ANALYSIS OF ENDOMETRIAL GENE AND PROTEIN ALTERATIONS FOLLOWING ENDOCRINE DISRUPTION IN THE PREGNANT GILT

By
MORGAN DEAN ASHWORTH JR

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ANALYSIS OF ENDOMETRIAL GENE AND PROTEIN ALTERATIONS

FOLLOWING ENDOCRINE DISRUPTION IN THE PREGNANT GILT

Thesis Approved:

Dr. Rodney Geisert
Thesis Adviser

Dr Jerry Malayer

Dr. Jerry Ritchey

Dr. Udaya DeSilva

Dr Gordon Emslie
Dean of the Graduate College

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Chapter I

Introduction

During estrus, the pig will ovulate 14-16 viable oocytes, however, the national average litter size is usually 10 piglets (Perry and Rowlands, 1962; Pope and First 1985). Placentation in the pig involves a noninvasive epithliochorial type attachment to the uterine epithelial surface microvilli that becomes slightly altered during implantation (Dantzer et al., 1985). Covering the surface of the uterine microvilli is the glycocalyx, a thick, carbohydrate rich coat that serves as an attachment matrix for the elongating conceptus to anchor to the uterus (Keys and King, 1990).

Estrogen, originating from the elongating conceptuses on Day 11.5 of pregnancy, serves as a multi-purpose signal to prepare the uterus for the opening of the implantation window (Thatcher and Bazer, 1977). Conceptus estrogen stimulates uterine folding and the release of uterine nutrients required by the conceptus for survival (Geisert et al., 1982b; Roberts et al., 1993). However, if the pregnant pig uterus is prematurely exposed to exogenous estrogen (Days 9 and 10) prior to the normal release by conceptuses on Day 12 of pregnancy, complete embryonic mortality will occur (Pope et al., 1986). The mechanism through which this endocrine disruption occurs remains to be elucidated. Studies by our laboratory established that the administration of estradiol to gilts on Days 9 and 10 of pregnancy resulted in complete conceptus degeneration by Day 16 of pregnancy (Morgan et al., 1987; Greis et al., 1989). Conceptus degeneration was

associated with removal of the glycocalyx from the uterine surface microvilli (Blair et al., 1991).

The purpose of the current investigation is to identify factors involved with the alterations in uterine secretory patterns resulting from early estrogen exposure in the pregnant gilt. The following review of pertinent literature will address issues in regards to proper timing and release of maternal contributions paramount for the opening of the uterine implantation window and establishment of pregnancy. Due to the importance of the glycocalxy for conceptus attachment, uterine growth factors and structural components will be reviewed.

Chapter II:

Literature Review

Endocrine synopsis of the porcine estrous cycle

The pig is a polytocous species which ovulates approximately 16-18 oocytes per estrus. The estrous cycle in the pig consists of two major phases (follicular, luteal) resulting from an orderly sequence of changes associated with the actions of uterine, ovarian, and higher brain hormones. Porcine estrous cycle, which is 21 Days in duration, is comprised of four major stages (proestrus, estrus, metestrus, and diestrus). Proestrus (Days 17-20) is initiated following the regression of the corpora lutea (CL) and the decline of plasma progesterone. Proestrus is distinguished by a rapidly growing pool of multiple ovarian, estrogen active follicles recruited by plasma follicle stimulated hormone (FSH). Estrus is dominated by ovarian follicular secretion of estradiol-17β, the major type of estrogen produced from the growing graafian follicles with peak plasma concentrations of 45 pg/ml occurring on Days 18-20 of the estrous cycle (Henricks, et al., 1972). Estrogen released from the growing follicles has a positive feedback on the basal medial nuclei of the hypothalamus to increase the frequency of pulse release of FSH and luteinizing hormone (LH). Ovulation is stimulated through the surge release of LH during estrus. In the pig, ovulation is variable, occurring during a 40 to 72 hour window during the expression of estrus. Metestrus follows the cessation of estrus and lasts about 1-2 days**.** Metestrus is the stage where transformation of corpora hemorrhagica to corpora lutea occurs. The development of fully functional CL initiates the stage of diestrous. Presence of elevated plasma progesterone concentrations exerts a negative feedback on the hypothalamic preovulatory center allowing only for the tonic release of

gonadatrophin releasing hormone (GnRH). Follicular growth and estrogen synthesis is suppressed through the low frequency release of FSH and LH until the CL regresses. High concentrations of progesterone in the blood inhibit myometrial contractions while exerting a positive response on the endometrial gland secretion to provide a suitable environment for potential embryonic development in the pregnant gilt. In the absence of conceptus stimulation, progesterone concentrations decline starting at Day 15 (Masuda et al., 1976), reaching a nadir (less than 1ng/mL) by Day 18 of the estrous cycle (Guthrie et al., 1972). The high concentration of plasma progesterone from the CL will in time (10 days) provide a mechanism from which uterine originated secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulates regression of the CL (Cavazos et al., 1969). Porcine CL synthesize and secrete oxytocin which increases in plasma during luteolysis. An increase in oxytocin is associated with an increase in uterine secretion of $PGF_{2\alpha}$ (Kotwica et al., 1990). The phosphoinositide pathway is postulated as the principal signaling pathway which endometrial oxytocin receptors initiate luteolysis in the cycling pig (Whiteaker et al., 1994).

Embryonic development in the pig

Porcine conceptus development is regulated through the uterine secretory changes that are driven by the first release of progesterone from the functional CL into the ovarian vein (Roberts et al., 1993). Following hatching of the porcine blastocyst from the zona pellucida on Day 8 of pregnancy, peri-implantation conceptus morphogenesis encompasses four critical developmental stages prior to trophoblast attachment to the uterine surface: spherical, ovoid, tubular, and filamentous (Anderson 1978). Free

floating conceptuses will equidistantly space themselves throughout the uterine lumen in preparation for trophoblastic elongation (Pope et al., 1982).

Porcine conceptuses undergo an ordered transformation in morphology during early gestation (Geisert et al., 1982b). Heuser and Streeter (1929) were the first to describe conceptus transformation from a spherical to filamentous morphology between Days 10 and 12 of gestation. On Days 10 and 11.5 of gestation, conceptus size can range from 4 mm to 10 mm spherical diameter. Growth or expansion of the spherical conceptus diameter occurs at an approximate rate of 0.25 mm/hr (Geisert et al., 1982b). Increase in spherical diameter of the conceptus occurs largely through cellular hyperplasia (Geisert et al., 1982b). Prior to period of the rapid trophoblast elongation on approximately Day 12 of gestation, 10 mm spherical conceptuses undergo a conversion to the tubular morphology. Once initiated, the tubular morphology is attained approximately 2 to 4 hr before rapid expansion of the trophoblast occurs Trophoblast expansion from tubular to the filamentous (reaching 100 mm to 200 mm) morphology will occur within 1 to 2 hr (Ross, Ashworth, and Geisert unpublished data). Upon initiation of rapid trophoblastic elongation, there is a temporal increase in conceptus steroidogenesis to increase estrogen synthesis through activation of aromatase expression (Conley et al., 1992). Increased aromatase activity allows conceptuses to have a surge release of estrogen into the uterine lumen, where it serves as the maternal recognition of pregnancy signal in the pig (Geisert et al., 1982, Bazer 1986). Mattson et al. (1990) proposed that the cytoskeletal changes for trophoblast expansion involved actin filaments which they classified into three different activities. The activities involving actin included: (1) rearrangement of the trophectodermal plasma membrane during conceptus

expansion, (2) generation of adequate force to bring about expansion of the conceptus, and (3) mobilization of cortical actin to assist in differentiation and maintenance through stabilization of epithelial transport properties. With the information at hand one could propose that the conceptus morphological changes occur through an autocrine mechanism which is independent of maternal uterine signaling and does not involve maternal signaling. Although early conceptus development and survival is dependent upon conceptus synchrony within the developing uterine secretory environment, rapid trophoblastic elongation is clearly conceptus driven. The presence of multiple conceptus morphologies can be observed in any single pregnancy indicating that trophoblast elongation is initiated by reaching a specific stage of spherical growth (10 mm). The process of conceptus expansion in the pig is critical to subsequent survival as each conceptus strives to increase the maximum uterine surface area possible for placental attachment as it competes against contemporary littermates. An increase in uterine placental contact will allow the conceptus to increase its nutrient uptake, giving it a selective advantage over slower developing conceptuses when ovulation rates exceed uterine capacity (Geisert and Yelich, 1997).

Although the developing conceptuses synthesize and secrete estrogen on Day 12 of gestation, uterine growth factors are required for competent conceptus signaling and proliferation. Insulin-like growth factor I (IGF-I) is synthesized and secreted into the uterine lumen by the endometrium prior to and during early conceptus periimplantation development (Tavakkol et al., 1988). The maximal concentrations of uterine luminal IGF's are present at the time of conceptus elongation which is coincidental with the initiation of the increase in cytochrome P450 aromatase ($P450_{arom}$) and cytochrome P450

17 α-hydroxylase ($P450_{17\alpha}$) activity (Green et al., 1995). Up regulation of IGF-I mRNA and IGF-I ligand released into the uterine lumen is synchronous with acute increase in conceptus estrogen production on Day 12 of gestation. Fischer et al. (1985) indicated the capacity of the endometrium to synthesize estrogen is negligible, therefore estrogen present in the uterine lumen is almost totally originates from the conceptuses. The biphasic patterns of conceptus estrogen synthesis and release during elongation and placental attachment to the uterine surface (Day 12 and Day 15 to 30) is paramount in prolonging the lifespan of the corpora lutea for maintenance of pregnancy throughout gestation in the pig. Initial estrogen synthesis by the conceptuses on Day 12 of pregnancy may serve a tri-fold role: equidistant spacing of conceptuses within the uterine horns, regulation of growth factors essential for conceptus differentiation and survival, and maternal recognition of pregnancy. The free floating conceptuses awaiting apposition to the uterine surface are exposed to a variety of uterine growth factors that are secreted following conceptus release of estrogen. Receptors for the various growth factors are expressed on the pre and post-elongated conceptus (Geisert and Yelich, 1997). For example, Vaughan et al. (1992) demonstrated epidermal growth factor (EGF) receptors were present on the trophoblast of the conceptus between Days 8 to 12 of pregnancy with greatest binding observed in Day 10 conceptuses. Appearance of high affinity binding regions for EGF suggest that conceptus development and growth may be dependent on an assortment of maternally derived growth factors. Clearly, although trophoblast elongation is under conceptus regulation, endometrial growth factor secretion and conceptus estrogen secretion are critical for proper signaling for embryonic growth and survival.

Maternal Recognition of Pregnancy

Short (1969) was the first to coin the term "maternal recognition of pregnancy" for the extension of the corpora lutea beyond normal length of an estrous cycle by the conceptus. Corpora lutea maintenance provides a continual source of progesterone needed to support conceptus development and survival throughout gestation in the pig (Nara et al., 1981). Bazer et al. (1982) first established that estrogen is the maternal recognition of pregnancy signal in the pig. Estrogen synthesis and secretion by the conceptuses occur in a biphasic pattern with an acute, intermediate peak on Day 12 of gestation, followed by a chronic sustained release of estrogen from Days 15 to 28 of gestation (Geisert et al., 1990). Dhindsa and Dziuk (1968) demonstrated that conceptuses must be present in both uterine horns until at least Day 18 of pregnancy to extend gestation beyond 30 days in the pig. Dzuik et al. (1968) indicated prior to Day 18 there must be at least two conceptuses per uterine horn for a successful pregnancy to establish in the pig. The need for a minimal number of conceptuses in each horn can be attributed to the extensive surface area of the uterine horns which synthesizes $PGF_{2\alpha}$ to regress the CL during the estrous cycle. Conceptuses equidistantly space themselves throughout the uterine horns prior to trophoblastic elongation so that following expansion, sufficient inhibition of the luteolysin $PGF_{2\alpha}$ occurs within the uterus. Adequate distribution of estrogen throughout the length of both uterine horns is therefore accomplished through the rapid trophoblast elongation on Day 12 of pregnancy. Conceptuses must inhibit $PGF_{2\alpha}$ in both uterine horns because the porcine lungs fail to metabolize about 82% of the

systemic $PGF_{2\alpha}$ that moves through in one passage. Thus, the pig has a systemic as well as local pathway for luteolysis (Davis et al., 1979)

The mechanisms and processes of maternal recognition of pregnancy in the pig have been comprehensively reviewed (Bazer et al., 1982, 1984, 1986, 1989; Geisert et al., 1990). Bazer and Thatcher (1977) presented the original theory that pregnancy recognition in the pig involved an estrogen stimulated alteration in $PGF_{2\alpha}$ movement within the uterus during early pregnancy. These authors proposed the classical endocrine/exocrine theory for CL maintenance in the pig which still holds today. These researchers suggest that conceptus estrogen synthesis redirects movement of endometrial $PGF_{2\alpha}$ release towards the uterine lumen in an exocrine pathway. In contrast, endometrial $PGF_{2\alpha}$ release is directed in an endocrine pathway through the stroma into the uterine vasculature network in the cyclic pig. Release of $PGF_{2\alpha}$ into the utero-ovarian portal vessels allows transport to the CL for induction of luteolysis.

Endometrial changes and ECM remodeling for receptivity of the porcine uterus.

The extracellular matrix (ECM) in the uterus is a complex network comprised of a plethora of molecules involved with cell migration and cell-to-cell interactions (Bost et al., 1998). In the pig, the endometrial ECM expression on the epithelial apical surface prevents conceptus invasion into the uterine endometrium forming the epitheliochorial type of placentation. However, conceptus exposure to tissue and matrices outside of the uterus results in cellular erosion and invasive activity (Samuel and Perry, 1972). These data indicated that the porcine conceptus is highly proteolytic and the maternal uterine environment inhibits active invasion through the uterine surface epithelium. The

architecture of the endometrial epithelium is dictated through changes in ovarian steroids, growth factors, cytokines and other regulatory molecules such as integrins.

Spatiotemporal alterations in uterine epithelium are paramount for successful remodeling of the uterine ECM in preparation for attachment of rapidly developing porcine conceptuses. Cell to cell and cell to ECM interactions are arbitrated through a family of cell transmembrane glycoproteins known as integrins (Lessey et al., 1995). Cell surface expression of the repertoire of integrins can be altered in response to inflammation (Heino et al., 1993), cell differentiation (Damsky et al., 1993) and are involved with tumor malignancy transformation (Plantefaber et al., 1989). Integrins are comprised of an α and β subunit that function as a transmembrane heterodimer receptor involved with cellular signaling proteins (Clark et al., 1995). Currently, 16 different α and 8 β subunits have been identified within the 22 possible integrin combinations identified in cells (Luscinskas et al., 1994). The various subunit combinations determine specificity for ECM ligands and functionality of the uterine integrins (Hynes et al., 1992). In the human, 9 integrin combinations have been characterized in uterine endometrium (Lessey et al., 1992). In the pig uterus, α_1, α_3, α_4, α_5, α_v β_1, and β_3 integrin subunits have been identified and characterized (Bowen et al. 1996). Conceptus trophectoderm and uterine epithelium express α_4, α_5, α_v, β_1 and β_3 in peak levels on Days 11 to15 of gestation which is the period of trophoblast attachment to the uterine surface epithelium. Heterodimers: $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_1$ serve as receptors for the fibronectin ligand, while $\alpha_v\beta_1$ and $\alpha_v\beta_3$ serve as receptors for the vitronectin ligand (Hynes et al., 1992). Day 12 and 15 conceptuses contain fibronectin on their trophoblast surface, which would allow adhesion to the luminal epithelial surface integrins (Tuo and Bazer, 1996). Furthermore,

Bowen et al. (1996) also demonstrated the presence of vitronectin at the site of conceptus attachment to uterine luminal epithelium, inferring interaction of integrins and their ligands between both the uterine surface and trophectoderm

Prior to the time of trophoblast attachment to the uterine luminal surface, the porcine uterine surface epithelial apical microvilli contains a thick extracellular glycocalyx, which becomes reduced in density following conceptus attachment (Dantzer et al., 1985). Mucin-1 (MUC-1) is a heavy glycosylated integral transmembrane glycoprotein expressed on the surface of epithelial cells which inhibit conceptus attachment to the uterine surface. Bowen et al. (1996) demonstrated that gene expression of MUC-1 is diminished by Day 10 of gestation in the pig. Decrease of MUC-1 expression on the uterine epithelium corresponds to down regulation of progesterone receptors within the uterine surface epithelium, which appears to be the opening of the implantation window for conceptus attachment. The decline observed in MUC-1 expression by Bowen and coworkers (1996) is consistent with loss of MUC-1 expression prior to implantation in rodents (Braga and Gendler, 1993). These results suggest a loss of MUC-1 expression may allow intimate contact of the conceptus trophectoderm with uterine epithelium for placental attachment.

Stabilization of the ECM is accomplished through a family of protease inhibitors known as the inter-α-trypsin-inhibitors (ITI) (see Bost et al., 1998). The ITI family is comprised of a combination of four heavy chains (HC1, HC2, HC3, HC4), and one light chain referred to as bikunin (Salier et al., 1996). All four heavy chains contain a Von Willebrand type A domain, which serves as a binding domain for heparin, proteoglycans, collagen, and integrins (Colombatti and Bonaldo, 1991). Bikunin, contains a 4-

chondrotin sulfate glucosaminoglycan (GAG) chain that binds to ITIH1, ITIH2, and ITIH3. Bikunin, binds to ITIH3 to form Pre-α-inhibitor a positive acute phase protein, or to ITIH1 and ITIH2 to form ITI which is a negative acute phase protein (Daveau et al., 1993). ITI's belong to the family of kunitz-type protease inhibitors (see Salier et al., 1996), which contain a domain that is inhibitory to serine proteases. In contrast, ITIH4 does not contain a bikunin binding region (Salier et al., 1996; Bost et al., 1998) and is readily cleaved by the serine protease tissue kallikrein (Nishimura et al., 1995). Geisert et al. (1998) suggested that the ITIH's serve to inhibit an acute phase reaction from occurring in the endometrium during the inflammatory type stimulation caused by the attaching conceptuses. These researchers indicated that ITIH4 expression in the endometrium was present throughout the estrous cycle and early gestation. Gene expression appeared enhanced under the influence of progesterone in the mid-luteal phase of the estrous cycle and during placental attachment in early gestation (Geisert et al., 1998). ITIH4 has been characterized as an acute phase protein (Gonzales-Ramon et al., 1995), which can be induced by interleukin-6 (IL-6), a transcriptional regulator associated with acute phase reactions (Sarafan et al., 1995). Bhanumathy et al. (2002) demonstrated that murine liver explants dramatically increased ITIH4 expression following treatment with IL-6. However, the proinflammatory cytokines interleukin 1β (IL-1β) and tumor necrosis factor alpha (TNF_{α}) do not invoke an increase in ITIH4 expression, suggesting it may play an integral part in stabilizing the ECM (Chen et al., 1994). Geisert et al. (1998) suggested a role for ITIH4 stabilization of the uterine epithelial surface glycocalyx to prevent conceptus cellular erosion and invasion into the uterine stroma.

The ITI's are involved with ECM stabilization through the binding to hyaluronic acid (HA), which is synthesized in the plasma membrane by the enzyme hyaluronan synthase (HAS) (Prehm et al., 1989). To date, three isoforms of HAS (HAS-1, HAS-2, HAS-3) have been identified (Itano and Kinata, 1996, Spicer et al., 1996, 1997). Hyaluronic acid size (polymer length) and the rate of synthesis may be attributed to the differential expression from the three distinct isoforms of the HAS enzyme (Itano et al., 1999). HAS-1 expression has been characterized in murine dermal fibroblastic cells, while HAS-2 expression has been observed in most tissues of the developing murine embryo (Sugiyama et al., 2002). Deletion of HAS-2 is fatal to mouse embryo by Day 9.5 of gestation mainly resulting from defects in cardiovascular morphogenesis (Camenisch et al., 2000). In the human, articular chondrocytes have been shown to express both HAS-2 and HAS-3 (Nishida et al., 1998). All three HAS enzymes have been localized in human cartilage (Recklies et al., 2001). Studies have demonstrated that insulin-like growth factor-1 (IGF-1) (Honda et al., 1991), tumor growth factor-β (TGF-β) (Sugiyama et al., 1998) and bone morphogenic protein 7 (BMP-7) (Nishida et al., 2000) stimulate HA synthesis through activation of HAS in a variety of cell types. Hyaluronic acid has been implicated in cell to cell, cell to matrix interactions, and cellular migration (Fraser et al., 1992). To date, only the ITI family referred to as serum derived hyaluronan associated proteins (SHAP) bind covalently to HA. The SHAP-HA complex (HA + HC1 or HC2) form when plasma and HA are mixed together (Huang et al., 1993). In the mouse ovary, the formation of the SHAP-HA complex further stabilizes the expanding cumulus ECM via releasing bikunin from ITI's during formation, enhancing its serine protease inhibitory activity. Coupling of ITI-HA is dependent on the presence of Ca^{+2}

ions and is paramount for normal fertility in the mouse (Sato et al., 2001). Studies with rheumatoid arthritis patients have demonstrated that increased concentrations of ITI-HA in synovial fluids are associated with chronic inflammation (Hutadilok et al., 1988). During inflammation tumor necrosis factor-stimulated gene 6 (TSG-6) is detected in synovial fluid (Wisniewski et al., 1993, 1997). Secretion of TSG-6 in rheumatoid arthritis patients allows binding to HA and either ITIH1 or ITIH2 (Mukhopadhyay et al., 2001), which can act as a crosslinker between different SHAP-HA molecules to stabilize the ECM in attempt to inhibit erosion of joints. The large mosaic hyaluronan-proteoglycan aggregated complexes may anchor to cells through hyaluronan binding to cell surface receptors such as CD44 (Chow et al., 1995). During stabilization of the ECM, HC1 and HC2 are released from its covalent bond with bikunin and transferred to HA (Jessen and Odem, 2003). Furthermore, bikunin released from the complex can suppress inflammation through inhibition of plasmin (Wisniewski et al., 1996). The proposed model for ECM stabilization is depicted in (Figure 2.1).

Site and specificity of uterine tissue remodeling and ECM turnover is accomplished primarily through the matrix metalloproteinase (MMP) system. The MMP system consists of an assortment of zinc and calcium dependent matrixins and their endogenous inhibitors TIMPS (tissue inhibitors of matrix metalloproteinases). Generally, MMP's are implicated in tissue remodeling due to their ability to degrade all ECM components (Woessner et al., 1991). A disruption in the balance of MMP's and TIMP's usually results in a serious pathological condition such as fibrosis (Nagase et al., 1997). MMP's are synthesized and secreted in a latent pro-form (Sato et al., 1994). Various pathological conditions (rheumatoid and osteoarthritis), demonstrates that excessive

amounts of activated MMP expression accelerates connective tissue breakdown (Aherns et al., 1996). Arthritis and cancer are pathological examples where the tightly regulated MMP system is functioning outside of normal conditions, and irreversible catastrophic degradation has occurred.

In the pig and most other ungulates, conceptus attachment does not involve deep trophoblastic invasion into the uterine mucosa (Dantzer et al., 1985). However, uterine MMP-2, MMP-3 and MMP-9 are the predominate matrixins in the cow (Maj et al., 1997, Salamonsen et al., 1995, Menino et al., 1997) and TIMP 1, TIMP-2, and TIMP 3 are the MMP inhibitors expressed during the estrous cycle and early pregnancy of the pig. MMP-2 and MMP-9 are primarily responsible for type IV collagen degradation, which is the predominant constituent of the cellular basement membrane (Lenhart et al., 2001). In the pig, TIMP expression has been localized in the trophectoderm and endometrial stroma during early gestation (Menino et al., 1997), heavy expression of TIMP's in uterine endometrial stroma and absent in the epithelium, may account for the lack of deep uterine invasiveness displayed by the conceptus in the receptive porcine uterus. In summary, it is apparent that the MMP system is involved with many aspects of uterine remodeling and conceptus attachment during early pregnancy. However, the specific action through which proteases permit remodeling while retaining specialized tissue functions still requires further investigation.

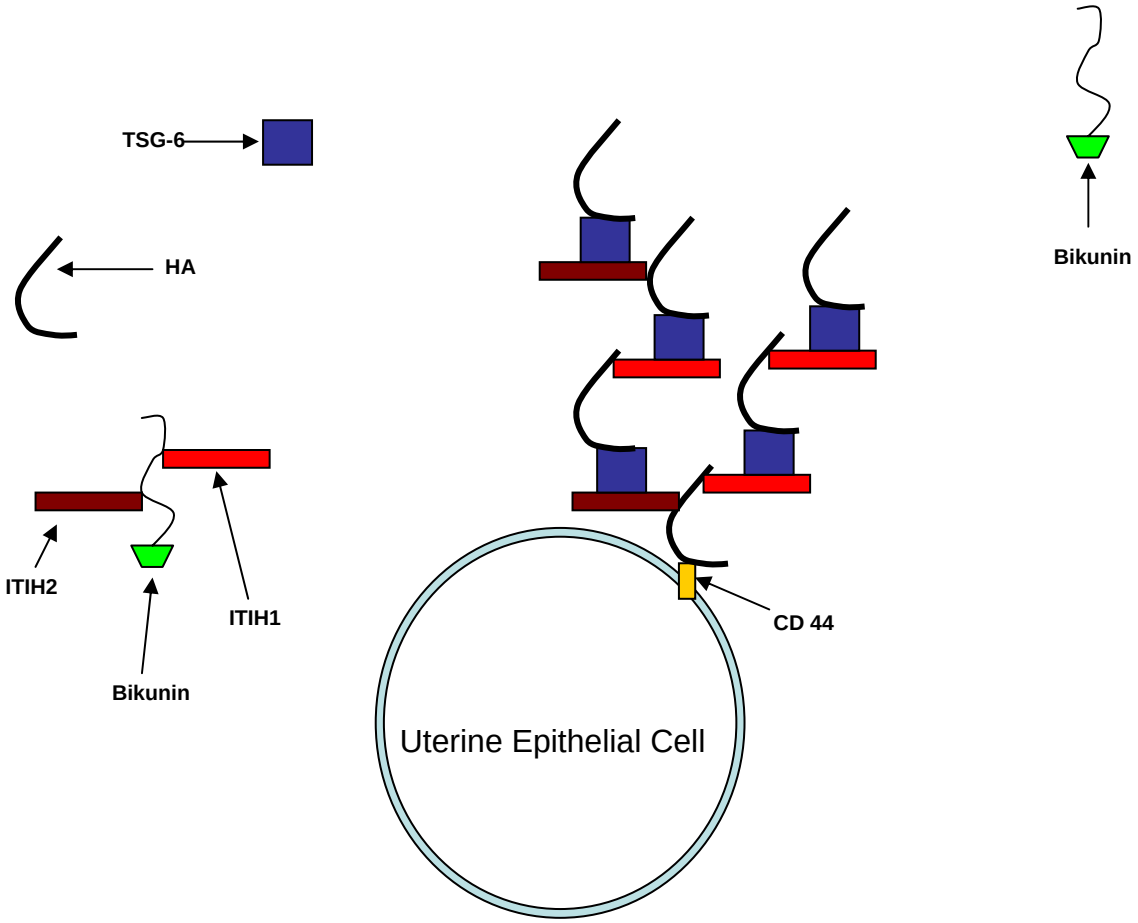


Figure 2.1 The inter-α-inhibitor is compreised of ITIH1 (75 kDa) and ITIH2 (80 kDa) covalently linked to bikunin through its chondroitin sulfate chain. The heavy chains of the inter-α-inhibitor will become covalently coupled to hyaluronan molecules that exhist in the uterus either bound to an epithelial cell surface receptor (CD44) or in the free form. During the assembly and stabilization process of the extracellular matrix (ECM), bikunin is released and its antiplasmin activity is greatly enhanced in the free form. Through an unknown process, the tumor necrosis stimulated gene 6 (TSG-6) protein displaces bikunin and becomes linked to the heavy chain molecules. TSG-6 contains an hyaluronan binding domain, it can cross-link hyluaronan molecules stabilizing the ECM on the uterine surface to mediate attachment between conceptus and uterine epithelium.

