretion through selective EDN1 binding sites called EBN_A (Girsh et al., 1996). However, the involvement of these factors in functional regression appears to be minimal. Up-regulation of EDN1 and Ang II occurs mainly during the structural

portion of luteal regression and promotes leukocyte migration and stimulates macrophages to release cytokines (Schams et al., 2003; Skarzynski, 2008).

Immune cells and cytokines are also important in structural luteolysis. Penny et al. (1999) showed that during regression of the CL, leukocytes, T-lymphocytes, and macrophages increase significantly. During luteolysis, a major role of macrophages appears to be degeneration of the extra-cellular matrix and phagocytosis of degenerating luteal cells (Niswender, 2000). An additional and critical role of immune cells that infiltrate the CL during regression is the secretion of a number of cytokines, such as tumor necrosis factor alpha (TNF), interleukin-1β (IL-1), and interferon-γ (IFN-γ).

During luteolysis, T-lymphocytes secrete IFN-γ and macrophages produce TNF (Fairchild and Pate, 1989; Fairchild and Pate, 1992). Intraluteal TNF increases significantly in both spontaneous and induced luteolysis in *in vivo* microdialyzed CL. Secretion of bioactive TNF begins after the loss of progesterone synthesis in the bovine CL suggesting that this peptide's role is complementary to the luteolytic activity of PGF (Shaw and Britt, 1995). It is likely that TNF stimulates synthesis of luteal PGF. Skarzynski et al. (2003)showed *in vivo* that lower doses of TNF increased PGF production within the bovine CL and increased nitrate/nitrite, stable metabolites of nitric oxide (NO). Interestingly, the study also showed that high doses of TNF increases in progesterone and prostaglandin E_2 (PGE_2) production, leading to prolongation of the luteal phase of

the estrous cycle (Skarzynski et al., 2003). There is also evidence that TNF must act in combination with other factors, such as IFN-γ, to induce luteolysis (Petroff et al., 2001; Korzekwa et al., 2006). Recent evidence has suggested that the combination of TNF and IFN-γ is highly cytotoxic (Petroff et al., 2001; Taniguchi et al., 2002). Taniguchi et al. (2002) showed that in bovine luteal cells IFN-γ increased the expression of *Fas* mRNA, a ligand inducing apoptosis via Fas receptors, and the presence of TNF augments this stimulatory effect. Furthermore, apoptotic bodies were observed in luteal cells treated with Fas ligand in the presence of IFN-γ and TNF, demonstrating the critical role of these cytokines in Fas L-Fas-mediated luteal cell death (Taniguchi et al., 2002).

Reactive oxygen compounds are also critically linked to apoptosis of bovine luteal cells during luteolysis. The primary reactive compounds found within steroidogenic luteal cells are superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide. During normal luteal function the harmful actions of these compounds are attenuated by antioxidants, such as the enzymes catalase and superoxide dismutase (Niswender et al. 2000). However, during structural regression of the bovine CL, expression of mRNA encoding these two enzymes is significantly decreased (Rueda et al. 1995). This down-regulation appears to be vital in promoting structural luteolysis within the bovine CL.

The mechanisms that lead to the destruction of the CL and the cow's subsequent return to estrus discussed in this chapter are summarized in Figure 1.2.1.

