IDENTIFICATION AND CHARACTERIZATION OF UTERINE TRANSCRIPTIONAL AND TRANSLATIONAL FACTORS CONTRIBUTING TO ENDOCRINE DISRUPTION OF THE PREGNANT AND PSEUDOPREGNANT MODEL PHENOTYPE IN PIGS

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TRANSCRIPTIONAL AND TRANSLATIONAL FACTORS CONTRIBUTING TO ENDOCRINE DISRUPTION OF PSUEDOPREGNANT PIGS

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"If the mind is to emerge unscathed
from this relentless struggle
with the unforeseen, two qualities
are indispensible: first, an intellect
that, even the darkest hour, retains some
glimmer of the inner light which leads
to the truth; second, the courage to follow this light
wherever it may lead."

Carl Von Clausewitz
Military Theorist

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

NOMENCLATURE

ACTN4	Actinin α4
AR	Aldose Reductase
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
CD24a	CD24 Antigen
cDNA	Complimentary Deoxyribonucleic Acid
CL	Corpora Lutea
CSF-1	Colony Stimulating Factor-1
CO	Corn Oil
C_T	Cycle Threshold
CXCL14	Chemokine Ligand 14
D12F	Day 12 Filamentous
D14F	Day 14 Filamentous
DAVID	Database for Annotation, Visualization and Integrated Discovery
dChip	DNA-Chip Analyzer
ddPCR	Differential Display Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EC	Estradiol Cypionate
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EMSA	Electrophoretic Mobility Shift Assay
EPC	Ectoplacental Cone
ERα	Estrogen Receptor α
ERβ	Estrogen Receptor β
ERKO	Estrogen Receptor Knock Out
EST	Expressed Sequence Tag
EV	Estradiol Valerate
F-actin	Filamentous Actin
FDR	False Discovery Rate
FSH	Follicle Stimulating Hormone
GCOS	GeneChip Operating Software
GE	Glandular Epithelium
GnRH	Gonadotropin Releasing Hormone
GO	Gene Ontology
GPAP3.0	GenePix Auto Processor 3.0
HA	Hyaluronic Acid
HAS	Hyaluronic Acid Synthatase
HSP27	Heat Shock Protein 27
ICM	Inner Cell Mass
IFN-γ	Interferon-γ

IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Proteins
IL-1RAP	Interleukin-1 Receptor Accessory Protein
IL-1RT1	Interleukin-1 Receptor Type 1
IL-1β	Interleukin-1β
IL-6	Interleukin-6
ITI	Inter-α-trypsin Inhibitor
IκBs	Inhibitors of Nuclear Factor κB
IκBβ	Inhibitor of κBβ
JAK-STAT	Janus Kinase- Signal Transducer and Activator of Transcription
LE	Luminal Epithelium
LH	Luteinizing Hormone
LIF	Leukaemia Inhibitory Factor
LPS	Lipopolysaccharide
MBEI	Model-Based Expression Indices
mRNA	Messenger Ribonucleic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NBA	Number Born Alive
NFκB	Nuclear Factor κB
NK	Natural-Killer
NMB	Neuromedin B
NMB-R	Neuromedin B Receptor
OD	Optical Density
P45017α	P45017α-hydroxylase
P450arom	Aromatase
PCOS	Polycystic Ovarian Syndrome
PCR	Polymerase Chain Reaction
PG	Prostaglandin
PGE	Prostaglandin E
PGF	Prostaglandin F
$PGF_2\alpha$	Prostaglandin $F_2\alpha$
PLA2	Phospholipase A2
PLAZ	Phospholipase AZ
PM	Perfect-Match
PR	Progesterone Receptor
PTGS1	Prostaglandin Synthetase-1 (also referred to as cyclooxygenase-1)
PTGS2	Prostaglandin Synthetase-2 (also referred to as cyclooxygenase-2)
PTHrP	Parathyroid Hormone Like Hormone
QT-RT-PCR	Quantitative Reverse-Transcriptase Polymerase Chain Reaction
RANK	Receptor Activator of NFκB
RANKL	Receptor Activator of NFκB Ligand
RAR	Retinoic Acid Receptors
RBP	Retinol Binding Protein
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction

SAGE	Serial Analysis of Gene Expression
SPP1	Secreted Phosphoprotein 1
sqPCR	Semi-quantitative Polymerase Chain Reaction
ST	Stromal Cells
STAR	Steroidogenic Acute Regulatory Protein
STAT	Signal Transducer and Activator of Transcription
TGC	Trophoblast Giant Cells
TGFα	Transforming Growth Factor-Alpha
TGFβ	Transforming Growth Factor-Beta
TH	T-Helper
TLR-4	Toll-like Receptor-4
TNF-α	Tumor Necrosis Factor α
TSG6	Tumor Necrosis Factor Stimulated Gene 6
UG	Uterine Glycocalyx

Chapter I

Introduction

During estrus, the pig will ovulate 14-16 oocytes while the national average litter size is approximately 10 piglets (Perry and Rowlands, 1962; Pope and First 1985). During days 10 through 15 of gestation, the porcine embryo undergoes three rapid and dynamic morphological changes (Heuser and Streeter, 1929). It is during this time that majority embryonic mortality occurs through an unidentified selection mechanism. To increase litter size it is imperative to understand the conceptus and endometrial factors involved in the establishment of pregnancy and survival of porcine embryos.

Essential functions of the endometrium during gestation include a prolific environment for embryonic development, a vital source of macro/micro nutrients for developing embryos, and a sufficient attachment site for apposition of the elongated conceptuses. Uterine capacity (Webel and Dziuk 1974) and the rapid changes in conceptus morphology (Giesert et al., 1982, Pope et al., 1990) have been theorized to be the predominate limiting factors associated with early embryonic mortality rates in the pig.

Embryonic mortality has been demonstrated to occur through endocrine disruption of the uterine environment in the pig. Exogenous estrogen administration to pregnant gilts on days 9 and 10 of pregnancy results in complete embryonic mortality by day 30 of gestation (Pope et al., 1986). Using this endocrine disruptor model, Morgan et

al. (1987) discovered that complete embryonic mortality actually occurs by day 16 of gestation and this event is associated with a dysfunctional breakdown of the uterine glycocalyx needed for conceptus attachment to the uterine surface (Blair et al., 1991).

The mechanism by which early administration of estrogen causes conceptus loss has yet to be elucidated in the literature. Furthermore, the origin of dysfunction has not been localized to the conceptus or maternal tissues specifically. The current investigation attempts to clarify the uterine dysfunction caused by early estrogen exposure in pigs through removing the conceptus contributions through construction of an endocrine disrupted psuedopregnant pig model. The following review of literature will focus on the areas of early gestation which is the temporal window in which a significant amount of embryonic mortality occurs, the potential mechanisms involved in those areas, and how endocrine disruption within those mechanisms may lead to reduced litter size in swine production.

Chapter II

LITERATURE REVIEW

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 release of follicle stimulating hormone (FSH) from the anterior pituitary. 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 stimulates 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 h window during the expression of estrus. Metestrus follows the cessation of estrus and lasts about 1-2 days post-ovulation**.** Metestrus is the stage where transformation of corpora hemorrhagica to corpora lutea occurs. The development

of fully functional CL initiates the stage of diestrous. Elevated plasma progesterone concentrations exert 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 (9ng/mL) 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 16 of the estrous cycle (Guthrie et al., 1972). The high concentration of plasma progesterone from the CL for 10 days of the estrous cycle will 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). The mechanism for this action will be extensively reviewed in a later portion of this literature review. Briefly, progesterone receptor (PR) exhibits an inverse expression in regards to the p65 subunit of the master transcription factor nuclear factor kappa B (NF-κB). NF-κB provides an ideal mechanism for the release of prostaglandins used during luteolysis. 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

Regulation of Conceptus Morhpology

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 space themselves equidistantly 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. Between 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/h (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 h 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 h (Geisert et al., 2006). 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.

Factors Associated with Embryonic Vitality and Survival

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).

Increase in uterine lumenal fluid content of insulin-like growth factor (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). Secretions of IGF could be important for conceptus differentiation and attachment to the uterine luminal surface after initial expansion through the uterine horns.

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-RI) (Corps et al., 1990). Although gene expression for IGF-RI was demonstrated, Chastant et al. (1995) was unable to detect the presence of IGF-IR in the trophoblast, but did localize the IGF-RII. Conceptus expression of the IGF-RI 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 days 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).

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 (Ashworth et al., 2005). 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 (Geisert et al., 1990). 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 suggests 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 in regards to 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) also indicated prior to day 18 there must be at least two conceptuses per uterine horn for a successful pregnancy to be established 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)

Endocrine ExocrineTheory for Porcine CL Maintenance

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.

Uterine Architectural Changes Produced During the Implantation Window

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 to 15 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.

Mucin Involvement During Conceptus Attachment

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.

The expression sialomucin complex (MUC-4) an additional cell surface marker, is also reduced in the uterine epithelium during implantation in rodents [McNeer et al., 1998; Carraway and Idris, 2001]. Furthermore, Ferrel et al. (2003) demonstrated an increased expression of MUC-4 protein expression during placental attachment in the pig. Placentation in the pig is a non-invasive attachment while the porcine conceptuses themselves are extremely invasive *ex utero* (Samuel and Perry, 1972). One could infer the harmonized expression of mucins such as MUC-1 and MUC-4 are both required to be tolerant for attachment but may play a regulatory role in controlling magnitude of conceptus proteolytic activity. In contrast to the pig, rodents appear to down-regulate both MUC-1 and MUC-4 which may be necessary for the more invasive murine phenotype placentation.

Inter-alpha-trypsin-inhibitors

Stabilization of the ECM is accomplished through a family of protease inhibitors known as the inter-α-trypsin-inhibitors (ITI) (see review 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 review 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.

Hyaluronin Acid Synthase Invovlment with ECM Stabilization

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 oocyte 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).

MMP/TIMP Remodeling of the Uterine Endometrium

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.

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Immunomodulation of Pregnancy

Universal Immune Function during Pregnancy

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 compliment cascade that generates components

that can bind tissues, recruits effector cells, and invokes 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 levels of proteins that inhibit complement component proteins (Tedesco et al., 1993). Furthermore, murine trophoblast devoid of these inhibitory proteins resulted in embryonic death (Xu et al., 2000). The adaptive system is reliant 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 theses specialized lymphocytes in very small numbers in the body when they are not needed and rapidly employ them through clonal expansion to eliminate pathogens of prior exposure in a very short time period. Successful pregnancies in the porcine uterine environment must circumvent both systems (innate and adaptive) in order to preserve gestation (Georgieva et al., 1984, Croy et al., 1987,)

Specialized Immune Cell Function during Early Pregnancy

In the pig uterus, it is well documented that during early pregnancy (early as D10), trophoblastic cells recruit lymphocytes (T-cells, NK cells) to the uterine endometrial stroma beneath 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 caused 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), (La Bonnardiere et al., 1991). Interferon gamma (IFN γ) and interferon delta (IFN-δ) are the two forms of IFN's in the pig, and unlike bovine, are not postulated as maternal recognition signals. IFN-γ is the most abundant type of IFN found in the porcine uterus, which is predominantly secreted by the trophoblast starting around day 13 of pregnancy. Tuo et al. (1993) demonstrated, INF-γ can have a stimulatory role on the activity of NK cells *in vitro*, suggesting NK cells may play a pivotal role in placentation. The function of IFN-γ has not been clearly defined in the pig uterus. 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. Furthermore, IFN-γ at its highest expression has been localized to the trophoblast on day 15 concomitant with greatest levels of uterine IL-18 (also termed IFN- γ inducing factor) (Joyce et al., 2005).

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 vascularzation 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 Th2 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 contemplation of this once accepted paradigm. Muranaka et al. (1998) demonstrated that pregnancy was lost when mouse IL-18 was co-administered with IL-12. Further examination of IL-18 revealed that mice that when selected based on abortive prone tendencies, they exhibited suppressed levels of IL-18 at the fetal-maternal interface. In contrast, non-abortive mice when mated 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.

Transcriptional Regulation of Gene Expression during Cyclicity and Early Pregnancy

Master Transcription Regulation and Activation

The NF-κB transcription factor superfamily regulates tissue immune function, the inflammasome, and acute phase reactions (McKay and Cidlowski, 1999). NF-κB activation targets a plehtora of genes, which include cytokines, growth factors, and cell adhesion molecules. NF-κB is comprised of a dimer from the Rel family of transcription factors (Ali and Mann, 2004). NF-κB receptor activation can come through a diversity of stimuli such as bacterial endotoxin lipopolysaccharide, oxidative stress, and the specific

cytokines such as tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β). NF-κB is constituentively located in the cytoplasm as a heterodimer compiled from the Rel family of proteins that include: p50, RelB (p100), p105 and RelA (p65), RelB (Ghosh *et al*., 1998). The heterodimer of p50 and p65 is generally thought of as the inducible NF-κB transcription factor found in eukaryotes, where as a homodimer of p50 is considered to be for constituent expression in the tissue. IκB's inhibitory effect on NF-κB renders it inactive while in the cytoplasm (Ali and Mann, 2004). Receptor activation of the known cytokines IL-1β and TNFα can also stimulate the pathway for phosphorylation of two serine residues contained within the IκB. Furthermore, IκB kinase is comprised of the two catalytic subunits: (IKKα and IKKβ) and a regulatory subunit IKKγ. NF-κB complex liberation is a direct result of phosphorylation of IκB which is ultimately targeted for polyubiquitination and degradation by the 26S proteasome (Ghosh *et al*., 1998). Upon liberation of the IκB allows the NF-κB heterodimer (p65:p50) translocation into the nucleus where binding to κB-sites will activate transcription. Nuclear κB site will stimulate IκB binding to the NF-κB complex where it will be inactivated and recycled back to the cytoplasm. κB sites contained within certain genes are transcriptionally regulated by NF-κB and may include a plethora of cytokines (TNFα, IL-1β, IL-6, and GM-CSF), chemokines (RANTES) and enzymes such as cyclooxgenase-2 (COX-2) (Ali and Mann, 2004). Steroid regulation of implantation/placentation and uterine receptiveness are modulated through activation of pathways involving adhesion molecules, cytokines, and growth factors which are ultimately NF-κB regulated (see Cross *et al*., 1994; Carson *et al*., 2000).

Implantation in the pig involves many events that mimic an acute inflammatory response. Regulation of of the estrous cycle and early pregnancy would be an ideal regulatory pathway of NF-κB. Furthermore, endometrial NF-κB activation is postulated as a key regulator of implantation in the human (Reznikov *et al*. 2000; King et al. 2001; Page *et al*., 2002) and mouse (Nakamura *et al*., 2004a,b). During the estrous cycle and early pregnancy in the pig, p65 is localized in the cytoplasm of the porcine uterine luminal and glandular epithelium (Ross and Gesiert, unpublished results). The porcine endometrium secretes a number of NF-κB regulated cytokines (IL-6 and LIF) and growth factors (KGF) during the period of rapid conceptus development on day 12 to 15 of pregnancy (see Geisert and Yelich, 1997). Ross et al. (2003) found that conceptus secretions of IL-1β are greatest on day 12 of gestation and falling to nadir level by day 15. An increase in epithelial KGF expression may be a resultant of conceptus estrogen, IL-1β, or a synergy caused by both. During early gestation, concentrations of peripheral progesterone are high (25 ng/ml), which provides the source for down regulation of uterine epithelial PR after 10 days of stimulation (Geisert et al., 1994). Down-regulation of PR in the uterine epithelium may indirectly up-regulate KGF expression through activation of NF-κB genes (Kalkhoven et al., 1996). The p65 subunit of the transcription factor NF-κB acts to repress the PR protein in a reciprocated negative fashion. As PR is down-regulated from uterine epithelium through presence of the ligand, the transcription factor NF-κB is activated. Although the specific mechanism of KGF in porcine gestation has yet to be elucidated, uterine stroma would appear to control the function of the uterine epithelium following down-regulation of the epithelial PR on day 10 of gestation. KGF-R expression of conceptus trophoblastic origin and KGF production by uterine

epithelial cells may indicate an embryo maternal crosstalk regulation of the conceptus and surface uterine extracellular matrix.

There is an 80-fold increase in endometrial COX-2 gene expression between days 5 to 12 of the estrous cycle and pregnancy (Ashworth et al., 2006). Moreover, the increase in endometrial COX-2 gene expression from days 10 to 15 of the estrous cycle and pregnancy is compartmentalized to the uterine luminal and glandular epithelium, which occurs after the window of epithelial PR down-regulation (Geisert *et al*., 1994). NF-κB localized in the uterine epithelium (Ross and Gesiert, unpublished results) with an increase in cyclooxgenase in the luminal and glandular epithelium (Ashworth et al., 2006), leads one to postulate NF-κB involvement in regulation of uterine function during the estrous cycle and early pregnancy.

During the attachment window of the placenta to the epithelium, embryonic IL-1β expressed concomitantly with estrogen secretion may establish an immunological dialog between the foreign conceptus tissues and the maternal endometrium. Caspase-1, also refered to in the literature as IL-1β-converting enzyme (ICE), cleaves the 31 kDa inactive form of IL-1β to its biologically active 18 kDa form. Although IL-1β secretion is severly attenuated by day 15 of pregnancy, there is a sustained second phase of conceptus estrogen secretion necessary to maintain CL function beyond 28 days of gestation (Geisert *et al*., 1987). This second phase of conceptus estrogen secretion is temporally associated with a pregnancy specific 8-fold increase in endometrial caspase-1 gene expression (Ashworth unpublished data). Increase in endometrial caspase-1 expression is not associated with endometrial release of IL-1β as lumenal content of IL-1β protein is greatly decreased on day 15 and 18 of pregnancy suggesting an alternative substrate may

be involved (Ross et al., 2003b). Pro-IL-18, which has structural similarities to IL-1β, is involved with modulation of the immune system through induction of interferon-γ is another substrate for caspase-1 (Fantuzzi and Dinarello, 1999). Although similar to IL-1β, IL-18 binds to its IL-18 receptor; however it does not activate the NF-κB pathway (Lee *et al*., 2004). IL-18 is expressed by murine endometrial epithelium (Yoshino et al., 2001) and has been localized to the porcine endometrial epithelium (Ashworth unpublished results).

Estrogen and Estrogen Receptor Involvement During Early Pregnancy

The general effects of estrogen on tissues have been studied dating as far back as the 1930's with the first isolation of the steroid molecule, which was followed by subsequent isolation of its receptor followed in the 1960's (Jensen et al., 1962). It has been well established that the estrogen signaling system plays a pivotal role in the physiology of the reproductive organs. Estrogens are a group of C_{18} sterol compounds that are derived from cholesterol. Numerous physiological ligands exist that are recognized by the ligand binding domain of the ER such as: 17β-estradiol, 17α-estradiol, estrone, and estriol (Kuiper et al., 1997) Estrogen receptors (ER) modulate transcription through ligand binding to a broad range of related ligands, including plant originated phytoestrogens, aflatoxins, as well as synthetic estrogens such as DES.

To date, two known estrogen receptors have been characterized: ERα, and ERβ. Bigsby and others in 2002 demonstrated that signaling through ERα, and ERβ play a critical role in proliferation of the endometrium. Furthermore, in the rodent uteri ERα has been determined to be the predominant receptor gene in the luminal and glandular

epithelium (Wang et al., 1999). In addition to proliferation, estrogens and ER's play an regulatory role of the inflammosome and specific cytokines such as TNFα (Yu et al., 1998); In contrast, IL-1β appears to be present in greatest abundance concomitantly with estrogen, but as observed in a recent study, appears to not be induced by estrogen (Ashworth, Geisert, and White unpublished results). Estrogen has been demonstrated to have some regulation over other key immune pathways such as: T-lymphocyte cytokine secretions, B-lymphocytes population energetics, and deemed critical for inducible nitric oxide synthase (iNOS) levels (Pfeilschifter et al., 2002).

In the early porcine conceptus, the machinery is present to rapidly convert steroid precursor molecules into estrogen (Perry et al., 1973, 1976). Geisert et al., 1990 determined that estrogen appearance during early pregnancy in the pig is bimodal with a very transient appearance around day 12, and reappearing as a sustained secretion around day 16 of pregnancy. Furthermore, this increase in conceptus estrogen has been attributed to the biological increases in uterine blood flow during early pregnancy (Ford and Christenson 1979). As previously discussed, Bazer and Thatcher determined that the ligand estrogen is responsible for the sequestering of prostaglandins within the uterus and avoiding luteolysis and termination of the pregnancy.

Porcine ER Expression during the Estrous Cycle and Early Pregnancy

Geisert et al. (1995) characterized changes in the expression patterns of ER in the porcine endometrium during the estrous cycle and early pregnancy. Concentrations of nuclear ER in the endometrium increased from days 0 to 12 when followed by a decline in ER starting around day 15 regardless of status (cyclic, pregnancy). Furthermore,

quantification of mRNA confirmed the ER nuclear findings. Immunocytochemical localization of protein revealed intense ER expression in the stomal cells and only moderate expression in the surface and glandular epithelial cells during estrus (Day 0 and Day 18). However, ER expression in the stroma was absent from days 5 to 15 of the estrous cycle but began to increase on day 18. In contrast to the cyclic pigs, on day 18 of pregnancy, ER remained low on day 18 of pregnancy (Geisert et al., 1995).

Porcine Embryonic Mortality and Endocrine Disruption

Estrogenic micotoxins 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 to 6 or 11to 15 of gestation, does not disrupt 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) (Table 2.1) to the steroid receptors. Exposures to 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.

In the pig, the majority of embryonic mortality occurs prior to day 30 of gestation (Perry et al., 1954). Embryonic losses during early gestation of the pig was first recorded by Corner et al. (1923), and was estimated by counting corpora lutea present on the ovary to determine ova ovulated, and viable conceptuses flushed from uterine horns at various

stages of gestation. Using the 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 of embryo in the pig. The periimplantation period (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 from differences in conceptus size and development based on order and timing between ovulations due to the variable length of estrus 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). Further, Geisert et al. (1982) demonstrated that exogenous estrogen administered on day 11 or 12 of the estrous cycle advanced uterine secretions. Based on Geisert's findings, more developed conceptuses in the same litter may possess the ability to initiate steriodogensis (producing E_2) first may advance uterine secretions 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.