Uterine inflammatory and immunoregulation

In the pig, the role and regulation of uterine immunity is poorly understood. Successful pregnancy in the pig is associated with acute and long term inflammation (Bischof et al., 1994). Proinflammatory mediators, such as IL-1β, are secreted maximally on Day 12 of gestation by the rapidly developing conceptuses, inferring local immunity involved in uterine responses to pregnancy (Ross et al., 2003). Studies in the pig have suggested that during pregnancy, the uterine immune system plays an active role in establishing pregnancy (Koch et al., 1985).

Distinguishing "self" from "nonself" to remove a potential pathogen is a rudimentary function of the mammalian immune system. However, during pregnancy, the maternal immunological system must be diverted to accept the nonself allogenic fetus, as self. Exposure of potential pathogenic substances begins with copulation and exposure to male semen (Lovell and Getty, 1968). Upon mating, the female reproductive tract must be tolerant of two non-self products in semen: 1) spermatozoa, which contain paternal cell antigen markers, and 2) "nonself proteins" originating from the seminal plasma. During gestation, the female must be tolerant to the contribution of the antigens presented to the maternal immunological system. Although one would theorize rejection of the fetus and its extraembryonic membranes should be a high probability, maintenance of the mammalian pregnancy is normally highly successful.

Immune responses are classified into two major categories called innate and adaptive. Innate immunity serves as the first line of defense against pathogens in the mammalian body; however this system lacks the ability of long term immunity such as

reinfection prevention. Activation of the complement cascade can generate components that bind tissues, recruits effector cells, and invoke an inflammatory response. Activation of the complement system has emerged as a potential factor in recurrent fetal loss (Chaucheteux et al., 2003). Studies have demonstrated that murine trophoblast produces high amounts of proteins that inhibit complement proteins (Tedesco et al., 1993). Furthermore, murine trophoblast devoid of these inhibitory proteins results in embryonic death (Xu et al., 2000). The adaptive system is relies on specialized lymphocytes that have been selected based on their tolerance for non-reactive self recognition and a diverse repertoire of cell surface receptors. The adaptive system can store specialized lymphocytes in very small numbers in the body when they are not needed and rapidly deploy them through clonal expansion to eliminate pathogens to which they had prior exposure. Successful pregnancies in the porcine uterine environment must circumvent both systems (innate and adaptive) in order to preserve the pregnancy (Georgieva et al., 1984, Croy et al., 1987)

It is well documented that during the early porcine pregnancy (early as Day 10), trophoblastic cells recruit lymphocytes (T-cells, NK cells) to the endometrial stroma beneath conceptus attachment sites (Englehardt et al., 2002). Guimond et al. (1997) demonstrated that NK cells not T-cells, controlled trophoblastic cytokine production. Further, the above author demonstrated mice deficient of NK cells have placental hypotrophy. These data suggest uterine cells involvement in allorecognition may belong to the uterine NK cells rather than the traditional T-cells.

The porcine trophoblast produces and secretes large amount of two types of interferons (IFN) during early pregnancy (La Bonnardiere et al., 1991). Interferon

gamma (IFN γ) and interferon delta (IFN-δ) are the two forms of IFN's in the pig, but unlike bovine, do not function as a maternal recognition signal in the pig. Porcine trophoblastic cells producing substantial amounts of IFN-γ beginning on Day 13 of gestation may suggest a possible role in implantation based on the timing of its appearance, since Day 13 is the opening of the implantation window. Tuo et al. (1993) demonstrated INF-γ can have a stimulatory role on the activity of NK cells *in vitro*, suggesting NK cells play a pivotal role in placentation. The standing T-cell shift (Th_1 to Th_2) paradigm suggest that successful pregnancies are associated with an alteration in the maternal T-cell repertoire from Th_1 (IFN-γ, IL-2) to predominantly Th_2 (IL-4, IL-10), bathing the conceptus in a series of Th_2 cytokines (Lin et al., 1993). The Th_1/Th_2 paradigm significantly oversimplifies the immunoregulatory action at the conceptus-maternal interface. The identification of NK cell involvement has undermined the foundation of the Th_1/Th_2 shift paradigm. Data generated from Croy's lab has demonstrated that NK cells have a two-fold involvement with pregnancy establishment; 1) maternal vascularization through its secretion of angiopoietin 2 (ANG-2) (Croy et al., 2000) and 2) NK cell regulation of trophoblastic interleukin 10 (IL-10) production, which is a Th_2 cytokine (Chaouat et al., 1998). NK cell involvement abrogated the Th_1/Th_2 paradigm, but it was through characterization of interleukin 18 (IL-18) that instigated rethought of this once accepted paradigm. Muranaka et al. (1998) demonstrated that pregnancy was lost when mouse IL-18 was co-administered with IL-12. Furthermore, mice selected based on abortive prone tendencies exhibit suppressed IL-18 levels at the fetal-maternal interface. In contrast, non-abortive mice produced elevated levels of IL-18 suggesting a Th_1/Th_2 divergence (Ostojic et al., 2003). In conclusion, most literature

questions the paradigm that a maternal shift from Th_1 to Th_2 immune cells is necessary for establishment of a successful pregnancy. Furthermore, the possibility exists that using the Th_1/Th_2 shift paradigm to explain what is happening in the uterus during early pregnancy may be oversimplifying a complex inflammatory process.

Uterine Conceptus Cross-Talk

Regulatory pathways and growth factors involved with timely porcine conceptus elongation and successful uterine attachment have been extensively studied (Perry et al., 1973, Geisert et al., 1982, Roberts and Bazer 1988, Mattson et al., 1990, Bazer et al., 1991, Vallet et al., 1996, Bowen et al., 1997). Conceptus attachment to the uterine surface epithelium and survival depends on proper synchrony with maternal uterine secretions and its interaction with the receptive endometrium.

The porcine conceptus synthesizes and secretes a variety of growth factors and expresses receptors for a variety of different cytokines and growth factors. Insulin-like growth factors I and II (IGF) are one of the most well characterized growth factors studied. The IGF family is comprised of insulin, IGF-I and IGF-II, for which these three ligands will bind their respected receptors (IGF-IR, IGF-IIR, IR). Although there is structural homology between insulin and IGF-I, these cognate ligands bind specifically to their cell surface receptors. IGF-II weakly binds to the IGF-IR, but at an affinity 15-fold lower than the IGF-I ligand (Germaine et al., 1992). The major biological regulation of these ligands are accomplished through seven known IGF binding proteins (IGFBP 1-7). IGF's can act through an endocrine or paracrine mechanism to regulate proliferation, differentiation and cellular metabolism.

During the early stages of pregnancy, time specific changes in uterine secretion occur in relation to ovarian steroids that released into the uterine vasculature. Peak release of IGF's into the uterine lumen occurs prior to and coincident with conceptus elongation and initiation of estrogen synthesis during early pregnancy (Simmen et al., 1990). Treatment of ovariectomized gilts with progesterone daily for 10 days, followed with estrogen increased release of uterine IGF-I (Hofig et al., 1991). Steroid treatment of ovariectomized gilts also stimulates an increase in uterine estrogen receptor (ER) mRNA suggesting that estrogen may regulate IGF-I release in the endometrium through its receptor (Sahlin et al., 1990,1994). Increase of IGF release and up-regulation of uterine ER is temporally associated with downstream regulation of luminal and glandular epithelial PR. Geisert et al. (1994) reported that uterine epithelial PR diminished by Day 10 of pregnancy and PR was virtually void in the epithelium by Day 12 of gestation. Loss of PR from the epithelium maybe important for the up-regulation of epithelial ER and the release of IGF-I.

For the maternal derived IGF's to potentially effect uterine and conceptus development; the endometrium and conceptus must express IGF receptors (Hofig et al., 1991). IGF-IR mRNA has been detected in the porcine endometrium, myometrium and the periimplantation porcine trophoblast (Hofig et al., 1991). *In vitro* culture of different developmental porcine conceptus morphologies with IGF-I suppressed aromatase activity in the spherical and tubular conceptuses, but increased aromatase activity in the filamentous conceptuses (Green et al., 1995). In contrast to Green's data, Chastant et al. (1994) were unable to immunologically localize IGF-IR to the porcine trophoblast but did detect the presence of the IGF-IIR/M6P. Although IGF-IIR is void of the major

intracellular signaling machinery, its primary function appears to be IGF-II inhibition and lysosomal targeting *in utero* (Ludwig et al., 1996). Furthermore, Ludwig's study demonstrated when the IGF-IIR was knocked out, IGF-II null mice exhibited a lethal over growth syndrome *in utero*. However, a single knock-out of the IGF ligand (IGF-I or IGF-II), results in only reduced birth weights (Liu et al., 1993).

IGF tissue bioavailability is dependent on the presence or absence of the IGFBP's. The porcine endometrium produces IGFBP 2-6 (Simmen et al., 1992). Geisert et al. (2001) demonstrated that uterine IGFBP's present in the porcine uterine luminal fluids (UTF) on Day 5 to 10 of the estrous cycle and pregnancy are cleaved on Day 12 to 18. Proteolysis of the IGFBP's is clearly timed with the period of conceptus elongation (Lee et al., 1998). These researchers demonstrated that UTF's from uteri containing elongated conceptuses, caused proteolysis of the IGFBP's contained in the UTF's. However, Geisert et al. (2001) demonstrated that nonpregnant, cycling contemporaries experienced the same loss of IGFBP's as the pregnant pig, inferring maternal loss of the IGFBP's are independent of the conceptuses presence. Furthermore, Vonnahme. et al (1999) demonstrated an increase in the serine protease tissue kallikrein occurs in the uterine lumen on Day 10 of pregnancy and the estrous cycle in the pig, which can cleave IGFBPs. Appearance of tissue kallikrein in the uterine lumen occurs during the period for disappearance of IGFBP's in UTF's. Early removal of IGF from its respective binding proteins may cause a rapid metabolism of free IGF's, as has been demonstrated using ^{125}I labeled IGF (Ballard et al., 1991). Ballard's group demonstrated that the free ^{125}IGF-I half-life of 10 min was extended 10-15 hr when bound to an IGFBP complex.

The tissue growth factor-beta (TGFβ) super family is another growth factor of extreme importance in uterine conceptus crosstalk. The TGFβ superfamily consist of 3 ligands (TGFβ1,TGFβ2, and TGFβ3) and two receptors (Type I, Type II). All three TGFβ isoforms are synthesized as an inactive or latent form. Gupta et al. (1996) localized all three TGFβ isoforms in the Day 10 to 14 periimplantation conceptuses. Although all TGFβ isoforms were present in the conceptus tissues, TGFβ1 staining was weak (Gupta et al., 1996). In contrast, intense immunocytochemical staining for TGFβ2 and TGFβ3 was predominately localized to trophectoderm. These authors determined that type I and II TGFβ receptors were expressed by the conceptus (Days 10-14), indicating that TGFβ's serve an important role in early conceptus growth and development. Furthermore, these authors provided additional evidence of uterine conceptus cross talk via TGFβ, based on the presence of the TGFβ type I and II receptor expression in the endometrium of pregnant gilts, which was absent in cycling gilt endometrium. Given the timing of TGFβ production and expression during early pregnancy, TGFβ may be involved in ECM remodeling of uterine tissues as formation and expansion of the extraembryonic membrane occurs between Days 15 to 30 of gestation.

Keratinocyte growth factor (KGF), also known as fibroblasic growth factor-7 (FGF-7) is one of only three growth factors known to be secreted by stromal cells, which can function to stimulate epithelial cells (Rupin et al., 1995). In the pig, progesterone down regulates its own receptor in the surface epithelium, requiring progesterone to regulate the epithelium via the stroma which still retains the PR (Geisert et al., 2001). In the rat, studies have shown KGF expression is under direct influence of the ovarian

steroid hormones estrogen and progesterone (Koji et al., 1994). In the primate, KGF message was 100-fold higher in the endometrial stroma during the luteal phase compared to follicular phase epithelium (Takehiko et al., 1994). In contrast to the invasive type implantation, the porcine uterine endometrial epithelium expression of KGF is greatest on Day 12 of gestation, and Day 15 of the estrous cycle (Ka et al., 2000), which is closely associated with the time of elongation and maternal recognition of pregnancy (Geisert et al., 1982). Additionally, Ross et al. (2003) found that conceptus secretion of IL-1β is greatest on Day 12 of gestation. An increase in epithelial KGF expression may be caused by conceptus E_2, IL-1β, or a synergistic combination of both. The KGF receptor (KGFR) is localized to the porcine endometrial epithelium and the conceptus trophectoderm implicating a potential function in uterine conceptus communication during epitheliochorial placentation (Ka et al., 2000). Although the specific action of KGF in porcine gestation has yet to be elucidated, stromal may control KGF function of the uterine epithelium following uterine epithelial down regulation of the PR on Day 10 of gestation. KGF-R expression of conceptus trophoblastic origin and KGF production by uterine epithelial cells may indicate conceptus regulation of the temporal uterine window and surface uterine extracellular matrix.

Leukemia inhibitory factor (LIF) is a member of the interleukin 6 (IL-6) super family and is a key cytokine produced in large quantities by the porcine endometrium at the time of conceptus elongation (Anegon et al., 1994). Stewart et al. (1992) demonstrated that LIF is a maternally controlled event not requiring the presence of a conceptus in the mouse. However, removal of the LIF gene from the female mice results in implantation failure. However, implantation can be restored through exogenous

administration of recombinant LIF or transferring the embryo to a pseudopregnant contemporary (Cullinan et al., 1996). Furthermore, human studies have demonstrated peak LIF expression by glandular epithelium corresponds to the calculated moment of implantation during the mid-luteal phase (Chen et al., 1995).

LIF exerts biological actions through a specific membrane-bound receptor. LIF-R heterodimerizes with IL-6 signal transducing component GP130, which involves the actions of the janus-kinase signal-transducing receptor and activator of transcription (JAK-STAT) pathway (Kunisada et al., 1996). Charnuck-Jones et al. (1994) determined that in humans, LIF gene transcripts peaked in the endometrium during blastocyst implantation. Furthermore, maximal blastocyst LIF-R levels were also observed during this period, suggesting fetal-maternal crosstalk during this period. Mice genetically deficient in LIF are viable except for implantation where a LIF-R abrogated mouse is a parturient lethality (Stewart et al., 1992).

In the pig, LIF, LIF-R, and IL-6 have been characterized in the conceptus and endometrium (Modric et al., 2000). Periimplantation elongated porcine conceptuses expressed transient increased levels of LIF and IL-6 on Day 12 of pregnancy (Anegon et al., 1994). Porcine conceptus E_2 secretion may act on the endometrium to stimulate LIF transcription on Day 12 of gestation, as been reported in human studies where E_2 increases LIF secretions *in vitro* (Sawai et al., 1997). Furthermore, Yelich et al. (1997) demonstrated that LIF-R was present in the conceptus at 7 mm spherical stage of development and decreased until reaching the elongated filamentous morphophology, which suggests a role in early maternally derived LIF secretions in embryonic development.

Porcine Embryonic Mortality and Endocrine Disruption

In the pig, the majority of embryonic mortality occurs prior to Day 30 of gestation (Perry et al., 1954). Using slaughter house material, Perry et al. (1954) indicated an average loss of embryos in the pig was between 20-48% based on the live number of embryos present and corpora lutea on the ovary.

There are two time periods during pregnancy which the majority of embryonic loss occurs in the pig. The periimplantation (Days 10 to 15) and post-placental attachment and expansion (Days 18 to 40) have been shown to be the periods of greatest loss of the possible litter size. Pope et al. (1994) estimated 30% of conceptus death occurs in early gestation (Days 10-18). Early conceptus loss has been postulated to occur because of differences in conceptus size and development resulting from varible timing between ovulations in the sow (Pope et al., 1982). When conceptuses were transferred to a more advanced uterine environment, lesser developed conceptuses were not as responsive to the advanced uterine secretions (Pope et al., 1990). Furthermore, Geisert et al. (1982) demonstrated that exogenous estrogen administered on Day 11-12 of the cycle advanced uterine secretion. Based on Geisert's findings, more developed conceptuses in the same litter may posses the ability to initiate steriodogensis (producing E_2) first could advance uterine secretion prior to lagging conceptuses ability to acquire and use these secretions. Another disadvantage for the retarded conceptuses resides in the fact that once contemporaries produce estrogen, the uterus begins to compartmentalize into discrete segments limiting uterine surface availability. Uterine capacity and surface area of the dam has been postulated as another limiting factor in controlling the number of

embryos that can survive to term (Webel and Dziuk 1974). Christenson et al. (1987) defined uterine capacity as: maximum number of fetuses adequately supported by uterine tissues with unlimited viable conceptuses available. Further studies suggest that placental efficiency may be a regulating factor in regards to maximum uterine capacity of the pig (Wilson et al., 1999). In the Meishan sow, conceptuses develop at a slower rate than standard European breeds of swine. The slow sustained release of Meishan conceptus estrogen may allow more time to establish properly segmented sections (micro-environments) in the Meishan uterus aiding in conceptus uterine synchrony.

Estrogenic mycotoxins in moldy corn results in complete embryonic loss when present in the diet of pregnant sows fed between Days 7-10 of pregnancy (Long and Diekman 1986). However, administration of estrogenic compounds on either Days 2-6 or Days 11-15 of gestation, does not disrupt normal pregnancy. Early estrogen exposure does not affect spacing or elongation of conceptuses, but rather modifies the uterine environment (Long et al., 1987). Endocrine disruptors can modulate ovarian steroid hormone function on the uterus. Changes in steroid hormone actions can be altered due to the antagonistic binding of endocrine disrupting compounds (EDC) to the steroid receptors. EDC's affect the functions of E_2 and P_4 on the uterus and the hypothalamic-hypophyseal axis as well. EDC's have the potential to alter the estrous cycle, ovulation, and gestation in the domestic pig if exposure occurs.

Pope et al. (1986) demonstrated that administration of estradiol-17β (8 mg/Day) on Days 9 and 10 of pregnancy resulted in complete embryonic mortality by Day 30 of gestation. Morgan et al. (1987) indicated that pregnant gilts exposed to estrogen on Days 9 and 10 of pregnancy resulted only in fragmented and degenerated conceptuses

recovered by Day 16 of gestation. On Days 14-16 of gestation, Gries et al. (1989) observed alterations in uterine protein content in the flushings of gilts receiving early estrogen. Embryonic death due to early estrogen exposure is highly correlated to a complete breakdown in the uterine glycocalyx occurring approximately Day 14 of gestation (Blair et al., 1991). Thus, these data suggest that early estrogen acts as an endocrine disrupter through alterations in the uterine epithelial suface at the level of the extracellular matrix around the time of placentation.

Statement of the Problem

The holy grail for the porcine embryologist resides in reducing the embryonic mortality during pregnancy in the pig to enhance reproductive efficiency. Estrogen exposure to the pregnant pig on Days 9 and 10 of gestation causes complete embryonic loss 4 to 6 Days post-exposure. The goal of the present studies are to characterize dysfunction in maternal uterine growth factors and inflammatory mediators associated with early estrogen exposure.

Due to the importance of prostaglandins production in successful pregnancies, we evaluated two isoforms of cyclooxygenase (COX-1 and COX-2). We first established the normal patterns of COX-1 and -2 expression during the estrous cycle and early pregnancy. A second study evaluated the effects of early estrogen exposure on COX-1 and -2 in pregnant gilts.

Endometrial IGF-I and IGF-II is maximally secreted into the uterine lumen at the time of conceptus estrogen synthesis (Simmens et al., 1992) suggesting E_2 may directly or indirectly enhance IGF secretion patterns via $P450_{aroma}$ during early gestation.

Evaluation of endometrial IGF-I and IGF-II ligand production and secretion, and their biological regulators (IGFBP's) need to be evaluated following endocrine disruption with E_2 in the pregnant pig. The expression pattern of the endometrial IGF-IR, which provides IGF signaling capabilities, is also present and should be evaluated following endocrine disruption in early gestation.

The current study attempts to provide some insight into disruption of the normal inflammatory process during early gestation following endocrine disruption. The loss of the glycocalyx is a post-translational event occurring down stream of possible premature activation of the uterine protease network caused by the early exposure to estrogen.

Chapter III.

CHARACTERIZATION OF ENDOMETRIAL CYCLOOXYGENASE (COX) EXPRESSION IN THE PIG DURING THE ESTROUS CYCLE, EARLY PREGNANCY AND FOLLOWING ENDOCRINE DISRUPTION OF PREGNANCY

Abstract

In the pig, rapid changes in conceptus morphology and attachment to the uterine surface during early gestation are associated with an increase in conceptus and endometrial production of prostaglandins (PG). Prostaglandin synthesis is mediated by cyclooxygenase (COX), for which COX-1 and COX-2 are the constituent and inducible forms, respectively. Conceptus estrogen synthesis on Day 12 of gestation serves as the signal for maternal recognition of pregnancy in the pig. However, early exposure (Day 9 and 10) of pregnant gilts to exogenous estrogens can result in complete embryonic mortality following Day 15 of gestation. Objectives for the present study were to 1) establish the pattern of endometrial COX-1 and COX-2 expression during the estrous cycle and early pregnancy of gilts and 2) determine the effect of endocrine disruption (estrogen treatment) on endometrial COX gene expression and uterine luminal content of prostaglandins in pregnant gilts. In experiment 1, endometrium and uterine flushings were harvested from gilts on Days 0, 5, 10, 12, 15 and 18 of the estrous cycle and Days 10, 12, 15 and 18 of pregnancy. In experiment 2, bred gilts were administered either 5 mg estradiol cypionate (E) or vehicle (Veh) on Days 9 and 10 of gestation. Endometrium and uterine flushings were harvested from the E and Veh treated gilts on Days 10, 12, 13, 15 and 17 of gestation. Endometrial COX-1 and COX-2 gene expression was determined

by Real-Time PCR. Western blot analysis was utilized to determine endometrial COX-1 and COX-2 protein expression. Prostaglandin (PGE_2, $PGF_{2\alpha}$) synthesis was quantified in the uterine flushings of Veh and E treated pregnant gilts to determine the effects of E on PG synthesis. In Experiment 1, there was a 3 to 10-fold increase in endometrial COX-2 protein expression from Day 5 to 18 of the estrous cycle and pregnancy. Endometrial COX-1 gene expression increased approximately 2 to 4-fold while COX-2 gene expression increased 10 to 160-fold between Day 5 and 15 of the estrous cycle and pregnancy. *In situ* hybridization indicated that COX-2 transcript increased in the luminal epithelium of pregnant and cycling gilts between Days 5 to 12. In Experiment 2, E treatment increased endometrial COX-2 protein on Day 10, but decreased expression on Day 12 of gestation compared to Veh treated gilts. COX-1 gene expression increased in E compared to Veh treated gilts on Day 13 of gestation. Uterine luminal flushing content of PGE_2 and $PGF_{2\alpha}$ increased from Day 10 to 18 of pregnancy; however E treatment did not alter uterine luminal content of prostaglandins. The present study demonstrates that an increase in uterine epithelial COX-2 expression occurs after Day 10 of the estrous cycle and pregnancy in the pig. Early E treatment on Days 9 and 10 of pregnancy alters the pattern of endometrial COX-1 and COX-2 gene expression which may provide a potential mechanism by which total embryonic loss occurs during implantation in the pig.

Introduction

The noninvasive trophoblastic attachment of the porcine conceptus forms an epitheliochorial type of placentation through its adhesion to the extracellular glycocalyx present on apical surface microvilli of the uterine luminal epithelium (King et al., 1982; Stroband and Van der Lende, 1990). Following removal of the antiadhesive factors present on the uterine apical surface epithelium, trophoblast attachment to the uterine luminal epithelium involves a coordinated program of events in the progesterone stimulated uterine environment that are mediated by actions of estrogen in mice, rats and pigs [Yoshinaga et al., 1966; Psychoyos, 1986; Bowen et al., 1996; Geisert and Yelich, 1997]. Trophoblast attachment to the uterine surface in the pig immediately follows rapid conceptus elongation and the acute increase of conceptus estrogen synthesis on Day 12 of pregnancy (see review Geisert et al., 1990). Release of estrogen by the elongating conceptuses induces endometrial receptivity for placental attachment to the uterine surface (Keys et al., 1990; Burghardt et al., 1997; Jaeger et al., 2001). The transient acute increase in estrogen release during rapid trophoblast elongation is concomitant with conceptus release of the proinflammatory cytokine, interleukin-1β (IL-1β), which has been proposed to serve as the initial stimulus for conceptus attachment to the uterine surface in the pig (Ross et al., 2003).

Implantation in mice and rats require PG synthesis in the estrogen primed uterus. The importance of endometrial COX-2 gene expression during implantation and decidualization was established using COX-2 null mice that have an altered inflammatory response resulting in reproductive defects in ovulation, fertilization and implantation [Dinchuk et al. 1995; Lim et al. 1997]. Defects in reproduction are COX-2 specific

because COX-1 deficient mice are fertile and only exhibit problems at parturition (Gross et al., 1998). During apposition of the blastocyst to the uterine surface, ovarian estrogen increases synthesis of PGE_2 at the site of implantation (Kennedy et al., 1994), which is essential for implantation to occur (Lim et al., 1997). Endometrial IL-1β gene expression increases in the peri-implantation mouse uterus on day 4.5 of pregnancy (De et al., 1993). Increase in endometrial IL-1β secretion may stimulate PGE_2 production through elevated COX-2 expression in the uterine epithelium (Chakraborority et al., 1996, Ospina et al., 2003).

The increase in porcine conceptus IL-1β gene and ligand expression immediately precedes the morphological transformation during trophoblast elongation (Ross et al., 2003). The pig conceptus and endometrium actively secrete PGs (Geisert et al., 1982; Guthrie and Lewis, 1986; Gross et al., 1988) and conceptus expression of COX-2 gene increases following trophoblast elongation and conceptus attachment to the luminal epithelium (Wilson et al., 2002). Pharmacological inhibition of PG synthesis does not affect trophoblast elongation in pigs (Geisert et al., 1986). However, inhibition of PG synthesis during the period of trophoblast attachment does increase embryonic mortality (Kraeling et al., 1985). These studies suggest that the acute release of conceptus IL-1β during trophoblast elongation may have an obligatory role to enhance COX-2 synthesis for endometrial release of prostaglandins during the establishment of pregnancy in the pig.