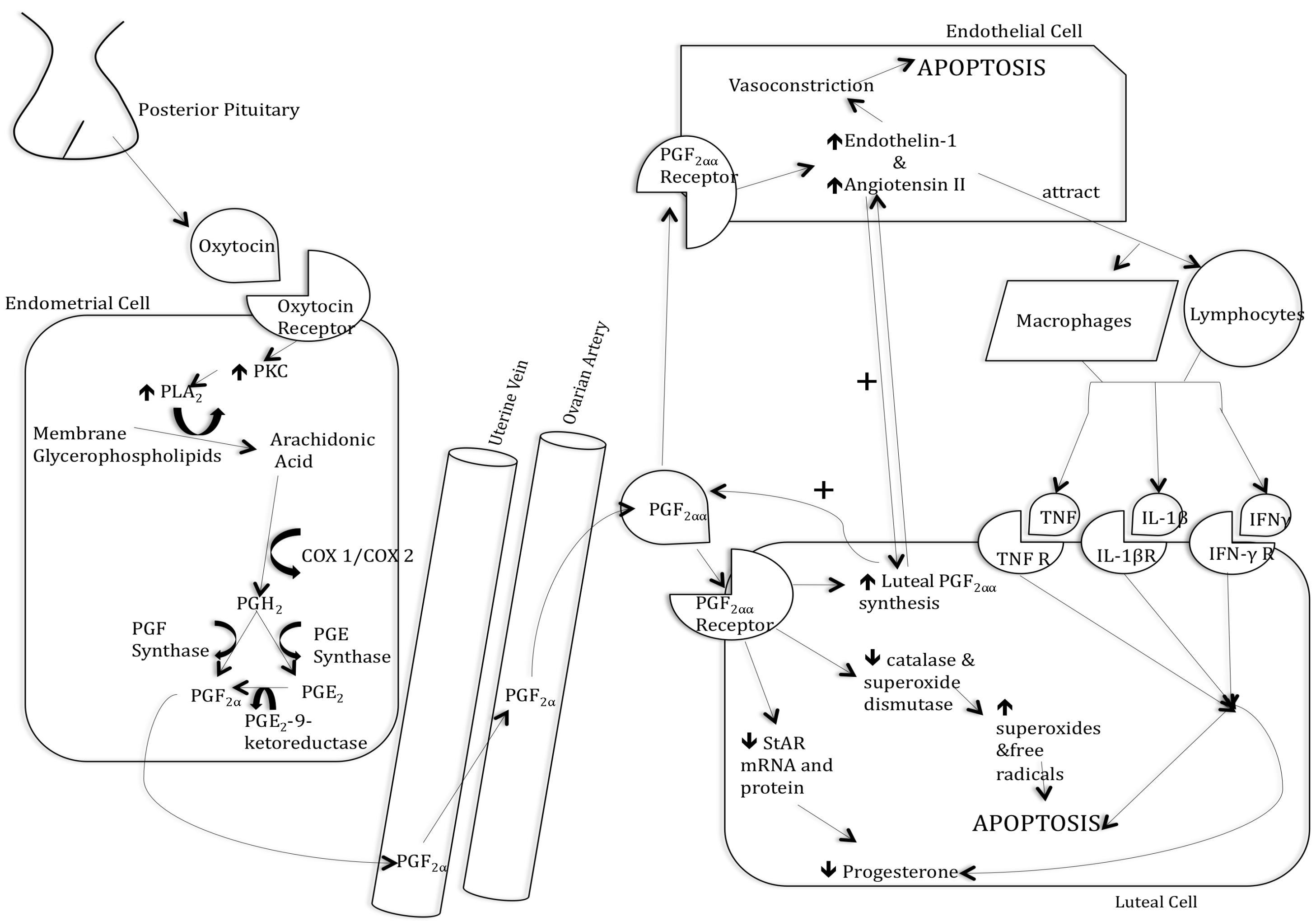

Figure 1.2.1 Model of luteolysis cascade

1.3 Maternal Recognition of Pregnancy

The developing embryo requires progesterone support for establishment and maintenance of pregnancy. Signaling of the conceptus to the maternal system resulting in prevention of the luteolytic cascade and prolongation of the lifespan of the CL is known as maternal recognition of pregnancy. In cattle, the pregnancy recognition signal is the type I interferon, interferon-tau (IFN-τ) (Bazer, 1992; Roberts et al., 1992).

Synthesis of IFN-τ is under the control of a number of factors. The transcription factor Ets-2 is a key component for production of IFN-τ. Ezashi and co-workers (1998) identified an Ets2 binding site in the promoter region of the ovine *IFN-τ* gene. This finding was soon expanded to include the bovine *IFN-τ* gene as well (Ezashi et al., 1998). At least two additional trophectodermal transcription factors are involved in regulation of IFN-τ production. Caudal-type homeobox 2 (Cdx2) has been shown to stimulate *IFN-τ* promoter activity in the presence of both Ets-2 and activator protein-1 (AP1) (Imakawa et al., 2006). The second factor is distal-less 3 (DLX3), whose expression has been shown in a bovine trophectoderm cell line (CT1). DXL3 acts in cooperation with Ets2 to optimize *IFN-τ* transcription (Ezashi et al., 2008).

Secretions from the uterine lumen can also regulate the production of IFN-τ. The first uterine factor discovered to play a possible role in IFN-τ regulation

was granulocyte-macrophage colony-stimulating-factor (GM-CSF), a product of the endometrial epithelium and stroma (de Moraes et al., 1999). Michael et al. (2006a) demonstrated that supplementation of CT1 cells with GM-CSF increases IFN-τ secretion. The second uterine factor affecting IFN-τ secretion is fibroblast growth factor 2 (FGF2). CT1 cells and bovine blastocysts supplemented with recombinant bovine FGF2 have increased *IFN-τ* mRNA levels and increased secretion of biologically active IFN-τ (Micheal et al., 2006b; Rodina et al., 2008). Therefore regulation of IFN-τ production is under the influence of factors both embryonic and maternal in origin.

IFN-τ production occurs during a defined period of embryonic development in cattle. *IFN-τ* mRNA and IFN-τ protein can be detected in the uterus as early as the hatched blastocyst stage (Day 11) (Hernandez-Ledezma et al., 1992). Large quantities of mRNA begin to be produced at Days 14-15 of pregnancy and continue to increase until approximately Day 19, when production appears to slow (Ealy et al., 2001; Ealy and Yang, 2009). The increase in *IFN-τ* mRNA coincides with blastocyst elongation, which increases the mass of the trophectoderm, leading to the profound increase of IFN-τ found in uterine flushes during this period. At approximately Day 21 of pregnancy, *IFN-τ* mRNA declines sharply, concurrent with trophectoderm attachment to the uterine lining, until it is no longer detectable by Day 25 (Ealy and Yang, 2009). Therefore

the maternal recognition of pregnancy period in cattle is associated with Days 14 to 25 of pregnancy.

The mechanism by which IFN-τ acts on the uterus to disrupt the luteolytic process has been much debated, but an accepted theory has been developed. IFN-τ binds to the type I interferon receptor (IFNAR), located on the apical border of the luminal and glandular endometrial epithelium (Hans et al., 1997). The interferon receptor is composed of two subunits: IFNAR1 and IFNAR2. IFNAR2 is the ligand-binding subunit of the receptor. IFN-τ binding leads to the recruitment of the signal-transducing subunit, IFNAR1, resulting in selective gene regulation through activation of JAK tyrosine kinases and signal transducers and activators of transcription (STAT) factors (Thatcher et al., 2001).

The primary way by which IFN-τ halts the luteolytic cascade is prevention of the up-regulation of the oxytocin receptor (OTR), which must be bound by oxytocin for sythesis of the luteolytic pulses of PGF, and suppression of the up-regulation of the estrogen receptor, ERα (Robinson et al., 1999; Robinson et al., 2001). Recombinant IFN-τ inhibits OTR expression both *in vivo* and *in vitro* (Spencer and Bazer, 1995; Spencer et al., 1998). However, it is unclear whether IFN-τ acts directly on the OTR gene or through suppression of the ERα gene to prevent transcription of OTR. Some evidence indicates that suppression of OTR is most likely a result of down-regulation of ERα (Bazer et al., 1997; Fleming et al., 2006) However, direct action of IFN-τ on OTR is supported by interferon

response elements located in the OTR promoter region (Telgmann et al., 2003). In pregnant cattle, down-regulation of the OTR occurs prior to any changes in ERα abundance, suggesting a possible role for IFN-τ in regulation of ERα expression and activity within the bovine endometrium (Robinson et al., 1999; Robinson et al., 2001).