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. Uterine capacity and endometrial surface area of the dam in the uterus 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).

Porcine Pregnancy Specific Endocrine Disrupted Systems

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 break down in the uterine glycocalyx occurring approximately day 14 of gestation (Blair et al., 1991).

Presence of ERα in the endometrial LE and GE and the possible role of estrogen in modulating NF-κB stimulated genes suggest that premature exposure to estrogen may disrupt pregnancy through aberrant gene expression between days 12 to 15 of pregnancy as occur in the mouse (Ma *et al*., 2003). Ross et al. (2008) utilized a porcine 15,000

Table 2.1. Endocrine disruptor chemicals with E_2 activity present in food and the environment

Chemical	Method of Exposure	Mechanical Action
Aflatoxins	Moldy corn, environment	Estrogen agonist-ER alpha
Polychlorobiphenyls (PCP)	Food, environment	Alters steroid metabolism /transport
DDT	Food, environment, workplaces	Estrogen activity
Organochlorine insecticides	Food, environment, workplace	Homeostasis of estrogen
Triazoles, Imidazoles	Food, environment, workplace	Inhibition of steroid hormone biosynthesis
Nonyl-phenols and octylphenols	Detergent by-products, consumer products	Estrogen agonist-ER alpha
Bisphenol A	Plastics, consumer products	Estrogen agonist-ER alpha
Polybrominated flame	Food, environment, workplaces, consumer products	Steroid homeostasis
Phthalates	Food, consumer products	Steroid biosynthesis
Parabens	Cosmetics, toiletries, and pharmaceuticals	Estrogen agonist- ER alpha and beta
UV-screen	Consumer products	Estrogen agonist- ER alpha
Isoflavones, lignans	Food (Soy-based food), consumer products	High affinity for ER beta

Adapted from Caserta et al., 2008

unigene cDNA array constructed from porcine uterine, ovarian and conceptus tissues (Whitworth *et al*., 2005) to evaluate alteration in endometrial gene profile following treatment of gilts with estrogen on days 9 and 10 of pregnancy. The vast majority of endometrial genes altered by early estrogen treatment occurred on day 13 and 15 of pregnancy. Many of the genes identified are factors involved with cell attachment, immunology, transcription regulation and metabolism. Osteopontin (OPN) mRNA expression is advanced in estrogen-treated gilts, which was confirmed in the LE by *in situ* hybridization (White et al., 2005). Osteopontin, a proposed mediator of trophoblast adhesion to the endometrial LE through integrin receptors present on the LE apical surface (Johnson *et al*., 2003), is stimulated by IL-1β, TNF-α, interferon-γ and estrogen. Alternation in OPN expression (NF-κB regulated gene) may cause failure of proper glycocalyx formation needed for conceptus attachment.

Early estrogen administration also alters the pattern of COX-2 expression in the uterine LE (Ashworth *et al*., 2006) supporting a mistiming of NF-κB activation. The importance of COX-2 expression in LE during establishment of pregnancy has not been investigated. 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 implantation (Lim *et al*., 1997). Pharmacological inhibition of PG synthesis through inhibition of COX does not affect trophoblast elongation in pigs (Geisert *et al*., 1986). However, it does increase embryonic mortality during the period of placental attachment to the uterine surface (Kraeling *et al*., 1985). Induction of COX-2 expression promotes PGE synthesis

(Murakami and Kudo, 2004). Endometrial secretion of PGE may function in stabilization of the extracellular matrix, inflammation and immune function in the pig

Early exposure of pregnant gilts to estrogen disrupts the normal synchrony in the presence of insulin-like growth factors in uterine lumen during the time of rapid conceptus elongation on day 12. Concentration of IGF-I is elevated within the uterine lumen prior to and during rapid trophoblast expansion on days 10 to 12 of pregnancy (Simmen *et al*, 1995; Geisert *et al*., 2001). The high uterine content of IGF-I 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*., 1995). Content of IGF-I in uterine flushings decreases dramatically following conceptus elongation and the initiation of placental attachment to the uterine surface epithelium on day 13 of gestation. The high amounts of IGF-I in the uterine lumen of the pig are closely associated with detection of insulin-like growth factor binding proteins in the uterine lumen (Lee *et al*., 1998; Geisert *et al*., 2001). However, there is 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 collected in uterine flushings after day 13. It is possible IGFBPs helps to sequester IGF-I in the uterine lumen for release during the

sensitive period of conceptus differentiation and trophoblast elongation. The precise nature of the loss of uterine luminal IGFs following conceptus elongation suggest that the release of IGFs during days 12 and 13 of pregnancy is very critical for down-stream development and survival of pig embryos. Administration of estrogen to gilts on days 9 and 10 of pregnancy causes premature proteolysis of uterine luminal IGFBPs on day 10 and an earlier (48 h) decline of IGF-I on days 10 and 11 (Ashworth et al., 2005). Although the study cannot demonstrate a causal effect of premature loss of IGFs with later conceptus death, early estrogen administration clearly causes a dramatic decline in IGFs before the critical period of conceptus elongation and differentiation. Mice devoid of the IGF-RII undergo *in-utero* mortality during gestation (see review Jones and Clemmons, 1995). Studies have demonstrated that the IGF-RII 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.

Regulatory RNA (miRNA) involvement during early pregnancy

General Regulatory Action

In order to elicit a biological response in the cell, mRNA translation into a protein is normally required; these proteins are regulated at the transcriptional and translational level through single stranded hairpin loop and dsRNA (miRNA/siRNA respectfully).

Furthermore, the quantity and temporal expression of these proteins are a critical control point regulated through a servant (protein) and master (ncRNA) relationship (Neilson et al., 2007).

Lee et al. (1993) was the first to discover and report miRNA transcripts. miRNA are an endogenous RNA species that range between 18-24 nt in length (Bartel et al., 2004). The miRNA task and organization begins with transcription of the endogenous miRNA transcript into a hairpin shaped oligonucleotide of around 90 nt termed pri-miRNA (primary miRNA) (Lee et al., 2002). This transcription process is very similar to mRNA transcription where RNA polymerase II is the predominate polymerase driving the transcription process (Bartel et al., 2004). The phenotype of the pri-miRNA is only of polyadenylation with methyl caps similar to mRNA (Lee et al., 2002). Step 1 of nuclear processing of the miRNA is accomplished through the enzyme Drosha, which separates the 60 nt stem-loop structure (miRNA) from the total pre-miRNA sequence; furthermore, these pri-miRNA transcripts can be located in an intron or exon of the mRNA coding sequence and are most likely cut out after the transcriptional processing. Moreover, the exact time and way these miRNA's are cut out, and wether it's during or after splicing has yet to be elucidated. Once the miRNA is processed (now termed pre-miRNA), the protein receptor Exp-5 (exportin 5) facilitates transport of the pre-miRNA from the nucleus to the cytoplasm (Lund et al., 2004). Once in the cytoplasm, the enzyme Dicer processes the ds loop-stem structure resulting in a mature miRNA (Lee et al., 2004). The mature miRNA is relatively short ranging between 18-22 nt in length. A helicase enzyme will come in and separate the miRNA strand from its complementary strand from the mature miRNA, the complementary ds

portion more than likely inhibits Drosha from processing it all the way down in the nuclear processing portion. The newly activated mature miRNA is now ready to bind to the mRNA transcript and either repress translation or inhibit the process all together depending on binding homology. The mature miRNA apposition and attachment to the miRNA binding domain is facilitated through a complex of interacting proteins to form the RNA Silencing Complex (RISC) (Lee et al., 2004). RISC, using the miRNA as a navigating tool, contains an endonuclease which will cut the mRNA in the middle of the miRNA binding site (which may contain numerous sites) (Tang et al., 2003). In contrast to miRNA, siRNA diverges in its origin and processing. While siRNA and miRNA share a similar expression end point, the major difference is that siRNA's are products that have been created from the formation of dsRNA, transposons, and viruses, while mRNA origin is from loci located within the genome (Bartel et al., 2004).

The miRNA mechanism was first clarified using the amount of lin-4 transcript that is complementary to lin-14 and lin-28. These genes play a developmental role in early to late *C. elegans* larva stage (Lee et al., 1993). As the author measured the translated product he noticed about a 88-90% attenuation, while lin-14 mRNA was unchanged and lin-28 mRNA was reduced to less than 50%. These findings seemed confounded at the beginning; however the study nicely demonstrated that silencing worked at the transcription and translational levels. Target recognition occurs through basically the first 8 residues of the miRNA (5'end) which is complementary to the 3' untranslated region (UTR) of the mRNA target, which has been tagged for posttranscriptional gene silencing (PTGS). Furthermore, the strength of complementary Watson-Crick base paring of the miRNA's to the 3'UTR mRNA determines which type

of silencing will be used; strong complementary paring will usually activate mRNA degradation, where a lower degree of complementary binding will target the gene for repression at translation level.

Regulatory RNA Involvement during the Female Menstrual Cycle

The review of literature for this section will reside primarily to humans due to a void in the literature. To date, approximately 600 miRNA's have been confirmed and around 1000 have been predicted (Berezikov et al., 2006). Furthermore, each single miRNA could potentially target hundreds of genes while several hundred miRNA's could target the same gene (Brennecke et al., 2005).

In the human, the endometrium undergoes very distinct and dynamic biochemical and morphological changes due to changes in sex hormone appearances and concentrations. Numerous studies have demonstrated that gene expression and regulatory functions are tightly controlled throughout the menstrual cycle (Horcajadas et al., 2007). Recently, Pan et al., (2007) indentified hundreds of miRNA's specifically localized to the human epithelial and stromal layers of the uterine endometrium. In the previous study, approximately 300 hundred miRNA's were isolated in the human endometrium where 65 miRNA's where localized to the epithelial cells. Moreover, half of the 65 miRNA's profiled were in the glandular epithelial cells and the other half in the lumenal epithelial cells suggesting a different mechanism in regards to gene regulation in the same cell type but involved with different functions. Using a defined cell culture system model, endometrial cells were cultured and used to characterize the regulatory role ovarian steroids (E_2 and P_4) have on miRNA populations (Pan et al., 2007). The

endometrial cell culture system was subjected to E_2 then subsequently exposed to P_4 to establish miRNA's influence under steroid regulation. Upon examination of selected miRNAs the authors carefully predicted target genes which included: TGF-β, TGF-β-receptor, ER, PR, and CYP-19A1 (aromatase) (Pan et al., 2007). TGF-β is one of the predominate uterine cytokines and a key regulator of inflammatory responses which are instrumental in regards to regulation of protease activity and extracellular matrix integrity (Chegini et al., 2002).

RNA Regulation during the Inflammatory Response

A plethora of miRNAs have been identified as playing a regulatory role in the immune and inflammatory response under mediators such as: let7, miR-17-5P, miR-20a, miR-106a, miR-125b, miR-146, miR-155 (Meng et al., 2007, Tili et al., 2007). Of these newly discovered miRNAs, miR-125b and miR-155 are of great importance due to their involvement in proper T and B lymphocyte development (Tili et al., 2007).

miR-155 has been shown to target the lipopolysaccaride (LPS) system and its ancillary signaling proteins (Tili et al., 2007). Furthermore this study demonstrated that with the addition of miR-155 enhanced the translation of TNF-α, while adding miR-125b repressed TNF-α translation. Endometrial appearance of these miRNAs expressed concomitantly with proinflammatory cytokines increased NF-κB activity (Pan et al., 2007). In monocytes, miR-146 is induced through the NF-κB system and causes an inhibition of TNF-α receptor associated factor 6 (TRAF6) and interleukin-1 associated kinase (IRAK1) expression. Furthermore, both of these factors are downstream from toll like receptors (TLR) and cytokine signaling pathways (Taganov et al., 2007). How the

NF-κB system is able to induce the miR-146 molecule has yet to be elucidated, however some studies suggest it is triggered through cell surface receptors and not intracellular receptors such as TLRs. Moreover, the study also postulated that initial activation of miRNAs within the uterine environment may originate from a nucleic acid viral source (O'Connell et al., 2007). Given these data, one could easily speculate the viral source to be endogenous.

RNA Regulatory Control of Endometrial Apoptosis

Selam et al., (2001) indicated that apoptosis within the endometrium was controlled through ovarian steroid exposure and a delicate balance between pro-apoptotic genes and anti-apoptotic genes. During early pregnancy and the window of implantation, a controlled inflammatory response is obligatory for success of the pregnancy. Many miRNAs have been identified or predicted to regulate apoptosis, cell cycle, and various types of cancer through controlling expression of certain genes (Matasubara et al., 2007). A plethora of miRNAs associated with triggering apoptosis are: miR-10a, miR-28, miR-196a, miR-337, miR-96, miRNA-145, miR-150, miR-155. Furthermore, altered expression patterns of miR-155 has been shown in studies to differentially regulate some of the caspases and Fas-Asscociated Death Domain proteins (FADD) which are both key machinery involved with apoptosis activation and signaling (Garafalo et al., 2008). Lastly, one of the hallmark signs of apoptosis control is the release of the Bcl2 family of transport proteins from the electron transport complex termed "cytochrome C". Recent studies have demonstrated that aberrant expression of the Bcl2 transport protein during

apoptosis is regulated through the expression of miR-15a and miR-16-1 (Cimmino et al., 2005).

Regulatory RNA Control of Implantation

Implantation and maternal acceptance of the fetal tissues is a complex issue that requires precise synchrony from maternal and embryonic dialog, and has yet to be fully elucidated. Ovarian hormones and their receptors present in a spacio-temporal fashion are mandatory for a successful pregnancy. Crosstalk between fetal and maternal tissues involves growth factors, chemokines, and cytokines which are all plausible constituents for miRNA regulation during this period. Specific miRNAs have been identified in the murine pregnancy model during peri-implantation and pre-implantation periods which may serve as potential candidates that regulate implantation (Chakrabarty et al., 2007). Furthermore, of the 32 miRNAs indentified in connection with implantation, 3 (miR-101, miR-144, miR-199a) were specifically predicted to target cyclooxygenase-2 (COX-2). The expression of these 3 miRNAs displayed a direct correlation with the expression of COX-2 during delayed implantation in the mouse, inferring a potential role with implantation (Chakrabarty et al., 2007). In addition to these direct miRNA regulators there are a plethora of other molecules that regulate COX-2 expression like cytokines, growth factors, and chemokines.

Statement of the Problem

Over the years, there have been a plethora of conceptus and endometrial factors identified that contribute to the successful establishment pregnancy and yield larger litter size in pigs. Unfortunately, from a reproductive standpoint in the United Statesthe pig is far from optimal. Of the 20 to 46 % conceptus mortality rate realized throughout gestation in the pig, a majority is directly related to the events during the peri-implantation stage of development. Three critical events must occur during early pregnancy: 1) the rapid elongation of the conceptus trophoblast and delivery of the maternal recognition signal, estrogen; 2) the uterine response to conceptus secreted estrogen, and 3) the down-regulation of the luminal epithelial progesterone receptor and the uterine receptivity associated with it. Temporally, these three biological processes all occur within a very narrow time frame (Days 10 to 18 of gestation). As important as they are regarding the establishment of pregnancy in the pig, little biological information is available delineating the mechanisms that regulate these events.

Ford et al. (1997) attributes the larger litter size of the Meishan sows to the ability of the conceptuses to regulate the uniformity and the extent to which they expand their trophoblast. Geisert and Schmitt (2000) suggested that the ability to regulate the simultaneous initiation of trophoblastic elongation would result in more consistent placental size and uterine space among littermates potentially increasing litter size.

Estrogen, while a required component of pregnancy establishment in the pig also plays integral roles during pregnancy establishment. Copious amounts of estrogen delivered by the conceptus during days 11 to 12 of gestation appear to be required as too few conceptuses are incapable of establishing pregnancy. While amount of estrogen

stimulation appears to have a requirement, the timing of the estrogen stimulation is also critical as ill-timed administration or ingestion of estrogen has been shown to cause a total loss of pregnancy in the pig. While certain events such as the redirecting of endometrial $PGF_{2\alpha}$ secretion are attributed to conceptus estrogen stimulation, very little is known regarding the endometrial molecular mechanisms and events following estrogen exposure. A deeper understanding of the regulatory roles that estrogen serves during pregnancy establishment in the pig will lend insight to steroidogenic factors affecting pregnancy outcome in pigs. Furthermore, the biological understanding of estrogen's impact on endometrial function may also be applicable to women who suffer from preeclampsia and endometriosis which are pathological conditions associated with elevated estrogen and progesterone circulation and dysfunctional blood flow and implantation window.

Lastly, the critical molecular events associated with the opening of the implantation window and the period of uterine receptivity is poorly described in most species. While the up-regulation of the Muc genes and down-regulation of the progesterone receptor in the luminal epithelium is associated with uterine receptivity in most species, the biological forces driving that phenomenon are not well described. Certainly, the regulation of specific transcription factors and the downstream activation of their regulated products contribute to the establishment or disruption of pregnancy in the pig.

Approach to the problem

While biotechnological advancements have been rapid over the past few years, bioinformatics resources have made exceptional progress recently. The ability to link microarray data acquisition platforms to bioinformatics tools has dramatically changed the capability to identify differentially expressed genes in a biological system and then accurately annotate the biological themes associated with the alterations in the system evaluated. Also, the significant amount of expressed sequence tags from the pig deposited into GenBank has prompted the development of the GeneChip® Porcine Genome Array by Affymetrix®. The 23, 937 probe set interrogates 23, 256 transcripts representing 20, 201 porcine genes. Utilization of array platforms and modern bioinformatics approaches will help identify the biological processes that are associated with both endometrial response to estrogen stimulation as well as the factors that are involved with opening of the window of implantation during early endometrial development. Identification and characterization of the changes in mRNA expression that are related to trophoblastic elongation, endometrial estrogen stimulation and uterine receptivity as a result of transcription factor activation will provide a better understanding of the biological events necessary for embryonic survival, implantation and a successful pregnancy in the pig.

Chapter III

Porcine Conceptus Induction of Endometrial Caspase 1 Expression and Interleukin-18 Release During the Period of Placental Attachment

ABSTRACT

Interleukin (IL)-18 is a pleiotropic cytokine expressed in the reproductive tract of the mouse and human during the establishment of pregnancy. The role of uterine IL-18 expression during the noninvasive type of conceptus implantation and placental development in the pig has not been investigated. Pro-IL-18 is a 24-kDa inactive product which can be secreted following cleavage to the active 18 kDa form by caspase-1. In the current study, we characterized endometrial IL-18 and caspase-1 expression during the estrous cycle and early pregnancy of the pig. Gilts were hysterectomized on either day 0, 5, 10, 12, 15 and 18 of the estrous cycle or day 10, 12, 15 and 18 of pregnancy. Endometrial IL-18 and caspase-1 gene expression was evaluated with quantitative RT-PCR and IL-18 in uterine flushings by ELISA. Caspase-1 endometrial gene expression did not change across the estrous cycle; however, endometrial caspase-1 gene expression was 6 and 10-fold greater on day 15 and 18 of pregnancy. IL-18 endometrial gene expression was not affected by reproductive status but increased more than 5-fold from day 10 to 18. Total uterine luminal content of IL-18 increased in pregnant compared to cyclic gilts on day 15 and 18. The current study provides evidence for a conceptus induced activation of endometrial caspase-1 and release of IL-18 during the period of placental attachment in the pig. We propose that IL-18 may play an important role in regulation of the maternal immune system during the establishment of pregnancy.

INTRODUCTION

The noninvasive attachment of the porcine conceptus forms an epitheliochorial type of placentation through its adhesion to the extracellular glycocalyx present on the apical surface microvilli of the uterine luminal epithelium (King et al., 1982; Stroband and Van der Lende, 1990). Following removal of the anti-adhesive factors expressed on the uterine surface epithelium (Bowen et al. 1996), trophoblast attachment to the uterine luminal epithelium involves a coordinated program of events in progesterone stimulated uterine environment that are mediated by actions of estrogen in the pig (Geisert and Yelich, 1997). Trophoblast attachment to the uterine surface in the pig immediately follows the rapid elongation of the trophoblast and the acute increase of conceptus estrogen synthesis on day 12 of pregnancy (Geisert et al., 1990). Release of estrogen by the elongating conceptuses will induce endometrial receptivity for placental attachment to the uterine surface (Keys et al., 1990; Burghardt et al., 1997; Jaeger et al., 2001).

Transient release of estrogen 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). Furthermore, Ross et al. (2003) demonstrated that the periimplantation porcine conceptuses secrete the greatest amount of IL-1β into the uterine lumen during the filamentous stage of morphology (day 12-15), which rapidly decreases to nadir levels by day 18 of gestation. The decline in conceptus IL-1β secretion suggests that another closely related cytokine may function at the conceptus and maternal uterine surface interface to continue regulation of the immunological interactions necessary for establishment of pregnancy in the pig. A possible candidate

cytokine would be IL-18, which is a member of IL-1 family (Okamura et al. 1995). IL-18, formerly known as interferon-γ inducing factor, is a pro-inflammatory cytokine that is believed to play a significant role in implantation. Implantation in the rodent requires the presence of IL-18 at the maternal fetal interface as indicated by abortive prone mice which have suppressed IL-18 levels at the fetal-maternal interface (Muranaka et al. 1998). In contrast, non-abortive mice produced elevated levels of IL-18 at the maternal fetal interface suggesting a Th_1/Th_2 divergence (Ostojic et al., 2003). Like Il-1β, the proform of IL-18 is stored in the cytoplasm and must be proteolyically cleaved to be secreted and exhibit biological activity. Caspase-1 cleaves and activates the proforms of IL-1β and IL-18 (Hentze et al., 2003) to the biologically active molecule with signaling capacity. Expression of IL-18 by the conceptus and/or endometrium is suggested as interferon γ (IFN-γ) increases following trophoblast elongation in the pig (Cencic and Bonnardier, 2002).

The present study was undertaken to determine the expression of caspase 1 and IL-18 during the estrous cycle and early pregnancy in the pig. We also investigate the possible alteration of caspase 1 and IL-18 expression following early exposure of pregnant gilts to estrogen which causes conceptus degeneration after Day 15 of pregnancy.