Exposure of pregnant gilts to exogenous estrogens 48 h prior to the normal secretion by conceptuses on Day 12 results in total embryonic mortality before Day 30 of gestation (Pope et al., 1986). Our lab previously demonstrated that conceptuses

degenerate between Days 15 to 18 of gestation following early estrogen treatment on Days 9 and 10 of pregnancy (Morgan et al., 1987; Gries et al., 1989; Blair et al., 1991). Although conceptus degeneration is correlated with the spaciotemporal loss of the microvilli glycocalyx on the endometrial surface epithelium (Blair et al., 1991), the factors involved with the uterine alteration and conceptus death are unknown. We propose early administration of estrogen may alter the normal synchrony of uterine COX-2 induction by conceptus IL-1β secretion, which causes defects in conceptus development and/or endometrial receptivity for placental attachment to the luminal surface.

Since endometrial COX-1 and COX-2 gene and protein expression have not been previously established during the estrous cycle and early pregnancy in the pig, the first objective of our study was to determine porcine endometrial expression of COX-1 and COX-2 during the porcine estrous cycle and early pregnancy. The objective of the second study was to determined endometrial COX-1 and COX-2 expression following treatment of gilts with estrogen on Days 9 and 10 of gestation.

Methods and Materials

Animals

Crossbred cycling gilts of similar age (8-10 mo) and weight (100-130 Kg) were checked twice daily for estrus behavior with intact males. Gilts assigned to be bred were naturally mated with fertile crossbred boars at first detection of estrus and subsequently at 12 and 24 h post-estrus detection.

Experiment I: Endometrial COX 1 and COX-2 Expression in Cyclic and Pregnant Gilts.

Gilts were hysterectomized on either Days 0, 5, 10 12, 15 and 18 of the estrous cycle (n =24) or Days 10, 12, 15 and 18 of pregnancy (n =16) as previously described by Gries et al., (1989). Following induction of anesthesia with 1.8 ml i.m. administration of a cocktail consisting of 2.5 ml (Xylazine: 100mg/ml: Miles Inc., Shawnee Mission, KS) and 2.5 ml Vetamine (Ketamine HCl: 100 mg/ml Molli Krodt Veterinary, Mundelein, IL) in 500 mg of Telazol (Tiletamine HCl and Zolazepum HCl: Fort Dodge, Syracuse, NE), anesthesia was maintained with a closed circuit system of halothane (5% flurothane) and oxygen (1.0 liters/min). The uterus was exposed via midventral laparotomy and a randomly selected uterine horn and its ipsilateral ovary, excised. Uterine horns were injected with 20 mL phosphate buffered saline (PBS, pH 7.4) via the isthmus and flushings were recovered in a petri dish. Conceptuses were removed from flushings of pregnant gilts, snap frozen in liquid N_2 and stored at -80°C. Uterine flushings were centrifuged (1000 x g @ 4°C for 10 min), supernatant collected and uterine flushings stored at -20°C. Endometrial tissue was removed from the antimesometrial side of the uterine horn, immediately snap frozen in liquid nitrogen and stored at-80°C until utilized for extraction of protein and RNA.

Uterine Tissue Fixation Procedures

Tisue sections were excised from the uterine endometrium and placed in 40 mL of freshly prepared 4% paraformaldehyde in phosphate buffered saline (PBS). Tissues were gently agitated at room temperature (rt) for 24 h, and the fixing solution was replaced with 40 mL of 70% EtOH (v/v in H_20), and stored at rt.

In situ hybridization

COX-1 and COX-2 mRNAs were localized in paraffin-embedded porcine uterine tissue by *in situ* hybridization using methods previously described by (Johnson et al., 2000). Briefly, deparaffinized, rehydrated, and deproteinated endometrial tissue cross-sections (5 μm) were hybridized with radiolabeled antisense or sense porcine COX-1 and/or COX-2 cRNA probes synthesized by *in vitro* transcription with [α-^{35}S]uridine 5-triphosphate (PerkinElmer Life Sciences). After hybridization, washes, and RNase A digestion, autoradiography was performed using NTB-2 liquid photographic emulsion (Eastman Kodak, Rockchester, NY). Slides were exposed at 4°C for 6 Days, developed in Kodak D-19 developer, counterstained with Harris' modified hematoxylin (Fisher Scientific, Fairlawn, NJ), dehydrated, and protected with coverslips.

Experiment II: Endometrial COX-1 and COX-2 Gene and Protein Expression following Early Exposure of Pregnant Gilts to Estrogen

Bred gilts were randomly assigned one of the following treatment groups: 2.5 ml i.m. injection of corn oil (Veh) on Days 9 and 10 of gestation (n = 20), or 5 mg i.m. injection of estradiol cypionate (E) (A.J. Legere, Scottsdale, AZ) on Days 9 and 10 of gestation (n=20). Gilts were unilaterally hysterectomized on either Days 10, 12, 13, 15 and 17 of gestation as described in Experiment 1. Uterine horns were flushed with 20 ml PBS, conceptuses removed, flushings centrifuged to remove cellular debris, and the supernatant stored at -20°C. Endomterial tissue was harvested from the uterine horn and

either fixed for *in situ* hybridization or snap frozen as previously described in Experiment 1.

RNA Extraction

Total RNA was extracted from endometrial tissue using RNAwiz™ reagent (Ambion, Inc. Austin, TX). Approximately 0.5 g of endometrial tissue was homogenized in 5.0 mL of RNAwiz using a Virtishear homogenizer (Virtis Company Inc., Gardiner, NY). RNA was resuspended in nuclease free water and stored at -80°C. Total RNA was quantified with a spectrophotometer at an absorbance of 260 and purity was verified using the 260/280 ratio.

Quantitative 1-Step RT-PCR

Quantitative analysis of endometrial COX-1 and COX-2 mRNA were analyzed using quantitative real time reverse polymerase chain reaction (RT-PCR) as previously described in our laboratory (Hettinger et al., 2001). The PCR amplification was performed in a reaction volume of 15 μl using an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The transcripts were evaluated using dual labeled probes with 6-Fam (5' reporter dye), and TAMRA (3' quenching dye). Primer and probe sequences for the amplification of COX-1, and COX-2 (Table 3.1) were generated from porcine sequences available in the NCBI database. Total RNA (100 ng) was assayed in duplicate using thermocycling conditions for one-step cDNA synthesis of 30 min at 48°C and 95°C for 10 min, followed by 45 repetitive cycles of 95°C for 15 sec and 60°C for 1 min. Ribosomal 18S RNA was assayed in each sample to normalize RNA loading as previously described by Ross et al. (2003).

Table 3.1 PCR Primer and probe sequences used for quantitative RT-PCR of endometrial COX-1 and COX-2 gene expression.

Gene		Forward Primer/Reverse Primer/Probe	GeneBank Accession
COX-1	Forward	5’ - CAACACTTCACCCACCAGTTCTTC-3’	**AF207823**
	Reverse	5’ - TCCATAAATGTGGCCGAGGTCTAC-3’	
	Probe	5’ - TTCTGGCAAGATGGGTCCTGGCTTCACCAA-3’	
COX-2	Forward	5’- TCGACCAGAGCAGAGAGATGAGAT-3’	**AF207824**
	Reverse	5’- ACCATAGAGCGCTTCTAACTCTGC-3’	
	Probe	5’- AATACCGCAAGCGCTTTCTGCTGAAGCCCT-3’	

Template amplification was quantified by determining the threshold cycle (C_T) based on the fluorescence detected within the geometric region of the semilog plot. In the geometric region, one cycle is equivalent to the doubling of PCR target template. Using the comparative C_T method (Hettinger et al., 2001), relative quantification and fold gene expression difference between treatment and day were determined for the endometrial samples (Table 3.2). Differences in mRNA expression of COX-1 and COX-2 were determined by subtracting target C_T of each sample from its respective ribosomal 18S C_T value, which provides the sample ΔC_T value. Calculation of the $\Delta\Delta C_T$ involves using the highest sample ΔC_T value as an arbitrary constant to subtract from all other ΔCT sample values. Fold differences in gene expression of the target gene are equivalent to $2^{-\Delta\Delta CT}$.

Western Blot analysis of COX-1 and COX-2

Cytoplasmic protein was extracted from endometrial tissue using T-PER™ reagent (Pierce Inc., Rockford, IL) to which a protease inhibitor cocktail, HALT™ (Pierce Inc., Rockford, IL) was added. A total of 250 mg of endometrial tissue was homogenized in 5 mL T-PER™ reagent on ice using a Virtishear homogenizer (Virtis Company Inc., Gardiner, NY). Following homogenization samples were centrifuged at 10,000 x g for 5 min, supernatant collected and stored at -20°C until utilized for western blot analysis. Cytoplasmic protein in the supernatant was assayed by Bradford Assay. Samples containing (30 μg protein) were boiled at 95°C for 90 sec in equal volumes of sample denature buffer (0.125 M Tris-HCL [pH 6.8], 205 mg glycerol, 4% SDS, 10% β-mercaptoethanol, and 0.0025% bromophenol blue and protein separated in a 10% SDS

Table 3.2. Comparison of endometrial COX-1 or COX-2 gene expression across days of the estrous cycle and early pregnancy using the comparative threshold (C_T) method.

Target	Status	Day	Average Target C_T*	Average 18s C_T*	ΔC_T†,*
COX-1	Cyclic	0	27.96 ± 0.81	18.46 ± 0.19	09.50 ± 0.47[ab] -1.27
	Cyclic	5	29.29 ± 0.25	18.52 ± 0.19	10.77 ± 0.38[a] 0.00
	Cyclic	10	28.98 ± 0.20	18.58 ± 0.11	10.40 ± 0.24[a] -0.37
	Pregnant	10	28.86 ± 0.27	18.57 ± 0.07	10.29 ± 0.31[a] -0.48
	Cyclic	12	28.81 ± 0.62	18.79 ± 0.58	10.02 ± 0.29[bc] -0.75
	Pregnant	12	27.12 ± 0.47	18.32 ± 0.09	08.80 ± 0.53[bc] -1.97
	Cyclic	15	27.35 ± 0.58	18.31 ± 0.05	09.04 ± 0.56[bc] -3.32
	Pregnant	15	27.35 ± 0.53	18.40 ± 0.12	08.94 ± 0.46[bc] -1.83
	Cyclic	18	27.46 ± 0.14	18.37 ± 0.05	09.09 ± 0.18[bc] -1.68
	Pregnant	18	27.61 ± 0.32	18.51 ± 0.09	08.47 ± 0.49[bc] -2.30
COX-2	Cyclic	0	26.31 ± 0.47	18.46 ± 0.19	07.85 ± 0.39[a] -3.67
	Cyclic	5	30.04 ± 0.63	18.52 ± 0.19	11.52 ± 0.62[a] 0.00
	Cyclic	10	26.73 ± 0.95	18.58 ± 0.11	08.15 ± 0.24[a] -3.37
	Pregnant	10	27.60 ± 0.81	18.57 ± 0.07	09.03 ± 0.31[a] -2.49
	Cyclic	12	24.04 ± 0.63	18.79 ± 0.58	05.26 ± 0.30[b] -6.26
	Pregnant	12	23.80 ± 0.67	18.32 ± 0.09	05.48 ± 0.74[b] -6.04
	Cyclic	15	22.55 ± 0.43	18.31 ± 0.05	04.24 ± 0.41[b] -7.28
	Pregnant	15	24.44 ± 0.92	18.40 ± 0.12	06.04 ± 0.92[b] -5.48
	Cyclic	18	24.47 ± 0.22	18.37 ± 0.05	05.42 ± 0.24[b] -2.49
	Pregnant	18	25.07 ± 0.94	18.51 ± 0.09	05.93 ± 0.49[b] -5.59

C_T* = Cycle Threshold: Cycle number where amplification crosses the threshold set in the geometric portion of the amplification curve.

ΔC_T† = Target (COX-1 and COX-2) – 18S Ribosomal CT: Normalization of PCR cycles for the target with 18S ribosomal RNA. Values with different superscripts for each of the target genes differ significantly ($P<0.05$)

$\Delta\Delta C_T$§ = Mean ΔC_T – highest mean ΔCT value: The mean value of Day 5 cyclic (highest ΔC_T; lowest gene expression for COX-1 and COX-2 genes) was used as a calibrator for COX-1 and COX-2 to set the baseline for comparing differences in ΔC_T values across all days.

polyacrylamide gel. Proteins were transferred to a PVDF membrane (Millipore Corp. Bedford, MA) by using a semi-dry immunoblot apparatus (MilliBlot™-SDS System) at 350 V for 3 h. Membranes were washed with Tris-buffered saline (TBS, 10mM Tris, 150 mM NaCl) containing 0.05% Tween 20 [TTBS] and then blocked with 5% nonfat dried milk for 1 h. After washing 3X with TTBS for 10 min, the membrane was incubated for 2 h with a rabbit antihuman COX-2 polyclonal antibody (1:2000), or sheep antihuman COX-1 polyclonal (1:2000) (Calbio-Chem Inc. La Jolla, CA). After washing 3X with TTBS for 10 min each, the membrane was incubated with goat antirabbit secondary antibody (COX-2) (1:1000), or mouse antisheep secondary (COX-1) (1:1000) (Bio-Rad, Hercules CA) for 1 h. Membranes were washed with TTBS 3X for 10 min followed by addition of color development solution (Bio-Rad Immuno-Blot kit: Bio-Rad Hercules, Ca). Densities of the resultant stained bands were measured with a Bio-Rad densitometer 2100 (Bio-Rad: Hercules, CA).

Enzyme-Linked PG Competitive Binding Assays

Total content of PGE_2 and $PGF_{2\alpha}$ in the uterine flushings collected in experiment 2 were quantified using a commercial ELISA (R&D Systems, Minneapolis, MN) in accordance with manufacturer's specifications. Samples were analyzed in duplicate with a single assay. The intra-assay coefficient of variation for the PGE_2 and $PGF_{2\alpha}$ assay were 5.1% and 4.9%, respectively.

Statistical Analysis

Data were analyzed by least squares ANOVA using the Proc Mixed model of the

Statistical Analysis System (SAS, 1988). The statistical model used to analyze cyclic and pregnant endometrial COX-1 and COX-2 gene and protein expression in Experiment 1 included the effects of day, reproductive status (cyclic, pregnant) and day x reproductive status interaction. The statistical model used to analyze endometrial COX-1 and COX-2 gene expression and uterine flushing PG content in Experiment 2 included the effects of day, treatment (E, Veh), and the day x treatment interaction. Due to unequal variances of PGE_2 and $PGF_{2\alpha}$ content in the uterine flushings, data was transformed using log and square root transformations for the statistical analysis.

Results

Experiment I

Endometrial COX-1 and COX-2 Gene Expression

Endometrial COX-1 gene expression (Table 3.2) was effected by Day ($P<0.0008$). Endometrial expression of COX-1 increased approximately 3-fold from Day 5 to 15 of the estrous cycle and between Day 10 to 12 of pregnancy (Figure 3.1). The increase in endometrial COX-1 gene expression remained elevated to Day 18 of the estrous cycle and pregnancy.

Endometrial COX-2 gene expression (Table 3.2) was effected by Day ($P<0.0001$) but not by reproductive status. Endometrial COX-2 gene expression was lowest on Day 5 of the estrous cycle increasing 10-fold on Day 10 and approximately 80-fold by Day 12. Expression of COX-2 was 160-fold greater on Day 15 compared to Day 5 of the estrous cycle. A similar increase in COX-2 gene expression was detected pregnant gilts

Figure 3.1. Fold difference in porcine endometrial COX-1 gene expression during the estrous cycle (Yellow) and early pregnancy (Blue). Fold differences in gene expression were calculated using the highest CT value (Day 5 cycle) as the baseline as described in the *methods and materials*. Bars with different superscripts represent statistical differences.

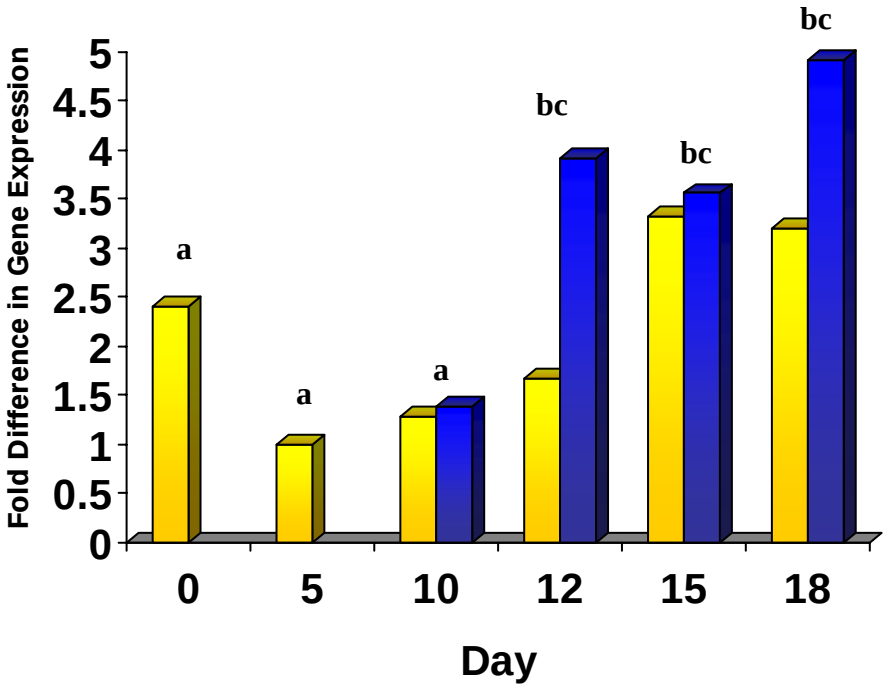
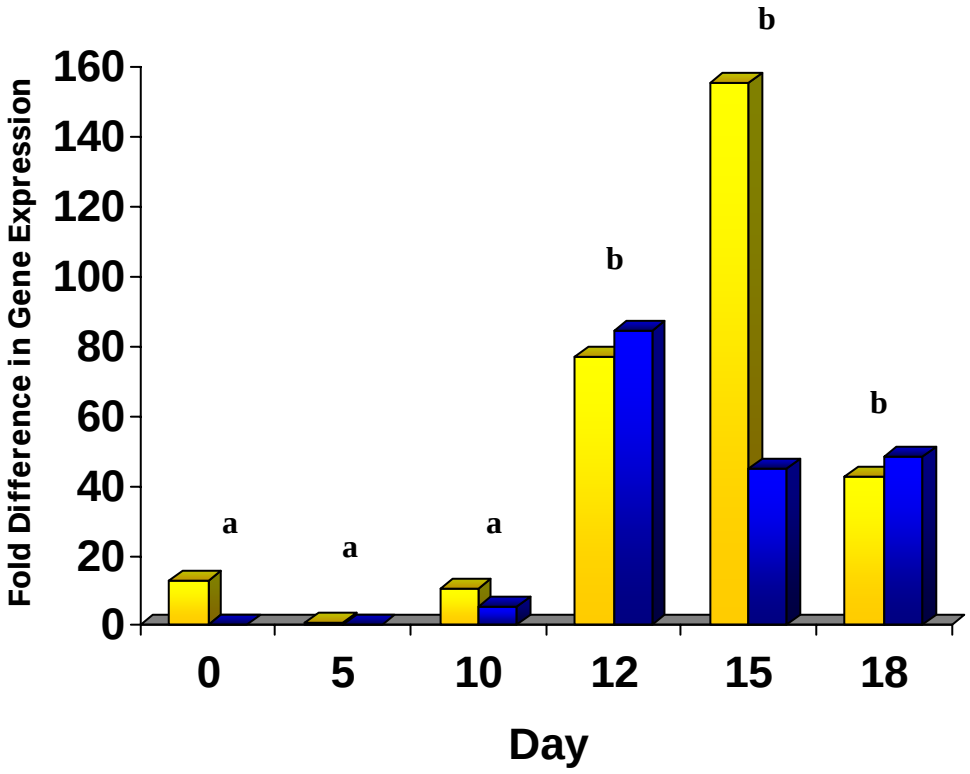

Fold Difference in Gene Expression
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a
a
bc
bc
bc
0
5
10
12
15
18
Day

Figure 3.2. Fold difference in porcine endometrial COX-2 gene expression during the estrous cycle (Yellow) and early pregnancy (Blue). Fold differences in gene expression were calculated using the highest C_T value (Day 5 cycle) as the baseline as described in the *methods and materials*. Bars with different superscripts represent statistical differences


Fold Difference in Gene Expression
160
140
120
100
80
60
40
20
0
a
a
a
b
b
b
0
5
10
12
15
18
Day

(Figure 3.2). Gene expression increased 9-fold between Day 10 and 12 of pregnancy and remain elevated across the days evaluated during early pregnancy.

In Situ Hybridization Analysis

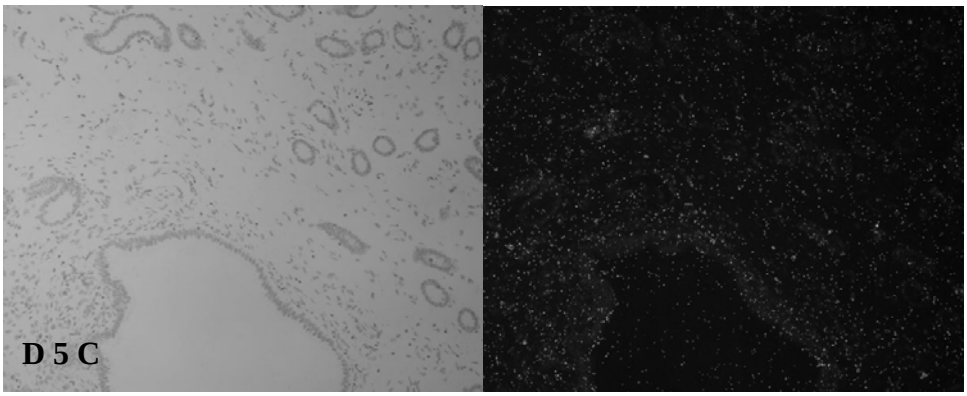
In situ hybridization analysis of the COX-1 transcript appeared very weak in the uterine stroma, glandular and luminal epithelium across all days of the estrous cycle and early pregnancy (data not shown). There were no apparent changes across day.

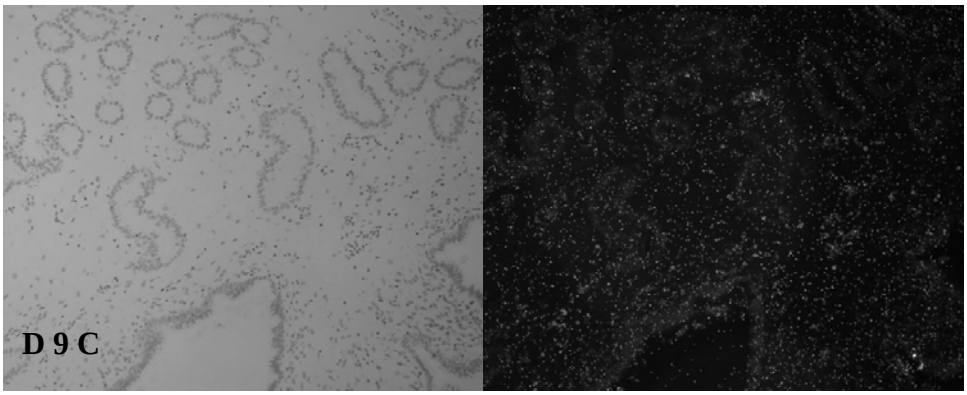
In situ hybridization revealed that uterine luminal and glandular epithelium of the endometrium was devoid of COX-2 mRNA on Day 5-9 of the estrous cycle (Figure 3.3). However, COX-2 mRNA expression with in the luminal epithelium of the uterine endometrium increased on Day 12 of the estrous cycle (Figure 3.3). Abundant COX-2 mRNA expression is detected in the luminal epithelium of the endometrium by Day 15 of the estrous cycle, but was relatively negligible in the uterine glandular epithelium. In the pregnant gilt endometrium (Figure 3.4), COX-2 mRNA cellular localization was faint in the glandular and luminal epithelium on Day 10. In contrast, COX-2 message was substantially increased in the luminal epithelium Day 13 of pregnancy. Expression of COX-2 mRNA remained highly abundant in the luminal epithelium while remaining faint in the glandular epithelium on Day 15.

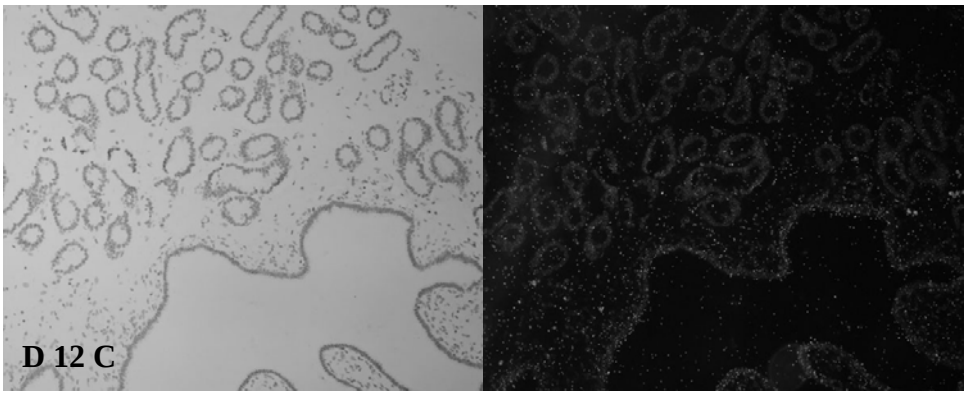
Western Blot analysis

Western blot analysis of the COX-1 enzyme (72 kDa) indicated that expression remained relatively constant through Day 10 of the estrous cycle (Figure 3.5A) and

Figure 3.3 *In situ* hybridization analysis of COX-2 mRNA expression in the uterine luminal and glandular endometrium from cyclic (a) (D 5, 12, 15) and pregnant (b) (D 10, 15) gilts. Protected transcripts were visualized by liquid emulsion autoradiography for one week and imaged under brightfield or darkfield illumination.