Though this mechanism appears to be the primary action of IFN-τ in establishing pregnancy, this signal also appears to modulate a number of other factors within the uterus. One system of particular interest is the IGF family. During early pregnancy IGF-1 and IGF-2 mRNA is increased compared to non-pregnant animals (Geisert et al., 1991; Kirby et al., 1996; Robinson et al., 2000; Bilby et al., 2006). Furthermore, endometrial-derived IGF-1, combined with embryonic-derived IGF-2, stimulates production of IFN-τ (Ko et al., 1991; Robinson et al., 2008). These observations make it likely that the conceptus upregulates expression of IGF-1 and IGF-2 within the endometrium during maternal recognition of pregnancy.

Interferon-stimulated genes (ISGs) are another class of factors modulated by IFN-τ. Within bovine endometrium, STATS-1, 2 and 3, IRF-1, ISG17, granulocyte chemotactic protein-2, and 2'S' oligoadenylate synthetase have been identified as modified by IFN-τ (Thatcher, 2001). Although it has not been definitively shown, it is logical to hypothesize that some of these proteins may be involved in suppression of PGF synthesis.

In addition to preventing the luteolytic, pulsatile release of PGF, IFN-τ further modulates expression of uterine prostaglandins. Administration of low doses of IFN-τ to bovine endometrial cultures results in inhibition of both PGF and PGE_2 production. However, when high doses of IFN-τ were administered, PGF production was unaltered while PGE_2 synthesis increased significantly (Binelli et al., 2000; Parent et al., 2003; Guzeloglu et al., 2004). PGE_2's role as a luteotropic hormone has led to the suggestion that IFN-τ alters prostaglandin production within the endometrium to favor PGE_2, thereby exerting a luteoprotective effect (Arosh et al., 2004a; Arosh et al., 2004b). This mechanism is supported by Xiao et al. (1998) who demonstrated increased prostaglandin secretion by IFN-τ through up-regulation of *COX-2* mRNA in endometrial stromal cells, the primary source of PGE_2. Furthermore, IFN-τ suppresses prostaglandin secretion via down-regulation of COX-2 expression in endometrial epithelial cells, the primary site of PGF synthesis.

PGE_2 of embryonic origin may also have a role in rescue of the CL. Bovine embryos produce detectable quantities of PGE_2 as early as Day 6 of pregnancy (Hwang et at., 1988). Lewis et al. (1982) and Wilson et al. (1992) also demonstrated the ability of the bovine conceptus to produce PGE_2 on Days 16 and 10, respectively. It is likely PGE_2 concentrations found in the uterus are the result of both IFN-τ-induced endometrial PGE_2 and PGE_2 of embryonic origin.

Luteal prostaglandin synthesis is also likely to be involved in maintenance of the CL. During the cow estrous cycle, a positive correlation between luteal PGE_2 and progesterone has been demonstrated (Kotwica et al., 2003). PGE_2 stimulates progesterone production with an efficiency comparable to LH in both bovine and ovine CL (Weems et al., 1997; Kim et al., 2001; Weems et al., 2002). During early pregnancy, prostaglandin E_2 synthase protein is significantly up-regulated and the ratio of PGE_2/PGF in luteal tissue is increased (Arosh et al., 2004b). These results suggest a role for luteal PGE_2 in maintenance of the early pregnant CL.

Researchers have begun to investigate the possibility of an effect of maternal recognition of pregnancy on gene modulation at the level of the CL for luteal maintenance. This hypothesis is supported by a study in cattle where each animal's uterus was infused with IFN-τ to mimic the effects of maternal recognition of pregnancy. Corpora lutea from IFN-τ-infused cows had a significant increase in prostaglandin E_2 synthase and a higher PGE_2/PGF ratio than control cows on Day 16 of pregnancy (Arosh et al., 2004b).

Production of PGE_2 by the bovine conceptus and increase in endometrial PGE_2 synthesis as a result of IFN-τ release has led to the hypothesis that embryonic PGE_2 may travel from the uterus to the CL by the same countercurrent exchange mechanism as PGF. Silva and co-workers (1984) showed a dramatic increase in utero-ovarian venous plasma PGE_2 concentrations on Days 13 and 14

in pregnant ewes. Presence of steady-state low levels of PGE_2 in the caudal vena cava of pregnant cows was demonstrated by Schallenberger and co-workers (1989). These results suggest that PGE_2 may travel from the uterus to the CL to have a direct effect on prolongation of luteal lifespan through alteration of luteal prostaglandin production and prostaglandin ratios.

Several studies in sheep have also suggested another direct effect of the conceptus on CL rescue. Oliveira and co-workers (2008) observed up-regulation of interferon-stimulated genes, ISG15 and OAS-1, in the CL during early pregnancy, and hypothesized it was the result of IFN-τ release from the uterine vein. This hypothesis was further supported in a recent study where antiviral assays demonstrated that the antiviral activity reported by Oliveira et al. (2008) was indeed caused by IFN-τ (Bott et al., 2010). This study also showed that IFN-τ infusion into the uterine vein extended luteal lifespan and increased ISG15 expression in pregnant compared to non-pregnant CL (Bott et al., 2010). During early pregnancy in cattle, ISG15 is also up-regulated within the CL (Yang et al., 2010). However, unlike in sheep, cultured bovine luteal cells did not respond to IFN-τ, implying that the pregnancy-dependent up-regulation of ISG1 within the CL is dependent on something other than IFN-τ in the bloodstream (Yang et al., 2010).

Collectively, these studies provide compelling evidence that there may be more significant regulation at the level of the CL during maternal recognition of pregnancy than was previously thought.