MATERIALS AND METHODS

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 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 Caspase-1 and IL-18 Expression in Cyclic and Pregnant Gilts.

Gilts were hysterectomized through midventral laparotomy on either days 0, 5, 10 12, 15 and 18 of the estrous cycle (n =24) or days 10, 12, 13, 15 and 18 of pregnancy (n =16) as previously described by Gries et al. (1989). Following induction of anaesthesia with 1.8 ml i.m. administration of a cocktail consisting of 2.5 ml cocktail (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 uterue and ovaries 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, 10 min, 4°C), supernatant collected and clarified 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.

Experiment II: Endometrial Caspase-1 and IL-18 Gene and Protein Expression following Early Exposure of Pregnant Gilts to Estrogen

Bred gilts were randomly assigned one of the following treatment groups: Control (n = 20), i.m. injection of corn oil (CO) (2.5 ml) on days 9 and 10 of gestation or estrogen (E) (n = 20), 5 mg i.m. injection of estradiol cypionate (A.J. Legere, Scottsdale, AZ) on days 9 and 10 of gestation. Gilts belonging to the CO and E treatment groups were hysterectomized on either day 10, 12, 13, 15 and 17 of gestation as described in experiment 1. Uterine horns were flushed with 20 ml PBS, conceptuses were collected, flushings centrifuged to remove cellular debris, and the superatnant stored at -20°C. Endometrial tissue was harvested from the uterine horn and either fixed for *in situ* hybridization or snap frozen in liquid nitrogen and stored at-80°C until utilized for extraction of protein and RNA.

Uterine Tissue Fixation Procedures

Endometrial tissue sections (~1.0 cm) were excised from the bottom 40 cm of the uterine horn which was not flushed with PBS. Tissue sections were placed in freshly prepared 4% paraformaldehyde in PBS (pH 7.2) and gently agitated at r.t. for 24 h. Solution was replaced with 70% EtOH (v/v in H_20), gently agitated for an additional 24 h. Fixed endometrial tissue was dehydrated in a series of graded ethanol changes

followed by xylene, and then embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO).

In situ hybridization

Caspase-1 mRNAs were localized in paraffin-embedded porcine uterine tissue by *in situ* hybridization using methods previously described (Johnson et al., 2000). Briefly, deparaffinized, rehydrated, and deproteinated uterine cross-sections (5 µm) were hybridized with radiolabeled antisense or sense porcine caspase-1 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.

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 Nanodrop® spectrophotometer at an absorbance of 260 nm and purity was verified using the 260/280 ratio.

Quantitative 1-Step RT-PCR

Quantitative analysis of endometrial caspase-1 and IL-18 mRNA were analyzed using quantitative real time reverse polymerase chain reaction (RT-PCR) as previously described by our laboratory (Hettinger et al., 2001, Ashworth et al., 2006). The PCR amplification was performed in a reaction volume of 15 μl using an ABI PRISM 7500 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 caspase-1 and IL-18 (Table 3.1) were generated from porcine sequences obtained using the NCBI genebank 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.

Using the comparative C_T method (Ashworth et al., 2006), relative quantification and fold gene expression difference between treatment and day were determined for the endometrial samples (Table 3.2 and Table 3.3). Differences in mRNA expression of caspase-1 and IL-18 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 $2^{-\Delta\Delta CT}$.

Enzyme-Linked Caspase-1 Competitive Binding Assays

Total luminal content of caspase 1 in the uterine flushings was quantified using a commercial ELISA kit (BenderMed Burlingame, CA) in accordance with manufacturer's specifications. Samples were analyzed in duplicate with a single assay. The intra-assay coefficient of variation for the assay was 5.6%.

Enzyme-Linked IL-18 Competitive Binding Assays

Total luminal content of IL-18 in the uterine flushings was quantified using a commercial ELISA kit (BenderMed Burlingame, CA) in accordance with manufacturer's specifications. Samples were analyzed in duplicate with a single assay. The intra-assay coefficient of variation for the assay was 5.1%.

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 evaluate endometrial caspase-1 and IL-18 mRNA expression and uterine flushing content in expeiriment I included the effects of day (0, 5, 10, 12, 13, 15, and 17), reproductive status (cyclic, pregnant), and the day x status interaction. If the status, day, or day*status interactions were significant, ($P < 0.05$), means were separated by using the PDIFF option of SAS. Additionally, due to unequal variances of caspase-1 and IL-18 protein in the uterine flushings of the cyclic and pregnant gilts, the original data was log transformed.

Table 3.1 PCR primer and probe sequences used for quantitative RT-PCR of endometrial Caspase-1 and IL-18 mRNA expression.

Gene	Forward Primer/Reverse Primer/Probe	GeneBank Accession
Caspase-1		
	Forward 5' – CAA CAC TTC ACC CAC CAG TTC TTC-3'	**NM214162**
	Reverse 5' – TCC ATA AAT GTG GCC GAG GTC TAC-3'	
	Probe 5' – TTC TGG CAA GAT GGG TCC TGG CTT CAC CAA-3'	
IL-18		
	Forward 5'- ATG GCT GCT GAA CCA GAA GA -3'	**NM213997**
	Reverse 5'- TGG TCG TTC AGA TTT CGT ATG ATT-3'	
	Probe 5'- CCT GGA ATC GGA TTA CTT TGG CAA GC-3'	

The statistical model used to evaluate endometrial caspase-1 and IL-18 mRNA expression and uterine flushing content in experiment II included the effects of day (10, 12, 13, 15, and 17), treatment (Con, E), and the day x treatment interaction. If the day, treatment or day*treatment interaction was significant, ($P < 0.05$), treatment means were then separated by using the PDIFF option of SAS. Additionally, due to unequal variances of caspase-1 and IL-18 protein in the uterine flushings of the pregnant gilts, the original data was log transformed for the statistical analysis.

Results

Experiment I: Cyclic and Pregnant Gilts.

Conceptus Caspase-1mRNA Expression

Caspase-1 mRNA abundance (Figure 3.1) increased from day 12 to 13 followed by its lowest esxpression on day 14. Conceptus caspase-1 increased from day 14, and its peak expression was on day 17.

Table 3.2. Comparison of endometrial Caspase-1 and IL-18 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†,*	$\Delta\Delta C_T$§
Caspase-1						
	Cyclic	0	27.18 ± 0.81	18.32 ± 0.19	8.04 ± 0.47^{ab}	-1.41
	Cyclic	5	27.17 ± 0.25	18.52 ± 0.19	9.18 ± 0.38^{a}	0.54
	Cyclic	10	27.99 ± 0.20	18.58 ± 0.11	9.53 ± 0.24^{a}	-0.21
	Pregnant	10	29.64 ± 0.27	18.60 ± 0.07	9.02 ± 0.31^{a}	-0.00
	Cyclic	12	29.12 ± 0.62	18.79 ± 0.58	8.99 ± 0.29^{bc}	-0.74
	Pregnant	12	27.99 ± 0.47	18.32 ± 0.09	9.33 ± 0.53^{bc}	-0.32
	Cyclic	15	27.35 ± 0.58	18.31 ± 0.05	9.27 ± 0.56^{bc}	-0.59
	Pregnant	15	25.65 ± 0.53	18.40 ± 0.12	7.20 ± 0.46^{bc}	-2.55
	Cyclic	18	27.12 ± 0.14	18.37 ± 0.05	8.99 ± 0.18^{bc}	-0.89
	Pregnant	18	25.31 ± 0.32	18.51 ± 0.09	6.52 ± 0.49^{bc}	-3.23
IL-18						
	Cyclic	0	28.63 ± 0.81	18.32 ± 0.19	10.17 ± 0.47^{ab}	-1.50
	Cyclic	5	30.17 ± 0.25	18.52 ± 0.19	11.67 ± 0.38^{a}	0.00
	Cyclic	10	30.22 ± 0.20	18.58 ± 0.11	11.65 ± 0.24^{a}	-0.02
	Pregnant	10	29.64 ± 0.27	18.60 ± 0.07	11.12 ± 0.31^{a}	-0.55
	Cyclic	12	29.12 ± 0.62	18.79 ± 0.58	10.49 ± 0.29^{bc}	-1.18
	Pregnant	12	28.12 ± 0.47	18.32 ± 0.09	10.24 ± 0.53^{bc}	-1.43
	Cyclic	15	27.35 ± 0.58	18.31 ± 0.05	9.25 ± 0.56^{bc}	-2.42
	Pregnant	15	28.15 ± 0.53	18.40 ± 0.12	9.77 ± 0.46^{bc}	-1.83
	Cyclic	18	28.12 ± 0.14	18.37 ± 0.05	9.76 ± 0.18^{bc}	-1.91
	Pregnant	18	27.61 ± 0.32	18.51 ± 0.09	8.52 ± 0.49^{bc}	-3.15

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

ΔC_T† = Target (Caspase-1 and IL-18) – 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 pregnant and Day 5 cyclic (highest ΔC_T; lowest gene expression for caspase-1 and IL-18 genes respectively) were used as a calibrator for caspase-1 and IL-18 to set the baseline for comparing differences in ΔC_T values across all days.

Endometrial Caspase-1mRNA Expression in the Cyclic vs Pregnant Gilts

Quantitative RT-PCR analysis of endometrial caspase-1 mRNA abundances in cyclic and pregnant gilts (Table 3.2) detected a day x status interaction ($P < 0.05$). Endometrial caspase-1 mRNA abundances were approximately 3 and 5-fold greater in the pregnant gilts when compared to the cycling contemporaries on days 15 and 18 respectively (Figure 3.2).

Uterine Luminal Content of Caspase-1 Protein in Cyclic vs Pregnant Gilts

Content of caspase-1 in the uterine flushings of cyclic and pregnant gilts (Figure 3.3) exhibited a day by status interaction ($P < 0.05$). Uterine luminal caspase-1 content in cyclic gilts was low on all days collected (27 to 123 pg). Caspase-1 in uterine flushings from pregnant gilts was similar to cyclic females on days 10 and 12 but there as a 5-fold increase on days 15 and 17 of pregnancy (595 and 541 pg, respectively).

Endometrial IL-18 mRNA Expression in the Cyclic vs Pregnant Gilts

PCR amplification for the IL-18 product (438 bp) indicated that IL-18 mRNA expression (Figure 3.4) is limited to the endometrium and not expressed by the conceptuses. Relative endometrial IL-18 mRNA abundances appeared greater on day 18 of pregnancy which is the period of placental attachment in the pig. A day effect ($P < 0.05$) was detected for RT-PCR endometrial IL-18 mRNA expression. Endometrial IL-

18 mRNA abundances were significantly greater on day 18 when compared to day 10 and day 12 (Figure 3.5).

Uterine Luminal Content of IL-18 Protein in Cyclic vsPregnant Gilts

Content of IL-18 in the uterine flushings of cyclic and pregnant gilts (Figure 3.6) exhibited a day by status interaction ($P < 0.05$). Uterine luminal IL-18 content in gilts was similar between cyclic and pregnant gilts on days 10 and 12 but there was an approximate 5-fold increase in uterine luminal IL-18 on days 15 and 17 pregnancy compared to cyclic gilts. The greatest amounts of IL-18 uterine luminal content occurred on day 15 and 17 of gestation (1,123 and 725 ng, respectively) were preceded by lowest IL-18 content on day 12 of pregnancy (28 ng).

Experiment II: Early Exposure of Pregnant Gilts to Estrogen

Conceptus development

Conceptuses were present in the uterine flushings collected from CO gilts across all days of pregnancy evaluated (day 10, 12, 13, 15, and 17). Normal 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.

Figure 3.1. A significant day effect on relative mRNA units (mean ± SEM) was identified for conceptus caspase-1 ($P<0.05$). Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference ($P<0.05$) between endometrium when comparing days12, 13, 14, 15, 16, and 17.

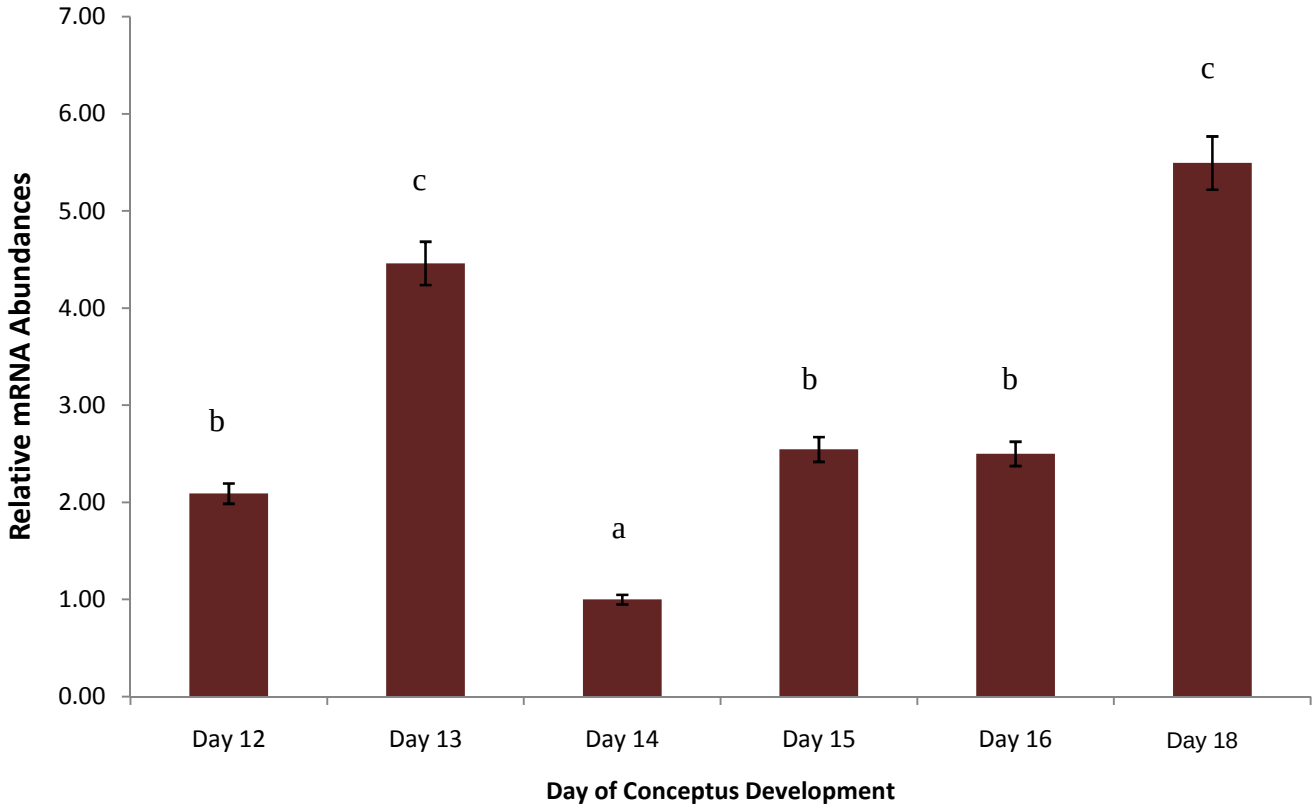

7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Relative mRNA Abundances
b
c
a
b
b
c
Day 12
Day 13
Day 14
Day 15
Day 16
Day 18
Day of Conceptus Development

Figure 3.2. A significant day by treatment interaction on relative mRNA units (mean ± SEM) was identified for endometrial caspase-1 ($P<0.05$). Fold differences in endometrial caspase-1 mRNA expression of cyclic (yellow bar), and pregnant (blue bar) gilt using real-time quantitative PCR. Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference ($P<0.05$) between cyclic versus pregnant endometrium comparing days 0, 5, 10, 12, 15, and 18 of the estrous cycle and days 10, 12, 15 and 18 of pregnancy (n = 4/day/status)

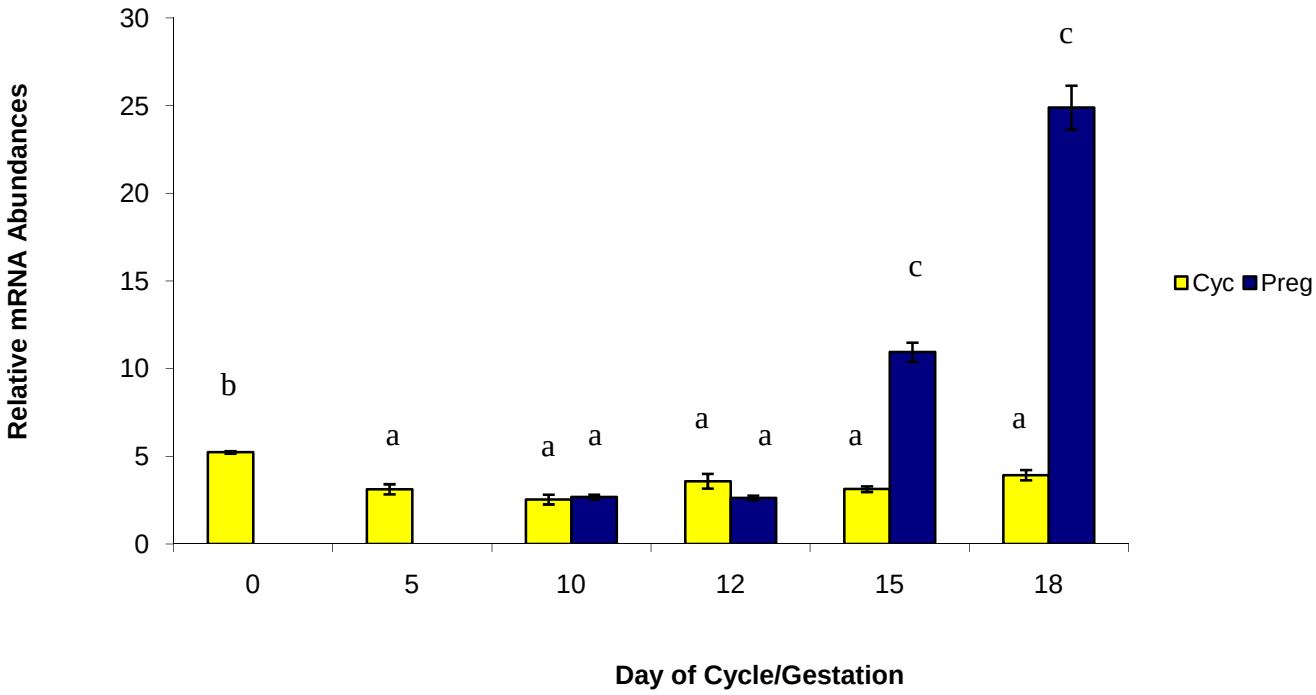

Relative mRNA Abundances
30
25
20
15
10
5
0
b
a
a
a
a
a
a
c
a
c
0
5
10
12
15
18
Day of Cycle/Gestation
Cyc
Preg

Figure 3.3 Relative amounts of total caspase-1 protein in uterine flushings from gilts on days 0, 5, 10, 12, 15 and 17 of the estrous cycle and days 10, 12, 15 and 17 of pregnancy (n = 4/day/status). Total content of caspase-1 (pg) in uterine flushing of cyclic (yellow bars) and pregnant (blue bars) gilts. Relative optical density for each sample was corrected for total protein concentration in the sample based on Serum Albumin levels. Data is presented as relative (pg). mean ± SEM

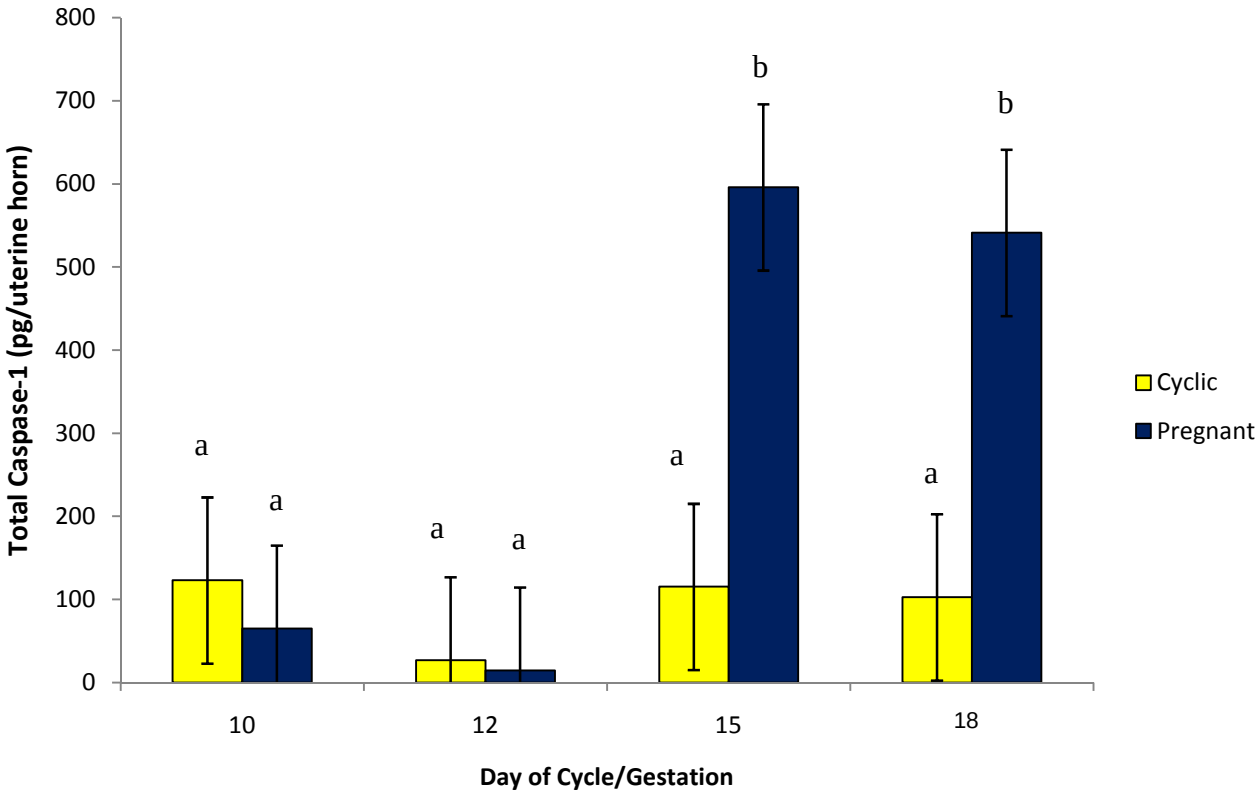

800
700
600
500
400
300
200
100
0
Total Caspase-1 (pg/uterine horn)
a
a
a
a
a
b
a
b
10
12
15
18
Day of Cycle/Gestation
Cyclic
Pregnant

Figure 3.4 PCR amplification for the *in situ* hybridization product (438 bp) indicated that IL-18 gene expression is limited to the maternal tissues (day 15 of estrous cycle and day 18 of pregnancy) and devoid of any IL-18 mRNA in the conceptus (days 14, 15, 16, and 17). Endometrial IL-18 mRNA abundance was greatest on day 18 of pregnancy which is in the window of attachment.