D 5 C


D 9 C


D 12 C

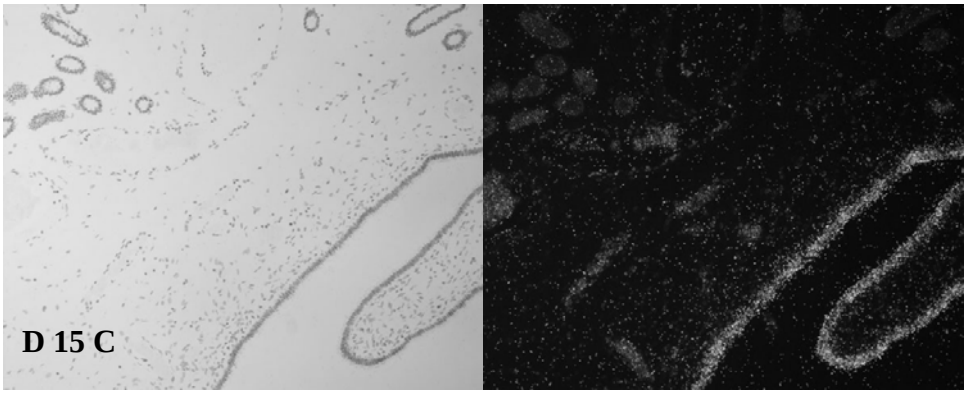
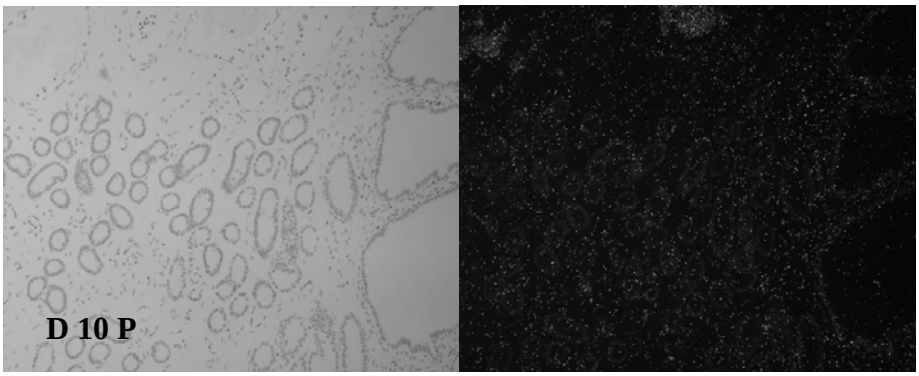
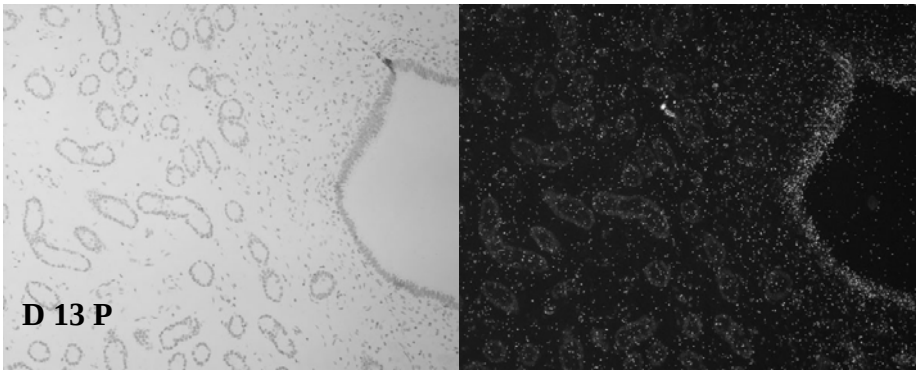
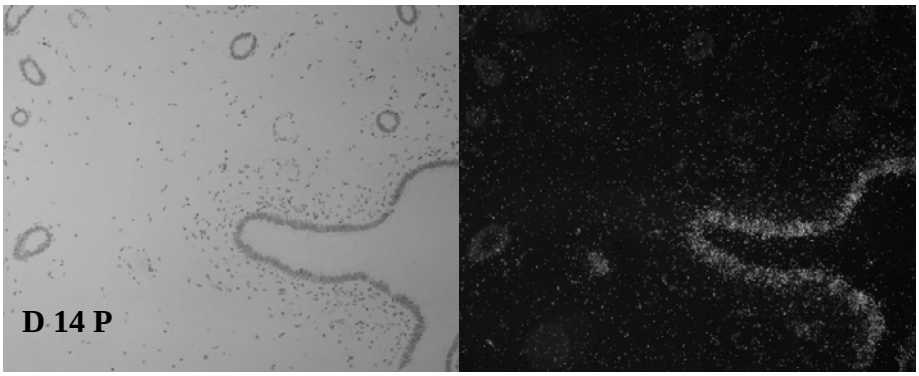

D 15 C

Figure 3.4 *In situ* hybridization analysis of COX-2 mRNA expression in the uterine luminal and glandular endometrium from pregnant (D 10, 15) gilts. Protected transcripts were visualized by liquid emulsion autoradiography for one week and imaged under brightfield or darkfield illumination.


D 10 P


D 13 P


D 14 P

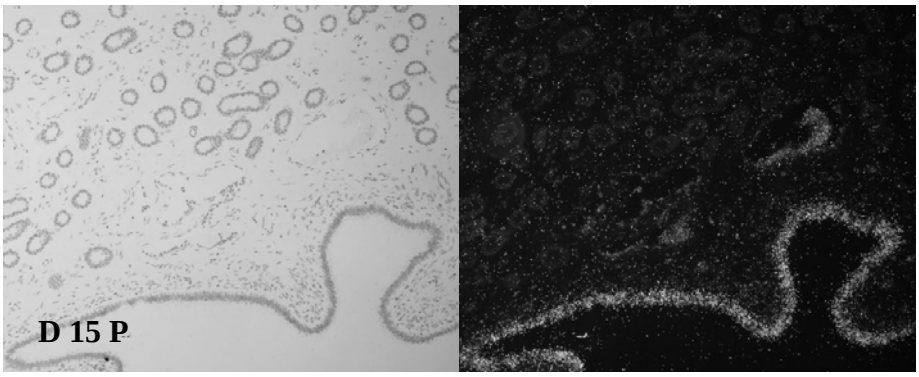
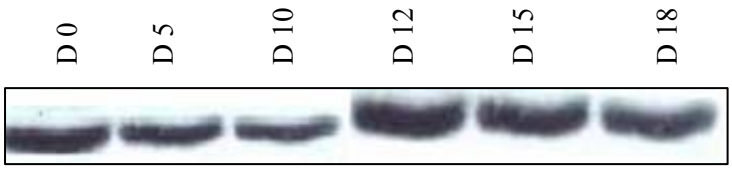

D 15 P

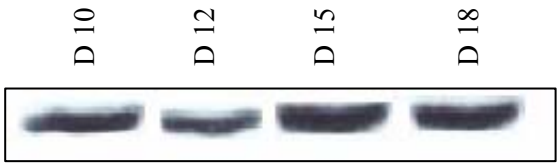
Figure 3.5 Western blot analysis of endometrial COX-1 expression of a 72 kDa immunoreactive product across the Days of the **(A)** estrous cycle and **(B)** early pregnancy.

(A)



Estrous Cycle

(B)



Pregnancy

pregnancy (Figure 3.5B). On Day 12 of the estrous cycle and pregnancy, there was an increase in density of the 72 kDa band for COX-1.

Western blot analysis of endometrial cytoplasmic protein detected a 72 kDa immunoreactive product to the COX-2 antiserum across days of the estrous cycle (Figure 3.6A) and early pregnancy (Figure 3.6B). Endometrial expression of the 72kDa product was effected by Day ($P<0.039$) and Status ($P<0.032$). COX-2 expression was faint on Day 5 of the estrous cycle with the intensity of COX-2 increasing 20-fold from Day 5 to 15 of the estrous cycle (Figure 3.6A). Endometrial COX-2 expression in pregnant gilts was 5-fold greater on Day 10 compared to cyclic gilts. Cytoplasmic content of COX-2 continue to increase 10-fold between Days 12 and 15 of pregnancy. Overall endometrial content of COX-2 was approximately 3-fold greater in pregnant compared to cyclic gilts.

Experiment 2

Endometrial COX-1 and COX-2 Gene Expression

Endometrial COX-1 gene expression in Veh and E treated gilts increased ($P<0.008$) from Day 10 to 13 of gestation (Table 3.3). Gene expression in Veh gilts increased 2-3 fold from Day 10 to 13, however COX-1 gene expression in E gilts was 3-fold greater than Veh gilts ($P<0.03$) on Day 13 of gestation (Figure 3.7).

A day x treatment interaction ($P<0.008$) was detected for endometrial COX-2 gene expression (Table 3.3). Gene expression increased 5-fold from Day 10 to 12 of pregnancy and remained elevated to Day 18 in Veh gilts (Figure 3.8). However, E treatment altered the normal expression pattern compared to Veh gilts. Endometrial

Figure 3.6 Western blot analysis of endometrial COX-2 expression of a 72 kDa immunoreactive product across the days of the **(A)** estrous cycle and **(B)** early pregnancy.


(A)
D0
D5
D10
D12
D15
D18
72 kDa
Estrous Cycle

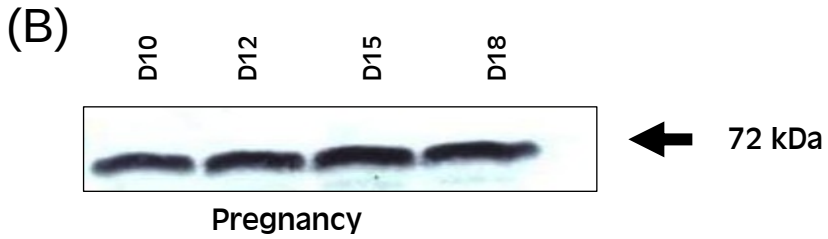

(B)
D10
D12
D15
D18
72 kDa
Pregnancy

Table 3.3. Comparison of endometrial COX-1 and COX -2 gene expression in Veh and E treated across days of pregnancy using the comparative threshold (C_T) method.

Target	TRT	Day	Average Target C_T *	Average 18S C_T *	ΔC_T † *	$\Delta\Delta C_T$ §
COX-1	Vehicle	10	28.44 ± 0.12	18.95 ± 0.50	9.49 ± 0.58[a]	0.00
	Estrogen	10	27.31 ± 0.12	18.67 ± 0.38	8.64 ± 0.63[a]	-0.85
	Vehicle	12	27.13 ± 0.14	18.32 ± 0.21	8.81 ± 0.21[a]	-0.68
	Estrogen	12	26.74 ± 0.47	17.84 ± 0.24	8.90 ± 0.31[a]	-0.59
	Vehicle	13	26.44 ± 0.29	18.19 ± 0.21	8.24 ± 0.31[**a**]	-1.25
	Estrogen	13	25.75 ± 0.22	19.13 ± 0.82	6.54 ± 0.88[**b**]	-2.96
	Vehicle	15	26.78 ± 0.12	18.54 ± 0.20	8.24 ± 0.56[**b**]	-1.25
	Estrogen	15	26.55 ± 0.10	18.86 ± 0.18	7.68 ± 0.56[**b**]	-1.81
	Vehicle	17	26.93 ± 0.14	18.57 ± 0.24	8.36 ± 0.79[ab]	-1.13
	Estrogen	17	26.90 ± 0.23	18.84 ± 0.41	8.06 ± 0.61[ab]	-1.43
COX-2	Vehicle	10	25.66 ± 0.75	18.95 ± 0.50	6.71 ± 0.74[ab]	-0.23
	Estrogen	10	23.85 ± 0.81	18.67 ± 0.38	5.17 ± 0.95[c]	-1.77
	Vehicle	12	22.90 ± 0.63	18.32 ± 0.21	4.58 ± 0.58[**c**]	-2.36
	Estrogen	12	24.78 ± 0.72	17.84 ± 0.24	6.94 ± 0.33[a]	0.00
	Vehicle	13	23.47 ± 0.75	18.19 ± 0.21	5.27 ± 0.89[bc]	-1.67
	Estrogen	13	25.36 ± 0.81	19.13 ± 0.82	6.22 ± 0.97[bc]	-0.72
	Vehicle	15	23.91 ± 0.50	18.54 ± 0.20	5.05 ± 0.39[**c**]	-1.89
	Estrogen	15	23.79 ± 0.40	18.86 ± 0.18	4.85 ± 0.35[**c**]	-2.09
	Vehicle	17	23.33 ± 0.57	18.57 ± 0.24	4.58 ± 0.61[**c**]	-2.37
	Estrogen	17	23.51 ± 0.43	18.84 ± 0.41	4.40 ± 0.30[**c**]	-2.54

*C_T = Cycle Threshold: Cycle number where amplification crosses the threshold set in the geometric portion of the amplification curve.

†ΔC_T = Target (COX-1,-2) – 18S Ribosomal CT: Normalization of PCR cycles for the target with 18S ribosomal RNA. Values with different superscripts for each of the target genes differ significantly ($P<0.05$)

§$\Delta\Delta C_T$ = Mean ΔC_T – highest mean ΔCT value: The mean value of Day 10 control (COX-1), and Day 12 E treated (COX-2) (highest ΔC_T; lowest gene expression for COX-1,-2 genes) was used as a calibrator for COX-1 and -2 to set the baseline for comparing differences in ΔC_T values across all days.

Figure 3.7. Fold difference in porcine endometrial COX-1 gene expression in the Veh (Orange) and E treated (Black) gilts. Fold differences in gene expression were calculated using the highest CT value (Day 10 Con) as the baseline as described in the *methods and materials*. Bars with different superscripts represent statistical differences.
.

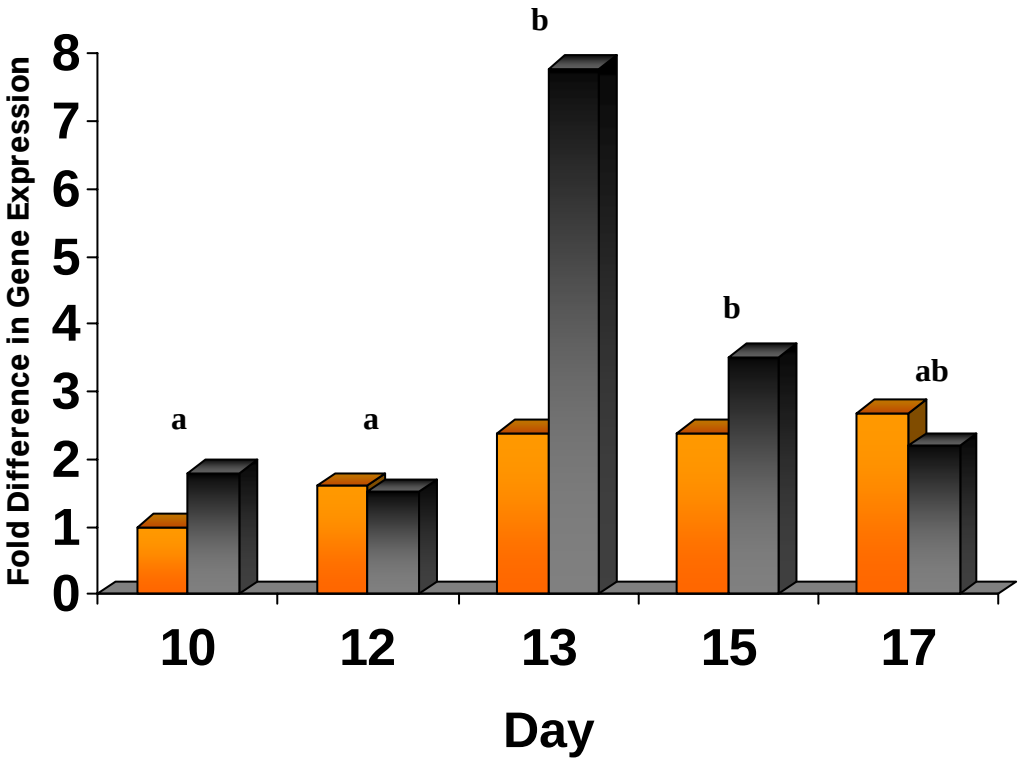
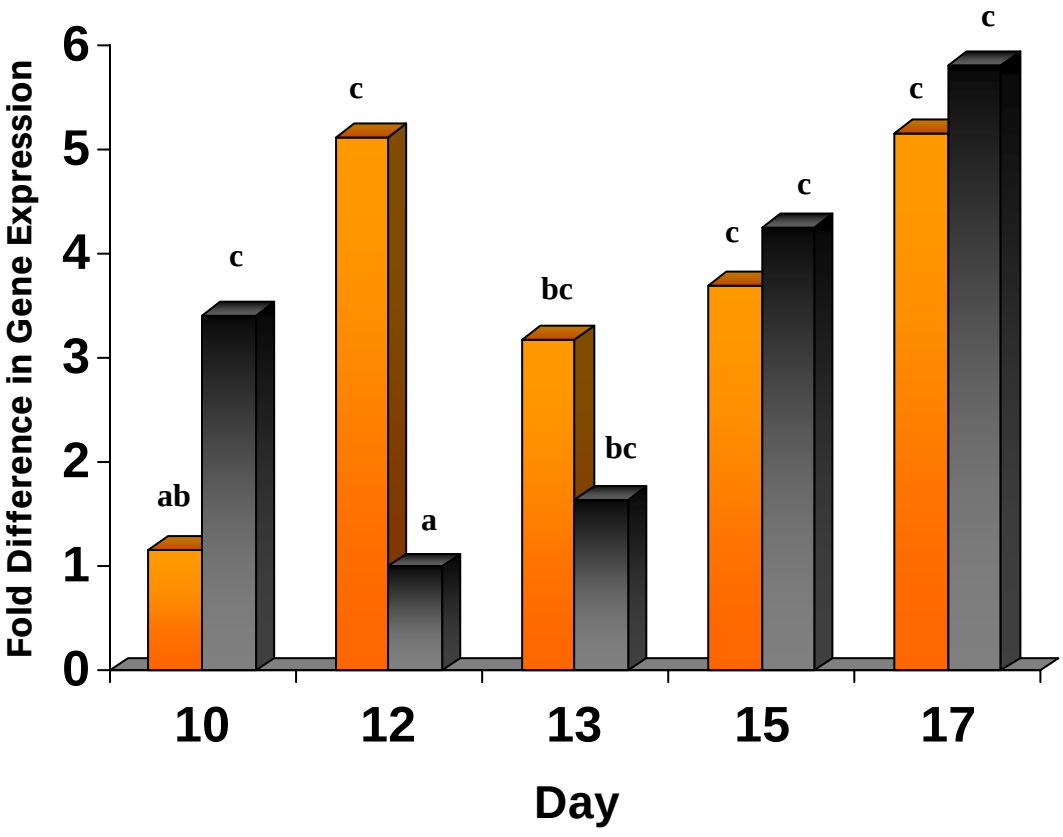

Fold Difference in Gene Expression
0
1
2
3
4
5
6
7
8
a
a
b
b
ab
10
12
13
15
17
Day

Figure 3.8. Fold difference in porcine endometrial COX-2 gene expression in the Vehl (Orange) and E treated (Black) gilts. Fold differences in gene expression were calculated using the highest CT value (Day 12 E) as the baseline as described in the *methods and materials*. Bars with different superscripts represent statistical differences.


Fold Difference in Gene Expression
0
1
2
3
4
5
6
ab
c
c
a
bc
bc
c
c
c
c
10
12
13
15
17
Day

COX-2 gene expression in E gilts was 3-fold greater on Day 10 of pregnancy compared to Veh gilts. However, COX-2 gene expression decreased on Days 12 and 13 of pregnancy in E gilts. There was 5-fold decrease in COX-2 gene expression on Day 12 of pregnancy in E compared to Veh gilts. Endometrial COX-2 gene expression was similar between treatment groups on Days 15 and 17 of gestation.

Western Blot Analysis of E and Veh Gilt Uterine Flushings

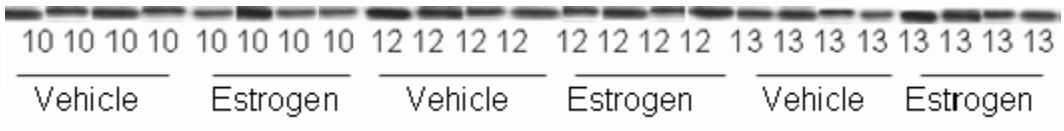
Western blot analysis did not indicate any differences in COX-1 protein in the uterine endometrium of the E and Veh treated gilts (Figure 3.9), however analysis of endometrial 72 kDa cytoplasmic COX-2 (Figure 3.10) was different between Veh and E treated gilts. Treatment of pregnant gilts with E altered the pattern of COX-2 expression. In Veh gilts, COX-2 increased on Day 12 and remained elevated through out Day 17 of early pregnancy. Administration of E caused a shift in COX-2 expression as the product expression increased on Day 10 followed by a decrease of COX-2 on Day 12. Endometrial content of COX-2 increased from Day 13 to 17 in the E treated gilts.

ELISA Assay of Prostaglandins in Uterine Flushings

Treatment did not alter the total uterine content of PGE_2 and $PGF_{2\alpha}$ in uterine flushings of pregnant gilts. Total luminal content of $PGF_{2\alpha}$ increased across the days of pregnancy ($P<0.001$). Uterine content of $PGF_{2\alpha}$ was lowest (0.11 ng) on Day 12 of gestation (Figure 3.11), increasing 9-fold (9.1 ng) by Day 17 of gestation. Content of PGE_2 in the uterine flushings increased across the days of gestation ($P<0.001$). Uterine PGE_2 content was lowest on Day 10 (6.23 ng) of gestation (Figure 3.12),

Figure 3.9 Western blot analysis of endometrial COX-1 expression of a 72 kDa immunoreactive product across the days of gestation in Veh and E treated gilts.

72 kDa


10 10 10 10 10 10 10 10 12 12 12 12 12 12 12 12 13 13 13 13 13 13 13 13
Vehicle
Estrogen
Vehicle
Estrogen
Vehicle
Estrogen

72 kDa

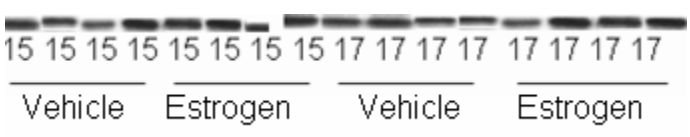
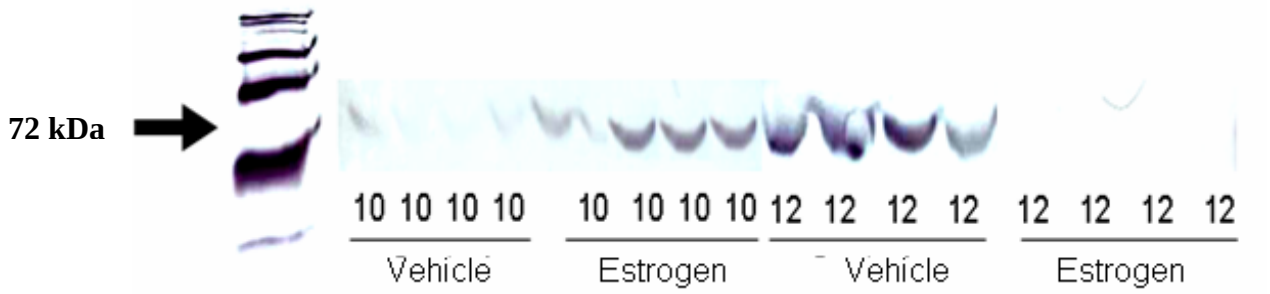

15 15 15 15 15 15 15 15 17 17 17 17 17 17 17 17
Vehicle
Estrogen
Vehicle
Estrogen

Figure 3.10 Western blot analysis of endometrial COX-2 expression of a 72 kDa immunoreactive product across the days of gestation in the Veh and E treated gilts.


72 kDa
10 10 10 10
10 10 10 10
12 12 12 12
12 12 12 12
Vehicle
Estrogen
Vehicle
Estrogen

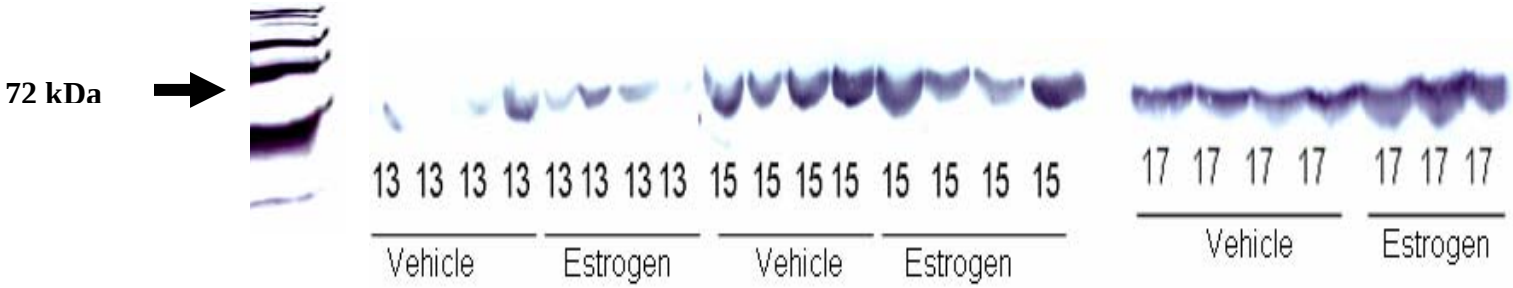

72 kDa
13 13 13 13
13 13 13 13
15 15 15 15
15 15 15 15
17 17 17 17
17 17 17
Vehicle
Estrogen
Vehicle
Estrogen
Vehicle
Estrogen

Figure 3.11. Total uterine lumenal content ± SEM of $PGF_{2\alpha}$ in the uterine luminal flushings from Veh (Orange) and E (Black) treated gilts across early pregnancy. Days with different superscripts represents significant differences ($P<0.05$).