Chapter 2 Introduction

The corpus luteum (CL) is an endocrine gland that develops on the mammalian ovary following ovulation. Proper formation of the CL is critical for successful establishment and maintenance of early pregnancy in mammals . The main function of the CL is to synthesize and secrete the steroid hormone progesterone, which is critical in preparing the uterus for successful establishment of pregnancy (Mann et al., 1999; Mann and Lamming, 2001; Green et al., 2005). If no embryo is present on Day 16 of the estrous cycle in the cow (Day 0 is the onset of estrus), increased estrogen concentrations result in the release of oxytocin from the posterior pituitary (Hixon and Flint, 1987; Asselin et al., 1996; McCracken et al., 1996). Oxytocin binds endometrial oxytocin receptors and induces release of luteolytic pulses of prostaglandin $F_{2\alpha}$ (PGF). PGF is released into the venous drainage where it travels to the CL via a countercurrent exchange mechanism and initiates a luteolytic cascade resulting in CL regression. During pregnancy, the embryo will signal its presence to the maternal system via secretion of interferon tau (IFN-τ). IFN-τ is secreted by the trophectoderm of the elongating bovine blastocyst from Day 12 to Day 25 (Roberts et. al., 2003). The mechanism by which IFN-τ acts to prevent luteolysis has been much debated. It is hypothesized IFN-τ establishes pregnancy by suppressing up-regulation of oxytocin and estrogen thereby preventing the pulsatile release of PGF and

prolonging CL lifespan. Additional studies have proposed an effect of maternal recognition of pregnancy within the CL. Arosh and co-workers (2004b) infused cow uteri with IFN-τ and observed a significant up-regulation within the CL of prostaglandin E_2 synthase (PGES), an enzyme critical for the production of PGE_2. A significant change in the ratio of PGES to PGF synthase in favor of PGES was also demonstrated (Arosh et al. 2004b). These data strongly suggest that at the time of maternal recognition of pregnancy changes in gene expression occur within the CL.

Furthermore, evidence suggests that the conceptus directly signals the corpus luteum. The developing bovine embryo produces PGE_2 (Lewis et al., 1982; Hwang et al., 1988; Lewis et al., 1992). Silva and co-workers (1984) showed a dramatic increase in utero-ovarian venous plasma PGE_2 concentrations during maternal recognition of pregnancy (Days 13 and 14) in pregnant ewes. Presence of steady-state low levels of PGE_2 in the caudal vena cava of pregnant cows was demonstrated by Schallenberger and co-workers (1989). These results suggest that PGE_2 may be one factor traveling to the CL from the uterus to directly affect CL maintenance.

Oliveira and co-workers (2008) tracked the movement of ovine IFN-τ from the uterine vein to the ovarian artery via a countercurrent exchange mechanism and eventually to the CL where it upregulated IFN-τ induced proteins (Oliveira et al. 2008). Bott et al. (2010) confirmed that the anti-viral activity in

the uterine vein was indeed caused by IFN-τ and that infusion of IFN-τ into the uterine vein increased luteal lifespan in ewes. Furthermore, Yang et al. (2010) observed an up-regulation of the interferon-induced protein, ISG15 in CL recovered from early pregnant cattle. However, unlike sheep, cultured bovine luteal cells did not respond to IFN-τ, implying that the pregnancy-dependent up-regulation of ISG1 within the CL is dependent on a factor other than IFN-τ (Yang et al. 2010). Based on these data it seems likely there is significant modulation of genes within the corpus luteum during maternal recognition of pregnancy in order to maintain its function. Therefore, the objectives of this study were to: 1) identify genes in the CL that are differentially expressed during maternal recognition of pregnancy, 2) characterize the direction and magnitude of that change, and 3) determine if luteotropic genes were up-regulated and luteolytic genes were down-regulated as a result of maternal recognition of pregnancy.

Chapter 3 Materials and Methods

3.1 Collection of Corpora Lutea

All animal care and experimental procedures were conducted with the approval of the Oregon State University Institutional Animal Care and Use Committee. Five non-lactating, multiparous cows (two Jersey, three cross-bred Angus) were estrous-synchronized with two 25-mg injections of $PGF_{2\alpha}$ (Lutalyse, Pfizer, New York, NY, USA) 12 days apart. From each individual cow two CL were scheduled for collection at Day 14 of the estrous cycle and one at Day 14 of pregnancy. In three cows non-pregnant CL were collected first, whereas in the other two cows the pregnant CL were collected first. Estrus detection was conducted at 12-h intervals and cows were inseminated 12 and 24-h after estrus onset. Cows were inseminated with semen from one of two Jersey bulls. Fourteen days later CL were collected transvaginally. During this procedure cows were anesthetized using 5-10 mL of 2% lidocaine hydrochloride (AgriLabs, St. Joseph, MO, USA). Each CL was rinsed briefly in ice-cold PBS, cut into quarters, snap frozen in liquid nitrogen, and stored at -80°C. Cows were rested for 45 days between collections.

3.2 Embryo Recovery

Non-surgical embryo recovery was performed on inseminated cows to confirm pregnancy. Each cow's uterus was flushed with 1L of DPBS containing 2mL heat-treated fetal calf serum (HyClone, Logan, UT, USA) and 10mL antibiotic, antimycotic solution (penicillin, streptomycin, amphotericin) (Sigma Aldrich, St. Louis, MO) using a Foley catheter and a gravitational flow system. Recovered flush solution was filtered through a teflon screen and the embryo was visualized using a dissecting microscope. Embryos were washed three times in DPBS containing 0.1% BSA, placed in cryovials, snap frozen in liquid nitrogen, and stored and -80°C.

3.3 RNA isolation and cDNA synthesis

Total RNA was extracted from CL as previously described (Chomczynski and Sacchi, 1987). Briefly, tissue was homogenized (Brinkmann Instruments, Canada) in GIT buffer + β-mercaptoethanol and centrifuged (Beckman-Coulter, Brea, CA). The aqueous phase was removed and ethanol (95%) was added to precipitate RNA overnight in a -20°C freezer. Ethanol was removed and total RNA was solubilized in distilled H_2O at 70°C. Quantity and quality of total RNA was determined by A_{260}/A_{280} ratio using a NanoPhotometer (IMPLEN, Munich,

Germany). RNA cleanup was performed using the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions.

Embryonic RNA was isolated using an RNAqueous® Micro Scale RNA Isolation Kit (Ambion®, Austin, TX) according to manufacturer's instructions. Reverse transcription of mRNA was performed using Applied Biosystem's High Capacity cDNA Reverse Transcription Kit according to manufacturers instructions (Foster City, CA).