IL-18 Gene Expression

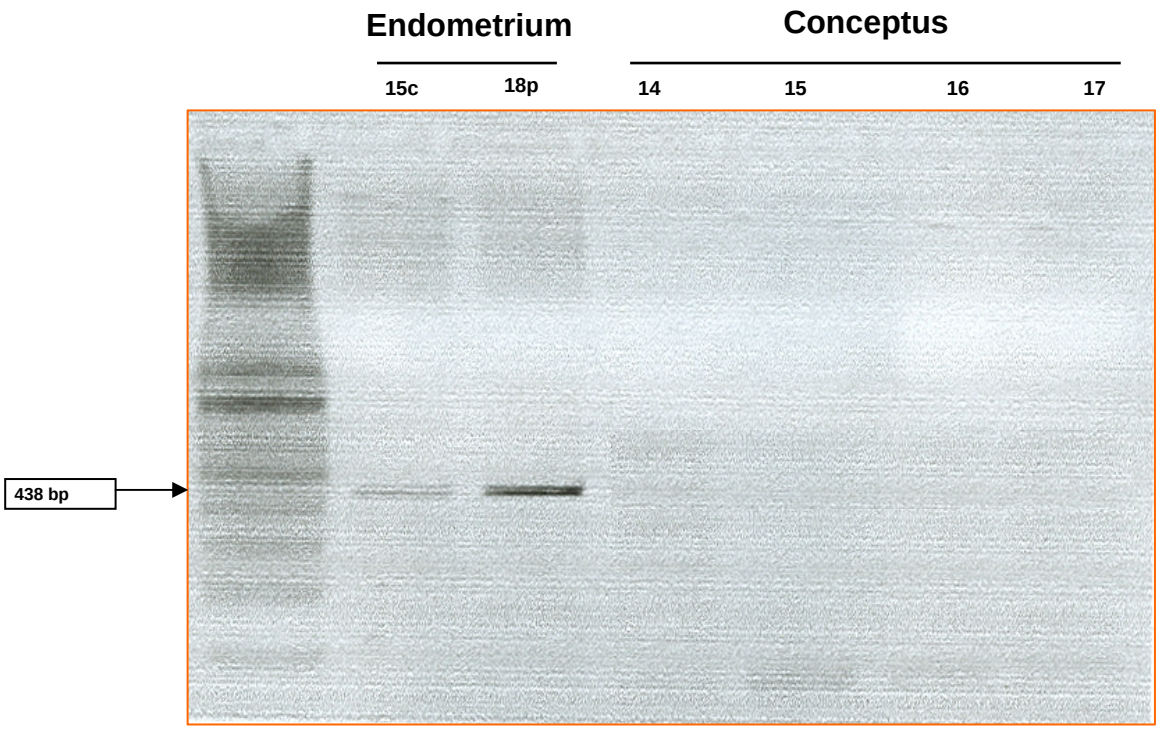


Figure 3.5. A significant day effect on relative mRNA units (mean ± SEM) was identified for endometrial *IL-18* ($P<0.05$). Fold differences in endometrial *IL-18* gene expression of cyclic (yellow bar), and pregnant (blue bar) gilt using real-time quantitative PCR. Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference ($P<0.05$) between cyclic versus pregnant endometrium comparing days 0, 5, 10, 12, 15, and 18 of the estrous cycle and days 10, 12, 15 and 18 of pregnancy (n = 4/day/status)

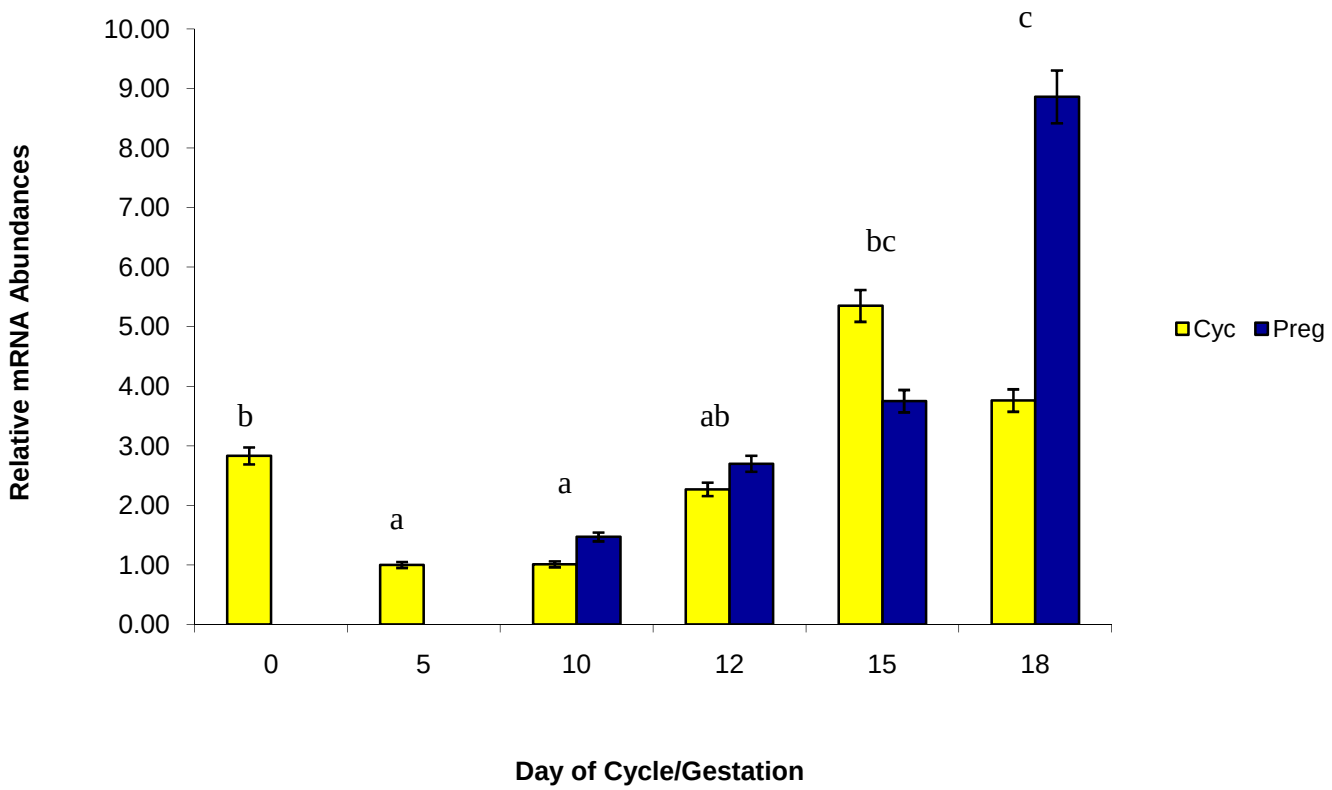
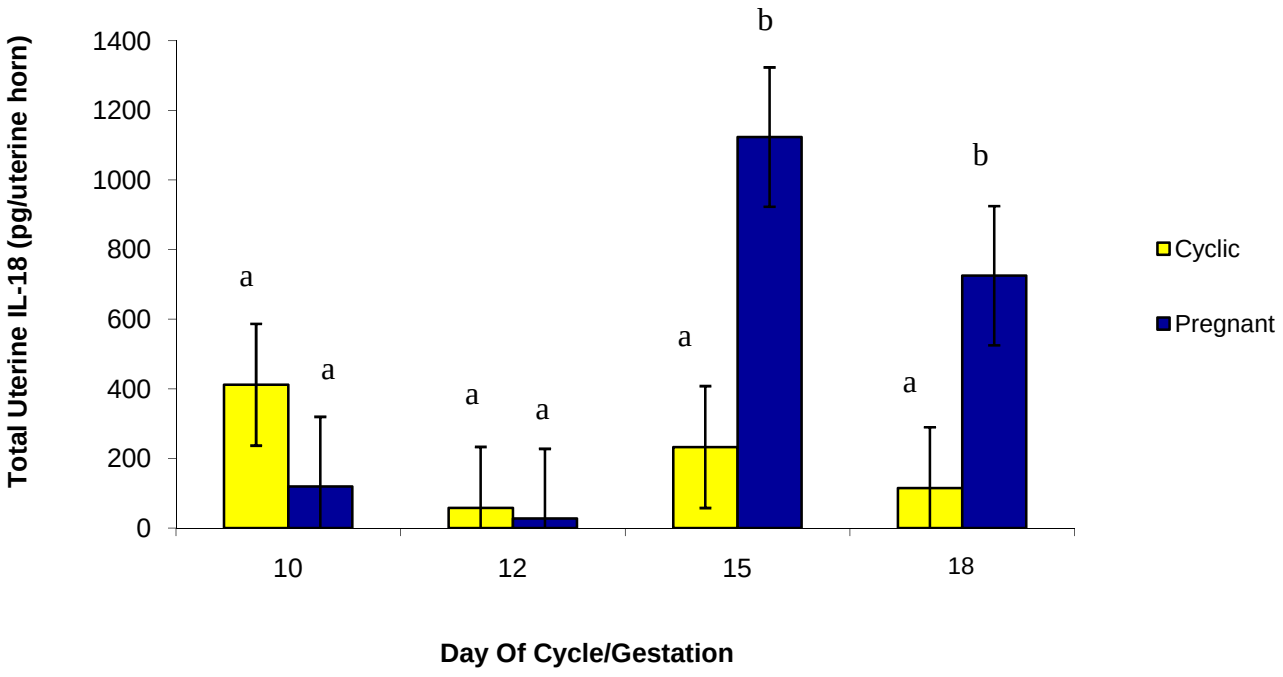

Relative mRNA Abundances
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
b
a
a
ab
bc
c
0
5
10
12
15
18
Day of Cycle/Gestation
Cyc
Preg

Figure 3.6 A day x treatment interaction ($P < 0.05$) was observed for relative amounts of total IL-18 protein in uterine flushing collected from gilts on days 0, 5, 10, 12, 15 and 17 of the estrous cycle and days 10, 12, 15 and 18 of pregnancy (n = 4/day/status). Total content of IL-18 (pg) in uterine flushing of cyclic (yellow bars) and pregnant (blue bars) gilts. Days 10, 12, 15, and 18 were statistically similar; however, during pregnancy IL-18 protein in the uterine luminal fluid were significantly elevated on days 15 and 17 as compared to pregnant gilts on days 10 and 12.


Total Uterine IL-18 (pg/uterine horn)
1400
1200
1000
800
600
400
200
0
a
a
a
a
a
b
a
b
10
12
15
18
Day Of Cycle/Gestation
Cyclic
Pregnant

Endometrial Caspase-1 mRNA Expression in the Endocrine Disrupted Gilts

A day by treatment interaction ($P < 0.05$) was detected for endometrial caspase-1 mRNA abundance in E compared to CO gilts (Table 3.3). Caspase-1 mRNA abundance was lowest in E and CO gilts on days 10 and 12. However, caspase-1 mRNA abundance was increased on days 15 and 17 of pregnancy.

Uterine Luminal Content of Caspase-1 Content in E and CO Treated Gilts

Content of caspase-1 protein in the uterine flushings of CO and E-treated gilts (Figure 3.8) exhibited a day by treatment interaction ($P < 0.05$). Caspase-1 was elevated in both CO and E-treated gilts on days 15 and 17 of pregnancy. However, E-treated gilts exhibited a premature increase in uterine luminal caspase-1 content on day 13 (650 pg) when compared to day 13 CO gilts (50 pg).

Endometrial Caspase-1in situ mRNA Localization in the Endocrine Disrupted Gilts

In situ hybridization indicated that caspase-1 mRNA expression was localized mostly to the uterine surface and glandular epithelium on Day 13 of pregnancy in endometrium of E gilts (Figure 3.9). Caspase-1 mRNA expression was localized to the uterine epithelium on day 13 of pregnancy in E gilts, and day 17 of CO gilts.

Endometrial IL-18 mRNA Expression in the Endocrine Disrupted Gilts

There were no transcriptional differences ($P > 0.05$) in IL-18 endometrial mRNA expression (Table 3.3) in response to the early E-treatment (Figure 3.10).

Uterine Luminal Content of IL-18 in E and CO Treated Gilts

Content of IL-18 in the uterine flushings of CO and E treated pregnant gilts (Figure 3.11) exhibited a day by treatment interaction ($P < 0.05$). Uterine luminal IL-18 content in CO gilts increased on days 15 and 17 compared to day 13 (7500, 6000 and 1300 pg, respectively). Uterine luminal content of IL-18 in E gilts did not increase on days 15 and 17 as compared to their CO contemporaries resulting in 50% less concentrations of IL-18 on days 15 and 17.

Discussion

The periimplantation period (Days 10 to 15), and post-placental attachment and expansion (Days 18 to 40) have been shown to be the periods of greatest embryonic loss in the pig. Pope et al. (1994) estimated 30% of conceptus death occurs in early gestation (Days 10-18). In the pig, initial placentation occurs between days 13 and 18 of gestation (King et al., 1982) in which day 13 appears to be the crucial point at which attachment is initiated by the conceptus and the implantation cascade proceeds. Placental attachment in the pig is a noninvasive epitheliochorial type placentation 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).

Table 3.3 Comparison of endometrial Caspase-1 and IL-18 mRNA expression across days of gestation following either early estrogen or corn oil administration to pregnant gilts using the comparative threshold (C_T) method.

Target	Status	Day	Average Target C_T *	Average 18s C_T *	ΔC_T †,*	$\Delta\Delta C_T$ §
Caspase-1						
	Control	10	26.98 ± 0.81	18.46 ± 0.19	7.63 ± 0.47^{ab}	-0.77
	Estrogen	10	26.62 ± 0.25	18.52 ± 0.19	7.46 ± 0.38^{ab}	-0.94
	Control	12	25.39 ± 0.81	18.46 ± 0.19	6.95 ± 0.47^{bc}	-1.45
	Estrogen	12	27.53 ± 0.25	18.52 ± 0.19	8.40 ± 0.38^{a}	0.00
	Control	13	26.25 ± 0.81	18.46 ± 0.19	7.62 ± 0.47^{ab}	-0.78
	Estrogen	13	25.74 ± 0.25	18.52 ± 0.19	5.37 ± 0.38^{d}	-3.03
	Control	15	23.62 ± 0.81	18.46 ± 0.19	4.87 ± 0.47^{d}	-3.53
	Estrogen	15	25.14 ± 0.25	18.52 ± 0.19	5.79 ± 0.38^{cd}	-2.61
	Control	17	23.57 ± 0.81	18.46 ± 0.19	5.27 ± 0.47^{d}	-3.13
	Estrogen	17	26.42 ± 0.25	18.52 ± 0.19	6.11 ± 0.38^{cd}	-2.29
IL-18						
	Control	10	26.57 ± 0.81	18.46 ± 0.19	8.11 ± 0.47^{a}	0.00
	Estrogen	10	28.04 ± 0.25	18.52 ± 0.19	9.52 ± 0.38^{a}	-0.83
	Control	12	26.79 ± 0.81	18.46 ± 0.19	8.33 ± 0.47^{a}	-0.34
	Estrogen	12	31.08 ± 0.25	18.52 ± 0.19	12.56 ± 0.38^{a}	-0.83
	Control	13	26.82 ± 0.81	18.46 ± 0.19	9.50 ± 0.47^{a}	-1.09
	Estrogen	13	28.11 ± 0.25	18.52 ± 0.19	10.77 ± 0.38^{a}	-0.66
	Control	15	26.17 ± 0.81	18.46 ± 0.19	9.50 ± 0.47^{a}	-1.74
	Estrogen	15	27.47 ± 0.25	18.52 ± 0.19	10.77 ± 0.38^{a}	-1.23
	Control	17	29.32 ± 0.81	18.46 ± 0.19	9.50 ± 0.47^{a}	-0.80
	Estrogen	17	28.52 ± 0.25	18.52 ± 0.19	10.77 ± 0.38^{a}	-1.33

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

ΔC_T † = Target (Caspase-1 and IL-18) – 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 caspase-1 and IL-18 genes) were used as a calibrator for caspase-1 and IL-18 to set the baseline for comparing differences in ΔC_T values across all days.

Figure 3.7. A significant day by treatment interaction was observed on relative mRNA units (mean ± SEM) was identified for endometrial *caspase-1* ($P<0.05$). Fold differences in endometrial caspase-1 mRNA expression of control (orange bar), and early estrogen exposure (black bar) gilts using real-time quantitative PCR. Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference ($P<0.05$) between control and estrogen treated endometrium comparing days 10, 12, 15 and 17 of pregnancy (n = 4/day/status)

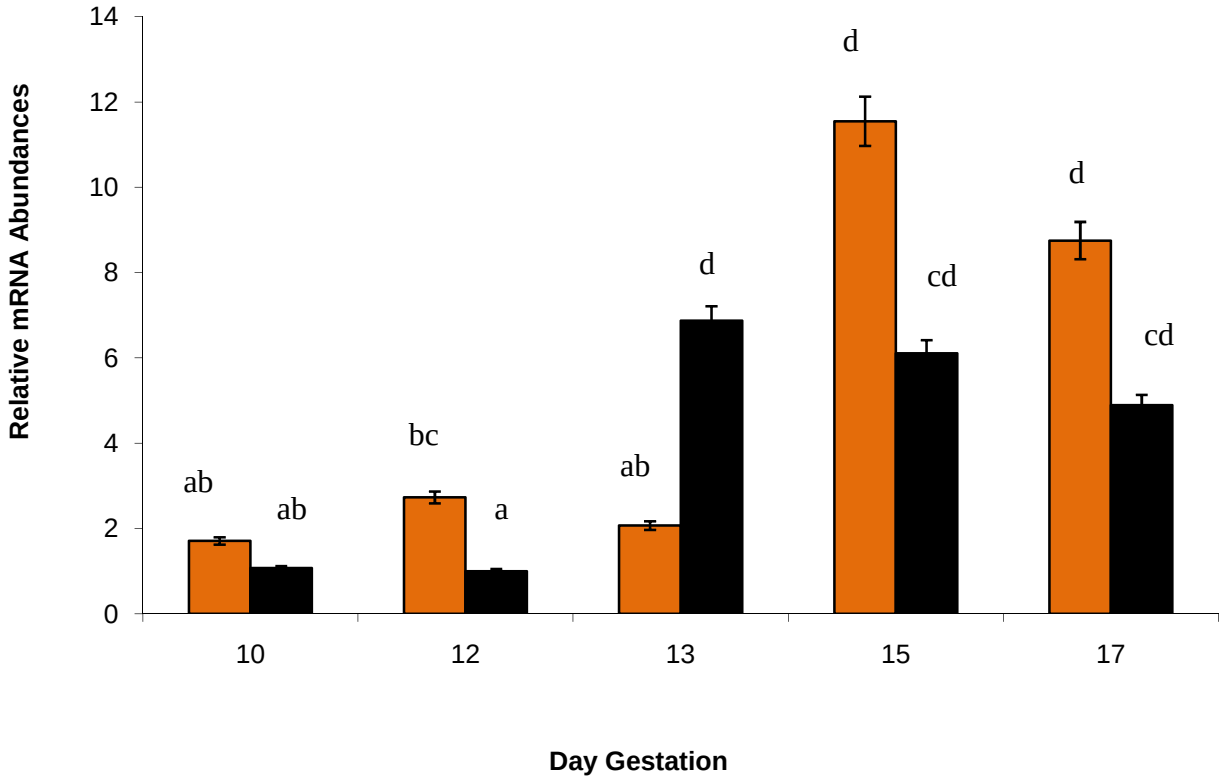

Relative mRNA Abundances
14
12
10
8
6
4
2
0
ab
ab
bc
a
ab
d
d
cd
d
cd
10
12
13
15
17
Day Gestation

Figure 3.8. A day x treatment interaction ($P < 0.05$) was observed for relative amounts of total caspase-1 protein in uterine flushing collected from gilts on days 10, 12, 13, 15 and 17 of pregnancy (orange bars) and early estrogen treated (black bars) gilts (n = 4/day/treatment). Days 10, 12, and 13 were statistically similar, however on day 13 administration of early estrogen significantly elevated caspase-1 protein in the uterine luminal fluid from 50 pg (day 13 CO) to 650 pg (day 15 E). Days 15 and 17 were similar in concentrations of Caspase-1 regardless of treatment.