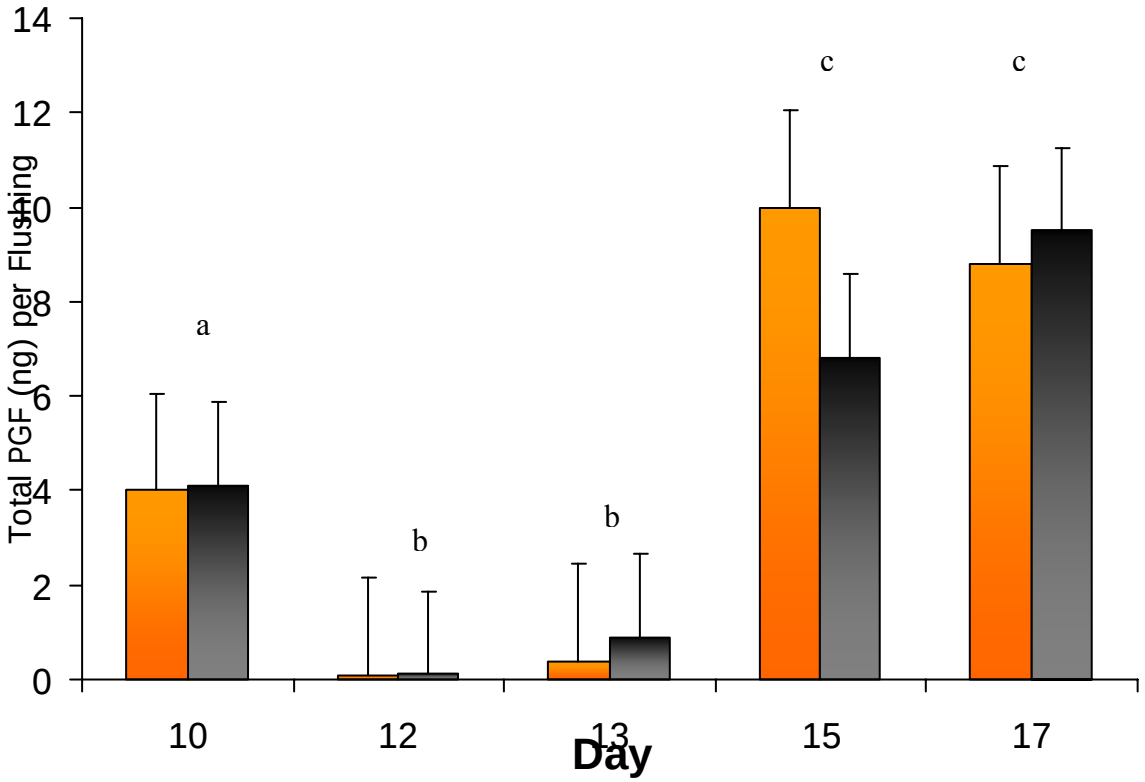
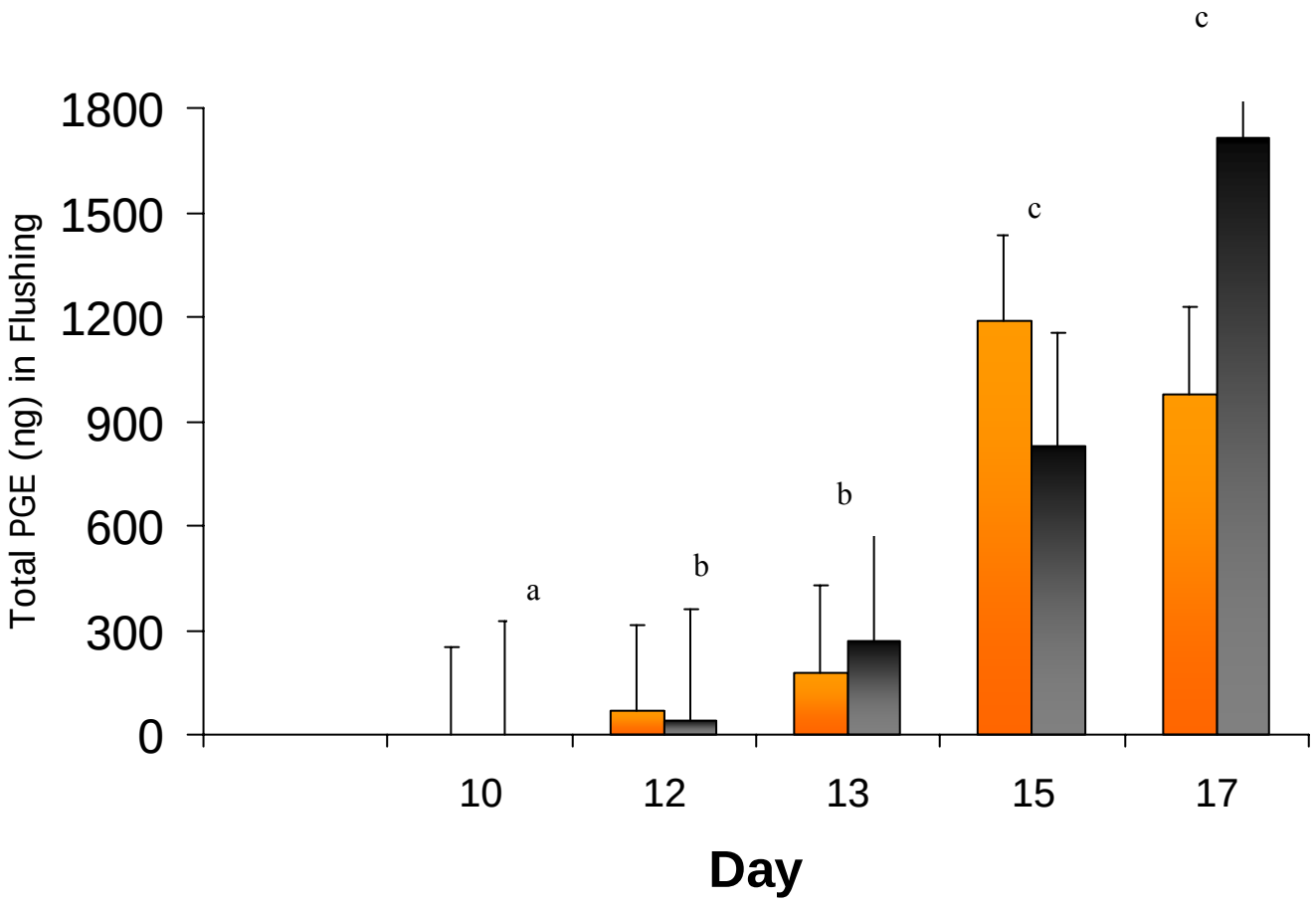

Total PGF (ng) per Flushing
14
12
10
8
6
4
2
0
a
b
b
c
c
10
12
13
15
17
Day

Figure 3.12. Total uterine lumenal content ± SEMof PGE_2 in the uterine luminal flushings from Veh (Orange) and E (Black) treated gilts across early pregnancy. Days with different superscripts represents significant differences ($P<0.05$).


Total PGE (ng) in Flushing
1800
1500
1200
900
600
300
0
a
b
b
c
c
10
12
13
15
17
Day

increasing 100-fold after conceptus elongation (Day 13). Uterine content of PGE_2 reached a maximum (1700 ng) on Day 17 of gestation.

Discussion

In most mammalian tissues, prostaglandin synthesis is regulated by either the constituent (COX-1) or the inducible (COX-2) form of cyclooxygenase (Smith et al., 2000). In the mouse, PG's have been implicated as a mediator of implantation due to elevated expression at attachment sites (Malathy et al., 1986). Lau et al. (1973) demonstrated in the mouse that administration of PG inhibitors disrupted implantation while coadministration of PG's restored the ability of the blastocysts to implant. A number of growth factors and cytokines stimulate prostaglandin synthesis through induction COX-2 gene expression (Baily et al., 1985). Both COX isoforms are expressed on the day of implantation in the mouse uterus (Chakraborty et al., 1996). The presence of COX-2 expression at the fetal-maternal interface in the rodent is crucial for blastocyst attachment to the uterine surface (Chakraborty et al., 1996). In the progesterone primed mouse uterus, ovarian estrogen is paramount for activation of downstream prostaglandin synthesis via COX-2 activation and conceptus implantation (Paria et al., 1998). Loss of COX-2 expression results in compounded reproductive failures in the mouse (Lim et al., 1997, Davis et al., 1999, Loftin et al., 2001). Disruption of PG synthesis inhibits implantation and maintenance of pregnancy which can be reestablished with administration of PG's in COX-2 knockout mice (Lim et al., 1997, Dinchuk et al., 1995). Prostaglandins are involved with changes in vascularity, immuno-inflammatory responses (Smith et al., 1996), and fluid movement (Biggers et al 1978). Prostaglandin

synthesis is essential for establishment of pregnancy in species with both invasive and noninvasive forms of implantation. Conceptus PG synthesis is not obligatory for rapid trophoblastic elongation in the pig on Day 12 of gestation (Geisert et al., 1986), however treatment of gilts with indomethacin during the period of placental attachment to the uterine surface (Days 13-17) causes embryonic death (Kraeling et al., 1985).

Implantation in the pig occurs through noninvasive attachment between the trophoblast and uterine surface epithelial microvilli (Dantzer et al., 1985). During trophoblastic elongation, conceptuses secrete estrogen into the uterine lumen which is the proposed signal for maternal recognition of pregnancy in the pig (Bazer and Thatcher, 1977). Prior to and during trophoblastic elongation, conceptuses secrete the pro-inflammatory cytokine IL-1β (Ross et al., 2003) prior to the period of conceptus attachment to the uterine luminal epithelium, which may trigger the inflammatory response for endometrial receptivity to implantation in the pig. IL-1β has been localized to the mouse embryo just prior to and during implantation (Huang et al., 1997, Krussel et al., 1997). Moore et al. (2004) demonstrated that murine exposure to recombinant IL-1β *in vivo* induces COX-2 gene expression. Furthermore, Haung et al. (1998) demonstrated that IL-1β exposure will induce COX-2 gene expression in human stromal cervical cells *in vitro*.

Studies have demonstrated COX-1 $^{-/-}$ deficient mice are fertile but experience a delay for initiating parturition (Langenbach et al., 1995). However, COX-2 $^{-/-}$ knockout mice exhibit multiple reproductive failures ranging from poor fertilization, increased implantation failure, and poor decidualization (Olofsson et al., 1996, Tsafriri et al., 1996). In the pig, PG production has been localized to the conceptus and endometrium (Geisert

et al., 1982, Gross et al., 1988). Kraeling et al. (1985) demonstrated inhibition of PG's does not alter elongation of conceptuses but rather increases embryonic mortality during the period of placental attachment. Although PG production is paramount for establishment of pregnancy, mechanisms regarding their roles have yet to be established clearly in domestic farm species. Although there are numerous papers describing the uterine content and endometrial synthesis of PG's in the pig, there was a void in the literature describing endometrial expression of cyclooxygenase during estrous cycle and early pregnancy.

Our study indicated that there is an 80-fold increase in COX-2 gene expression on Day 12 of pregnancy would be consistent with maximum secretion of conceptus derived IL-1β. However, the increase in endometrial COX-2 gene expression is not "pregnancy specific" as a similar increase in mRNA and protein expression occurred in cyclic females. Increase in endometrial COX-2 gene expression is isolated to the uterine surface epithelium during the estrous cycle and early pregnancy. Present data indicates both endometrial gene and protein expression increase at a time critical for conceptus development in the pig. Increased expression in COX-2 gene and protein expression in the gilt appears to be temporally associated with the loss of the progesterone receptor (PR) from the uterine surface and glandular epithelium (Geisert et al., 1994). One possible factor for induction of endometrial COX-2 gene expression may be through activation of the nuclear factor-κB (NF-κB) pathway (Page et al., 2002). A mutual negative interaction between NF-κB (subunit P-65) and PR has been suggested (Mckay and Cidlowsk, 1999), and loss of the PR in the endometrial epithelia is associated with increased activation of NF-κB (King et al., 2001). Changes in endometrial COX-2 gene

expression maybe regulated through cell specific changes in PR and NF-κB, which play an important role in implantation and the maintenance of pregnancy. Nakamura et al. (2004) has indicated that NF-κB activation is important for opening the window of implantation in the mouse uterus. It is possible that conceptus estrogen and IL-1β serve to regulate an alternant NF-κB pathway, so NF-κB stimulated cytokines and COX-2 expression permit normal conceptus development and survival. Estrogen has been demonstrated to serve an important role in regulating the NF-kB pathway as well (Chadwick et al., 2005). Therefore it is possible that timing of conceptus estrogen and IL-1β both play an important function in regulating the NF-kB system for a successful pregnancy. The possible role of endometrial NF-κB in the establishment of pregnancy in the pig needs to be investigated.

Pope et al., (1986) was the first to demonstrate the detrimental effects of early estrogen exposure on embryo survival. The mechanism by which early exposure to estrogen induces complete embryonic mortality has yet to be elucidated. Although slight reductions in the uterine surface glycocalyx occur during placentation in the pig (Dantzer et al., 1985), early estrogen exposure results in uterine surface microvilli devoid of gylcocalyx by Day 16 of gestation (Blair et al., 1991). Breakdown of the glycocalyx is not an immediate response, but rather occurs 5 days after the early estrogen exposure (Blair et al., 1991). The present study is the first to provide evidence that exogenous E exposure (D 9 and 10) to pregnant gilts prior to the normal appearance (D 12) by the conceptus, results in an alteration in the gene and protein expression of the inducible cyclooxygenase system (COX-2). Although uterine PG content in uterine flushing was not different between Veh and E treated gilts, conceptus production of PGs is high during

this early period of pregnancy (Kraeling et al., 1985). Therefore, uterine contribution of PGs from the porcine conceptuses cannot be separated from the endometrium. Geisert et al. (1987) also did not detect any difference in uterine $PGF_{2\alpha}$ between Veh and E treated gilts. It is interesting that although endometrial COX-2 gene expression is as high in cyclic gilts as pregnant the lumenal content is low. It is possible that endometrial production in cyclic gilts is moving into the uterine vascular through the stroma rather than into the uterine lumen as proposed by the endocrine-exocrine model of Bazer and Thatcher (1977). However, cyclic gilts given exogenous estrogen on Day 11 also do not have an increase in lumenal PGs until Day 15 (Geisert et al., 1982). Thus, presence of elevated endometrial COX-2 gene does not necessarily correspond to changes in the uterine lumen. The premature increase of COX-2 on Day 10 provides evidence that the estrogen can stimulate an early increase in the uterine epithelium prior to Day 12. The normal pattern observed in the COX-2 protein levels increase on Day 12, diminish on Day 13, and then continue to remain high on Days 15 to 17. However, gene analysis remains unchanged (RT-PCR, *in situ* data), which may open the possibility of differential regulation of gene and protein expression. Moreover, cytokines secreted from the conceptus on Day 12, such as IL-1β (Ross et al., 2003) may serve as a master transcription regulator of COX-2 through conceptus IL-1β stimulation of the NF-κB pathway, while stimulated by another cytokine (possible IL-18) on days 15-17. However, translation expression of the COX-2 protein appears to be an estrogen driven event based on the temporal appearance and disappearance of COX-2 expression in the uterus. The other possibility is that the uterine secretions are advanced 48 h ahead of the normal temporal and special pattern of early gestation. Gesiert et al. (1982) demonstrated

that exogenous E administration on Day 11 of the estrous cycle stimulated an increase in various uterine secretions needed for conceptus development. Porcine conceptuses are very sensitive to the uterine environment as recipient females must be within ± 24 h of synchrony with the donor to establish pregnancy in the pig (Polge, 1982). Transfer of embryos 48 h behind of synchrony recipient gilts results in very poor fertility. Recent studies in our lab have indicated that early estrogen exposure to the pregnant gilt on Days 9 and 10 results in a premature proteolysis of the IGFBP's, and early loss of the IGF-I and IGF –II ligands 48 h before normal disappearance (Ashworth, unpublished results). These data combined with the premature loss of the IGF's may be indicative of an advancement of the uterine environment by 48 h. The early estrogen administered to the pregnant gilts provides a similar window of advancement before normal conceptus estrogen, which corresponds to the temporal alteration of COX-2 in the present study. Thus, loss of embryos during the period of trophoblast attachment following early estrogen exposure maybe caused by an advancement of uterine gene programming that distrupts the normal timing of conceptus attachment to the uterine surface resulting in later embryonic death.

Chapter IV

Endocrine Disruption of Uterine Insulin-Like Growth Factor (IGF) Expression in the Pregnant Gilt

Abstract

On day 12 of pregnancy, porcine conceptuses signal their presence in the uterus through the release of estrogen, the signal for pregnancy recognition in the pig. Upon establishment of pregnancy, the rapidly expanding conceptuses require endometrial secretion of nutrients and growth factors to maintain the rapidly developing placental membranes and fetal growth. Insulin-like growth factors (IGF) are key growth factors involved in cellular development and expansion. Early exposure of pregnant gilts to estrogen, prior to the normal period of porcine conceptus estrogen secretion, disrupts the uterine environment resulting in complete embryonic mortality during the period of placental attachment and expansion. Current study evaluates the uterine IGF system following endocrine disruption of early pregnancy in gilts through early exposure estrogen. Mated gilts were administered either 5 mg estradiol cypionate (E) or vehicle (Veh) on Days 9 and 10 of gestation. Endometrium and uterine flushings (UTF) were harvested from Veh and E gilts on either Days 10, 12, 13, 15, and 17 of gestation. Endometrial IGF-I and IGF receptor type I (IGF-IR) gene expression was determined by Real-Time PCR. Uterine luminal content of IGF-I and IGF-II in UTF was determined by radioimmunoassay and ligand blot analysis was utilized to determine UTF content of the insulin-like growth factor binding proteins (IGFBP). Treatment of gilts with E altered endometrial gene expression as IGF-I mRNA expression was suppressed 2.5-fold in E

compared to Veh treated gilts on Day 12 of pregnancy. Endometrial gene expression for the IGF-IR was lowest on Day 13 for Veh gilts, while E treated gilts had a 3-fold increase in IGF-IR gene expression on Day 13 of gestation. Uterine content of IGF-I and, IGF-II were greatest on Days 10, 12, and 13 followed by a 4 to 6-fold decrease on Day 15 of gestation. Administration of E caused an earlier (24 h) decline in IGF's compared to Veh treated gilts. Early E administration resulted in the premature proteolysis of IGFBPs with in the pregnant pig uterus on Day 10 of gestation however, Veh treated pregnant gilts still contained functional intact IGFBPs on Day 10 The present study demonstrates that early exposure of pregnant gilts to estrogen causes a premature loss of uterine IGF's during the period of conceptus elongation. These results indicate that the timing for the release of uterine IGF's during early porcine conceptus development may play an important function in the ability of the conceptuses to attach and survive following maternal recognition of pregnancy.

Introduction

In the pig, establishment of pregnancy involves a specific sequence of uterine developmental and secretory events that are essential for continued conceptus growth and survival. Early porcine conceptus development involves a rapid transformation of the trophoblast from a spherical to filamentous morphology between Day 11 to 12 of gestation (Geisert et al., 1982). During rapid trophoblast elongation on Day 12, conceptuses secrete estrogen which serves as the signal for maternal recognition of pregnancy in the pig (Bazer and Thatcher 1977). There is a spatiotemporal relationship between the increase in uterine luminal content of insulin-like growth factors (IGF-I and

IGF-II) and early conceptus expansion and estrogen synthesis on Days 10 through 13 of gestation (Simmen et al., 1992; Geisert et al., 2001). Increase in uterine lumenal fluid content of IGF's on Days 11 to 12 of gestation has been proposed to be involved with stimulation of conceptus aromatase ($P450_{arom}$) activity to enhance conceptus estrogen synthesis during the period of trophoblast elongation (Letcher et al., 1989) and could be important for conceptus differentiation and attachment to the uterine luminal surface after initial expansion through the uterine horns.

Implantation of pig conceptuses involves a superficial attachment of the trophoblast to the microvilli located on the uterine apical surface epithelium (Perry et al., 1981; Dantzer, 1985). Trophoblast attachment to the uterine luminal surface epithelium is mediated by a number of endometrial cytokines, growth factors, and interactions between the developing conceptus and apical expression of endometrial integrins on the surface epithelium (Burghardt et al., 1997; Geisert and Yelich, 1997). The IGF system has been characterized in a multitude of biological systems (Simmens et al., 1992; Choi et al., 1997; Irwin et al., 2001; Sato, et al., 2002) and is composed of three ligands (IGF-I, -II, and insulin), five regulatory binding proteins (IGFBP-2 through 6), and three cell surface receptors (IGF-I receptor, IGF-II receptor, insulin receptor) (Jones et al., 1995). The porcine conceptus has been reported to express mRNA for insulin-like growth factor I receptor (IGF-IR) (Corps et al., 1990). Although gene expression for IGF-IR was demonstrated, Chastant et al. (1995) was unable to detect the presence of IGF-IR in the trophoblast, but did localize the IGF-IIR. Conceptus expression of the IGF-IR mRNA and the presence of trophoblast IGF-RII indicate that uterine IGF secretion could serve an integral part in early porcine conceptus development and survival.

Importance of IGF's in early porcine conceptus development and uterine receptivity for implantation is demonstrated by the precise alteration in the presence of IGFBPs that occurs during the period of conceptus expansion (Lee et al., 1998; Geisert et al., 2001). Uterine IGFBP's are present in the porcine uterine lumen from Day 5 to 10 of the estrous cycle and during early gestation (Lee et al., 1998; Geisert et al., 2001). However, the porcine uterine luminal IGFBP's are proteolytically cleaved after Day 11 in both cyclic and pregnant gilts. Activation of proteolytic enzymes such as the serine protease, tissue kallikrein and the matrix metalloproteinases degrade IGFBPs in the uterine lumen allowing IGF stimulation of the conceptuses during a critical period of development in the pig (Vonnahme et al., 1999; Geisert et al. 2001).

Our laboratory has established that conceptus secretion of estrogen between Days 11 and 13 of gestation plays a critical role in the normal process of implantation in pigs, and we have demonstrated that estrogen can function as an endocrine disruptor of implantation if administered on Days 9 and 10, i.e., 48 h prior to the normal period of secretion of estrogen and 96 h prior to initiation of implantation. Exposure of pregnant gilts to exogenous estrogens before the normal physiological secretion of conceptus estrogens on Day 12 results in complete embryonic mortality before Day 30 of gestation (Pope et al., 1986). Early exposure of gilts to estrogen on Days 9 and 10 of pregnancy causes conceptus degeneration and fragmentation between Days 15 to 18 of gestation (Blair et al., 1991; Gries et al., 1989). Recently, the concentration and timing of estrogen stimulation was demonstrated to function within a very narrow range to open the window for uterine receptivity in the mouse (Ma et al., 2003). High concentrations of estrogen shorten the window of receptivity and cause implantation failure as a result of aberrant

uterine gene expression during blastocyst attachment. We propose that estrogen treatment of pregnant gilts on Days 9 and 10 alters the timing of IGF-I presence within the uterine lumen, which could be critical for continued embryonic development. The current study evaluated the uterine IGF system following administration of estrogen on Days 9 and 10 of pregnancy in the pig.

Methods and Materials

Animals

Research was conducted in accordance with the Guiding Principles for Care and Use of Animals promoted by the Society for the Study of Reproduction and approved by the Oklahoma State Institutional Care and Use Committee. Crossbred cycling gilts of similar age (8-10 mo) and weight (100-130 Kg) were checked twice daily for estrus behavior (estrus onset = Day 0 of estrous cycle) with intact males. Gilts were mated with fertile crossbred boars at first detection of estrus and again 24 h post-detection. Bred gilts (n=40) were randomly assigned one of the following two treatment groups: Vehicle (Veh), i.m. injection of corn oil (2.5 ml) on Days 9 and 10 of gestation or estrogen treatment (E), 5 mg i.m. injection of estradiol cypionate (A.J. Legere, Scottsdale, AZ) on Days 9 and 10 of gestation.

Surgical procedure

Bred gilts were hysterectomized on either Days 10, 12, 13, 15 and 17 of gestation as previously described by Gries et al. (1989). Following initial induction of anaesthesia with an 1.8 ml i.m. administration of a cocktail consisting of 2.5 ml (Xylazine:

100mg/ml: Miles Inc., Shawnee Mission, KS) and 2.5 ml Vetamine (Ketamine HCl: 100 mg/ml Molli Krodt Veterinary, Mundelein, IL) in 500 mg of Telazol (Tiletamine HCl and Zolazepum HCl: Fort Dodge, Syracuse, NE), anesthesia was maintained with a closed circuit system of halothane (5% flurothane) and oxygen (1.0 liters/min). The uterus was exposed via midventral laparotomy and a randomly selected uterine horn, and its ipsilateral ovary, excised. Uterine luminal contents and conceptuses were flushed from the horn by infusing 20 mL phosphate buffered saline (PBS, pH 7.4) through the lumen and collecting flushings into a petri dish. Conceptuses were removed from flushings, snap frozen in liquid nitrogen and stored at -80°C. Uterine flushings were centrifuged (1000 x g, @ 4°C for 10 min), supernant collected and stored at -20°C. Endometrial tissue was removed from the antimesometrial side of the uterine horn, immediately snap frozen in liquid nitrogen and stored at -80°C until utilized for RNA extraction.

Endometrial RNA Extraction

Total RNA was extracted from endometrial tissue using RNAwiz™ reagent (Ambion, Inc. Austin, TX). Approximately 0.5 g of endometrial tissue was homogenized in 5.0 mL of RNAwiz using a Virtishear homogenizer (Virtis Company Inc., Gardiner, NY). RNA was rehydrated in nuclease free water and stored at -80°C. Total RNA was quantified with a spectrophotometer at an absorbance of 260 and purity was verified using the 260/280 ratio.

Quantitative 1-Step RT-PCR

Quantitative analysis of endometrial IGF-I and IGF-IR mRNA were conducted by using quantitative real time reverse polymerase chain reaction (RT-PCR) as previously described by our laboratory (Hettinger et al., 2001). The PCR amplification was

performed in a reaction volume of 15 μl using an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The transcripts were evaluated using dual labeled probes containing 6-Fam (5' reporter dye), and TAMRA (3' quenching dye). Primer and probe sequences for the amplification of IGF-I and IGF-1R (Table 4.1) were generated from porcine cDNA sequences available in the NCBI database. Total RNA (100 ng) was assayed in duplicate using thermocycling conditions for one-step cDNA synthesis of 30 min at 48°C and 95°C for 10 min, followed by 45 repetitive cycles of 95°C for 15 sec and 60°C for 1 min. Ribosomal 18S RNA was assayed in each sample to normalize RNA loading as previously described by Ross et al. (2003).

Template amplification was quantified by determining the threshold cycle (C_T) based on the fluorescence detected within the geometric region of the semilog plot. In the geometric region, one cycle is equivalent to the doubling of PCR target template. Using the comparative C_T method (Hettinger et al., 2001), relative quantification and fold gene expression difference between treatment and day were determined for the endometrial samples (Table 4.2). Differences in mRNA expression of IGF-1 and IGF-1R were determined by subtracting target C_T of each sample from its respective ribosomal 18S C_T value, which provides the sample ΔC_T value. Calculation of the $\Delta\Delta C_T$ involves using the highest sample ΔC_T value as an arbitrary constant to subtract from all other ΔCT sample values. Fold differences in gene expression of the target gene are equivalent to $2^{-\Delta\Delta CT}$.

Table 4.1 PCR Primer and probe sequences used for quantitative RT-PCR

Gene		Forward Primer/Reverse Primer/Probe	GeneBank Accession
IGF-I	Forward	5'-GCCTTGACTGTGATATGCGTGGTT-3'	**X64400**
	Reverse	5'-AGAAAGACAAGTTAGCGTCCGGAGT-3'	
	Probe	5'-ACACTGACGGATGCTGAAGGCGGGCACCAT-3'	
IGF-IR	Forward	5'-GCATGGCATACCTCAACGCCAATA-3'	**TC131942**
	Reverse	5'-TGTGAAGTCTTCGGCCACCATACA-3'	
	Probe	5'-TTTGTCCACAGAGACCTCGCTGCCCGGAA-3**'**	

Radioimmunoassay of uterine flushing IGF content

Uterine content of immunoreactive IGF-I in uterine lumenal flushings was determined in a single radioimmunoassay after acid-ethanol extraction (16 h at 4^{o}C) as previously described by Echternkamp et al. (1990). Intra-assay coefficient of variation was 2.8%. Content of IGF-II in uterine flushings was determined in a single assay by radioimmunoassay as described by Spicer et al. (1995). The intra-assay coefficient of variation was 3.0%.

Ligand Blotting

Uterine flushing samples were prepared for ligand blotting by concentrating 4 ml of uterine flushing using a Centricon 10 concentrator (Amicon Beverly, MA) with a molecular wt cut off of 10 000. Protein content in the concentrated uterine flushing sample was determined by Bradford protein assay using BSA as the protein standard. IGFBPs in the uterine flushings were analyzed by one-dimensional SDS-PAGE as described by Echterncamp et al. (1994). Protein (50 μg) from concentrated uterine flushing was mixed with 21 μL non-reducing denaturation buffer (Bio-Rad, Hercules CA.). Bovine follicular fluid, diluted 10-fold, was utilized as a positive control to identify band size and IGFBPs in the porcine uterine flushings. Samples were denatured by heating to 100°C for 3 min, centrifuged at 4657 x g for 3 min and separated using 12% (w/v) PAGE for 65 min at a constant current of 25 mA per gel. After separation, proteins in the gel were electrophoretically transferred to nitrocellulose paper (Midwest Scientific, St Louis, MO) and subsequently ligand blotted (16 h at 4°C) using recombinant bovine [^{125}I]-labelled IGF-I and IGF-II. The next day, the nitrocellulose blots were washed, dried, and exposed to X-ray film at -80°C for 21 days. Individual band intensity on

audioradiographs was determined using scanning densitometry with Molecular Analyst (BIORAD).