3.4 Microarrays

RNA integrity screening, probe synthesis, hybridization and scanning were conducted by the Center for Gemome Research and Biocomputing Core Laboratories at Oregon State University. Briefly, 5 µg of total RNA were used to generate biotinylated complementary RNA (cRNA) for each treatment group using the One-Cycle Target Labeling protocol (Affymetrix, Santa Clara, CA) from the GeneChip® Expression Analysis Technical Manual (701021 Rev. 5). Prior to hybridization, the cRNA was purified with GeneChip® Sample Cleanup Modules (Affymetrix, Santa Clara, CA) and fragmented. Ten micrograms from each CL sample and the Affymetrix eukaryotic hybridization controls were hybridized for 16 hours to GeneChip® bovine genome arrays in an Affymetrix GeneChip® Hybridization Oven 640. Affymetrix GeneChip® Fluidics Station 450 was used to

wash and stain the arrays with streptavidin-phycoerythrin (Moleculer Probes, Eugene, OR) and biotinylated anti-streptavidin (Vector Laboratories, Burlingame, CA) according to the standard antibody amplification protocol for eukaryotic targets. Arrays were scanned with an Affymetrix GeneChip® Scanner 3000 at 570nm. The Affymetrix eukaryotic hybridization control kit and Poly-A RNA control kit were used to ensure efficiency of hybridization and cRNA amplification. All cRNA was synthesized at the same time. Hybridizations were conducted with one replicate of all times and treatments concurrently. Each array image was visually screened to discount for signal artifacts, scratches or debris.

3.5 Real-Time RT-PCR of luteal RNA

Gene expression was measured by real-time PCR using custom-designed TaqMan Gene Expression Assays, TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and 2.0 μg of reverse-transcribed total RNA. Reaction volumes were 20μl and RT-PCR were performed using the Applied Biosystems 7300 Real-Time PCR System (Foster City, CA) according to manufacturer's instructions (TaqMan Gene Expression Assays Protocol). GAPDH expression was used as an endogenous control. The PCR conditions were as follows: 50 C for 2 min followed by 95 C for 10 min followed by 40 cycles of 95 C for 15 sec and 60C for 1 min.

3.6 Real-time RT-PCR of embryonic RNA

Expression of interferon-tau was measured by real-time PCR using an inventoried TaqMan Gene Expression Assay, TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and 2.0 μg of reverse-transcribed total RNA. Reaction volumes were 20μl and RT-PCR was performed using the Applied Biosystems 7300 Real-Time PCR System (Foster City, CA) according to manufacturer's instructions (TaqMan Gene Expression Assays Protocol). GAPDH expression was used as an endogenous control. The PCR conditions were as follows: 50 C for 2 min followed by 95 C for 10 min followed by 40 cycles of 95 C for 15 sec and 60C for 1 min. PCR products were visualized on a 2% agarose gel stained with SYBR green and a KODAK Electrophoresis Documentation and Analysis System 120 (Rochester, NY).

3.7 Progesterone ELISA

A progesterone ELISA kit (Cat. No. 1860, Alpha Diagnostic International, San Antonio, TX) was used to quantify the plasma progesterone concentrations in each cow at the time of surgery. Cross-reactivity of the anti-serum was 100% to progesterone with the next-highest cross-reactivity of 1.5% to 11-deoxycorticosterone and less than 1% for all others. Assays were performed

according to the manufacturers instructions and each progesterone sample was run in duplicate. Inter- and intra-assay coefficients of variation were 7.53% and 9.1%, respectively.

3.8 Statistics

Microarray data were analyzed using a paired T-test comparing pregnant tissue to non-pregnant tissue within the same animal. Results with a fold change ≥1.5 and $P < 0.05$ are shown. PCR results were also analyzed using a paired T-test comparing pregnant to non-pregnant tissue within the same animal, ($P < 0.05$ was considered significant).

Chapter 4 Results

4.1 Plasma progesterone

Plasma progesterone concentrations in pregnant and non-pregnant cows were ≥7 ng/mL (Figure 4.1.1) indicating CL were functional at the time of luteectomy. No significant differences in progesterone concentrations were observed between pregnant and non-pregnant cows.

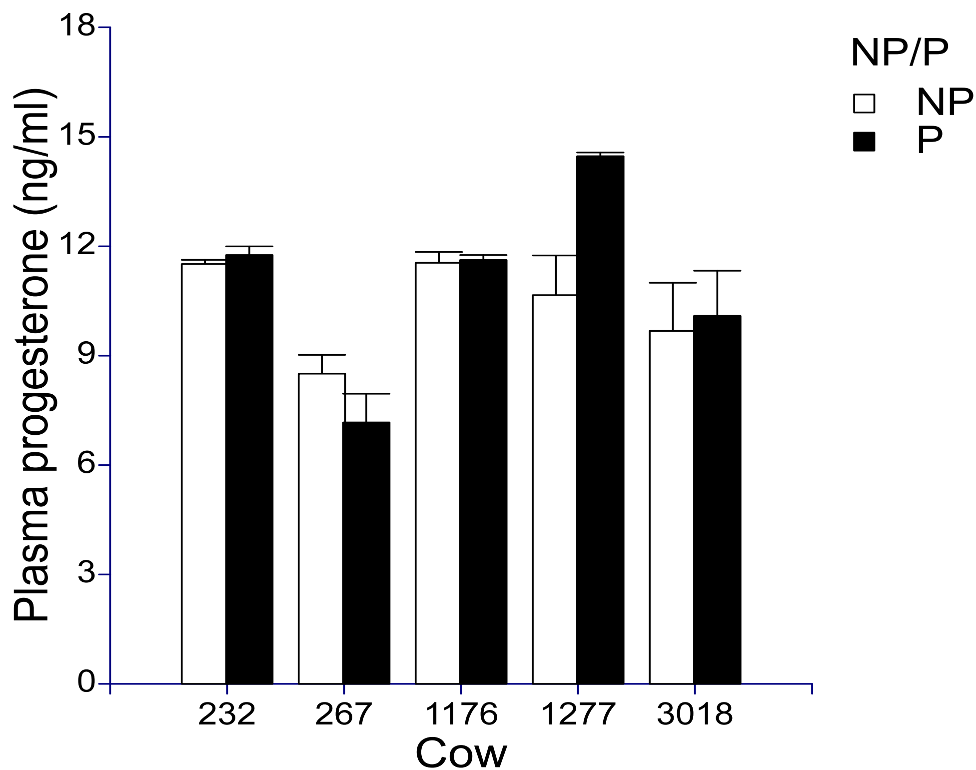

Figure 4.1.1. Plasma progesterone concentrations in pregnant and non-pregnant cows on Day 14.