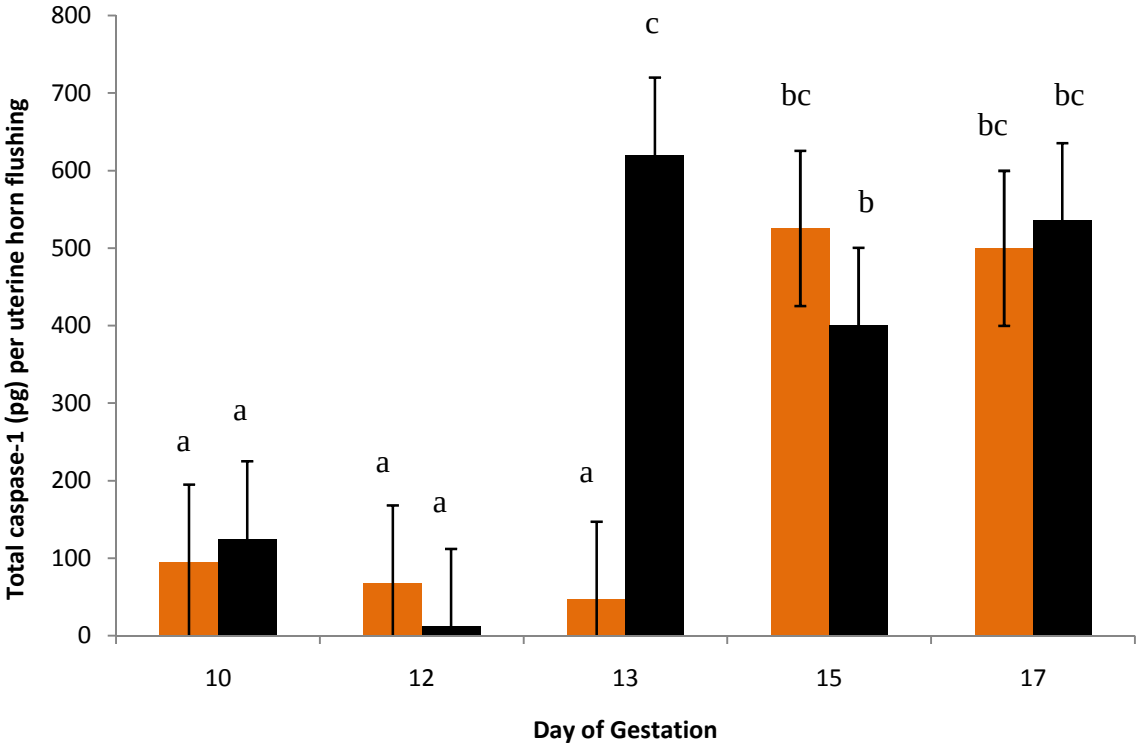

Total caspase-1 (pg) per uterine horn flushing
800
700
600
500
400
300
200
100
0
a
a
a
a
a
c
bc
b
bc
bc
10
12
13
15
17
Day of Gestation

Figure 3.9. *In situ* hybridization analysis of *caspase-1* mRNA expression in porcine endometrium during early pregnancy. Protected transcripts in endometrium from Days 13 early estrogen exposure, and day 17 of control pregnancy were visualized by liquid emulsion autoradiography and imaged under bright-field and dark-field illumination. *caspase-1* mRNA expression is abundant in the luminal epithelium (LE) and glandular epithelium (GE) during Days 13 and 17 while only in the LE on Day 17 of pregnancy.

Caspase 1

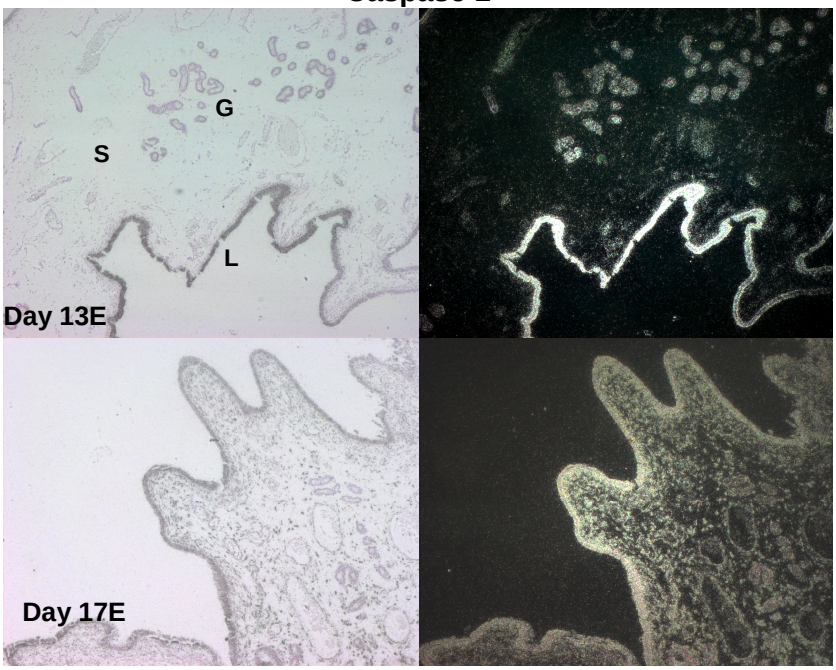


Figure 3.10. No significant main effects or interactions were observed on relative mRNA units (mean ± SEM) for endometrial *IL-18* ($P > 0.05$). Fold differences in endometrial *IL-18* mRNA expression of control (orange bar), and early estrogen exposure (black bar) gilts using real-time quantitative PCR. Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference ($P<0.05$) between control and estrogen treated endometrium comparing days 10, 12, 15 and 17 of pregnancy (n = 4/day/status)

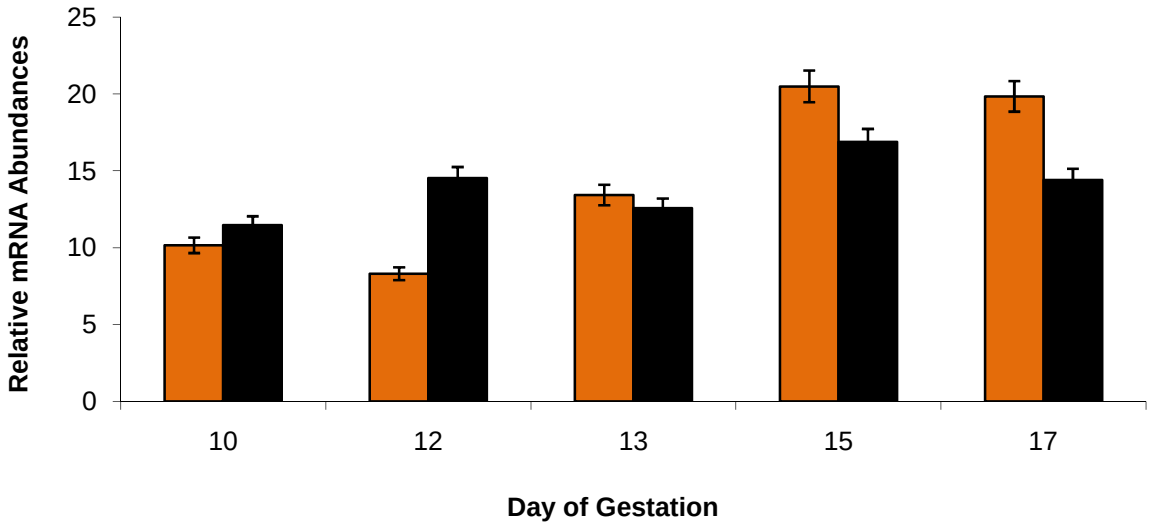
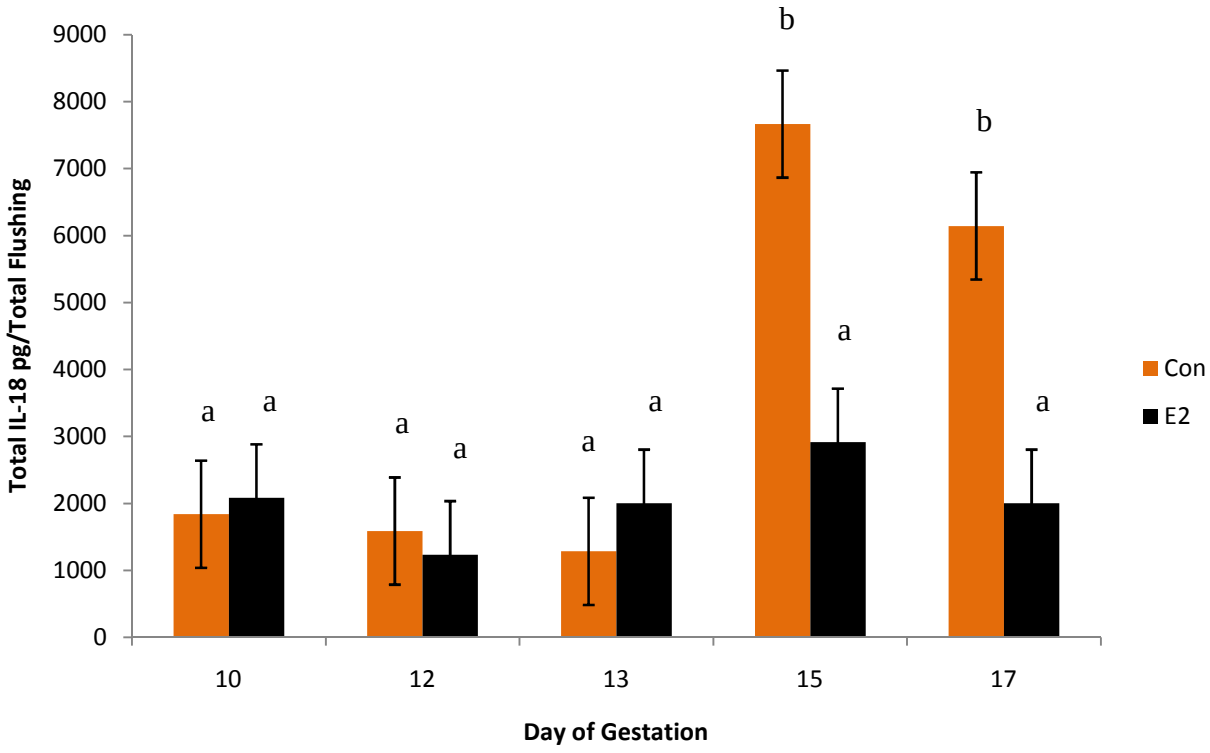

Relative mRNA Abundances
25
20
15
10
5
0
10
12
13
15
17
Day of Gestation

Figure 3.11. A day x treatment interaction ($P < 0.05$) was observed for relative amounts of total IL-18 protein in uterine flushing collected from gilts on days 10, 12, 13, 15 and 17 of pregnancy (orange bars) and early estrogen treated (black bars) gilts (n = 4/day/treatment). Days 10, 12, and 13 were statistically similar, however on days 15 and 17 the early estrogen significantly decreased the amount of IL-18 protein in the uterine luminal fluid by 50% compared to the CO gilts.


Total IL-18 pg/Total Flushing
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
a
a
a
a
a
a
b
a
b
a
10
12
13
15
17
Day of Gestation
Con
E2

Between days 11 to 12 of gestation the porcine conceptuses will begin to synthesize and secrete estrogen into the uterine lumen signaling maternal recognition and establishment of pregnancy (Bazer and Thatcher, 1977). If the pregnant pig receives estrogen stimulation prematurely, days 9 and 10 instead of days 11 to 12, complete embryonic mortality will occur by day 30 of gestation (Pope et al., 1986). Furthermore, other research (Morgan et al., 1987, Gries et al., 1989) has indicated that premature estrogen stimulation initiates conceptus mortality between days 14-18 of gestation. Since days 14-18 of gestation is the time frame that trophoblast attachment occurs, one may attribute the conceptus degeneration 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 developing conceptuses obliterated the glycocalyx covering on the uterine surface microvilli, eliminating the attachment substrate for the conceptus.

Recently, our lab has discovered the expression of the immune modulator, chemokine ligand 9 (CXCL9) in the endometrium using microarray technology (Ashworth, Geisert and White unpublished results). Moreover, increases in mRNA abundance for this chemokine is temporally expressed during the window of implantation in the pig, and is increases 8-fold from day 13 to day 15 of gestation. Recently, Coma et al. (2006) demonstrated IL-18's ability to induce an increase from basal levels of CXCL9 expression by 10-fold in human macrophages, and a synergistic 50-fold in the presence of both IL-18 and IL-12. Data from the fore mentioned study's would be congruent with the appearance of IL-18 in the present study, which would make IL-18 a plausible candidate for induction of CXCL9.

Ross et al. (2003) demonstrated that the periimplantation porcine conceptuses secrete large amounts of IL-1β into the uterine lumen during the transition to 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 conceptuses on day 12, IFN-γ has been observed to increase followed by substantial increase during day 15 of gestation, which may be attributed to the release of IL-18 from the endometrium. Moreover, Joyce et al. (2006) demonstrated that greatest levels of IFN-γ occur from the trophoblast during days 15 through 18 of the porcine pregnancy, which is clearly complimentary to IL-18 increase during days 15 through 18 of pregnancy in our study. Both the mRNA and protein expression patterns observed in caspase-1are temporally aligned for the proper processing of both IL-1β and IL-18 during early pregnancy in the pig. Conceptus caspase-1 mRNA expression is greatest in abundance during days 13 and 17. Furthermore, endometrial caspase-1mRNA expression was markedly elevated during days 15 and 17 of pregnancy. These data would infer IL-1β processing by caspase-1during liberation from conceptus during the initial release of IL-1β around day 12 of pregnancy is of conceptus origin since there is an absence of caspase-1 protein found in the uterine luminal flushings. Additionally, the increased expression of caspase-1 mRNA observed in the endometrium which mirrors the elevation of caspase-1 protein in uterine flushings would be consistent with the increase observed secretion of IL-18 during days 15 to 18 of pregnancy. Given these data, we have clearly established evidence for cytokine crosstalk between the developing conceptuses and endometrium based on gene

expression of IL-18 exclusively expressed in endometrium, while completely devoid in all days of conceptus during early pregnancy.

Exposure of pregnant gilts to exogenous estrogen 48 h prior to the normal appearance of estrogen by the conceptuses on day 12, results in embryonic mortality before day 30 of gestation (Pope et al., 1986). Our lab previously demonstrated that conceptuses degenerated between Days 15 to 18 of gestation following early estrogen treatment on days 9 and 10 of pregnancy (Morgan et al., 1987). Conceptus degeneration was correlated with the spaciotemporal loss of the microvilli glycocalyx on the endometrial surface epithelium (Blair, et al., 1991). Although the exactly mechanism by which this mortality occurs has yet to be elucidated, the present study was designed to characterize uterine changes in caspase-1 and IL-18, and investigate their association with endocrine disruption in the pregnant gilt and its effects on uterine-conceptus attachment and development.

Following endocrine disruption of the pregnant gilts, we observed marked changes in both IL-18 and caspase-1 mRNA and protein expression due to the early exposure to estrogen. Endometrial IL-18 expression was unaffected at the transcriptional level; however the actual secretion of the active IL-18 protein was significantly suppressed on days 15 and 17 of pregnancy in gilts exposed prematurely to estrogen. At first analysis these data appear confounding; however, these observed translational changes may be due in part to a role played through regulatory RNA's. Therefore, the levels of decreased IL-18 protein observed in uterine flushings of early estrogen exposed endometrium may serve as a source of dysfunction during conceptus attachment.

Moreover, in the mouse, decreased levels of IL-18 at the embryo-maternal interface have been shown to be supportive of recurrent spontaneous abortions (Muranaka et al. 1998).

Following early estrogen exposure, an increase in caspase-1 mRNA and protein was observed prematurely on day 13 of pregnancy. Thus, this would be consistent with other studies previously conducted in our lab demonstrating a 48 h advancement in various uterine markers (IGF-I, -II, and COX-1, -2) within the implantation window (Ashworth et al., 2005, 2006). Caspase-1was expressed maximally in the uterine luminal fluid during day 13 of pregnancy in estrogen treated gilts. Ashworth et al., (2005) demonstrated that early estrogen exposure to pregnant gilts resulted in an early proteolysis of the IGFBP's (-II, and -III) which caused IGF-I, -II ligands to disassociate from its respective binding proteins resulting in a disappearance 48 h earlier than normal. Furthermore, this premature vanishing of IGF ligands may account for the early elevation observed in caspase-1 given the apoptosis suppressive effects of the IGF ligands (Jung et al., 1996).

The current study has clearly demonstrated that the pregnant gilt expresses caspase-1 in conceptus and endometrial tissues, and they are temporally associated with the window of implantation based on appearance. Additionally, the current study also demonstrated that the early porcine pregnant endometrium expresses IL-18 exclusively suggesting a crosstalk system between IL-18 endometrial origin and IFN-γ predominantly conceptus origin (Joyce et al., 2005). Furthermore, the current study also demonstrated that early estrogen exposure to pregnant gilts resulted in a dysfunction in caspase-1 and IL-18 appearance during the critical window of implantation during early porcine pregnancy.

Chapter IV

ENDOCRINE DISRUPTION OF ENDOMETRIAL GENE EXPRESSION DURING THE PERIOD OF NORMAL CONCEPTUS ATTACHMENT USING THE PIG PSUEDOPREGNANT MODEL

Abstract

Synchronization of conceptus crosstalk with endometrial development is imperative for successful establishment of pregnancy in the pig. However, administration of estrogen earlier than normal conceptus secretion on day 12 of pregnancy in the pig is characterized by complete embryonic mortality by day 15. The objective of this study was to characterize the direct effects of early estrogen administration on endometrial gene expression during the period of conceptus attachment (Days 13-15). To isolate the direct effects of early estrogen administration on endometrial gene expression, the effects of conceptus secretions were removed by utilizing the established pig pseudopregnant model. The study evaluated the effects of estrogen when administered on days 9 and 10 (E9) of the estrous cycle (endocrine disruption of pregnancy) or day 11.5 (E11.5) which is the normal period of conceptus estrogen secretion. Endometrial gene expression was evaluated using the GeneChip® Porcine Genome Array. In all, different probe sets were statistically different between at least one comparison (D13 E9 vs. D15 E9, D13 E11.5 vs D15 E11.5). When restricted to day D13 E9 vs. D15 E9 comparison 55 genes were statistically different with greater than 1.7-fold change in expression. Utilization of k-means clustering, in addition to the Database for Annotation, Visualization, and Integrated Discovery (DAVID) identified

genes of interest. Quantitative RT-PCR expression profiles for chemokine ligand 11 (CXCL-11), chemokine ligand 9 (CXCL-9), guanylate binding protein 1 (GBP1), CD 163, and CD 18 were supportive of the GeneChip® Porcine Genome Array data. These data provide insight into global uterine transcriptional changes associated with endocrine disruption of the endometrium that are likely fundamentals for the proper establishment of pregnancy in the pig devoid of any conceptus effects.

Introduction

Early pregnancy loss is a major cause of human infertility with reports indicating 25-31% of women losing their pregnancy during the peri-implantation period (Wilcox et al. 1999). Since implantation is limited to a short and restricted time period, successful establishment of pregnancy in mammals requires precise communication and synchrony between the developing conceptus and uterine environment. The window of implantation can be defined as a remodeling of cell surface molecules allowing a close relationship of the conceptus and uterine surface epithelium (Denker, 1993; Glasser and Mulholland, 1993), which establishes of a specific cytokine environment with which the uterus promotes conceptus attachment and placenta development (Kliman, 2000). During early pregnancy in the pig, the peri-implantation period is one of the most critical stages of conceptus development. After ovulation and fertilization, prenatal mortality in the pig ranges from 20% to 46% by term (Pope, 1994) with the majority of the loss occurring during the peri-implantation stage of development between days 12 to 18 of gestation (Stroband and Van der Lende, 1990). Estrogen synthesis and secretion by the conceptuses occurs in a biphasic pattern with an acute intermediate peak on day 12 of

gestation followed by a second chronic sustained release of estrogen from days 15 to 28 of gestation (Geisert et al., 1990). Incompatibility between the developing conceptus and the rapidly changing uterine environment can result in a major constraint to conceptus survival. Asynchrony greater than 24 h between potentially viable conceptuses and the uterine environment has been demonstrated to cause conceptus death as early as day 8 of gestation (Polge, 1982; Geisert et al., 1991).

Exposure to estrogen through clinical treatment or endocrine disruptor chemicals contributes to human and animal infertility. Low pregnancy rates are evident in women following the use of various IVF/ET protocols. Reduced fertility maybe attributed to secretion of increased physiological levels of estrogen resulting from ovarian hyper-stimulation altering the window of uterine receptivity which affect conceptus attachment to the uterine surface. Consequences of asynchronous timing for uterine stimulation by estrogen raises concern for reproductive success of humans and animals subjected to environmental estrogens, which can function as endocrine disruptors (Cummings, 1997; Mendes, 2002; Caserta et al., 2008).

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 in the fragmentation and degeneration of conceptuses on day 16 of gestation. On days 14 to 16 of gestation, Gries et al. (1989) observed alterations in uterine protein content in the flushings of gilts receiving early exposure to 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). Additionally, early estrogen exposure has been shown to shift the implantation window for the appearance of several growth factors by 24 to 48 h such as the case with uterine secretion of insulin-like growth factor I (IGF-I) and -II, and their binding proteins (Ashworth et al., 2005). Furthermore, Ross et al., (2007) demonstrated that early exogenous estrogen administered to pregnant gilts on days 9 and 10 of pregnancy altered the timing for 77 endometrial expressed genes between the days of 10, 13 and 15 of pregnancy. These data indicate that early estrogen exposure acts as an endocrine disrupter through alterations in the timing of the implantation window and endometrial transcriptome at the level of the extracellular matrix around the time of placental attachment to the uterine epithelial surface.

Currently, limited information is available in regards to global gene expression responsible for dysfunctional endometrial transcriptional and translational expression with estrogen in the absence of conceptuses in the pig. The current objective utilizes the GeneChip® Porcine Genome Array from Affymetrix ® representing approximately 20,201 genes to identify differentially expressed endometrial genes involved during endocrine disruption following early estrogen administration to cyclic pigs. Treatment of gilts with estrogen, the maternal recognition signal in the pig, on day 11.5 extends life span of the corpora lutea which is useful as a psuedopregnant model to isolate the direct effects of estrogen on endometrial function. Identification and characterization of endometrial gene expression patterns during endocrine disruption using the psuedopregnant pig model will provide a better understanding of the events associated with dysfunction of the endometrium but in the unique absence of conceptus development or secretions

Materials and Methods

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.