Statistical analysis

Data were analyzed by the least square ANOVA using the Proc Mixed procedure of SAS (SAS, 1988). The statistical method used to analyze uterine gene expression for IGF-I, and IGF-IR, and IGF-I and IGF-II protein in the uterine flushing included effects of day, treatment, and day x treatment interaction.

Results

Conceptus development

Normal conceptuses were present in the uterine flushings collect from bred Veh gilts across the days of pregnancy evaluated. Normal appearing conceptuses were collected from E gilts on Days 10, 12 and 13 of gestation. However, conceptus tissues were in various stages of fragmentation when collected on Days 15 and 17 of gestation.

Quantitative RT-PCR

Quantitative RT-PCR analysis of endometrial IGF-I gene expression in E and Veh treated gilts (Table 4.2) detected a day x treatment interaction ($P<0.003$). Estrogen treatment on Days 9 and 10 of gestation decreased endometrial IGF-I gene expression approximately 3-fold compared to Veh gilts on Day 12 (Figure 4.1). However on Day 13 of gestation, endometrial IGF-I gene expression increased 2.5-fold in E compared to Veh

treated gilts (Figure 4.1). Endometrial IGF-I expression was similar between treatments on Days 10, 15 and 17 of gestation.

A day x treatment interaction ($P<0.0004$) was detected for endometrial IGF-IR gene expression (Table 4.2). Although there was a slight decrease in endometrial IGF-IR gene expression on Day 13, IGF-IR did not significantly change across the day of gestation in Veh gilts. However, endometrial IGF-IR gene expression increased 3-fold on Day 13 of gestation in E compared to Veh gilts (Figure 4.2). Gene expression was similar between E and Veh gilts on Days 15 and 17 of gestation.

Uterine Lumenal content of IGF-I and IGF-II Ligand

Content of IGF-I in the uterine flushings of E and Veh gilts (Figure 4.3) were effected by day ($P<0.01$) and treatment ($P<0.03$). Uterine luminal IGF-I content in Veh gilts was greatest on days 10, 12 and 13 (484, 611 and 690 ng, respectively) of gestation, which was followed by a 4 to 6-fold decline on Days 15 (150 ng) and 17 (109 ng) of gestation. Uterine flushing content of IGF-I in E-treated gilts was similar to Veh gilts on Day 10, but uterine luminal IGF-I content decreased 48 h earlier in E gilts ($P<0.03$). Amount of uterine IGF-I in E-treated gilts sharply declined on Day 12 and was 10-fold less on Day 13 (60 ng) of gestation compared to Veh (690 ng) gilts. Content of IGF-I in uterine flushings was similar across treatments on Days 15 and 17 of gestation.

A treatment x day interaction ($P<0.04$) was detected for the content of IGF-II in uterine flushings. Pregnant gilts treated with estrogen on Days 9 and 10 of gestation had a 7-fold decrease in uterine content of IGF-II on Day 13 of gestation compared to Veh gilts

(52 vs 370 ng). Uterine content of IGF-II was similar on all other days of gestation evaluated in the study.

Ligand Blotting

Presence of IGFBPs in the uterine lumen of Veh and E treated gilts were evaluated through ligand blot analysis with [^{125}I]-labeled IGF-I and IGF-II. The ligand blot revealed the presence of two major bands at 46 and 34 kDa (IGFBP-2, -3) in uterine flushings of Veh gilts on Day 10 of pregnancy but no bands were detected on Day 12, 13, 15 or 17 of gestation (Figure 4.5). Ligand blot indicated that E-treatment caused an earlier loss of IGFBPs as no bands were detected in Day 10 in contrast to Veh gilts.

Discussion

During conceptus trophoblast elongation and estrogen release to establish pregnancy in the pig, a number of endometrial cytokines and growth factors are released into the uterine lumen to support conceptus development and survival (see review Geisert and Yelich, 1997). The high concentration of IGF-I within the uterine lumen prior to and during rapid trophoblast expansion on Day 10 to 12 of pregnancy is thought to have a direct effect on augmenting conceptus steriodogenesis via enhancement of P-450$_{arom}$ gene expression and enhancing aromatase activity (Simmen et al., 1989, Hofig et al., 1991, Ko et al., 1994). Although Green et al. (1995) reported an increase in IGF-IR mRNA transcript in the expanding conceptuses, Chastant et al. (1991) were unable to localize the translated receptor protein to the trophoblast.

Table 4.2. Endometrial IGF-I and IGF-IR gene expression of vehicle and estrogen treated gilts

Target	TRT	Day	Average Target C_T *	18S C_T *	ΔC_T † *	$\Delta\Delta C_T$ §
IGF-I	Veh	10	28.25 ± 0.29	18.95 ± 0.50	9.30 ± 0.42^{bc}	-1.87
	E	10	27.84 ± 0.19	18.67 ± 0.38	9.17 ± 0.12^{bc}	-1.99
	Veh	12	28.30 ± 0.27	18.32 ± 0.21	9.98 ± 0.27^{bc}	-1.18
	E	12	29.00 ± 0.39	17.84 ± 0.24	11.16 ± 0.55^{a}	0.00
	Veh	13	28.66 ± 0.19	18.19 ± 0.21	10.47 ± 0.18^{b}	-0.69
	E	13	28.24 ± 0.53	19.13 ± 0.82	9.11 ± 0.28^{c}	-2.05
	Veh	15	28.01 ± 0.37	18.54 ± 0.20	9.47 ± 0.31^{bc}	-1.69
	E	15	27.72 ± 0.30	18.86 ± 0.18	8.86 ± 0.35^{c}	-2.30
	Veh	17	27.86 ± 0.25	18.57 ± 0.24	9.29 ± 0.28^{c}	-1.87
	E	17	27.72 ± 0.43	18.84 ± 0.41	8.88 ± 0.34^{bc}	-2.28
IGF-IR	Veh	10	21.22 ± 0.06	18.95 ± 0.50	2.27 ± 0.26^{bc}	-0.83
	E	10	21.36 ± 0.34	18.67 ± 0.38	2.69 ± 0.14^{ab}	-0.41
	Veh	12	21.05 ± 0.34	18.32 ± 0.21	2.73 ± 0.31^{ab}	-0.37
	E	12	20.82 ± 0.17	17.84 ± 0.24	2.98 ± 0.23^{a}	-0.12
	Veh	13	21.30 ± 0.15	18.19 ± 0.21	3.10 ± 0.13^{a}	0.00
	E	13	20.90 ± 0.50	19.13 ± 0.82	1.69 ± 0.20^{d}	-1.42
	Veh	15	20.99 ± 0.04	18.54 ± 0.20	2.36 ± 0.08^{b}	-0.74
	E	15	20.50 ± 0.13	18.86 ± 0.18	1.74 ± 0.15^{cd}	-1.37
	Veh	17	20.77 ± 0.18	18.57 ± 0.24	2.23 ± 0.19^{bc}	-0.07
	E	17	20.66 ± 0.19	18.84 ± 0.41	1.82 ± 0.20^{cd}	-1.28

*C_T = Cycle Threshold: Cycle number where gene amplification crosses the threshold set in the geometric portion of the amplification curve.

†ΔC_T = Target (IGF-I, IGF-IR) – 18S Ribosomal CT: Normalization of PCR cycles for the target with 18S ribosomal RNA. Values with different superscripts for each of the target genes differ significantly ($P<0.05$)

§$\Delta\Delta C_T$ = Mean ΔC_T – highest mean ΔCT value, the mean value for IGF-I and IGF-IR with the highest ΔC_T (lowest expression for target) were used as a calibrator to set the baseline for comparing differences in ΔC_T values across all treatments and days.

Figure 4.1 Fold difference in porcine endometrial IGF-I gene expression in Veh (Orange) and E treated (Black) pregnant gilts. The gene expression level for Day 12 E treated endometrium was set as baseline and fold difference was calculated as described in Material and Methods. Columns with different superscripts differ significantly ($P<0.003$)

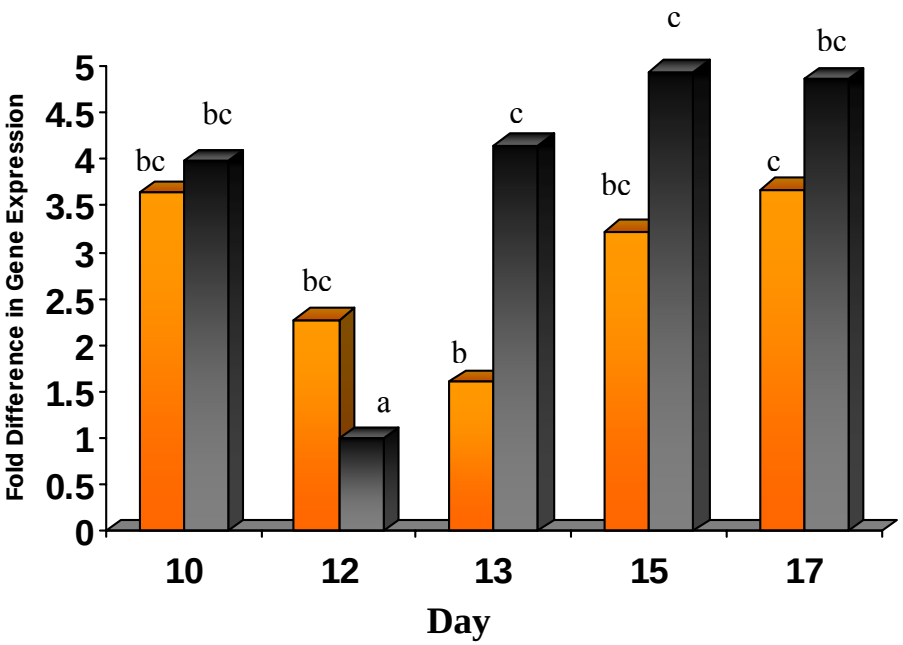

Fold Difference in Gene Expression
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
bc
bc
bc
a
b
c
bc
c
c
bc
10
12
13
15
17
Day

Figure 4.2 Fold difference in porcine endometrial IGF-IR gene expression in the Veh (Orange) and E treated (Black) pregnant gilts. The gene expression level for Day 13 Veh treated endometrium was set as baseline and fold difference was calculated as described in Material and Methods. Columns with different superscripts differ significantly ($P<0.004$)

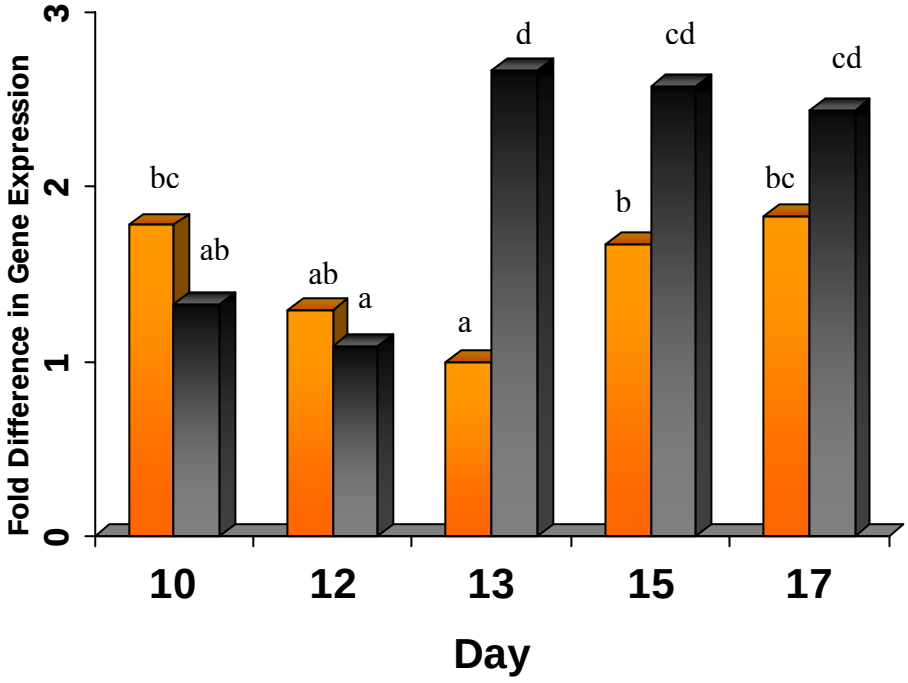

Fold Difference in Gene Expression
0
1
2
3
bc
ab
ab
a
a
d
b
cd
bc
cd
10
12
13
15
17
Day

Figure 4.3. Content ± SEM of IGF-I in the uterine flushings from Veh (Orange) and E treated (Black) pregnant gilts. A significant day ($P<0.01$) and treatment ($P<0.03$) effect was detected. Columns with different superscripts differ significantly (Day effect $P<0.01$) (Trt effect $P<0.03$).

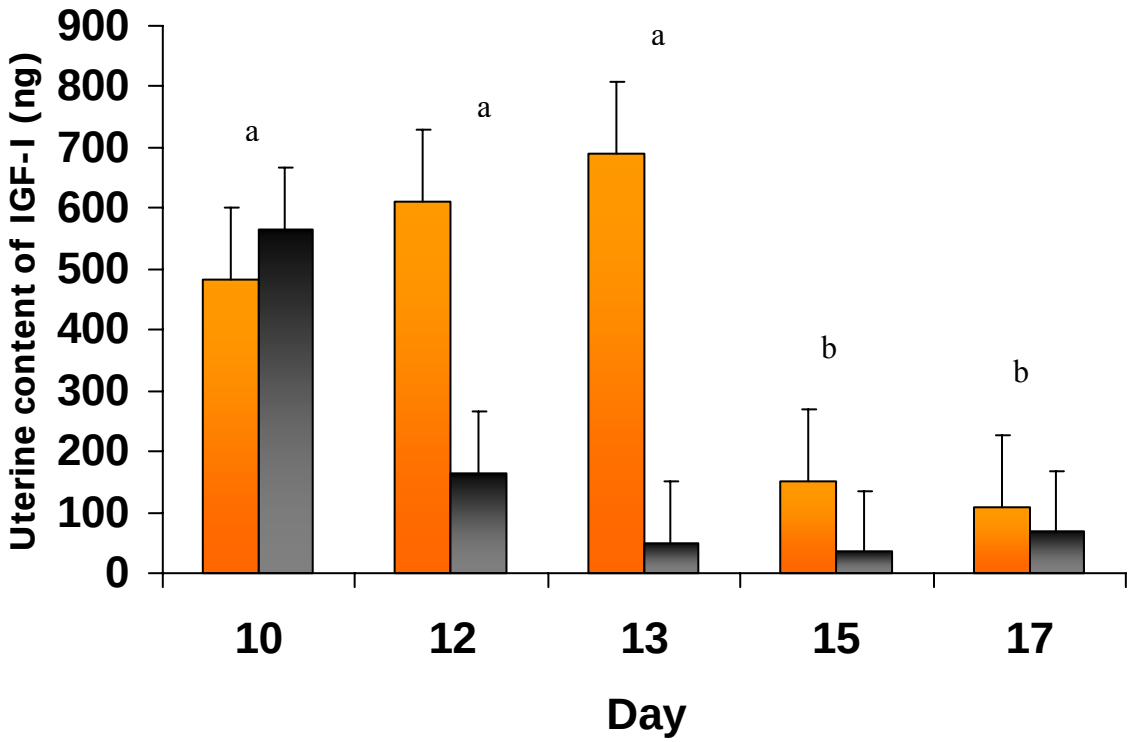

Uterine content of IGF-I (ng)
900
800
700
600
500
400
300
200
100
0
a
a
a
b
b
10
12
13
15
17
Day

Figure 4.4 Content $\pm$ SEM of IGF-II in the uterine flushings from Veh (Orange) and E (Black) treated pregnant gilts. A significant ($P<0.04$) day by treatment interaction was detected. Columns with different superscripts differ (day $*$ trt $P<0.03$).

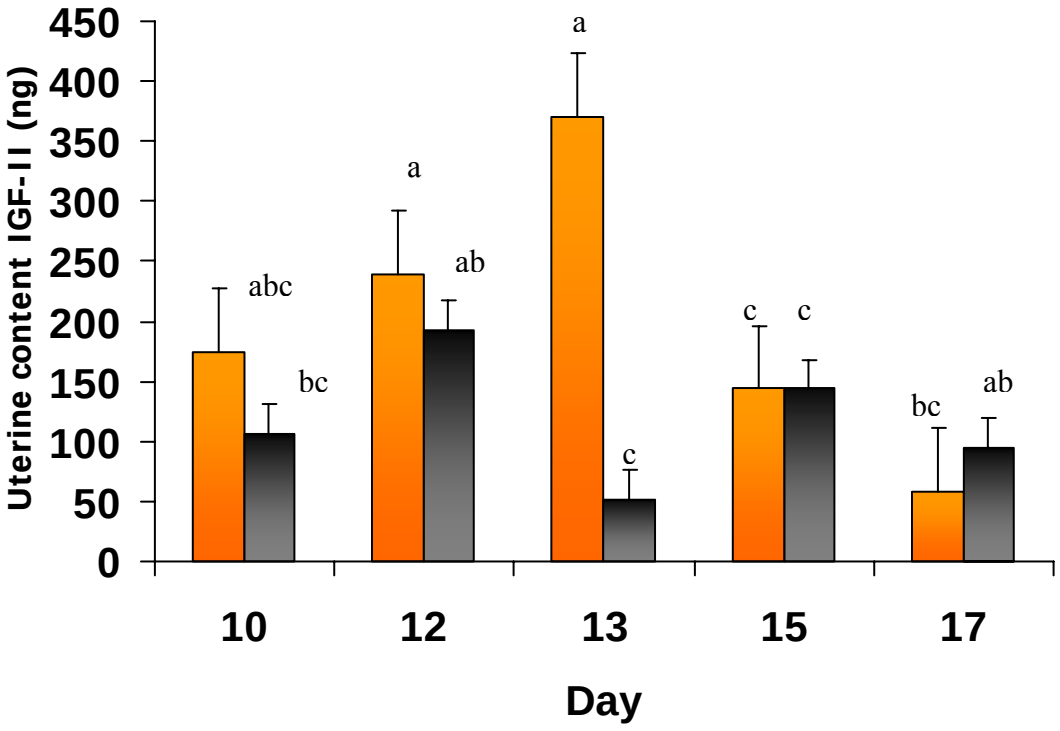
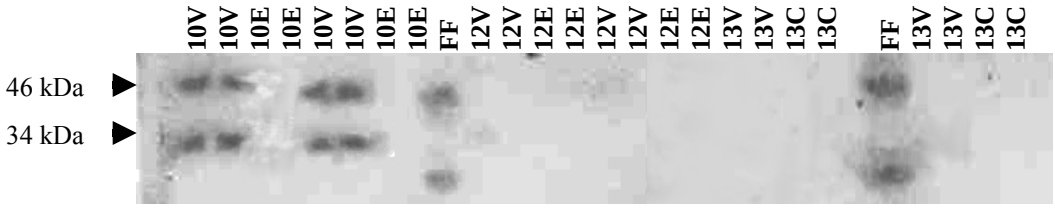

Uterine content IGF-II (ng)
450
400
350
300
250
200
150
100
50
0
abc
bc
a
ab
a
c
c
c
bc
ab
10
12
13
15
17
Day

Figure 4.5 Ligand binding of [^{125}I]-labelled IGF-I and IGF-II (insulin-like growth factor I and II) to insulin-like growth factor binding proteins (IGFBPs) in concentrated uterine flushings of individual gilts collected on selected days of pregnancy (Days 10, 12 and 13). Pre-stained colored markers were used to define the sizes of the bands and bovine follicular fluid (FF) served as a positive control, as well as to identify the various IGFBPs.


10V
10V
10E
10E
10V
10V
10E
10E
FF
12V
12V
12E
12E
12V
12V
12E
12E
13V
13V
13C
13C
FF
13V
13V
13C
13C
46 kDa
34 kDa

However, the presence of IGF-IIR in the trophoblast suggests that uterine and/or conceptus IGF secretion can regulate early conceptus development. In the present study, uterine luminal content of IGF-I and IGF-II peaked on the day of conceptus elongation (Day 11 to 12) in Veh gilts. Content of IGF-I and II in uterine flushings decreased dramatically following conceptus elongation and the initiation of placental attachment to the uterine surface epithelium (Day 13). Results from Veh gilts in the present study are consistent with previous publications on luminal uterine IGF content in cyclic and pregnant pigs (Lecther et al., 1989; Geisert et al., 2001). Although there is a slight decline in endometrial IGF-I gene expression on Days 12 and 13 of pregnancy, gene expression returns to similar levels to Day 10 on Day 15 and 17. Thus, the decrease of IGF-I in uterine flushings is not related to a depression in endometrial IGF-I gene expression.

The high amounts of IGFs in the uterine lumen of the pig are closely associated with detection of IGFBPs in the uterine lumen. Insulin-like growth factor binding proteins regulate the biological activity of the IGF ligands *in vivo* (Rechler et al., 1993). The IGFBP's share structural homology and have a high binding affinity for the IGF ligands, but are negligible toward insulin. On Day 10 of the estrous cycle and pregnancy, IGFBP-2 and -3 are detected in the uterine luminal fluids of cyclic and pregnant pigs when uterine luminal content of IGF-I and –II are high (Lee et al., 1998; Geisert et al., 2001). However, there is almost a complete loss of uterine luminal IGFBPs on Day 12 of either the estrous cycle or pregnancy (Geisert et al., 2001). Disappearance of IGFBP's observed in uterine luminal flushings on Day 12 is due to an increase in IGFBP proteolysis rather than down-regulation of IGFBP mRNA (Lee et al., 1998). The

proteolysis of IGFBPs in the porcine uterine lumen may occur through activation of the serine protease, tissue kallikrein and/or metalloproteinases (Lee et al., 1998; Geisert et al., 2001). IGF's are regulated and stabilized through tertiary binding to IGFBPs. The degradation of IGFBP's within the uterine lumen may in part be responsible for the decreased content of IGF-I and IGF-II collected in uterine flushings after Day 12. Ballard et al. (1991) demonstrated that the normal 10 min half-life of ^{125}I-IGF-I can be extended to greater than 15 h when bound to IGFBPs. Furthermore, IGFBP may play a role in prevention of premature binding and signaling of the ligands through the IGF-IR at the cellular level (Conover et al., 1990). It is possible IGFBPs help sequester IGF-I and -II in the uterine lumen for release during the sensitive period of conceptus differentiation and trophoblast elongation. The spatiotemporal association of uterine IGFs and IGFBPs at critical period in early porcine conceptus development and the alteration observed following estrogen administration in the present study, suggests that the uterine IGF system serves an important biological role in establishment and maintenance of pregnancy.

The abundant presence of IGF-I receptors in the endometrium indicates that IGFs have an autocrine role in uterine function during the period of conceptus expansion (Simmen et al., 1992). In the current study, endometrial IGF-IR gene expression increased after the decrease in luminal IGFs. Circulating concentrations of IGF-I and IGF-II are generally thought to depress expression of IGF-IR locally (Rosenfeld et al., 1982), which may explain the up-regulation of the IGF-IR mRNA on Days 15 and 17. Maintenance of endometrial IGF-I gene expression and loss of luminal IGFBPs may

allow IGFs to stimulate uterine tissue rather than sequestering the growth factors in the lumen during pregnancy.

Although estrogen plays a major function in regulating the block to luteolysis and uterine changes in secretion and morphology for implantation in the pig, inappropriate exposure to estrogen (i.e., delivered prior to the normal time of conceptus secretion on Day 11 to 12 of gestation) has a detrimental effect on conceptus survival. Consumption of feed containing the estrogenic mycotoxin, zearalenone, causes total embryonic loss in swine (Long & Diekman, 1986). Pope et al. (1985) first demonstrated that administration of estrogen to gilts on Days 9 and 10 of gestation resulted in complete embryo mortality before Day 30 of gestation; however, no effect was observed when estrogen was administered on Days 12 to 13 (time of endogenous conceptus estrogen secretion). Our laboratory demonstrated that premature exposure of the pregnant uterus to estrogen (Day 9 and 10) does not effect conceptus elongation on Day 12, but results in conceptus degeneration on Day 15 of pregnancy (Morgan et al. 1987, Gries et al. 1989). The cause of the early conceptus degeneration following endocrine disruption with estrogen is not known. Blair et al. (1991) indicated that early estrogen administration causes a loss of the uterine epithelial surface glycocalyx which could interfere with conceptus attachment to the uterine surface. Loss of porcine conceptuses at the time of placental attachment following estrogen treatment is similar to the implantation failure caused by estrogen in the mouse. Recently the concentration and timing of estrogen stimulation was demonstrated to function within a very narrow range to open the window for uterine receptivity in the mouse (Ma et al. 2003). High concentrations of estrogen shorten the window of receptivity and cause implantation failure as a result of aberrant uterine gene

expression during blastocyst attachment. This study provides direct evidence that estrogen not only opens the window of uterine receptivity in the mouse, but the concentration and timing are critical for ensuring proper down-stream events essential for blastocyst implantation and survival.

Our results indicate that administration of estrogen to gilts on Days 9 and 10 of pregnancy causes premature proteolysis of uterine luminal IGFBPs. The disappearance of IGFBP's may in part be responsible for the early decline of IGF-I and IGF-II on Days 12 and 13 in estrogen treated gilts. Corthorn et al. (1997) demonstrated that estrogen stimulated tissue kallikrein activation in the uterine epithelium of the rat. Estrogen activation of the uterine proteolytic enzymes such as tissue kallikrein and matrix metalloproteinases that degrade the IGFBPs (Geisert et al., 2001) could be the mechanism by which the early porcine conceptus releases IGFs for its development *in utero*. The advanced increase in endometrial IGF-IR gene expression in E treated (Day 13) compared to Veh (Day 15) gilts is consistent with the early decline of the IGFs in the uterine lumen. Precise nature of the loss of uterine luminal IGFs following conceptus elongation suggest that the release of IGFs during Day 12 and 13 of pregnancy is very critical for down-stream development and survival of pig embryos. Although we cannot demonstrate a causal effect of premature loss of IGFs with later embryonic death from our current study, early estrogen administration clearly causes a dramatic decline in IGFs before the critical period of conceptus elongation and differentiation. Mice devoid of the IGF-IIR undergo *in-utero* mortality during gestation (see review Jones and Clemmons, 1995). Studies have demonstrated that the IGF-IIR is fundamental in embryonic development in the mouse (Barlow et al., 1991), and provides major roles in tissue

remodeling and translocation of newly synthesized cathepsins to the lysosomes (Dahms et al., 1989). Thus, alteration in the normal synchrony of IGF release in the uterine lumen during early pregnancy may cause aberrant endometrial and/or conceptus gene expression during implantation. Microarray analysis of endometrium from estrogen treated gilts has indicated that a number of genes are upregulated on Day 13 compared to normal controls (Ross, Ashworth, and Geisert, unpublished results). Therefore, endocrine disruption is not solely limited to the IGF system although IGFs could play roles in the alteration of many other genes as well.