4.2 Real-time RT-PCR verification of embryonic expression of IFN-τ

Embryos were successfully collected from each of the pregnant animals on Day 14 (Figure 4.2.1). INF-τ mRNA was observed in all embryos collected on Day 14 (Figure 4.2.2), indicating maternal recognition of pregnancy was occurring at the time of luteal tissue collection.

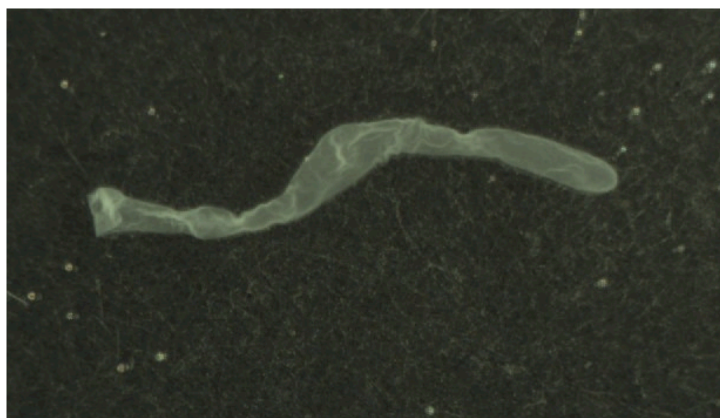

Figure 4.2.1. Day 14 embryo recovered from pregnant cow (13.5mm x 0.8mm).

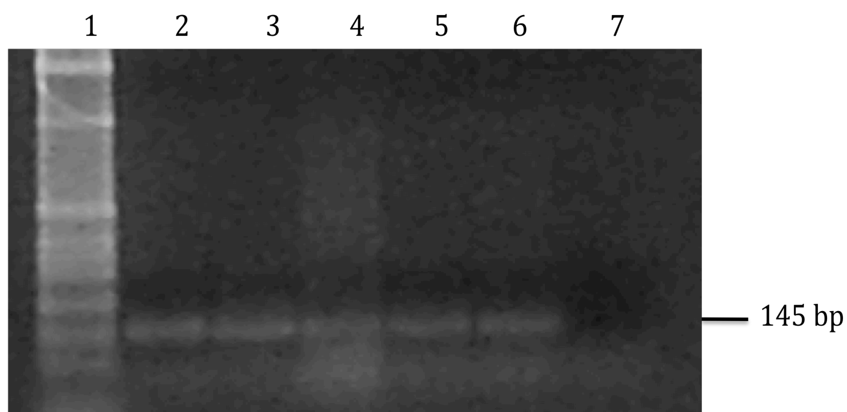

Figure 4.2.2. INF-τ mRNA in Day 14 embryos collected from pregnant cows. Lane assignments: 1- 1 Kb ladder, 2- embryo 1176, 3- embryo 1277, 4- embryo 3018, 5- embryo 232, 6- embryo 267, 7- Water

4.3 Differentially expressed genes identified by microarray

Microarray analysis revealed statistically significant differential expression in 35 genes, of which 29 increased and 6 decreased expression relative to non-pregnant CL (Tables 4.3.1 and 4.3.2). Fold changes ranged from 3.58 to 1.52 for up-regulated genes and -1.6 to -1.5 for down-regulated genes. Up-regulated genes cover a wide span of cellular and biological functions. Six genes have known functions in to transcriptional regulation, and five genes encode proteins associated with immune function. Other up-regulated genes have known functions associated with signal transduction, ion transport, DNA replication, metabolism, cell cycle progression, cytoskeletal components, angiogenesis, cell survival, cell differentiation, protein splicing, anti-adhesion properties, and hormone activity (Table 4.3.1). Similarly, the six down-regulated genes encoded proteins involved in ion transport, angiogenesis regulation, signal transduction, metabolism, and hormone activity (Table 4.3.2).

Table 4.3.1 Genes up-regulated ≥1.5-fold in the pregnant cow corpus luteum

Biological Function	Gene Symbol	Gene Name	Fold Change	P-Value
Transcription Regulation	BHLHE40	Basic helix-loop-helix family member e40	2.94	0.042
	ANKRD1	Ankyrin repeat domain 1	2.75	0.003
	PER1	Period 1	2.15	0.024
	BDP1	B double prime 1	1.59	0.038
	POLR1B	Polymerase I polypeptide B	1.51	0.014
	DDX5	DEAD box polypeptide 5	1.51	0.018
Immune Function	RFX2	Regulatory factor X 2	3.58	0.011
	F2RL1	Coagulation factor II receptor-like 1	2.28	0.024
	ZC3H8	Zinc finger CCCH-type containing 8	1.62	0.033
	IL4R	Interleukin 4 receptor	1.59	0.038
	SECTM1	Secreted and transmembrane 1	1.54	0.020
Ion Transport	NFATC1	Nuclear factor of activated T-cells, calcineurin-dependent 1	1.93	0.050
	KCNK17	Potassium channel, subfamily K, member 17	1.63	0.015
Signal Transduction	RASA2	RAS p21 protein activator 2	1.66	0.001
	BRWD1	Bromodomain and WD repeat domain containing 1	1.61	0.011
	RAPGEF1	Rap guanine nucleotide exchange factor 1-like, transcript variant 1	1.57	0.041
DNA Replication	BAZ1A	Bromodomain adjacent to zinc finger domain 1A	1.71	0.046
	CHD1	chromodomain helicase DNA binding protein 1	1.63	0.005

Table 4.3.1 (Continued) Genes up-regulated ≥1.5-fold in the pregnant cow corpus luteum