Experimental Design

Cycling gilts were randomly assigned one of the following treatment groups: 1) Estrogen treatment on day 11.5 (E11.5), gilts (n = 7) received an 2.5 mg i.m. injection of estradiol cypionate (A.J. Legere, Scottsdale, AZ) on day 11.5 of the estrous cycle or 2) Estrogen treatment on days 9 and 10 (E9), gilts (n = 7) were administered 2.5 mg i.m. injection of estradiol cypionate on days 9 and 10 of the estrous cycle (E9). Following induction of anaesthesia with 1.8 ml im 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. Uterine flushings were centrifuged (1000 x g, 10 min, 4°C), supernant collected and uterine flushings were stored at -20°C. Endometrial tissue was removed

from the antimesometrial side of the uterine horn, immediately snap frozen in liquid nitrogen and stored in at-80°C until RNA extraction.

Tissue Collection and Fixation

Uterine horns were flushed with 20 ml PBS, flushings were centrifuged to remove cellular debris, and the superatnant stored at -20°C. Endomterial tissue was harvested from the uterine horn and either fixed for *in situ* hybridization or snap frozen for RNA harvesting.

RNA Isolation

Total RNA was extracted from endometrial tissue using TRIzol™ reagent (Invitrogen, Inc). Approximately 0.5 g of endometrial tissue was homogenized in 5.0 mL of TRIzol 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 nm and purity was verified using the 260/280 ratio.

Microarray Analysis

Affymetrix Porcine Chip

The GeneChip® Porcine Genome Array (Affymetrix, Santa Clara, CA) contains 23,937 probe sets interrogating 23,256 transcripts, representing 20,201 genes. Four chips were used for each day and estrogen treatment of uterine endometrium for either: E11.5 (Day 13 and 15) or endocrine disrupted E9 (Day 13 and 15). RNA utilized for each chip represented a set of endometrial total RNA for the respective day or estrogen treatment.

Prior to target labeling, RNA was further purified (RNeasy MinElute Cleanup, Qiagen, Valencia, CA). Target labeling, GeneChip® hybridization, scanning and quantitation were conducted by The University of Tulsa Microarray Core Facility. Affymetrix GeneChip Operating Software (GCOS version 1.1.1, Affymetrix, Santa Clara, CA) was used to quantitate each GeneChip®. The summary intensities for each probe were loaded into DNA-Chip Analyzer (dChip), version 1.3 for normalization, standardization, and analysis.

Normalization and Standardization

For normalization, dChip's method of invariant set normalization in which the chip with the median intensity value was used as the baseline against which the brightness of the remaining chips were adjusted in order to be of a comparable level. To reduce variance of expression level estimates by accounting for probe differences, standardization was conducted by calculating model-based expression indices (MBEI) using dChip's Perfect-Match (PM)-only model.

Log Transformation and Statistical Analysis

The MBEI were log base 2 transformed to approximate a normal distribution for each gene and provide measures by which to conduct the statistical analysis. Unpaired t-tests were calculated using dChip to evaluate differences between two groups. Analysis of gene expression was done to compare expression changes between E9 and E11.5 endometrium utilized in the study. The false discovery rate (FDR) utilized to restrict the list of candidate genes was a *P* value less than 0.001 as determined by the unpaired t-test

and a numerical change in expression of at least 1.7-fold for each endometrial comparison evaluated.

GeneChip® Porcine Genome Array Re-annotation

The annotation was updated by utilizing the provided sequence from Affymetrix that was utilized in probe development. Through the utilization of BLAST [Altschul et al., 1990], a human accession number, based on homology, was assigned to each Affymetrix ID already correlated to each probe on the chip. The assignment of a human accession number allowed a more complete analysis of the biological processes being regulated during this endocrine disrupted psuedopregnancy, by enabling the more effective use of software such as the Database for Annotation, Visualization and Integrated Discovery (DAVID).

Clustering Analysis

All genes determined to be significantly different based on the *p*-value less than 0.001 indicated for all comparisons between day and estrogen treatment of uterine endometrium. Genes (n = 117) were utilized for analysis in TIGR MeV v4.3. We chose to sort the 117 genes that were both statistically ($P < 0.001$) and numerically (± 1.7-fold change) different into 7 clusters. The 7 clusters were identified using the k-means learning algorithm following 1000 replications.

Database for Annotation, Visualization and Integrated Discovery

Database for Annotation, Visualization and Integrated Discovery (DAVID) version 2.0 (http://david.niaid.nih.gov/david/version2/index.htm) is a program that enables the utilization of microarray gene lists to generate specific functional annotations of the biological processes affected by the treatment as determined through microarray experiments [Glynn et al., 2003]. DAVID was utilized to annotate biological themes occurring during the E9 day 13 vs E9 day 15 based on the most differential gene expression for this study occurring at this time. During early porcine pregnancy, initial conceptus-uterine attachment occurs between days 13 to 15 of pregnancy and is the period that early estrogen administration disrupts conceptus development. All genes identified to be both significantly different ($P < 0.001$) and biologically different (± 1.7-fold change) for the D13 E11.5 vs. D15 E9 comparisons, and successfully assigned a human accession number, were used in the analysis via DAVID. Utilizing gene ontology (GO) terms as identified through biological process, cellular component and molecular function; as well as protein domain, and biochemical pathway membership; biological themes were generated by grouping like terms thereby creating annotation clusters associated with each developmental transition.

Identification and Analysis of Network and Canonical Pathways

Gene expression significantly up regulated by estrogen at FDR<10% were analyzed using software from the Ingenuity Pathway Analysis (IPA) systems, version 4.0 (Ingenuity Systems, Redwood City, CA). Human annotation that was derived from the Affymetrix® Porcine generated gene annotation list compiled from DAVID and was used for the IPA analysis of related networks and conical pathways. As previously discussed

in the DAVID analysis, only the D13 E9 vs D15 E9 gene list comparison was used as input data in the IPA software. Each gene identifier to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These genes of specific focus were placed on a global template of molecular networks that have been developed from comprehensive information contained in the vast IPA database. Individual networks were generated based on the themes and relative connectivity of the focus genes inputted. A numerical score based on network ranking and gene relevancy is generated in order to rank multiple networks within the inputted data set. IPA uses a right-tailed Fisher's Exact Test to calculate the *P*-value for the networks. This generated *P*-value is indicative of the chance that a gene listed in the network is there based on chance. Genes and or gene products are represented in the networks as nodes, and the line connecting them represents a biological relationship. The nodes are colored coded as red and green (up and down respectfully) with intensity of color indicating magnitude of direction.

The most significant canonical pathways from our study were identified using the global template from the IPA library of canonical pathways database. Significance of the association between our input data set and the canonically derived pathways were determined based on two parameters: 1) A ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway, and 2) A calculated P-value using fisher's exact test determining the probability that the association between the focus genes in the data set and the canonical pathway is due to chance alone.

Quantitative One-Step RT-PCR

Quantitative RT-PCR analysis of transcripts of interest was conducted as previously described by our laboratory (Ashworth et al., 2006). RNA from endometrium were utilized for Affymetrix analysis and were aliquoted to be used for PCR analysis. Genomic DNA removal and the synthesis of cDNA to be used for quantitative analysis were done using the QuantiTect Reverse Transcription kit according to manufacturer's recommendations (Qiagen, Valencia, CA). Briefly, 0.8 μg of each uterine tissue (n = 4 gilts /day/trt) were added to a genomic DNA wipeout buffer for 5 min at 37^{o}C, followed by a 2 min incubation on ice, then proceeding to the addition of reverse transcription buffer, RT primer mix and reverse transcriptase; and incubated at 42^{o}C for 30 min. Individual endometrial samples were taken at days 13 and 15 from the E 11.5 gilts and E9 gilts (D13 E11.5, D15 E11.5, D13 E9, D15 E9). The PCR amplification was conducted using the Bio-Rad icycler Sequence Detection Systems (Bio-rad Systems). The real-time detection during each amplification cycle was done by using a specific sequence nested between the forward and reverse specific primers of the intercalating dye, SYBR green. All primers utilized for quantitative analysis for each target gene are presented in Table 4.1. One hundred nanograms of synthesized cDNA were assayed for each sample in duplicate. Thermal cycling conditions for SYBR green detection were 50^{o}C for 30 min and 95^{o}C for 15 min, followed by 40 repetitive cycles of 95 for 15^{o}C sec and variable annealing temperature for 30 sec, 72^{o}C for 33 sec and a variable temperature during fluorescent detection for 33 sec. Fluorescence detection temperature was determined by evaluating melting curve analysis for the samples and the no template control amplification plot. Detection temperatures were set at a temperature when the

intended target was the only contributing factor to fluorescence. 18S ribosomal RNA was assayed as a normalization control to correct for loading discrepancies. Following RT-PCR, quantitation of gene amplification was made by determining the cycle threshold (C_T) based on the fluorescence detected within the geometric region of the semilog view of the amplification plot. Relative quantitation of target gene expression was evaluated using the comparative C_T method as previously described (Hettinger et al. 2001; Ashworth et al., 2006). The ΔC_T value was determined by subtracting the target C_T of each sample from its respective ribosomal 18S C_T value. Calculation of $\Delta\Delta C_T$ involves using the single greatest sample ΔC_T value (the sample with the lowest expression) as an arbitrary constant to subtract from all other sample ΔC_T values. Relative mRNA units for each sample were calculated assuming an amplification efficiency of 2 during the geometric region of amplification, and applying the equation, $2^{\Delta\Delta Ct}$. Relative units in figures 4.1a-4.1e are presented as mean ± SEM. To compare the expression patterns determined through QT-RT-PCR with that determined

Table 4.1 Primer and probe sequence information for the quantitative amplification of each target gene.

Affymetrix ID[a]	Target[b]	Primers (Forward/Reverse)[c]	Fluorescent Reporter[d]	Amplicon Size[e]
Ssc.30027.1.A1_at	CXCL-11	GCATGCTATAGTCTTGGCTGTCA GCAAAGACACCTTCCCGCTTTGAA	SYBR Green	174 bp
Ssc.26146.1.S1_at	CXCL-9	TGCATCAACACCAGCCAAAGGATG TTAGGCTGACCTGTTTCTCCCACT	SYBR Green	304 bp
Ssc.29054.1.A1_at	G-Binding protein	TGCTTTGCTTCTGACTCGGCTCTA TCTGTTTCACGGGAGGGTTTGACT	SYBR Green	159 bp
Ssc.5053.1.S1_at	CD 163	TACATGCCACAGGTCGCTCATCTT TCAGGCAAGAATTCATCTCCCGGT	SYBR Green	132 bp
Ssc.14561.1.S1_at	CD18 (Integrin B2)	AGGACAACAGCTCCATCATCTGCT ACTCGCAGAACTGGCCGTAAATCT	SYBR Green	196 bp

[a]The Affymetrix ID refers to the ID given by Affymetrix for which the probe set was designed.
[b]The target gene name was determined through manual annotation of the EST sequence used by Affymetrix during the generation of the probe set.
[c]Forward and reverse primers for each target gene. The forward primer sequence is above the reverse for each target gene. Forward and reverse do not necessarily indicate the *in vivo* direction of transcription.
[d]The intercalating dye, SYBR Green, was used to measure amount of amplified target during each cycle of quantitative RT-PCR.
[e]Amplicon size refers to the product size of the amplified PCR product.

using microarray analysis, the mean relative mRNA units determined for each gene were divided by the mean relative mRNA units to produce fold differences presented in Table 4.2

Quantitation and Statistical Analysis

Normalized QT-RT-PCR ΔC_T values were analyzed using the PROC MIXED of the Statistical Analysis System. The statistical model used in the analysis tested the fixed effect of estrogen on endometrial gene expression (D13 E11.5, D15 E11.5 and D13 E9, D15 E9). Separation of the means was completed using the PDIFF function in SAS. Significance ($P < 0.05$) was determined by probability differences of least squares means between early estrogen exposed endometrium and non-estrogen exposed endometrium gene expression.

Results

Affymetrix Analysis

Chips with more than 5% of probe sets flagged as array outliers are of suspect quality. dChip did not flag any of the arrays as an outlier (when fitted expression for the entire probe set has a standard error greater than 3 standard deviations from the mean when compared to the other chips). Accordingly, no tissue was re-hybridized to a new array nor was any array dropped from analysis. Single outliers are lone probes of unusual intensity within a chip. In this set of samples, outlier percentages ranged from 0.02% to 0.30%. Single outliers were treated as missing values in subsequent analyses. The percentage of genes called "present" by the GCOS software ranged from 65.49% to 70.37%. Table 4.3 lists the intensities, presence call percentages, and outlier percentages for each gene chip as produced by dChip.

Table 4.3. Intensities, percent present, and outliers for each AffyChip utilized during microarray analysis.

Array[a]	Median Intensity (unnormalized)[b]	"Present" Detection Call %[c]	Single Outlier %[d]
13 PPa, Chip 1	291	69.90	0.02
13 PPb, Chip 2	237	71.96	0.02
13PPc, Chip 3	282	70.83	0.04
13PPd, Chip 4	238	69.51	0.05
15PPa, Chip 5	159	68.48	0.04
15PPb, Chip 6	375	70.38	0.03
15PPc, Chip 7	238	69.49	0.01
13PPEa, Chip 8	254	71.05	0.04
13PPEb, Chip 9	207	67.40	0.09
13PPEc, Chip 10	271	70.93	0.02
13PPEd, Chip 11	282	70.10	0.05
15PPEa, Chip 12	307	70.93	0.10
15PPEb, Chip 13	330	70.10	0.04
15PPEc, Chip 14	260	69.80	0.01

[a]Each endometrium and status of endometrium was hybridized to three or four chips.
[b]Unnormalized median target intensity for each chip. Prior to analysis in dChip, intensity for each chip was normalized by adjusting the brightness of each chip to be comparable to the median intensity; Day 14, Chip 16.
[c]Detection call percentage refers to the percentage of targets that were identified present for each chip.
[d]Column represents the percentage of individual outliers for each chip. Five percent or greater of individual probe set outliers would indicate an array of poor quality

When the results from the four comparisons were combined, there were 117 altered probe sets of which 55 remained after deleting those with an "absent" detection call across all chips in all comparison groups. Of the 55 genes differentially expressed, D13 E9 compared to D15 E9 was comprised of 26 genes that were up regulated due to the estrogen treatment. Conversely, 18 genes displayed down regulated expression when comparing the D13 E9 versus the D15 E9. D15 E11.5 compared to D15 E9 produced 11 unique genes, however only 2 were statistically significant (1 up and 1 down regulated). D13 E11.5 compared to D13 E9 produced 8 differentially expressed genes; all genes for this comparison were up regulated. Lastly, D13 E11.5 compared to D15 E11.5 contained only 1 gene differentially expressed which was up regulated. When the results were restricted to unique GenBank Accession numbers, 40 were found to differ significantly in their expression in one or more of the four comparisons. The number of statistically and biologically different genes for each comparison between E11.5 and disrupted E9 are present in Table 4.4.

Database for Analysis, Visualization and Integrated Discovery

Utilization of DAVID resulted in the discovery of 5 functional annotation clusters representing biological systems affected by the administration of exogenous estrogen on days 9 and 10 of pregnancy (Table 4.5). General functional terms and process involved include: regulation of immune response and response to stimuli, leukocyte activation and cell localization, anatomical structure development.

Table 4.4. Numbers of statistically different mRNA abundance for genes identified between morphological comparisons

Comparison[a]	N unique Genes[b]	N unique, ≥ 1.7-Fold Difference[c]	N unique, ↑ ≥ 1.7-Fold Difference[d]	N unique, ↓ ≥ 1.7-Fold Difference[d]
D13 E11.5 vs D13 E9	19	8	8	0
D13 E11.5 vs D15 E11.5	4	1	0	1
D15 E11.5 vs D15 E9	11	2	1	1
D13 E9 vs D15 E9	84	44	26	18

[a]Represents the morphological comparison for each row.
[b]Number of genes with a unique identity based on the accession number utilized for the creation
[c]Number of unique genes that have at least a 1.7-fold difference in mRNA abundance between the E115.5 and E9.
[d]Number of genes increasing or decreasing in mRNA abundance for a given comparison.

Table 4.5. Functional annotation clusters of biological terms representing processes affected during endocrine disruption of endometrium comparing D13 to D15.

Annotation Cluster[a]	Enrichment Score[b]	Biological Terms[c]
1	4.37	Immune response (8), immune system process (8), response to stimulus (9)
2	1.16	Leukocyte activation (3), cell activation (3), multicellular organismal process (6), localization (4),
3	1.06	Signal transduction (8), cell communication (8), cellular process (11)
4	0.83	System development (5), anatomical structure development (5), multicellular organismal development (5), multicellular organismal process (6), developmental process (5), organ development (3)
5	0.60	Establishment of protein localization (3), protein localization (3),macromolecule localization (3), localization (4), establishment of localization (3)

[a]Five most significant annotation clusters identified based on the gene list submitted for analysis through DAVID.

[b]Enrichment score is determined through DAVID and ranks the significance of each annotation cluster based on the relatedness of the terms and the genes associated with them.

[c]Column represents terms in the annotation clusters. The gene ontology (GO) terms were gathered based on the known annotation of the submitted genes with respect to biological process only.

Clustering Analysis

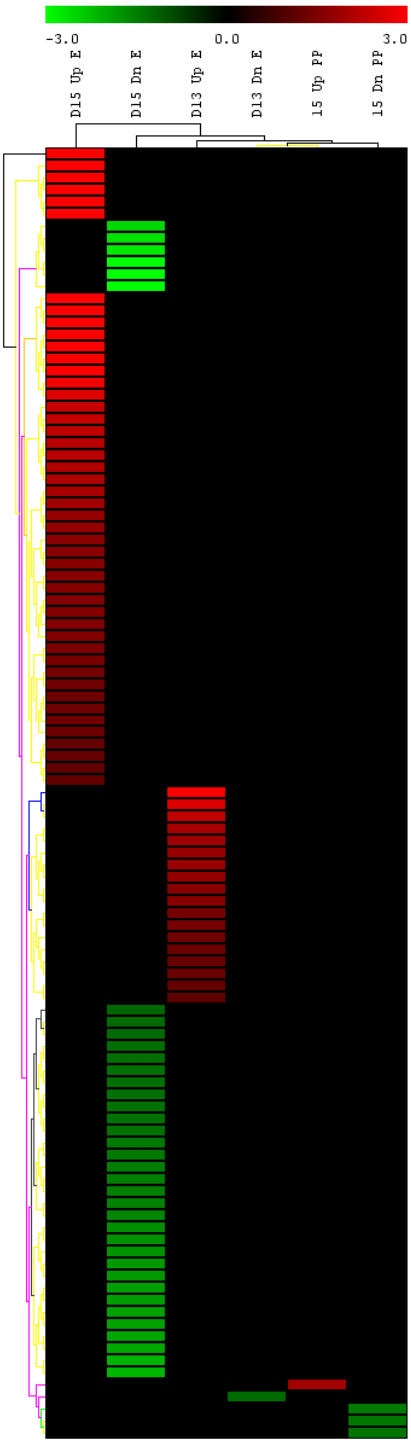
The 7 clusters generated utilizing the K-means clustering algorithm were distinguishable by their expression patterns (Figure 4.2 a,b,c,d,e,f, and g,) and represented the 117 genes that were determined to meet the FDR threshold for one or more of the comparisons between the days and endocrine disrupted status. The smallest cluster contained 1 gene while the largest contained 46 genes. The mean cluster size was nearly 16 genes; however, the median cluster size was 2 genes suggesting that most of the clusters represented normal distribution and also unique expression patterns and a few larger clusters represented general trends (Figure 4.2 h, and c). The affymetrix ID, associated human GenBank accession number, putative identity for each gene and expression profile is listed for each gene that was significantly ($P < 0.001$) and biologically different (> 1.7-fold change) between any day and estrogen status of endometrium are listed in association with their k-means cluster in the Appendix.

Conical Signaling Pathway Analysis

Figure 4.3 list the top 12 conical pathways regulated by estrogen in the early estrogen exposed gilts. The top five signaling pathways based on their significance ($P \leq 0.05$) included: antigen presentation pathway, role of NFAT in regulation of the immune response, IL-4 signaling pathway, CTLA4 signaling in cytotoxic T-lymphocytes, and CD28 signaling in T-Helper cells.

Figure 4.1 a-h. Seven clusters were generated using the k-means clustering algorithm to associate genes with similar expression patterns for the endocrine disruption caused by early estrogen exposure. 117 genes were determine to be significant and biologically different between at least one of the comparisons of treatment or day produced a mean cluster size of 15.2 genes, a median cluster size of 2 genes, and a mode of 1 and 2.