The present study suggests proteolysis of the IGFBP's is clearly an endocrine disrupted event caused by the administration of estrogen during early pregnancy in the pig. Our data suggest that the bioavailability IGFs prior to and during conceptus elongation and differentiation maybe essential for continued development and survival in the pig.

Chapter V

Summary and Conclusion

In the pig, placentation occurs between days 13 and 18 of gestation (King et al., 1982). Day 13 appears to be the crucial time in which attachment is initiated by the conceptus and the implantation cascade proceeds. Placental attachment in the pig is a noninvasive epitheliochorial which involves the attachment of the trophoblast to a sticky glycocalyx located on the surface of uterine epithelial microvilli (Dantzer et al., 1985). The glycocalyx may serve as an intermediate attachment substrate allowing the trophoblast to interdigitate with the uterine surface epithelium (Keys and King, 1990).

On Day 11.5 of gestation the porcine conceptus will begin to synthesize and secrete estrogen into the uterine lumen signaling maternal recognition and establishment of a successful pregnancy (Bazer and Thatcher, 1977). If the pregnant pig receives estrogen early, Days 9 and 10 instead of Day 11.5, complete embryonic mortality will occur by day 30 of gestation (Pope et al., 1986). Furthermore, other work (Morgan et al., 1987, Gries et al., 1989) narrowed the conceptus mortality window to days 14-18 of gestation. Since Day 14-18 is the time frame that trophoblast attachment occurs, one may attribute the mortality to attachment failure. Ultrastructure changes in the glycocalyx were documented by Blair et al. (1991), confirming that early exogenous administration of estrogen prior to the normal release by the conceptuses on Day 11.5 obliterated the glycocalyx covering on the uterine surface microvilli, eliminating the attachment substrate for the conceptus.

The present study was designed to characterize uterine changes in cylooxygenases, prostaglandins, and insulin-like growth factors associated with endocrine disruption in the pregnant gilt, and its effects on uterine-conceptus attachment and development. Adverse effects from estrogenic mycotoxic compounds (such as zearlenone) from fungi growing on plants can pose an extensive economic burden to today's high producing swine farms.

In most mammalian tissues, prostaglandin synthesis is regulated by either the constituent (COX-1) or the inducible (COX-2) form of cyclooxygenase (Smith et al., 2000). Both COX isoforms are expressed on the day of implantation in the mouse uterus (Chakraborty et al., 1996). The presence of COX-2 expression at the fetal-maternal interface in the rodent is crucial for conceptus attachment to the uterine surface allowing for subsequent invasion into the uterine tissue (Chakraborty et al., 1996). Loss of COX-2 gene expression results in compounded reproductive failures in the mouse (Lim et al., 1997, Davis et al., 1999, Loftin et al., 2001). Disruption of PG synthesis inhibits implantation and maintenance of pregnancy in can be reestablished with administration of PG's in COX-2 knockout mice (Lim et al., 1997, Dinchuk et al., 1995). Prostaglandin synthesis is essential for establishment of pregnancy in species with both invasive and noninvasive forms of implantation. Conceptus PG synthesis is not obligatory for rapid trophoblastic elongation in the pig on Day 12 of gestation (Geisert et al., 1986), however treatment of gilts with indomethacin during the period of placental attachment to the uterine surface (Days 13-17) causes embryonic death (Kraeling et al., 1985) .

Prior to and during trophoblastic elongation, conceptuses secrete the pro-inflammatory cytokine IL-1β (Ross et al., 2003). Conceptus production of IL-1β prior to

the period of conceptus attachment to the uterine luminal epithelium may serve to trigger the inflammatory response for endometrial receptivity to the conceptus. The 80-fold increase in COX-2 gene expression on Day 12 of pregnancy discovered in the present study are consistent with maximum secretions of conceptus derived IL-1β. However, the increase in endometrial COX-2 gene expression is not "pregnancy specific" as a similar increase in expression occurred in cyclic females. Furthermore, the lack of COX-2 transcripts observed through *in situ* hybridization on Day 15 of the pseudopregnant pig (Ashworth unpublished results) infers that a divergence in the COX-2 signaling pathway occurs in the pregnant gilt with functional conceptuses, as opposed to the withdrawal of PR from the uterine surface epithelium in the cycling contemoparies. Increased expression in COX-2 gene and protein is temporally associated with the loss of the progesterone receptor (PR) from the uterine surface and glandular epithelium (Geisert et al., 1994). Induction of COX-2 gene expression can be stimulated through activation of the nuclear factor-κB pathway (Page et al., 2002). A mutal negative interaction between NF-κB and PR has been suggested (Mckay and Cidlowsk, 1999), and loss of the PR in the endometrial epithelia is associated with increased activation of NF-κB (King et al., 2001). Changes in endometrial COX-2 gene expression maybe regulated through cell specific changes in PR and NF-κB, which play an important role in implantation and the maintenance of pregnancy.

In the present study, uterine luminal content of IGF-I and IGF-II peaked on the day of conceptus elongation (Day 11 to 12) in Veh gilts. Content of IGF-I and II in uterine flushings decreased dramatically following conceptus elongation and the initiation of placental attachment to the uterine surface epithelium (Day 13). On Day 10

of the estrous cycle and pregnancy, IGFBP-2 and -3 are detected in the uterine luminal fluids of cyclic and pregnant pigs when uterine luminal content of IGF-I and –II are high (Lee et al., 1998; Geisert et al., 2001). IGFBPs may play a role in prevention of premature binding and signaling of the ligands through the IGF-IR at the cellular level (Conover et al., 1990). It is possible IGFBPs help sequester IGF-I and -II in the uterine lumen for release during the sensitive period of conceptus differentiation and trophoblast elongation. The spatiotemporal association of uterine IGFs and IGFBPs at critical period in early porcine conceptus development and the alteration observed following early estrogen administration in the present study, suggests that the uterine IGF system serves an important biological role in establishment and maintenance of pregnancy. In the current study, endometrial IGF-IR gene expression increased after the decrease in luminal IGFs. Circulating concentrations of IGF-I and IGF-II are generally thought to depress expression of IGF-IR locally (Rosenfeld et al., 1982), which may explain the up-regulation of the IGF-IR mRNA on Days 15 and 17. Maintenance of endometrial IGF-I gene expression and loss of luminal IGFBPs may allow IGFs to stimulate uterine tissue rather than sequestering the growth factors in the lumen during pregnancy. The loss of the IGFBPs prematurely on Day 10 after the estrogen treatment is of particular interest due partly to the biostability and half-life of free IGF ligands in utero. The premature decline observed in the IGF-I, -II ligands within the uterine luminal fluids of the early estrogen treated gilts may be the result of IGFBP proteolysis prior to the normal time. This early proteolysis of IGFBPs remains unknown; however, there are several protease candidates that fit the theoretical profile. Tissue kallikreins are one family of proteases that are present in the uterus at this time (Vonnahme et al., 1999, 2003). Kallikrien 4 is

one of the many tissue kallireins present in the pig uterus around Day 10 of gestation, and is conspicuously controlled through the sex hormones estrogen and progesterone (Mayers and Clements, 2001). Recently, Ferdanado et al. (unpublished results) demonstrated a 4-fold increase in transcription from Days 10-13 of pregnancy compared to Days 13-17 of gestation. Furthermore, Geisert et al. (2001) demonstrated that kallikrein inhibitors administered to the pregnant and cycling pig results in the inhibition of IGFBP degradation. The early appearance or activation of kallikrein may be responsible for the premature disappearance of the IGFBPs in the early estrogen treated pregnant gilt.

Cathepsins are lysosomal cystein proteases (Barret and Kirschke, 1981) that have been associated with implantation in the rodent (Elangovan and Moulton, 1980). Roberts et al. (1976) reported the activity of various cathepsins (B_1, D, and E) in the uterine luminal fluid of the pregnant gilt. Although cathepsins contribute relatively small quantities to the secreted uterine protein spectrum, they may play a major function in conceptus signaling, processing, and uterine surface stabilization. Cathepsins can be synthesized and released by neutrophils (Gibson and Cohen, 1999), which are recruited to the site of inflammation in the wound healing process. Wound healing involves macrophages and fibroblast secretion of the IGF ligands to promote wound healing (Mueller et al., 1991). The presence of IGFBPs at the site of the wound maybe associated with distress signaling from cytokines and chemokines that are recruiting nuetrophils to the site for release of cathepsins to free the IGF ligand through proteolysis of IGFBPs.

Cathepsins may play a pivotal role in the regulation of the implantation window through the direction of cytokine processing. Vancomprernolle et al. (1998)

demonstrated that cathepsin B is specific for caspase-1 (ICE) activation, which will in part process the proforms of IL-1β and IL-18 (Hentze et al., 2003). Ross et al. (2003) demonstrated that the periimplantation porcine conceptuses secrete largest amounts of IL-1β into the uterine lumen during the filamentous morphology (Day 12-15), which rapidly decreases to nadir levels by Day 18 of gestation. IL-18 may be an attractive cytokine to compensate for the declining levels in IL-1β. IL-18 is also known as interferon inducing factor due to its ability to induce interferon gamma (IFN-γ). In the elongating porcine conceptus, IFN-γ has been observed to increase substantially during day 15 of gestation, which may be attributed to the release of IL-18 from the endometrium.

Further studies characterizing the types and temporal patterns of cathepsins warrant investigation. In the current study, early estrogen administration to pregnant gilts resulted in a drastic reduction in the presence of the IGF-II ligand, which binds to the IGF-IIR located on the elongating porcine trophoblast (Chastant et al., 1994). One of the few functions known about the IGF-IIR is to colocalize the newly synthesized pro-cathepsins to the lysosome for safe storage until needed (Capony et al., 1989). The IGF-IIR is a multifunctional protein believed to be associated with tumor suppression (Braulke et al., 1999), due to loss of the functional receptor observed in human breast cancer tumors (Hankins et al., 1996). Without the IGF-IIR function to transport the pro-cathepsins to the lysosomes, large quantities of the proteases are secreted from a breast cancer cell line using *in vitro* (Xie et al., 2002). The large secretions of procathepsins are responsible for the invasive nature of the tumor, due chiefly to the protease activity of the cathepsins. In the mouse, this process is paramount where adequate connection to the blood supply is essential for successful pregnancy. However, in the pig breaching of the

uterine epithelium and degradation of the underlying extracelluar matrix is unwarranted. Additionally, an abundance of cathepsin in the uterine lumen before the uterus is adequately prepared may allow premature activation of caspase-1 allowing untimely processing of IL-18. Further studies to clarify the signaling dysfunctions of the IGF-IIR will be needed determine the amount of IGF-IIR signaling.

In conclusion, the present thesis is the first to demonstrate that exposure of pregnant gilts to estrogen, before the normal conceptus secretion, acts as an endocrine disruptor to the cyclooxygenase and insulin-like growth factor systems. The disruption seen in the cyclooxygenases may result from altered timing from the loss of epithelial progesterone receptor, while the disruption in the IGF system appears to be early proteolysis of the IGFBPs.

Appendix

IGFBP LIGAND BLOT

SDA-PAGE Procedure:

Assembly of electrophoresis unit.

1. Clean glass plates with methanol and allow to air dry. Select plates with no chips along the edge of the glass, otherwise it won't seal good.
2. Place rubber seals in the bottom of the casting stand.
3. Lay one glass plate flat on the work bench.
4. Lay a gray spacer on each side of the glass plate. Align gray spacers with the outside edges of the glass plate.
5. Lay the other glass plate on tip and align both plates and spacers. This is called the sandwich.
6. Slide one clamp over one end of the sandwich. Tilt the sandwich slightly and do the same for the other side.
7. Stand plate upright on the bottom edge on a flat surface. Push the clamps toward each other so that the edge of the plates and the gray spacers fit all the way in the clamps.
8. Carefully snug the top and bottom screw of each clamp.
9. Slide the spacer mate (card board template) into the sandwich to check the space between the gray spacers. The spacer mate should slide in easily with no gap on either side.
10. Lay the sandwich down and tighten all screws. You want the screws to be tight so that there is no leakage, but you don't want them too tight.
11. Put a thin layer of **stop-cock grease** along the bottom and top edge of the sandwich to facilitate the seal.
12. Stand sandwich up and place it in the casting stand on top of the rubber seal, and remove spacer mate.
13. Insert two black locking cams (one for each side) into the corresponding holes at the bottom of the casting stand. Turn them in opposite direction to tighten the sandwich in the casting stand and create a seal along the bottom.

Preparing and pouring gels.

1. Prepare 10% ammonium persulfate (APS) as described in the solutions portion of this protocol.
2. Mix together reagents for the separating gel in an erlimyer flask (you want a 12% gel for IGFBPs). Add APS or TEMED last, because it will cause the gel to polymerize. It wont set up instantaneously, but you do need to work quickly. You do not need to degas the gel, because you will introduce the air right back into it as you pour. Wear gloves when handling the acrylamide.
3. Fill a 60 mL syringe with the gel mixture. It will take about 25 to 30 mL per gel, and you must pour two gels for the electrophoresis machine to work right. You can take

the stopper out of the syringe and invert it to fill, or you may draw it into the syringe through a needle.

4. Fit the syringe with a 16 ga needle and carefully place it between the glass sandwich towards one of the gray spacers. Fill the sandwich with 25 to 30 mL or to the second screw from the top of the clamp. It should take 40 to 50 min for the gel to polymerize. You can observe the time it takes the left over gel to set up in the flask.
5. Immediately after pouring the separating gel, use a 1 mL syringe fitted with a needle to overlay the gel with 1 to 2 mL of .1% SDS. You will have to make a stock of .1% SDS. You can use water, but it will mix with the gel a little and potentially mess up the top edge. You want a good smooth top edge.
6. While waiting for the gel to set up, mix up the reagents for the stacking gel (you want a 4% gel). DO NOT add APS or TEMED until ready to pour.
7. After the separating gel has polymerized, invert the sandwich and casting stand over the sink to drain off the overlay of .1% SDS.
8. After the sandwich has been inverted, fold a chem-wipe and put it between the two glass plates of the sandwich to blot the top of the separating gel.
9. Add APS and TEMED to stacking gel and pour to top as previously described.
10. After pouring the staking gel, put the comb in before it sets up. You do this by sliding the teeth of the comb in at an angel, from one end to the other.
11. After stacking gel has set up, pull the comb strait out. Be careful so you don't mess up the wells. The stacking gel is very gooy.
12. Wash the lanes with water about twice, inverting the assembly to drain each time.
13. After the lanes have been washed with water, fill them with electrophoresis buffer.
14. Load the gels with samples that have been previously prepared. Load as close to the bottom of the well as possible.
15. Set the top buffer chamber onto the top of the glass plates. Be sure that the rubber gasket is in place before you do this or it wont seal.
16. Remove black locking cams from the bottom of the casting stand.
17. Insert the black locking cams in the top buffer chamber and lock into place as described for the casting stand.
18. Fill top buffer chamber with electrophoresis buffer. It should NOT leak down the sides. If it does, you don't have a good seal. Dump the buffer and repeat the locking steps.
19. Prepare the electrophoresis chamber.
    a. Place the chamber on a stir plate.
    b. Put a medium sized stir bar in the bottom of the chamber.
    c. Attach hoses from the circulating water bath to the cooling apparatus. This is the white tube structure that fits in the middle of the tank.
    d. Turn on circulating water bath.
    e. Fill electrophoresis chamber **2/3 full** with electrophoresis buffer.
20. Lift the sandwich and top buffer chamber out of the casting stand and carefully place it in the electrophoresis chamber. The cooling apparatus will fit between the two glass sandwiches.

Finish filling top buffer chamber so that horizontal electrode is covered with buffer. If you do not, it will burn into and ruin the chamber.

PREPARATION OF FLUSHING SAMPLES:

1. Make assay sheet
   Lane 1 is molecular weight marker
   Lanes 2 - n are samples.
2. Label 1.5 mL micro-centrifuge tubes with sample number and place in rack.
3. Begin to thaw serum samples.
4. Pipette 50 μg protein from flushings into corresponding 1.5 mL micro-centrifuge tube.
5. Pipette 21 μL of Laemmli Sample Buffer (Cat # 161-0737; Bio-Rad Laboratories, Hercules, CA) into each tube.
6. Vortex to mix and then centrifuge for a few seconds.
7. Boil samples for 4 min.
   a. Fill 1000 mL beaker almost to top with water.
   b. Heat on hot plate till boiling.
   c. Cover with aluminum foil.
   d. Push micro-centrifuge tubes through the aluminum foil so that the tips are in the steam or water.
8. Remove samples and cool on ice.
9. Centrifuge for a few seconds to get the contents to the bottom of the tube.
10. Load gel with 25 μL of prepared sample from the micro-centrifuge tube. Use gel-loading tips for this.
21. To finish electrophoresis chamber assembly place lid on chamber..
22. Attach electrodes to the chamber (black to negative; red to positive) and attach them to the power supply.
23. Turn on power supply. You want to run it at 15 amps for 2 gels (30 amps for 4 gels) and run it overnight (5:00 pm to 8:30 am).
24. Check the gel in the morning. You want the dye to be almost off the gel, and then you can stop it. If it hasn't reached the bottom you can turn the current up to 50 amps.

TRANSFER FROM GEL TO MEMBRANE:

Equipment and Supplies Needed.

9 x 9 Rubber Maid containers (at least two).
   Rubber Maid corp.
14 x 14 cm square of 3 mm filter paper (4 per gel).
   Trans-Blot®, Bio-Rad Laboratories, Hercules, CA; Cat # 1703955.
14 x 12 cm square of .45 μm nitrocellulose membrane (1 per gel).
   Trans-Blot®, Transfer medium, Bio-Rad Laboratories, Hercules, CA; Cat # 162-0115.
Circulating water bath (4^{o}C).

Approximately 6 L of transfer buffer.
Electrophoretic transfer chamber. Transphor, Hoefer Scientific Instruments. San Francisco, CA)

Transfer Procedure.

1. Prepare and cut the 3 mm filter paper and the nitrocellulose membranes. Be careful to wear gloves when cutting membrane. Cut away the upper left corner of the membrane (corresponds to lane one). This is to orient the membrane with the gel and to allow you to know where lane one is. Label (with pencil) gel number and date on the side of the membrane that will contact the gel.

2. Stop electrophoresis and disassemble sandwich.
    a. The electrophoresis of the gel should be finished. Turn off the current to the electrophoresis unit.
    b. Turn off circulating water bath.
    c. Lift off lid from electrophoresis unit.
    d. Carefully pull out the gel sandwich-buffer chamber assembly.
    e. Drain electrophoresis buffer from the top buffer chamber into sink.
    f. Place gel sandwich-buffer chamber assembly into casting rack. At this point, it should look like it did when you assembled the buffer chamber onto the gel sandwich.
    g. Twist and remove black camber locks from buffer chamber assembly.
    h. Lift off buffer chamber and place in sink to wash.
    i. Pull one gel sandwich from casting rack and lay flat.
    j. Loosen all screws on each clamp, and remove clamps.
    k. Using the spatula tool (provided with electrophoresis unit) remove the gray spacers by pushing one end out from between glass plates. It can then be pulled out with you fingers. Repeat for other gray spacer. (**NOTE**: Always wear gloves when handling gel to prevent exposure to acrylamide).
    l. Slide spatula tool into space where one of the gray spacers was and lightly pull up so as to "pop" or separate the top glass plate from the gel. Place this glass plate in soapy water for washing.
2. Remove gel (Wear Gloves; remove gels in reverse order…4, 3, 2, 1).
    a. Using on of the gray spacers, carefully cut away the staking gel from the separating gel. It will be very sticky. You may have to lift plate over acrylamide waste and scrape it off. Be careful not to track acrylamide all over the lab. Cut the top corner of the gel, above lane one off diagonally so you will be able to maintain orientation of the gel throughout the rest of the experiement.
    b. Using a gray spacer, cut the top corner of the gel, that is above lane one, diagonally to orient the gel and membrane.
    c. Place spacers in soapy water for cleaning.
    d. Fill a rubbermaid pan about ½ full of transfer buffer.
    e. Using a disposable pipette, squirt a little transfer buffer on and around the gel.

f. Wet the end of the spatula tool and your fingers in transfer buffer. This prevents the gel from "sticking" to much. If it sticks it tears.
g. Slide spatula tool under one of the lower corners of the gel and lift up and carefully take hold of the gel. Repeat for the other lower corner.
h. Carefully peel up the gel from the glass plate. Gel will tear easily, so lift or peel, don't pull. If gel tears its no big deal, just be careful and get all the pieces into the buffer.
i. After peeling gel off glass plate, lay it in the rubbermaid pan in transfer buffer. Incubate gels in transfer buffer 30 min. This step equilibrates out the electrophoresis buffer.
j. Place last glass plate in soapy water.
k. Repeat for each gel.

3. Attach hoses from circulating water bath to transfer chamber. The chamber should be oriented with fill toward you.
4. Fill transfer box to start line with transfer buffer.
5. Place medium star bar in transfer chamber and place it on a stir plate.
6. Wet 3 mm filter paper and membrane in rubbermaid pan.
7. Prepare sandwich for transfer.
    a. Fill a 9 x 11 pyrex baking dish with a small amount of transfer buffer.
    b. Open a transfer cassette and lay the black side in the dish (see page 4 of Hoefer Transphor instrunctions; these instructions are backwards to the way we do it.).
    c. Place a sponge into dish, on top of cassette. Make push it down to make sure it is wet.
    d. Remove a square piece of filter paper from transfer buffer and place it on the sponge next. Take extreme care to smooth air bubbles out from between each layer with a pipette. Air bubbles prevent transfer to membrane.
    e. Remove a second square of filter paper from the transfer buffer and place it on top next. Take a pasture pipette with the tip broken off and smooth out any air bubbles.
    f. Remove the gel from the buffer, and place it on the stack next. Smooth out any air bubbles between the gel and filter paper.
    g. Remove the membrane from the transfer buffer and place it on stack next. Place it with the label side down, and notched corner corresponding to the gel. Smooth out any air bubbles between the gel and membrane.
    h. Remove a third square of filter paper from the transfer buffer and place it on the stack next. Smooth out any air bubbles between the filter paper and membrane.
    i. Remove the forth square of filter paper from the transfer buffer and place it on the stack next. Smooth out any air bubbles between the two filter papers.
    j. Wet final sponge in transfer buffer and place on top of stack.
    k. Close and lock transfer cassette.
8. Place transfer cassette into transfer chamber with hinged side up. Electricity runs from black to gray, so you want to put the transfer cassette in the same orientation (ie… the membrane to the gray side). This will move proteins onto the membrane.

9. Finish filling transfer chamber with transfer buffer (1/2 way between the MIN and MAX line). You want cassette and sponges completely covered.
10. Turn on the circulating water bath. Start stir plate.
11. Set transfer power pack on top of transfer chamber. The gray panel should face the red (+), and the black panel should face the black (-) lead.
12. Turn on the power pack. Run the power at .6 mAmps for 2 ½ hrs. You can run it 30 - 40 min longer.

Prehybridization.

Always wear gloves when handling the membrane.

1. Remove the transfer sandwich from transfer chamber.
    a. Turn off power to power pack (black button on back).
    b. Carefully pull power pack off of the top.
    c. Pull out transfer cassette and place it in the pyrex baking dish, gray side up.
    d. Put TBS saline in rubbermaid box. Just want enough to cover membrane.
    e. Open transfer cassette, and remove the two squares of 3 mm filter paper.
    f. Lift off membrane and place it in the TBA saline solution in rubbermaid box. The gel and filter paper can be discarded in the acrylamide waste. Change gloves.
2. Place the membrane on a rocking platform and incubate for 10 min. Afterwards the TBA-saline can be discarded down the drain.
3. Transfer membrane to a new rubbermaid box that contains 1% BSA-TBS saline. Incubate on rocking platform for 1 hr.
4. Drain off the 1% BSA-TBS saline down the drain.
5. Wash the blot two times for 15 min each in Tween 20-TBS saline. Fill rubbermaid box containing the membrane with enough Tween 20-TBS saline to cover membrane and incubate for 15 min on rocking platform. Drain and repeat for second wash.
6. Prepare radioactive tracer of ^{125}I-IGF-II for hybridization.

Radioactive tracer for hybridization.

You want 15,000 cpm / 100 μL. You will need to add 3 mL of hot stuff (tracer) for each membrane in the bag.

1. Locate the iodinated IGF-II in the refrigerator. You should already know where the peak is from the iodinantion sheet.
2. Pick the correct fraction and count 10 μL on the gamma counter.
3. Calculate the correct amount of hot to add to make the volume needed. Follow the calculation below.

    If you have 800,000 cpm / 10 μL of iodinated IGF-II,
    And you want 15,000 cpm / 100 μL of hot stuff,
    You multiply (800,000 cpm / 10 μL) * (10) = 8,000,000 cpm / 100 μL,
    (8,000,000 cpm) / (15,000 cpm) = 1:533 dilution.
    If you have two membranes you need 6 mL of hot stuff,
    So 6 mL = 6,000 μL
    (6,000 μL) / (533) = 11 μL of iodinated IGF-II to make 6 mL of hot stuff.

4. Pipette the iodinated IGF-II and bring to volume with 1% BSA-0.1% Tween 20-TBS Saline. You can use a 50 mL disposable centrifuge tube.

Hybridization (Wear Gloves).

1. After the last wash in Tween 20-TBS saline is finished, take membrane out.
2. Place membrane in plastic seal-a-meal bag (Scienceware, Bel-Art Products, Pequcennock, NJ). Place it as far to one corner as possible. Take care to smooth out the bag as much as possible and get as much air out as possible. There will be bubbles because the membrane is wet.
3. Use the impulse sealer (#Z10-12E, Clamco Corp., Cleveland, OH) to seal the bag about two inches above the membrane. You may also want to put an extra seal below the factor seal on the bottom of the bag just for insurance.
4. Turn the bag and seal the side to about ½ inch from the membrane.
5. Cut diagonally across the top corner of the bag. You want to cut just enough to be able to insert an 18 ga needle.
6. Fit a 10 mL syringe with an 18 ga needle and fill it with the radiolabled ^{125}I-IGF-II tracer (hot stuff) that was previously prepared. The original protocol called for 3 mL per membrane, and you could put two membranes in each bag. However, if the iodination is too old, we use 6 mL per bag even if we only have one membrane (according to Spicer).
7. Place the needle through the cut and align it down the side of the bag. Slowly dispense the hot stuff into the bag, taking care that it doesn't come out.
8. Push the hot stuff down to the bottom of the bag and seal the cut corner as close to the edge as possible.
9. Carefully push the air bubbles to the freshly sealed corner of the bag. You can use a pasture pipette or a rubber spatula. Try to seal the bag below the bubbles and trap them into the corner. The main object is to reduce the air bubbles in the bag so that there are no spots that limit the contact of the hot stuff with the membrane. You may want to use what ever variation of this technique that is easiest for you.
10. Press the hot stuff around in the bag so that the membrane is completely covered.
11. Place the bag in a radioactive labeled pan or tub so that if any hot stuff leaks out, it is contained.
12. Put the tub on a rocking platform in the walk in cooler.
13. Incubate membranes with hot stuff overnight at 4^{o}C.