Metabolism	B4GALT5	Beta 1,4 galactosyltransferase polypeptide 5	2.70	0.047
	INSIG1	Insulin induced gene 1	2.23	0.011
Cell Cycle Progression	MGC148992	Similar to RGC-32	1.54	0.015
	ATR	Ataxia telangiectasia and Rad3 related	1.54	0.014
Cytoskeleton	NDRG1	N-myc downstream regulated 1	1.73	0.013
Angiogenesis	ENPEP	Glutamyl aminopeptidase	1.58	0.021
Cell Survival	KLF11	Kruppel-like factor 11	1.53	0.043
Cell Differentiation	IFRD1	Interferon-related developmental regulator 1	1.88	0.027
Protein Splicing	SFPQ	Splicing factor proline-glutamine-rich	1.66	0.014
Anti-adhesion	TNC	Tenascin C	2.16	0.033
Hormone Activity	LHCGR	Luteinizing hormone receptor	1.52	0.037

Table 4.3.2. Genes down-regulated ≥1.5-fold in the pregnant cow corpus luteum

Biological Function	Gene Symbol	Gene Name	Fold Change	P-Value
Angiogenesis Regulation	TSPAN12	Tetraspanin 12	-1.504	0.004
Signal Transduction	GNGT1	Guanine nucleotide binding protein, gamma transducing activity polypeptide 1	-1.670	0.004
Ion Transport	KCNN2	Potassium intermediate/small conductance calcium activated channel, subfamily N, member 2 transcript variant	-1.531	0.002
	S100A8	S100 calcium binding protein A8	-1.548	0.050
Metabolism	FABP4	Fatty acid binding protein, adipocyte	-1.520	0.006
Hormone Activity	SST	Somatostatin	-1.660	0.003

4.4 Real-time RT-PCR analysis of selected genes

From the differentially expressed genes shown in the microarray, four genes were chosen for validation: LHCGR, IFRD1, INSIG1, and SST. Other genes chosen for further analysis by RT-PCR included genes involved in: prostaglandin synthesis (COX1, COX2, AKR1C4, PTGES, CBR1), growth hormone/IGF-1 signaling (GHR, IGF-1, IGF-1R, IGFBP3), interferon-related genes (ISG15, IFNAR1, IFNAR2), and reproductive hormone receptors (PGR, ESR1, ESR2, PTGFR, PTGER2) (Table

4.4.1). Real-time RT-PCR indicated that mRNA for COX2, PTGFR, AKR1C4, and LHCGR decreased($P<0.05$) in pregnant relative to non-pregnant CL (Table 4.4.1). Significant changes in expression due to pregnancy were not observed among the remaining genes.

Table 4.4.1. Fold changes in expression for genes selected for analysis by real-time RT-PCR.

		Real-Time PCR		Microarray	
Gene Symbol	Gene Name	Fold Change[+]	P-Value	Fold Change	P-Value
Prostaglandin Synthesis:					
COX1	Cyclooxygenase 1	-1.72	0.54	-1.06	0.367
COX2	Cyclooxygenase 2	-2.89*	0.014	-1.03	0.739
PTGES	Prostaglandin E synthase	-2.01	0.216	-1.01	0.942
AKR1C4	Prostaglandin F synthase	-1.50*	0.035	-1.12	0.11
CBR1	PGE-9-ketoreductase	-1.35	0.51	-1.25*	4.00E-04
Reproductive Hormone Receptors:					
PTGFR	Prostaglandin F receptor	-2.56*	0.033	-1.15	0.493
PTGER2	Prostaglandin E2 receptor	-9.5	0.05	1.09	0.308
LHCGR	Luteinizing hormone receptor	-2.73*	0.048	1.52*	0.037
ESR1	Estrogen receptor alpha	-1.6	0.298	-1.19*	3.81E-10
ESR2	Estrogen receptor beta	-1.79	0.238	-1.01	0.94
PGR	Progesterone receptor	-1.29	0.106	1.03	0.623

Table 4.4.1 (Continued) Fold changes of genes verified by real-time RT-PCR. .

Growth Hormone/IGF-1 signaling:					
GHR	Growth hormone receptor	-2.84	0.075	-1.12	0.091
INSIG1	Insulin-induced gene 1	-2.45	0.26	2.23*	0.011
IGF1	Insulin-like growth factor 1	-1.9	0.238	-1.01	0.918
IGF1R	Insulin-like growth factor 1 receptor	-2.4	0.086	-1.05	0.582
IGFBP3	Insulin-like growth factor binding protein 3	-2.25	0.22	1.38	0.44
SST	Somatostatin	1.11	0.79	-1.66*	0.003
Interferon-related genes:					
IFNAR1	Interferon receptor alpha chain	-1.77	0.08	1.03	0.615
IFNAR2	Interferon receptor beta chain	-2.7	0.07	1.17	0.175
IFRD1	Interferon-related developmental regulator 1	-2.5	0.17	1.88*	0.027
ISG15	Interferon-stimulated gene 15	-1.31	0.62	-1.36	0.062

* denotes significant fold changes (P <0.05).

+ GAPDH was used as internal control for RT-PCR

Chapter 5 Discussion

This study is the first to investigate differential gene expression on a genome-wide basis in the pregnant bovine CL during maternal recognition of pregnancy compared to the non-pregnant CL. Changes in luteal gene expression may be the result of a direct effect of the conceptus. Several authors have proposed that gene modulation within the CL of early pregnancy is the result of IFN-τ traveling from the uterus to the ovary (Oliveira et al., 2008; Bott et al., 2010). Other studies have not supported this mechanism in the bovine model as gene expression is not altered in cultured luteal cells when exposed to IFN-τ (Yang et al., 2010). Arosh and co-workers (2004b) suggested changes in expression of genes involved in prostaglandin synthesis in the CL were due to movement of PGE_2 from the endometrium to the CL. In the present study, the specific factor altering gene expression was not identified. However, the gene modulation that occurs is consistent with suppression of the luteolytic mechanism and maintenance of CL function.

Down-regulation of genes encoding the enzymes COX2 and prostaglandin F synthase (PGFS) are consistent with inhibiting PGF production. Conversion of arachidonic acid to the intermediate PGH_2 by the COX enzymes is the rate limiting step in prostaglandin production (Kim et al., 2003). COX is constitutively expressed and COX2 is an inducible form of the enzyme (Arosh et al., 2004a).