Figure 4.1a



CXCL-11
"guanylate binding protein 1, interferon-inducible,"
(C-X-C motif) ligand 9
Pleckstrin
CYP3A29: cytochrome P450 3A29
TYROBP: TYRO protein tyrosine kinase binding protein
Similar to KIAA0644 Gene product
Similar to Transmembrane protein 144
"solute carrier family 38, member 1 [Homo sapiens]"
proline/arginine-rich end leucine-rich repeat protein
phosphatidylcholine transfer protein
protocadherin transcript variant 3
 NKp80 NK receptor
 strongly similar to plexin C1 [Mus musculus]
"MRC1: mannose receptor, C type 1"
"MMP7: matrix metallopeptidase 7 (matrilysin, uterine)"
lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa)
"proteoglycan 1 precursor-like, Transcript Variant 1"
LMP7: proteasome subunit LMP7
Ssc.19943.2
"MRNA, clone:AMP010030C04, expressed in alveolar macrophage"
Bruton agammaglobulinemia tyrosine kinase (BTK)
 SLA-DMA alpha 2 domain
"TAP1: Transporter 1, ATP-binding cassette, sub-family B "
CD18: CD18 leukocyte adhesion molecule
Similar to CD53 (Bovine)
"C1S: complement component 1, s subcomponent"
"IL2R-G: interleukin 2 receptor, gamma "
Caspase-1 (ICE)
CD1 Cluster gene
"MHC class II, DM beta"
MHC class II antigen
"MRNA, clone:THY010107H05, expressed in thymus"
Transcribed locus
FCNB: ficolin
"Transcribed locus, moderately similar to XP_947303.1 PREDICTED: similar to unc-93 homolog B1 isoform 1 [Homo sapiens]"
Transcribed locus
SBAB-707F1.3: complement component 2
"MRNA, clone:SPL010046C07, expressed in spleen"
Transcribed locus
"Transcribed locus, strongly similar to NP_066363.1 hypothetical protein L0C57169 [Homo sapiens]"
Transcribed locus
"Clone xcad2lb_e4.y1.abd, mRNA sequence"
"MRNA, clone:AMP010064D06, expressed in alveolar macrophage"
"Transcribed locus, strongly similar to NP_061134.1 Jun dimerization protein p21SNFT [Homo sapiens]"
"Transcribed locus, moderately similar to XP_946275.1 PREDICTED: similar to Transmembrane 7 superfamily member 1 [Homo sapiens]"
"Transcribed locus, strongly similar to XP_981749.1 PREDICTED: similar to B-cell CLL/lymphoma 7C [Mus musculus]"
"MRNA, clone:0VRM10058D03, expressed in ovary"
CAMKCD: calcium/calmodulin-dependent protein kinase (CaM kinase) II delta
"MRNA, clone:LNG010060B03, expressed in lung"
"Transcribed locus, strongly similar to NP_059089.1 caseinolytic protease, ATP-dependent, proteolytic subunit homolog [Mus musculus]"
Transcribed locus
"MRNA, clone:SPL010050C11, expressed in spleen"
Predicted Soluble carrier family 34 (Sodium phosphate) Transcript Variant 2
FM0D: fibromodulin
Bovine Transmembrane Protein 144
Macaca mulatta ubiquitin specific protease 14 (tRNA-guanine Transglycosylase)
"MRNA, clone:AMP010061G05, expressed in alveolar macrophage"
strongly similar to NP_038919.1 SAM pointed domain containing ets transcription factor [Mus musculus]
"MRNA, clone:LVRM10061G07, expressed in liver"
moderately similar to NP_570968.1 cAMP responsive element binding protein 3-like 4 [Homo sapiens]
"MRNA, clone:AMP010057H08, expressed in alveolar macrophage"
"Transcribed locus, moderately similar to NP_001028886.1 nucleolar protein 1, 120kDa [Homo sapiens]"
Transcribed locus
"MRNA, clone:TES010023G04, expressed in testis"
"MRNA, clone:0VRM10041E01, expressed in ovary"
Transcribed locus
"MRNA, clone:0VRM10040C07, expressed in ovary"
Transcribed locus
"MRNA, clone:AMP010044G02, expressed in alveolar macrophage"
Transcribed locus
"Transcribed locus, moderately similar to XP_947327.1 PREDICTED: similar to Tripartite motif protein 16 (Estrogen-responsive B box protein) [Homo sapiens]"
"Antigen NY-BR-84-like protein mRNA, partial sequence"
EIF4A2: eukaryotic translation initiation factor 4A isoform 2
Similar to LPS Trafficiking Rheus macca
strongly similar to NP_997696.1 HLA-B associated transcript 5 [Rattus norvegicus]
TM0D3: tropomodulin 3 (ubiquitous)
Transcribed locus
"Transcribed locus, strongly similar to NP_060531.1 PRP38 pre-mRNA processing factor 38 (yeast) domain containing B [Homo sapiens]"
Transcribed locus
Transcribed locus
Transcribed locus
"Transcribed locus, strongly similar to NP_997696.1 HLA-B associated transcript 5 [Rattus norvegicus]"
Transcribed locus
"MRNA, clone:LVRM10138D07, expressed in liver"
Transcribed locus
Transcribed locus
Transcribed locus
Transcribed locus
Transcribed locus
"MRNA, clone:LNG010080E11, expressed in lung"
Transcribed locus
Transcribed locus
"MRNA, clone:AMP010061G05, expressed in alveolar macrophage"
"MRNA, clone:0VRM10211E06, expressed in ovary"
Transcribed locus
Transcribed locus
"Macaca mulatta ubiquitin specific protease 14, Transcript Variant 1"
Transcribed locus
asparagine-linked glycosylation 9 homolog
"Bos taurus ras homolog gene family, member U"
"protein tyrosine phosphatase, receptor"
CD 163
EIF4EBP1: eukaryotic translation initiation factor 4E binding protein 1
"similar to LPS-responsive vesicle trafficking, beach and anchor containing"
"MRNA, clone:LVRM10138D07, expressed in liver"
Transcribed locus

Figure 4.1b

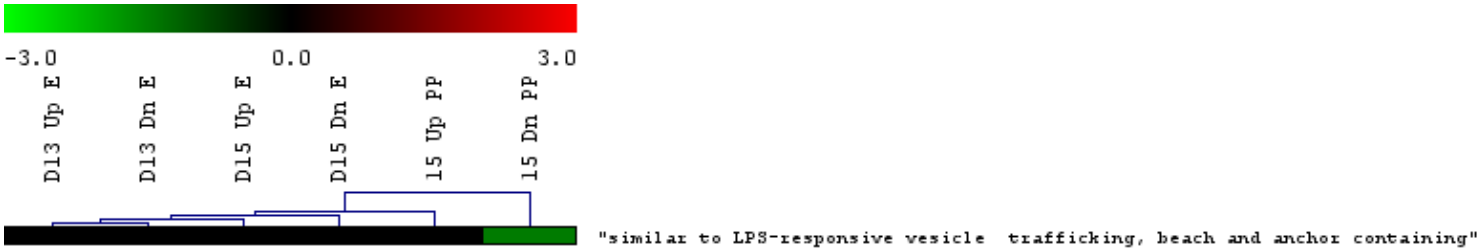


Figure 4.1c

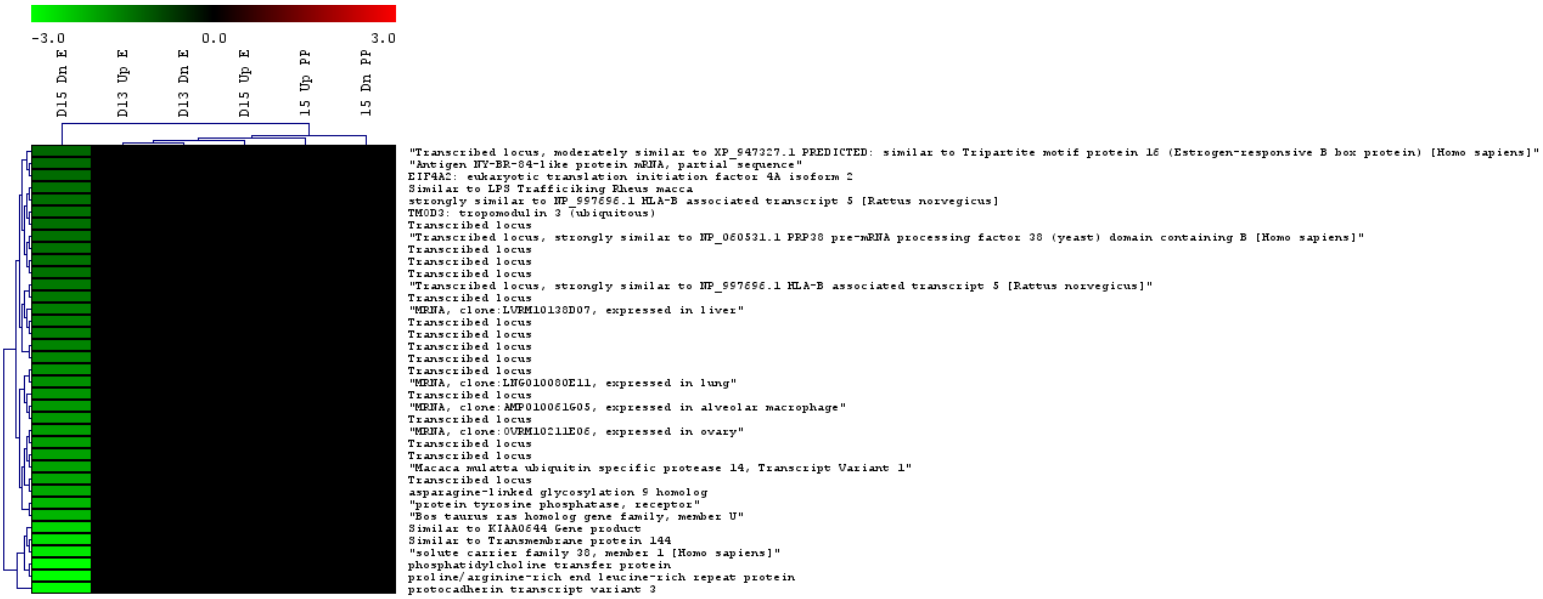


Figure 4.1d

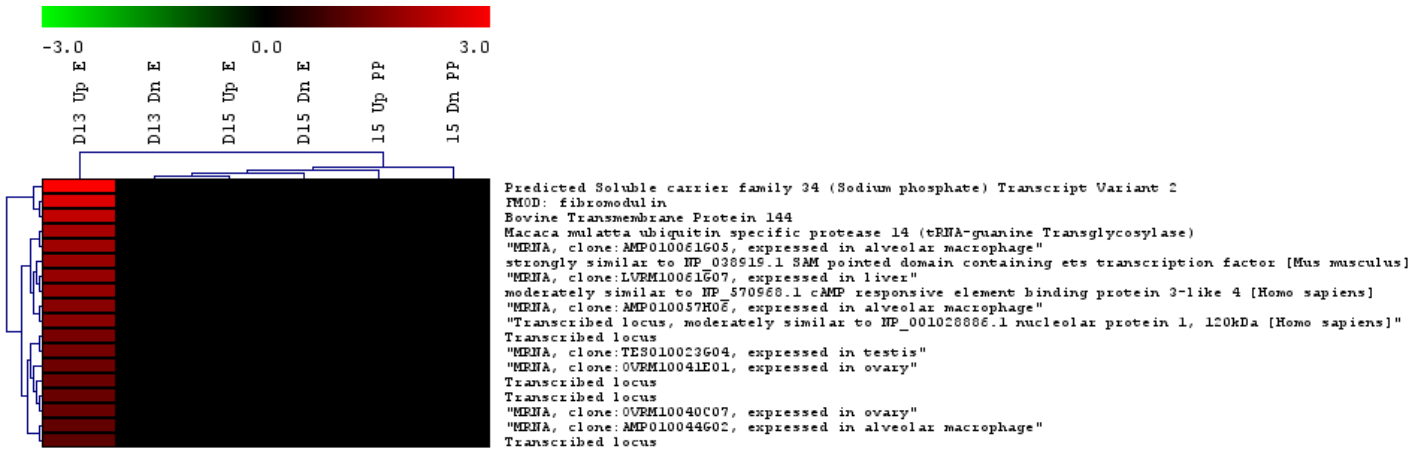


Figure 4.1e

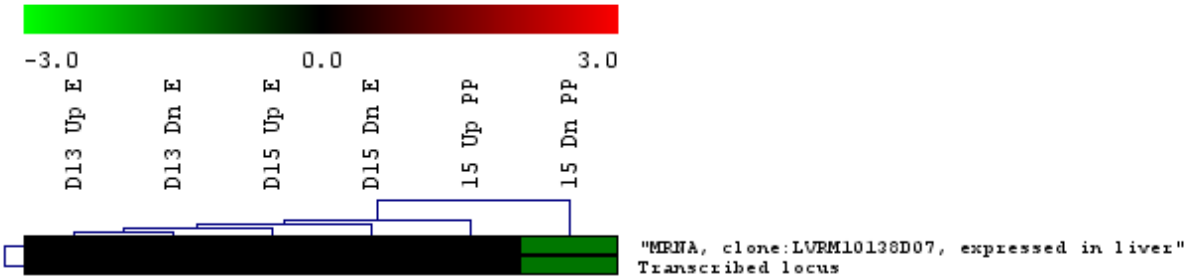


Figure 4.1f

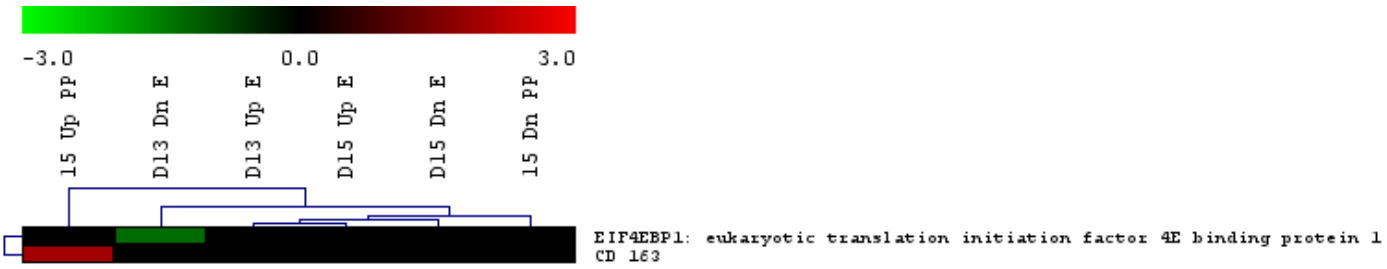


Figure 4.1g

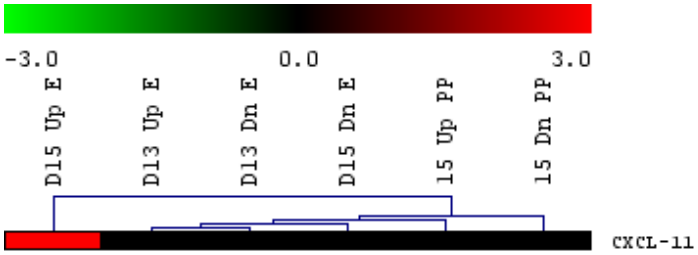
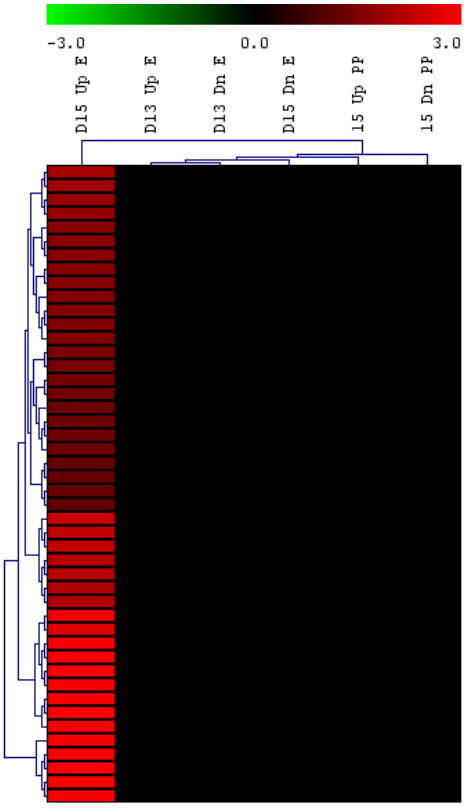
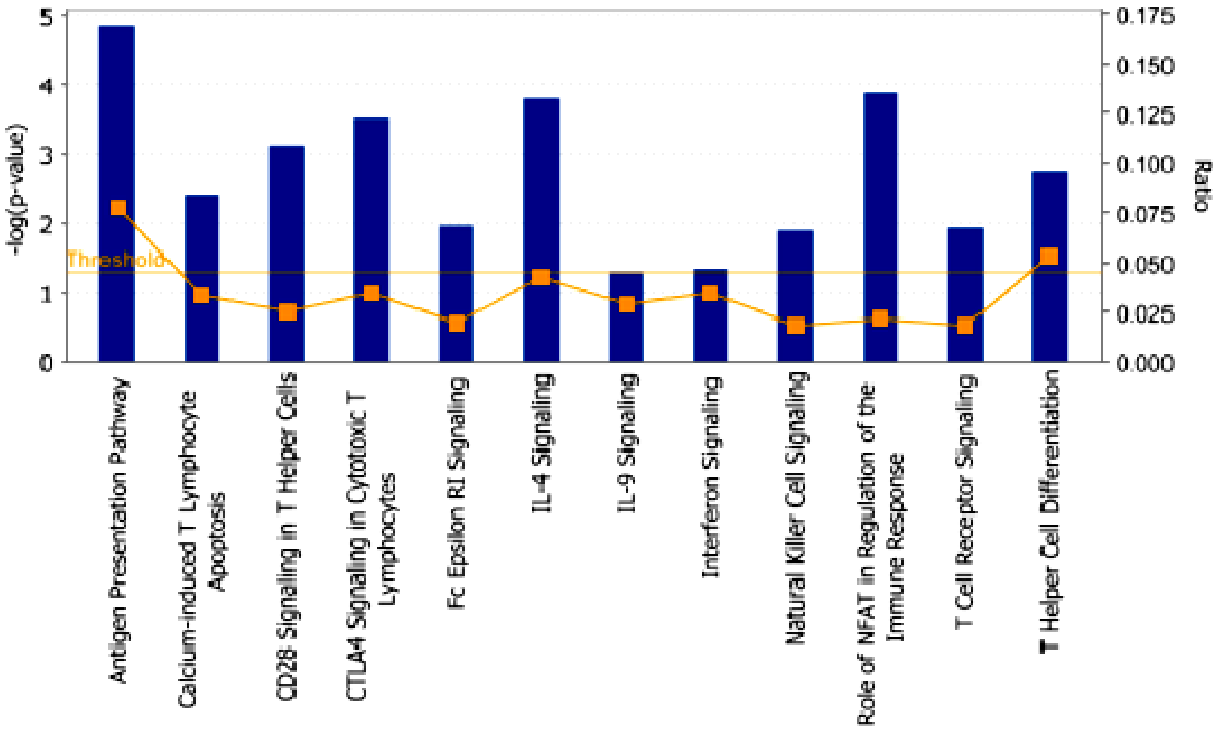


Figure 4.1h



Caspase-1 (ICE)
CD1 Cluster gene
MHC class II antigen
"MHC class II, DM beta"
"MRNA, clone:THY010107H05, expressed in thymus"
Transcribed locus
FCNB: ficolin
"Transcribed locus, moderately similar to XP_947303.1 PREDICTED: similar to unc-93 homolog B1 isoform 1 [Homo sapiens]"
Transcribed locus
"Transcribed locus, strongly similar to NP_066363.1 hypothetical protein L0C57169 [Homo sapiens]"
SBAB-707F1.3: complement component 2
"MRNA, clone:SPL010046C07, expressed in spleen"
Transcribed locus
Transcribed locus
"Clone rcad21b_e4.y1.abd, mRNA sequence"
"MRNA, clone:AMP010064D06, expressed in alveolar macrophage"
"Transcribed locus, strongly similar to NP_061134.1 Jun dimerization protein p21SNFT [Homo sapiens]"
CAMK2D: calcium/calmodulin-dependent protein kinase (CaM kinase) II delta
"Transcribed locus, strongly similar to XP_991749.1 PREDICTED: similar to B-cell CLL/lymphoma 7C [Mus musculus]"
"MRNA, clone:0VRM10058D03, expressed in ovary"
"Transcribed locus, moderately similar to XP_946275.1 PREDICTED: similar to Transmembrane 7 superfamily member 1 [Homo sapiens]"
"MRNA, clone:LNG010060B03, expressed in lung"
"Transcribed locus, strongly similar to NP_059089.1 caseinolytic protease, ATP-dependent, proteolytic subunit homolog [Mus musculus]"
Transcribed locus
"MRNA, clone:SPL010050C11, expressed in spleen"
Bruton agammaglobulinemia tyrosine kinase (BTK)
 SLA-DMA alpha 2 domain
"TAP1: Transporter 1, ATP-binding cassette, sub-family B "
CD18: CD18 leukocyte adhesion molecule
Similar to CD53 (Bovine)
"C1S: complement component 1, s subcomponent"
"IL2R-G: interleukin 2 receptor, gamma "
Ssc.19943.2
"MRNA, clone:AMP010030C04, expressed in alveolar macrophage"
"MMP7: matrix metallopeptidase 7 (matrilysin, uterine)"
lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa)
"proteoglycan 1 precursor-like, Transcript Variant 1"
LMP7: proteasome subunit LMP7
 strongly similar to plexin C1 [Mus musculus]
"MRC1: mannose receptor, C type 1"
 NKp80 NK receptor
"guanylate binding protein 1, interferon-inducible,"
CYP3A29: cytochrome P450 3A29
TYR0BP: TYR0 protein tyrosine kinase binding protein
Pleckstrin
(C-X-C motif) ligand 9

Figure 4.2. Top pathways regulated by estrogen based on their significance (*p*-value) calculated using the right tailed Fisher's Exact Test using the complete comparison of early estrogen exposure endometrium comparing D13 vs D15


-log(p-value)
Ratio
Threshold
0
1
2
3
4
5
0.000
0.025
0.050
0.075
0.100
0.125
0.150
0.175
Antigen Presentation Pathway
Calcium-induced T Lymphocyte Apoptosis
CD28 Signaling in T Helper Cells
CTLA4 Signaling in Cytotoxic T Lymphocytes
Fc Epsilon RI Signaling
IL-4 Signaling
IL-9 Signaling
Interferon Signaling
Natural Killer Cell Signaling
Role of NFAT in Regulation of the Immune Response
T Cell Receptor Signaling
T Helper Cell Differentiation

Regulatory Networks

Using data analysis from early estrogen exposed endometrium on day 13 vs day 15, a total of 5 networks were linked to an estrogen response (Figures 4.4). These networks had an IPA score of: 28, 8, 3, 2, and 2 respectively. These scores represent a numerical value of approximate fit for each network and determine focus genes based on relativity to the input data set. The network data we obtained allows us to prioritize and fuel new directions based on some of these "key" molecules identified as central hubs in these networks.

Networks 1, 2, 3

Network one consisted of 11 focus molecules which contained CXCL-11 as the major hub in this network (Figure 4.5a). This network contained the associated function to include antigen presentation, cell mediated immune response, and humoral immune responses. Network 2 consisted of 4 focus genes and an IPA score of 8 (Figure 4.5b). The genes in this network have ascribed functions such as cell death, hematological disease, and immunological disease. Network 3 consisted of 1 focus genes and an IPA score of 3 (Figure 4.5c). The genes in this network have ascribed functions such as cancer, cardiovascular disease, cardiovascular system development and function.