Preparation of blots for film exposure (Wear Gloves).

1. Prepare a rubbermaid pan by filling it with enough Tween 20-TBS saline to cover the membrane.
2. Remove the seal-a-meal bag from the radioactive pan and carefully cut one corner of seal a meal bag open and drain liquid into the liquid radioactive waste.
3. Cut open three sides of the bag and remove the membrane. Be careful to perform all procedures with radioactivity over a mat that can be thrown away. In addition, the scissors will need to be decontaminated. I do this in the hood just for saftey.

4. Place the membrane in the rubber maid container of Tween 20-TBS saline.
5. Discard all waste in appropriate radioactive waste container or hot box.
6. Incubate the membrane in Tween 20-TBS saline twice, each time for 15 min. Discard Tween 20-TBS saline in radioactive waste after each washing.
7. Incubate blots in TBS saline three times, each for 15 min. Discard TBS saline into radioactive waste after each washing.
8. After all washings are complete, place membrane on 3 mm filter paper to dry. Place the side of the membrane that was in contact with the gel down. It shouldn't have been turned over since you took it from the transfer sandwich.
9. After membrane is dry, cut a piece of plastic wrap large enough to wrap the membrane, and place it on the work bench.
10. Place the membrane face down on the plastic wrap, and fold the edges of the plastic wrap onto back of membrane so that it is sealed.
11. Tape membrane, face up, to an old piece of x-ray film with scotch tape.
12. Put the taped membrane into a film cassette, and proceed to dark room to load film.

Loading X-ray film.

1. In a dark room (lights are on right now), open the film cassette, remove the intensifying screen, and lay it on the lid.
2. Locate x-ray film and set the box at your feet.
3. Turn on red lights.
4. Open the x-ray film box, unfold the packaging, and pull out a x-ray film.
5. Locate the notched corner (if no notched corner, make one with a pair of scissors) and place the film into the cassette.
6. Fold x-ray film packaging back down and put lid back on box.
7. Put intensifying screen in place, close cassette lid, and lock into place. Lights can now be turned on.
8. Put a piece of label tape on the film cassette to identify the membrane and experiment.
9. Put film cassette in –80^{o}C for appropriate time (2 d). You determine the time, but don't want it to long because it increases background.

To Develop Film.

1. Remove film cassette out of -80^{o}C and allow it to warm to room temperature (about 1 hr).
2. Go to 3rd floor Nobel Research Center to Biochem dept.
3. Go into the dark room. Light switch to the right of the turn stile will turn on the "Dark Room In Use" sign. The other light switch to the left of the turn stile runs the lights in the dark room.
4. Instruction for developing machine are on the wall to the right of it. These are the weekday instructions. Don't use the weekend instructions. Breifly…
    a. Turn standby switch on and wait for the ready light to come on.
    b. Log in your run on the og in sheet. You will need an account number for this because biochem charges $1 for developing.

c. Get an exposed x-ray film from the locker on the wall.
d. Place it on the input tray on the right of the machine. Align the film to the top of the tray so that it is square.
e. Gently push the film in so that the machine catches it.
f. The machine will develop the film and spit it out the left side. This basically gets the machine warmed up and the chemicals running through it good.
g. Turn on the red lights, and turn off the overhead lights.
h. Open your film cassette and remove the e-ray film.
i. Place it on the machine as described above. Once the film is developed, you can turn the lights back on again.
j. Turn standby switch off again.

Western Blot Analysis

1. After Electrophoresis, remove gel from glass plates by gently prying off the top glass with a spacer. Cut off the stacking gel and dispose. Nick the left hand corner and place gels in transfer buffer on a rocking plat form for 20-25 mins.
2. While gel is in transfer buffer, prepare the PVDF membrane by soaking in:
    a. Methanol, 1-3 mins
    b. Millipore water, 1-2 mins
    c. Transfer buffer, 2 mins

    Do not touch membranes with bare hands! Do not allow the membrane to dry out any time during the procedure.
3. Prepare the transfer unit on the semi-dry blotting apparatus. Place the sandwich on the anode base as follows:
    a. 1 sheet (6 cm x 8.5 cm) 3MM paper soaked in anode buffer 1
    b. 2 sheets (6 cm x 8.5 cm) 3MM paper soaked in anode buffer 2
    c. PVDF membrane (6 cm x 8.5 cm) soaked in transfer buffer
    d. Gel soaked in transfer buffer
    e. 3 sheets (6 cm x 8.5 cm) 3MM paper soaked in cathode buffer
4. Place lid (cathode) on top of sandwhich with screws loosely attached to prevent desiccation.
5. Electroblot for 2.5 h at 350 mA (constant current) for 2 sandwiches.

Immunostaining

Both cyclooxygenase 1 and cyclooxygenase 2 were used in the same fashion, but with varying amounts of antibody concentration added (see below). All amounts are for 2 membranes.

1 After electroblotting, immerse the membranes in TBS for 2 mins. Place the gels in fixative overnight. Stain the next day with coomassie blue for 2 hours then destain.
2 Pour off TBS and add 60 ml of the 1st blocking solution (3% gelatin in TBS) and incubate for 1 h.
3 Wash membranes in TTBS one time for 10 mins.

4 Pour off the TTBS and add the second blocking solution (5% powdered milk in TTBS).
5 Wash membranes in TTBS one time for 10 mins.
6 Add primary antibody buffer; COX-1, COX-2 (1:2500 and 1:1000 respectively) and incubate 1 h.
7 Wash membranes in TTBS 3 times for 10 mins each.
8 Pour of TTBS and Add secondary antibody. Incubate for 2 h.
9 While the second antibody is incubating measure out 66 ml of HRP color development buffer and allow it to reach room temperature. Thaw and measure 13.33 ml of HRP color reagent A and protect it from the light.
10 Wash the membranes in TTBS 2 times each
11 Pout off TTBS and add color development buffer. Do not incubate longer than 15 min
12 Pour off color developer and wash in Millipore water. Membranes can be stored in H_20 protected from the light.

Solutions

Tranfer Buffer
25 mM Tris, 192 mM glycine in 15% (v/v) methanol; pH 8.2-8.3 for 2 membranes : 400 ml containing 1.21 g tris, 5.77g Glycine

Anode Buffer 1
0.3 M Tris in 10% (v/v) MeOH; pH 10.4
200 ml contains 0.61 g Tris

Anode Buffer 2
0.0025 M Tris in 10% (v/v) MeOH; pH 10.4
200 ml conatins 0.61 g Tris

Cathode Buffer
0.025 M Tris, 40 mM glycine in 20% (v/v) MeOH; pH 9.4
200 ml contain 0.61 g Tris, 0.60 g Glycine

Tris Buffered Saline (TBS)
20 mM Tris, 500 mM NaCl; pH 7.5
For 2 membranes, 800 ml contains 1.94 g Tris, 23.38 g NaCl

Wash Solution (TTBS)
20 mM Tris, 500 mM NaCl; pH 7.5
For 2 membranes, 800 ml contains 1.94 g Tris, 23.38 g NaCl
Add 250 ml of tween twenty

First Blocking solution
3% gelatin in TBS, heat to dissolve (not over 60 C)
For 2 membranes 60 ml TBS conatines 1.86 g Gelatin

Dual Labeled Probe and Primer Design

1. Guidelines for designing both primers and probes:
    a. Select the probe first and design the primers as close to the probe with out overlapping it (amplicons of 50-150 base pairs are strongly recommended)
    b. Kep G/C content in the 30-80% range for both the primers and the probe.
    c. Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
2. guidelines specific for designing the probe:
    a. When using primer express®software, the T_m should be 68-70 C (~8-10 C higher than T_m of the primers)
    b. No G on the 5' end
    c. Select the strand that gives the probe more C than G bases.
    d. Design the probe to have 6-FAM as the 5'-reporting dye.
        i. Multiple reporting dyes must be used if multiplexing, however, FAM is the most stable dye and should be used whenever assaying for a single target gene within a single reaction.
3. Guidelines specific for designing primers
    a. When using primer express®software, the T_m should be 58-60 C (~8-10 C lower than T_m of the probe)
    b. The five nucleotides at the 3' end should have no more than two G and/or C bases.

In Situ Hybridization

Tissue Fixation

1. Uterine tissue should be excised and immediately processed. In general, a section of the uterine horn (not greater than 1.0 cm) is placed in 40 ml of freshly 4% paraformaldehyde in phosphate buffered saline (PBS). It is important to keep a very high fixative volume to tissue ratio.

2. The section of uterine horn in a fixative should be gently agitated on a rocker or orbital shaker for 24h @ RT.

3. After 24 h, the fixative shall be drained out, and replaced with 40 ml of 70% EtOH (v/v in H_20) and gently agitated overnight at RT.

4. After 24 h, the 70% EtOH should be replaced with fresh 70% EtOH, and tissue permanly stored at RT.

Solution preparations

1 Heat 2/3 final volume to 60 C in fume hood with stir bar.
2 Add granulated paraformaldhyde slowly, add 1 to 2 drops of 1N NaOH (this will help clear soln)
3 When soln is clear, remove from heat
4 Add 1X PBS to make final desired volume
5 After soln is cool, adjust pH to 7.2

Tissue Sectioning

1.. Trim blocks so that they are about 1cm square on the block surface. This is easier to handle in long strips (Ribbons)

a. Face Blocks to get tissue (Cut at 20 μM) embedded in paraplast
b. After facing blocks turn the blok face down on ice (freeze H_20 in a flat dish). Place 5-6 blocks on the ice at a time.
c. Use 37 C water to expand sections in the ribbons. (If possible place 4 sections on the side if possible). Split ribbons with forceps.
d. Slides are possitivly charged: Fisher brand-superfrost**/Plus, Cat # 12-550-15; 12 X 75 X 1.0mm
e. Section with a fresh section of the blade (use new region of knife for each block). Should be able to cut four blocks /knife blade. Sections need to be 5μM.
f. Place ribbon (4 sections) on slide and blot on paper towel. Place slides in a tray with wet paper towels in an incubator (37 C) overnight. **this is critical to dry slides so they stick on the slide** Label slides with SHUR/Mark pen-triangle BioMedical Sciences (Do Not use Sharpie). After drying overnight, place the slides in a slide box and store at 5 C until used for hybridization.

Preparation of Slides

Get formaldehyde out of freezer to thaw for solutions

1. Xylene (CitrSolve CAT# 22-143975) CitriSolve will have a layer on the bottom, so be careful not to pour in the tanks! Make sure tank solvent is clean between runs. Treat for 5 mins and agitate every 1-2 mins "Critical" repeat 2X. Check to make sure the paraplast is cleared from slides!
2. 100%EtOH fro 1 min -2X (agitate slides through procedure)
3. 95% EtOH 1 min-2X
4. 70% EtOH 3 min- 1X
5. PBS for 5 min-2X

10X PBS (1 Liter)

80g NaCL

2g KCL

14.4g Na_2HPO_4 or (11.5g $Na_2HPO_4 \cdot 7\ H_20$)

2.4g KH_2PO_4

6. Fresh 4% paraformaldhyde (PAF) 20 min-1X[Needs to go in PAF waste when done with step 9] Dissolve 4g PAF/100ml of PBS-Need 400-500mL
   To Make: Fill beaker with ddH_20 to about 60% of final volume (Do Not Heat Water). Add PAF (20g/500mL) and about 1.25g NaOH pellets/500 mL. Stir on stir plate in hood until it goes into solution~10 mins, then add enough 10X PBS to make final concentration 1X. pH to 7.2 with 12N NaOH "slowely" and bring to volume with ddH_20
7. PBS for 5 min-2X
8. Proteinase K for 7.5 min-1X

   500mL

   25 mL 1M Tris (pH 8.0)

   5 mL 0.5 M EDTA

   1 mL Proteinase K (10mg/mL) stock kept in freezer

   Q.S. with ddH_20
9. 4% PAF for 15 min 1X(Can reuse the PAF from first wash in step 6)-Dispose PAF in waste bottle
10. ddh20 for 1 min-1X
11. PBS for 5 min-2X
12. 70% EtOH for 3 min-1X
13. 95% EtOH for 1 min-2X
14. 100% EtOH for 1 min-2X
15. Air dry slides at RT (10 min) on paper towels in tray. While drying slides start denaturation of probe.

Linearization of Plasmids for *in vitro* Transcription (IVT)

1. Digest 20 μg of DNA with appropriate restriction enzyme for >2hr at appropriate temperature.

DNA	20μg
10X REB	20 λ
Enzyme	10 λ
Water	to 200 λ

2. Extract once with an equal volume of PCI (Phenol:Chloroform:Isoamly alchohol) and once again with chloroform
3. Precipitate DNA with 3 vol 100% EtOH, 1/10 vol 3M NaOAc and 5 λ of Dextrane T500 (10mg/ml)
4. Place at -80 C for 15 min, then spin down for 10 min at MAX speed @ RT
5. Remove EtOH, and wash pellet with 150 λ of 70% EtOH
6. Remove all 70% EtOH and resuspend pellet in 40 λ of Rnase-free water. The DNA is at approximately 0.5 μg/λ

Preparation of Probe

1. set up probe synthesis reaction (enough for about 10 slides) in a 0.5 mL tube (larger rxn's use 1.5 mL tube). Use Rnase free tubes and pipet tips (Pipette tips should have filter –ART-Molecular BioProducts)

Order	Solution	10 Slides	20 Slides	40 Slides
1	Water (Ambion DEPC)	4.0 uL	8.0	16.00
2	5X Transfer Buffer (Vortex)	2.5 uL	5.0	10.0
3	100 mM DTT	1.25	2.50	5.0
4	2.5 mM rACG	1.25	2.50	5.0
5	DNA (10mg/ml)	1.0 ug	2.00	4.0
6	Rnasin (Keep Cold)	0.5 uL	1.00	2.0
7	UTP (^{35}S)-40 mCi/mL	1.25	2.50	5.0
8	Either T7, SP6 or T3 Poly	0.75	1.50	3.0

Remember you need to know directions of the insert from the plasmid (T7 or SP6) to get antisense or sense gene (Sense is Control!!).

2. Vortex tube, Quick spin and then incubate for 2 h at 37 C in heat block.
3. Prepare CENTRI-SEP column (Princeton Seprations, Inc-CAT# CS-90

   Need two columns for 4X reaction. The column can only handle 100 uL of fluid. Need to rehydrate the column atleast 1 hr before the probe reaction is added to the column.

   a) Add 80 uL Ambion water to the column

   b) Invert and gently vortex

   c) Sit upright for atleast 30 mins –leave bottom cap on (don't start flow yet).
4. After 2 h incubation add 3uL RQ1-DNAse I (Promega) and 0.5 uL RNasin to tubes. If 4X Rxn (40 slides) double the volume-6uL RQ-1-DNase, 1.0 uL RNasin. Vortex, quick spin and incubate for 15 min at 37 C

5. After incubation add 20 uL yeast tRNA (10mg/ml) and 40 uL Phenol:Chlorofrom:Isoamyl (Ratio 25:24:1)-Soln stored at – 4 C warapped in foil (its light sensitive). 4X Rxn 40 uL yeast tRNA and 80 uL PCI. Vortex well!! ****When pipetting from PCI, make sure to get lower layer where phenol is bottom layer, and water is on top****

****Water Saturated Phenol is for RNA, Tris buffered phenol is for DNA****

6 a) centrifuge at full speed on a microcentrifuge (27,000 x g) for 5 min
b)Remove top layer with a pipet (filtered) and place in a new bullet tube (Don't get bottom layer)

7. Add a volume of chloroform to match PCI

1X=40uL

3X=80uL

Vortex and spin at full speed for 5 min

Complete SEP column preparation: Take top and bottom cap off. Place in cup and use finger to push (top pressure) a drop from the bottom to start flow!!

After spinning tubes with probe, SEP columns in centrifuged and position the column so the notch on the rim faces out (top of rotor). Spin at 750 g for 2 min. Use column immediately- do not let it dry out!!

8 Add top layer of bullet tube (probe) to column (place in middle of matrix and don't touch matrix with pipet).

9 Precipitate with 60 uL 3M NaOAc (pH 5), 1 uL yeast tRNA, 5uL Dextran T500 (10mg/ml) and 300uL EtOH

10 Spin bullet tubes at maximum speed for 10 min Pour off liquid in radioactive waste.

11 Wash pellets with 70% EtOH

12 Wash pellets in 50 uL (1X-2X) or 100 uL (3X-4X) of 100 mM DTT. Can use pipet to break-up pellet.

13 Count in β-counter (1-2 uL)!

Calculation for probe Hybridization

Antisense -20 slides: 5 X 10^6 cpm/slide

20 slides X 60 uL hybridization soln/slide=1200 uL (may add for 1-2 more slides)+Volume of probe (Need 5 million cpm/slide)=50 uL

1200 uL + 50 uL = 1200 uL

Need 10% DTT (100 mM DTT) so get 10 % of volume-1250 uL X 0.10=125 uL of 1M DTT

Need only 2 slides for sense Probe!!!

Vortex, quick spin and incubate at 70 C for 10 mins to denature probe!!!

Summary

cRNA probes (5 X 106 cpm/slide) with hybridization Solution containing 100 mM DTT at 70 C for 10 min.

Store hybridization soln in freezer – Place in 55 C incubator before mixing with probe and DTT. 1M DTT is stored in freezer, make sure has smell to know it is working!

Need 60 uL solution/slide

Probe Hybridizations

1. Following the denaturation of the probe (70 C), add the Hyb soln to the middle of the slide. Set pipetman at 65 uL, but when adding don't blowout last fluid to avoid air bubbles (KEY!!!)
2. Put on coverslip
    a. Touch middle soln with coverslip and drawback to edge. Slowly drop cover slip on slide!
3. Place a layer of 3MM paper in a pyrex baking dish that has been wetted with 250 mL of 50% Formamide / 5X SSC
    a. Fromamide is stored in freezer as stock! Must thaw out before use. Once thawed keep in the cooler.

4. Cover pyrex dish with plastic wrap and seal. Place in an incubator at 55 C for at least 16 h
5. Make 50% formaldehyde /2X SSC/50 mM BME-*Leave BME out until just before use the next day [make sure BME is not old]
    a. Make 1 liter =500 mL formaldehyde /100 mL 20X SSC=h20 to 1000mL (add 3.5 mL BME next day)
    b. Make 5X SSC/10 mM BME-*Leave BME out until just before use next day. 500 mL=125 mL 20X SSC+H20 to 500 mL (add 350 uL BME Next Day)
    c. Place soln in incubator at 55 C for next day use!!!
6. Add BME to solutions the next day
    a. 50% Formaldhyde/2X SSC/50 mM BME (3.56 mL/liter)
    b. 5X SSC/10 mM BME (350 uL/500 mL) keep in incubator at 55 C
    c. Place the 50% formaldehyde /2X SSC/50 mM BME in 65 C water bath or incubator. Use glass slide holders.
7. Gently remove coverslips by sliding down into radioactive waste container (use empty NaCl container). If coverslip sticks to slide, dip in buffer and slide off. Place slides in rack with 55 C 5X SSC/10 mM BME.
    a. <u>Place in 55 C incubator for 30 min-agitate every 10 mins</u>
8. Dump out baking dish (In isotope sink) Wash with 10% Count-off (rinse bottle) then rinse with DDH20. dry table and rinse large double taped pipets
9. Dump first hybridization wash into radioactive liquid waste [35S]. Add 50% Formaldhyde/2X SSC/50 mM BME and place in 65 C incubator or water bath for 20 min
10. Dump second wash of hybridization in radioactive liquid waste.Add TEN (0.5M NaCl/10mM Tris(pH 8)/5mM EDTA) for 10 min at RT
    a. 10X TEN (1 Liter)
    b. 292.2g NaCL
    c. 10 mL 1M TrisCL (pH 8)
    d. 10 mL 0.5 M EDTA
11. TEN 10 min 37 C-3X

12. TEN with RNase A (10ug/mL) -500 mL (0.5 mL Rnase stock 10mg/ml) in the freezer). Incubate 37 C for 30 min
13. TEN 15 Min at 37 C
14. 50 % Formamide /2X SSC/50 mM BME at 65 C for 20 min
15. 2X SSC[500 mL=50mL 20XSSC+450mL H20] at RT for 15 min
16. 0.1X SSC [500mL-2.5 mL 20X SSC 497.5 mL H20] RT for 12 min
17. 70% EtOH/0.3 M Ammonium Actetate at RT for 5 mins-2X
18. 95% EtOH/0.3 M Ammonium Acetate for RT for 1min
19. 100% EtOH at RT for 1 min 2X
20. Ait dry and expose to bomax film overnight. Devlop next day to estimate length needed to develop slides
    a. Place slides in order in film case-tape down corners to keep from moving
    b. Darkroom turn on safe light, which has an orange filter, use kodiac BioMax MR film-packaged in singlesheets**make sure emulsion side down (Non-shiney side)-notched should be in upper left corner. Expose at laest 16 h

In Situ Hybridzation

Solutions

5X SSC/10 mM BME (500 mL)
125 mL 20X SSC
350 µl BME
q.s. ddH_2O

50% Formamide/2X SSC/50 mM BME (800 mL)
400 mL Formamide
80 mL 20X SSC
2.72 mL BME
q.s. ddH_2O

20X SSC (1 liter)
175.4 g NaCl
88.2 g Sodium citrate
Dissolve in 800 mL ddH_2O, adjust pH to 7.0 with a few drops of 10N NaOH. Bring to volume with ddH_2O and filter sterilze. DEPC treat and autoclave.

2X SSC (400 mL)
40 mL 20X SSC
q.s. ddH_2O

0.1X SSC (400 mL)
2 mL 20X SSC
q.s. ddH_2O

70% EtOH/0.3 M NH_4Ac (800 mL)
560 mL 100% EtOH
48 mL NH_4Ac
q.s. ddH_2O

95% EtOH/0.3 M NH_4Ac (400 mL)
24 mL 5M NH_4Ac
100% EtOH

10X TEN (1 Liter)
292.2g NaCL (5M)
10 mL 1M TrisCL (pH 8)- (100 mM)
10 mL 0.5 M EDTA – (50 mM)

1X TEN (0.5M NaCL/10 mM Tris/5 mM E
100 mL 10X TEN
q.s. ddH_2O to 1000 mL
*Need 1.5 L for all the washes!!!

Hybridization Solution (100 mL)
50 mL Formamide -(50%)
6 mL 5M NaCL - (0.3 M)
2 mL 1M Tris-HCl pH 8.0 – (20 mM)
1 mL 0.5 M EDTA pH 8.0 – (5 mM)
1 mL 1M Sodium Phosphate pH 8.0 – (10 mM)
2 mL 50X Denhardt's (1X)
10 g Dextran Sulfate (10%)
5 mL of 10 mg/mL Stock Yeast tRNA (0.5 mg/mL)
q.s. Water to 20 mL

50X Denhardt's (500 mL)
5 g Ficoll 400
5 g Polyvinylpyrrolidone
5 g BSA (Pentax Fraction V)
500 mL Water

Store Frozen in -20C -----**Before use add 1M DTT to a final concentration of 100 mM DTT for probes!!!!**

Audioradiography

1. Thaw Kodak NTB2 emulsion vials at 42 C in a light tight container (wrapped in foil).

   Emulsion is aliquaoted in vials (5mL) stored in a box covered in a foil pack in a black plastic bag in the refrigerator . MUST BE LIGHT TIGHT!!!

   Take strips of foil to wrap two vials in dark room. Don't use safe light, just wrap in total darkness. As an alternantive, use orange safe light. Make sure the vials are completely wrapped in foil and the lids are on tight.

   The slide dipper takes two vials to fill it. This is enough to cover 50-60 slides

   Make up the slide boxes to hols slides following covering with emulsion. Place a slide about 3-4 nothces from the top of the box. Take two kimwipes and cross them. Place some dridrite in the middle and roll up to place above the slide box. Put paper towel cut to the bottom of the box. Wrap up dridrite in paper towels to place in the bread box when slides have been dipped.

2. Need to bring 50 ml connicle tube with 10 mL ddH20 placed in 42 C water bath with vials (Make sure light is on). Can get by with as soon as it reaches TEMP./ FOR 20 SLIDES. In the dark room with the orange filter, gently mix the emulsion swith ddh20 in the connicle tube. No air bubbles (rotate tube side to side) Emulsion is 1:1 with water.

3. Fill slide dipper that has been placed in the 42 C H_20 bath.

4. Dip each slide to the bottom of the slide dipper. Wipe off excess, polish back to remove film from from edge.

5. After dipping , place towels in light tight box. Leave lids off during this time,.allowto dry for 6 to 8 hours. Rotate slides 3-6 hours.
   Tape door shut to indicate it is use.

6. After 3-6 hours drying go to dark room and place lids on slide boxes. Wrap in foil, store at 4C.
7. to develop allow slides to reach rt
8. Kodak D-19 developer (78.3g / 500 mL wrapper in foil) for 4 min-1x
9. water in 0.5 min-1
10. fixer for 5 min 1

Fixer
171 water
59.5 mL Sol A
6.5 mL Sol B

11. Water for 5 min
12. Hemaltoxin for 30-sec
13. Water for 30 sec
14. 70 EtOH
15. 95 EtOH
16. 100 EtOH
17. Xyxlene for 1 min 3X
18. Permount and coverslip
19. Place drop in middle of slide, allow to dry

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VITA

Morgan D Ashworth Jr

Candidate for the degree of

Master of Science

Thesis: ANALYSIS OF ENDOMETRIAL GENE AND PROTEIN ALTERATIONS FOLLOWING ENDOCRINE DISRUPTION IN THE PREGNANT GILT

Major field: Animal Science

Personal Data: born in Brownwood, TX on January 28, 1969, the son of Morgan and Dorthy Ashworth

Education: Graduated from Lone Grove High High School in May of 1988. Earned Bachelor of Science Degree from Oklahoma State University, Stillwater, Oklahoma in August 2001. Completed the requirements for the Master of Science with a major in Molecular Reproductive Physiology at the Oklahoma State University in May 2005.

Name: Morgan Dean Ashworth Jr Date: May 2005

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: ANALYSIS OF ENDOMETRIAL GENE AND PROTEIN ALTERATIONS FOLLOWING ENDOCRINE DISRUPTION IN THE PREGNANT GILT

Pages in Study:160 Candidate for the Degree of Master of Science

Major Field: Animal Science

In the pig, administration of estrogen to gilts on D 9 and 10 of pregnancy causes conceptus fragmentation and death between D 15 to 18 of gestation. *Experiment I* established normal patterns for gene and protein expression of cyclic and pregnant cyclooxygenase -1 (COX-1) and -2 (COX-2). *Experiment II* evaluated endometrial expression for COX-1 and -2, prostaglandins, insulin-like growth factor (IGF) -I and –II, and its receptor following early estrogen administration to pregnant gilts. The current study demonstrated early estrogen administration disrupts endometrial synthesis and release of cyclooxygenases and insulin-like growth factors during pregnancy providing a potential mechanism by which total embryonic loss occurs in the pig.

Advisor's Approval:_____