Luteal COX2 mRNA on Day 16 of pregnancy, the period of maternal recognition of pregnancy, did not significantly differ between animals treated with IFN-τ and control animals (Arosh et al., 2004b). However, in cultured bovine endometrial cells, low doses of IFN-τ inhibited both PGF and PGE_2 production, whereas high doses of IFN-τ increased PGE_2 production without affecting PGF production (Binelli et al., 2000; Parent et al., 2003; Guzeloglu et al., 2004). Day 14 is the first time point where significant quantities of IFN-τ can be detected in uterine flushes. Therefore, at this early time point the lower amounts of IFN-τ present may be down-regulating all prostaglandin production. Higher concentrations of IFN-τ at Day 16 may account for the selective increase in PGE_2 production in CL observed by Arosh et al. (2004b).

PGFS is also integrally involved in luteolysis as it converts PGH_2 to PGF. PGFS production is consistent throughout all phases of the estrous cycles (Arosh et al. 2004a). However, results presented here are the first to show down-regulation of PGFS in early pregnancy. This decrease in PGFS may allow luteal synthesis to favor PGE_2 production because despite no significant change in prostaglandin E_2 synthase (PGES), PGES converts PGH_2 to PGE_2 at a much faster rate (150-fold) than PGFS converts PGH_2 to PGF (Madore et al., 2003; Thoren et al., 2003). This observation supports the suggestion of other authors, where the ratio of PGE_2/PGF may play an important role in CL maintenance (Arosh et al., 2004b).

Down-regulation of the PGF receptor may also play an important role in rescue of the CL. During maternal recognition of pregnancy, the luteolytic pulses of PGF are inhibited, but basal production of PGF is unaffected by IFN-τ (Okuda et al., 2002). Down-regulation of PGF receptors in the early pregnant CL may be another mechanism by which sensitivity of the CL to luteolytic PGF is decreased.

Significant differential expression of several other novel genes in the Day 14 pregnant CL were identified after analysis of microarray results. These included insulin-induced gene 1 (INSIG1), interferon-related developmental regulator 1 (IFRD1), somatostatin (SST), and the luteinizing hormone receptor (LHCGR). However, upon validation using real-time RT-PCR these modifications were shown not to be significant, except for the LHCGR. When analyzed using real-time RT-PCR, expression of the LHCGR gene was shown to actually decrease significantly, as opposed to the increase as shown by the microarray. This inconsistency between the two techniques is most likely due to the microarray probes for LHCGR not binding all of the isoforms of LHCGR. The LH receptor has been reported to have four different isoforms (Kawate and Okuda, 1998; Nogueira et al., 2007). Of the four, the microarray probes were designed in such a manner that they did not bind the isoform with the exon 3 deletion. This may account for the differences in magnitude of the fold-changes between the microarray and real-time RT-PCR, and how each could be statistically significant.

As real-time RT-PCR accounted for all isoforms of LHCGR, on Day 14 an overall decrease in expression of the LH receptor was observed. This result is somewhat unexpected as LHCGR is important for production of progesterone from small luteal cells (Diaz et al., 2002). However, it is possible that this fold-decrease does not reflect a true change in transcription rate. The microarray identified an increase in mRNA for a number of novel genes on Day 14 . Therefore, it is possible that the transcription rate for LHCGR mRNA did not change as other genes were being up-regulated and as a result contributed to a lower percentage of the total RNA in the pregnant CL compared to the non-pregnant CL. Because both pregnant and non-pregnant PCR reactions were with an equal amount of total RNA, the lower concentration of LHCGR mRNA in the pregnant CL could be interpreted as a decrease in expression.

In conclusion, on Day 14 of pregnancy in the cow, genes involved in the production of PGF are down-regulated in the CL. This down-regulation may play a critical role in maintenance of the CL for successful establishment of pregnancy.

Chapter 6 General Conclusions

Maternal recognition of pregnancy is the physiological process whereby the embryo signals its presence to the maternal system. The pregnancy recognition signal in cattle is IFN-τ (Bazer, 1992; Roberts et al., 1992). IFN-τ is known to inhibit luteolysis through down-regulation of estrogen and oxytocin receptors within the endometrium, thereby inhibiting release of luteolytic pulses of PGF and rescuing the CL from destruction (Robinson et al., 1999, Robinson et al., 2001). Developing bovine embryos also produce PGE_2, which may play a role in luteal maintenance (Lewis et al., 1982; Hwang et al., 1988; Wilson et al., 1992). However, not much is known about direct effects of the embryo on the CL itself. The purpose of this study was to determine what kind of gene modulation occurs within the CL at the onset of maternal recognition of pregnancy.

Several studies have shown changes in gene expression in the early pregnant CL in sheep or at a later day of pregnancy in cattle (Arosh et al., 2004; Oliveira et al., 2008; Bott et al., 2010; Yang et al., 2010). Results of this study support the theory that there is gene modulation within the CL during maternal recognition of pregnancy. On Day 14 of pregnancy, expression of genes involved in intraluteal PGF synthesis are significantly down-regulated, perhaps contributing to CL maintenance. Other studies have theorized about the importance of intraluteal PGF synthesis for the completion of luteolysis (Tsai and

Wiltbank, 1997; Hayashi et al., 2003). Data presented here support a role for down-regulation of intraluteal PGF synthesis in CL rescue. Findings presented here also demonstrate the occurrence of gene modulation in the CL as early as Day 14 of pregnancy in cattle.

However, this question still remains: what factor is causing the modulation of genes within the CL during maternal recognition of pregnancy? Several investigators have suggested that it is IFN-τ, while others have proposed that communication between the conceptus and the CL is mediated by immune cells (Oliveira et al., 2008; Bott et al., 2010; Yang et al., 2010). Further studies need to be conducted to elucidate the mechanism by which the presence of the conceptus alters gene expression within the CL. Examination of global gene expression at other time points during early pregnancy would be useful to develop a comprehensive overview of the gene modulation occurring within the CL during maternal recognition of pregnancy.

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