Antigen Presentation Pathway Network

Antigen presentation pathway network (Figure 4.6) involved the up regulation of transporter 1, ATP-binding cassette, sub-family B (TAP I) expression for movement of degraded peptide antigens across extra- and intracellular membranes. Additionally, MHC

Figure 4.3a. Network 1 regulated by estrogen centered around CXCL-11. This network contained a set of focus genes (11) with a score of 28. The probability of a gene being there by chance would be 10^{-28}. The score is not an indication of the quality or significance of the network. Node (gene) and connecting lines (gene relationship) are described in the *methods and materials* section. The intensity of the color [red (up) and green (down)] of the nodes would be indicative of the magnitude of direction involved in regulation.

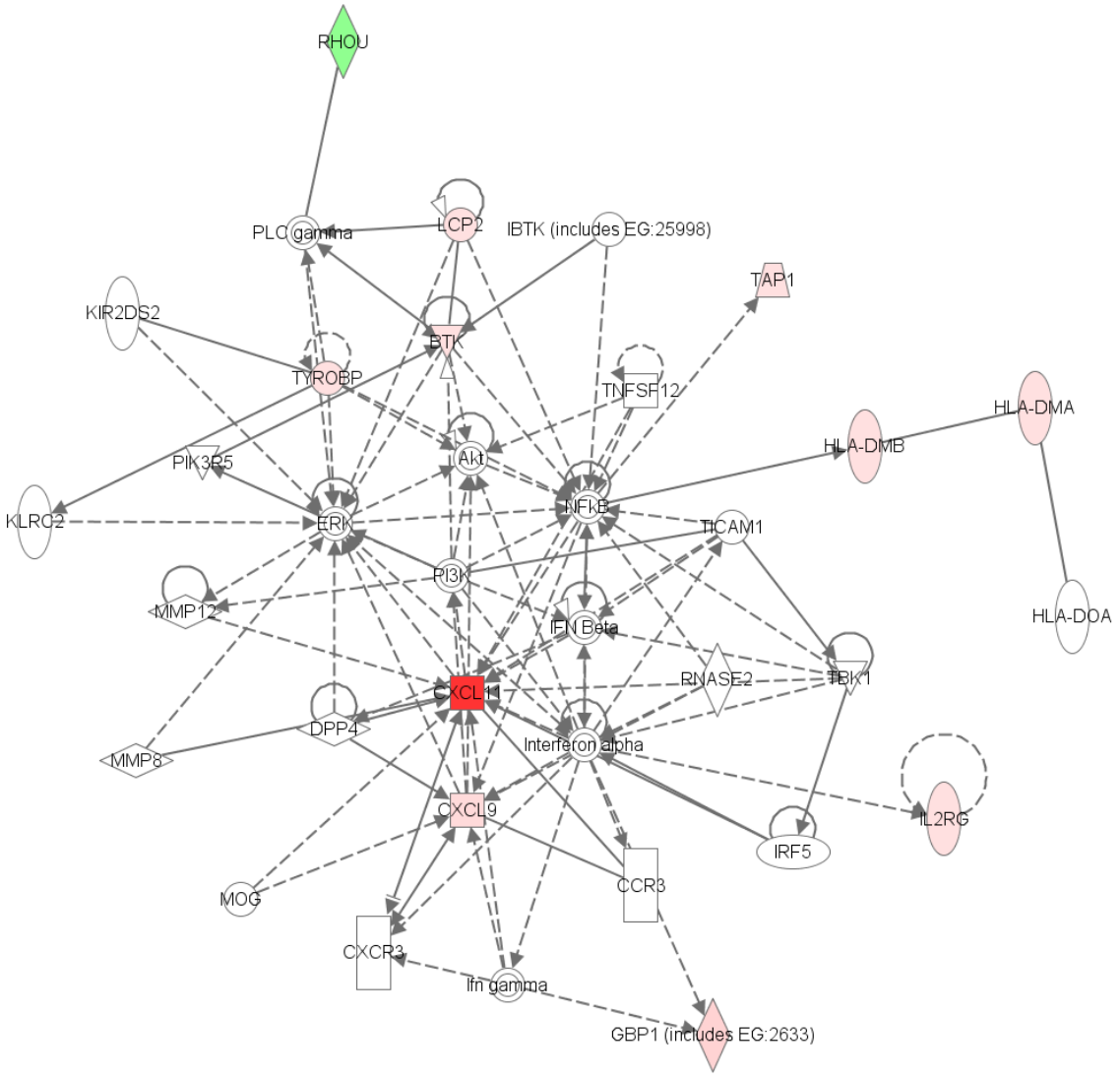

RHOU
PLC gamma
LCP2
IBTK (includes EG:25998)
TAP1
KIR2DS2
BTK
TYROBP
TNFSF12
HLA-DMA
HLA-DMB
PIK3R5
Akt
NFkB
KLRC2
ERK
TICAM1
PI3K
HLA-DOA
MMP12
IFN Beta
RNASE2
TBK1
CXCL11
DPP4
MMP8
Interferon alpha
CXCL9
IL2RG
IRF5
CCR3
MOG
CXCR3
Ifn gamma
GBP1 (includes EG:2633)

Figure 4.3b. Network 2 regulated by estrogen centered around IFN-γ. This network contained the maximum amount of focus genes (4) with a score of 8. The probability of a gene being there by chance would be 10^{-8}. The score is not an indication of the quality or significance of the network. Node (gene) and connecting lines (gene relationship) are described in the methods and materials section. The intensity of the color [red (up) and green (down)] of the nodes would be indicative of the magnitude of direction involved in the regulation.

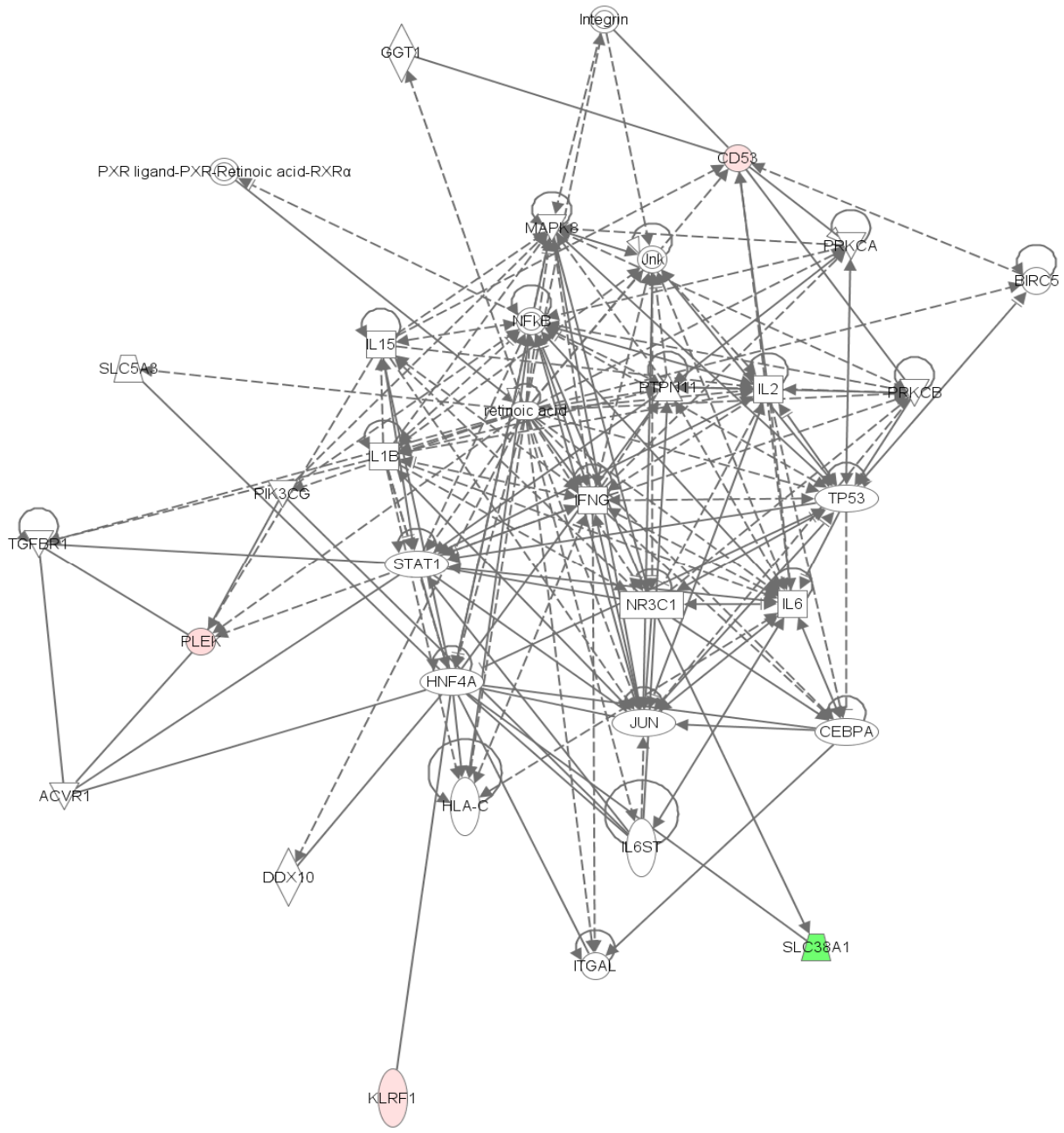

Integrin
GGT1
CD53
PXR ligand-PXR-Retinoic acid-RXRα
MAPK8
Jnk
PRKCA
BIRC5
NFKB
IL15
SLC5A8
PTPN11
IL2
PRKCB
retinoic acid
IL1B
PIK3CG
IFNG
TP53
TGFBR1
STAT1
NR3C1
IL6
PLEK
HNF4A
JUN
CEBPA
ACVR1
HLA-C
IL6ST
DDX10
SLC38A1
ITGAL
KLRF1

Figure 4.3c. Network 3 regulated by estrogen centered around HSPG2. This network contained the maximum amount of focus genes (1) with a score of 2. The probability of a gene being there by chance would be 10^{-2}. The score is not an indication of the quality or significance of the network. Node (gene) and connecting lines (gene relationship) are described in the methods and materials section. The intensity of the color [red (up) and green (down)] of the nodes would be indicative of the magnitude of direction involved in the regulation.

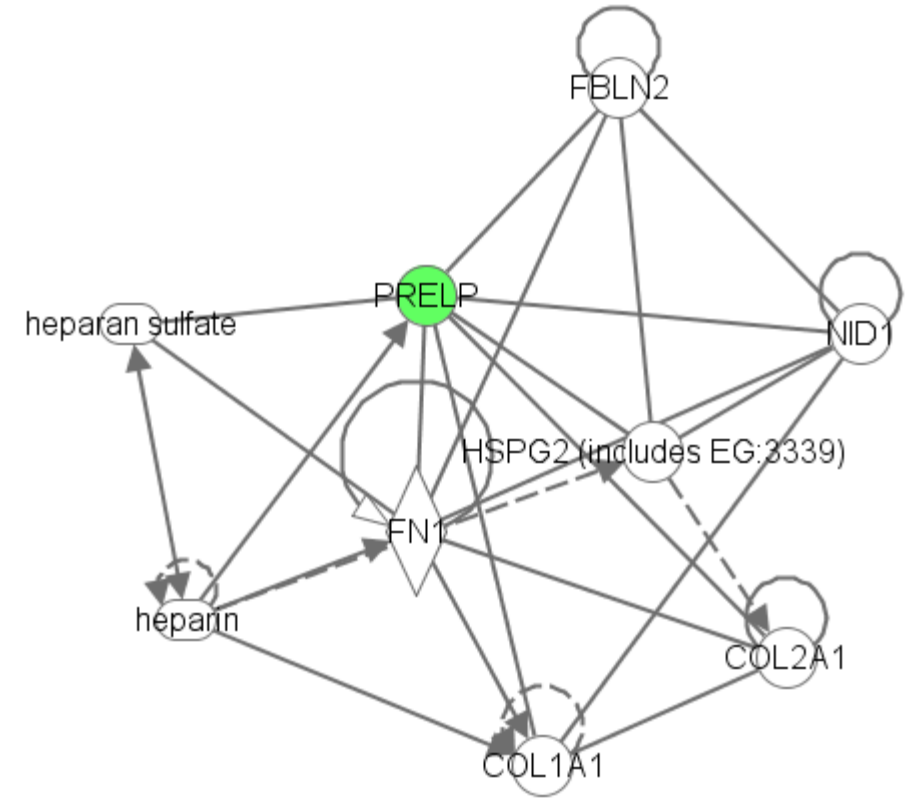
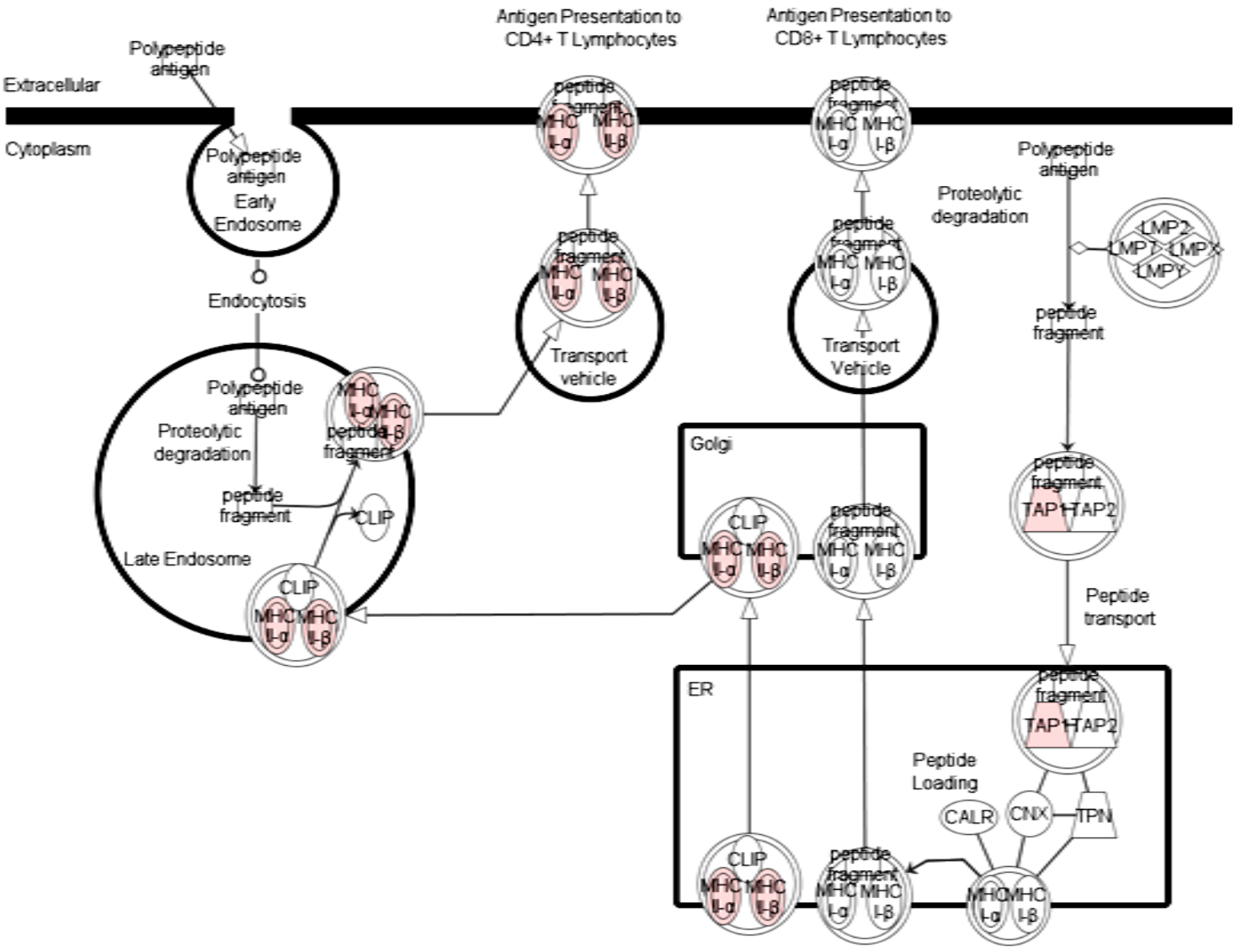

FBLN2
PRELP
heparan sulfate
NID1
HSPG2 (includes EG:3339)
FN1
heparin
COL2A1
COL1A1

Figure 4.4. Signaling Antigen Presentation Pathway regulated by estrogen centered on the MHC II pathway. Node (gene) and connecting lines (gene relationship) are described in the methods and materials section. The intensity of the color [red (up) and green (down)] of the nodes would be indicative of the magnitude of direction involved in the regulation.


Antigen Presentation to CD4+ T Lymphocytes
Antigen Presentation to CD8+ T Lymphocytes
Polypeptide antigen
Extracellular
Cytoplasm
Polypeptide antigen
Early Endosome
Endocytosis
Polypeptide antigen
Proteolytic degradation
peptide fragment
CLIP
Late Endosome
MHC II-α
MHC II-β
peptide fragment
Transport vehicle
peptide fragment
Transport Vehicle
Proteolytic degradation
Polypeptide antigen
LMP2
LMP7
LMP10
LMPY
peptide fragment
Golgi
CLIP
peptide fragment
TAP1
TAP2
Peptide transport
ER
Peptide Loading
CALR
CNX
TPN
MHC I-α
MHC I-β

II α and β molecules are up regulated in this pathway as well involving extracellular antigen presentation. Moreover, all the antigen presentation pathways involved in this signaling network due to premature exposure to exogenous estrogen all supported presentation to $CD4^+$ Cytotoxic T-Cells through up regulation of MHC II

Quantitative Real time PCR

Based on the evaluation through DAVID, k-means clustering, and IPA analysis; relative differences between selected mRNA populations was further quantified using the two-step qRT-PCR method. Messenger RNA's were evaluated using this method and are listed in regards with primer sequences used for detection (Table 4.1). Quantitation was completed to analyze the mRNA expression for *chemokine ligand 11* (CXCL-11), *chemokine ligand 9* (CXCL-9), *guaylate binding protein 1* (GBP1), c*luster of differentiation 163* (CD163), and c*luster of differentiation 18, known as integrin 2β* (CD18). In general, the expression patterns validated and confirmed the directional relation to the changes found on the array (Table 4.2).

Chemokine Ligand 11

A significant day by estrogen interaction ($P < 0.04$) on relative abundances of mRNA (mean ± SEM) was identified for CXCL-11 (Figure 4.5a). The mRNA expression was not different due to estrogen on day 15. However, there was a 256-fold increase in endometrial gene expression when comparing day 13 to day 15 following early estrogen exposure to the endometrium.

Chemokine Ligand 9

A significant effect of day ($P < 0.04$) was observed in relation to relative mRNA abundances of CXCL9 (Figure 4.5b). Although early estrogen administration had no effect on CXCL9 mRNA expression, day 15 gene expression increased 8-fold compared to day 13.

Guaylate Binding Protein 1

GBP1 was significantly affected by day ($P = 0.004$) and day of estrogen ($P = 0.04$) (Figure 4.5c). Early estrogen exposure of the endometrium contributed to an increased GBP1 gene expression resulting in a doubling in abundance of GPB1 mRNA on D15 when compared to D13.

Cluster of Differentiation 163

A slight but significant day of estrogen treatment ($P = 0.01$) effect for CD 163 gene expression was observed. The qRT-PCR results demonstrated about a 2-fold increase in CD 163 (Figure 4.5d) due to early estrogen exposure. Additionally, there was a 2-fold increase in CD163 mRNA when comparing on D15 when compared to D13.

Cluster of Differentiation 18 (Integrin β2)

A significant day by estrogen treatment interaction ($P = 0.009$) was observed for gene expression for CD 18 (Figure 4.1e). Greatest abundance of CD18 mRNA on day 15 endometrium occurred when exposed to estrogen on days 9 and 10, where as the

Figure 4.5a. A significant day by treatment interaction on relative mRNA units (mean ± SEM) was identified for ***CXCL-11*** ($P<0.03$) Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference ($P<0.05$) between endometrium exposed to early estrogen when comparing day 13 to 15.

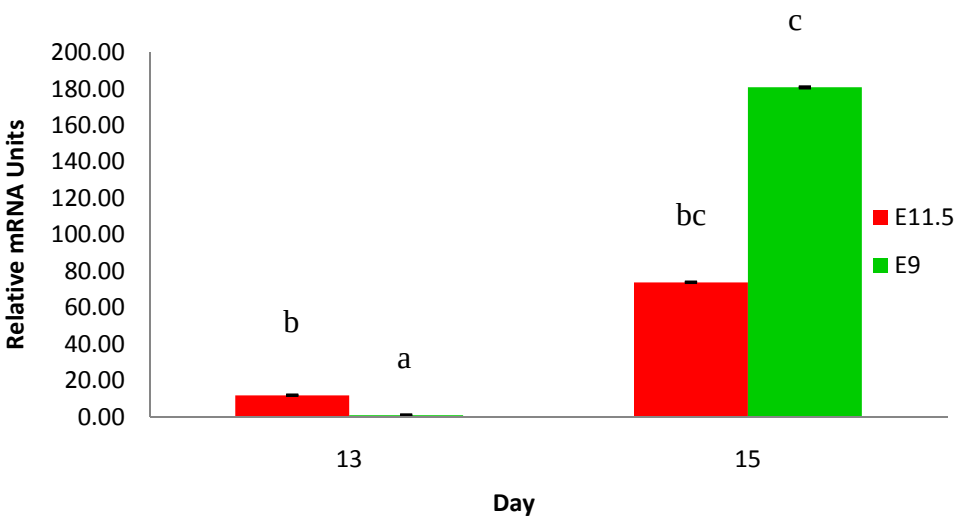

c
200.00
180.00
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Relative mRNA Units
bc
b
a
E11.5
E9
13
15
Day

Figure 4.5b. A significant day effect on relative mRNA units (mean ± SEM) was identified for ***CXCL-9*** ($P<0.04$). Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference ($P<0.05$) between day 13 and day 15 endometrium regardless of estrogen exposure.

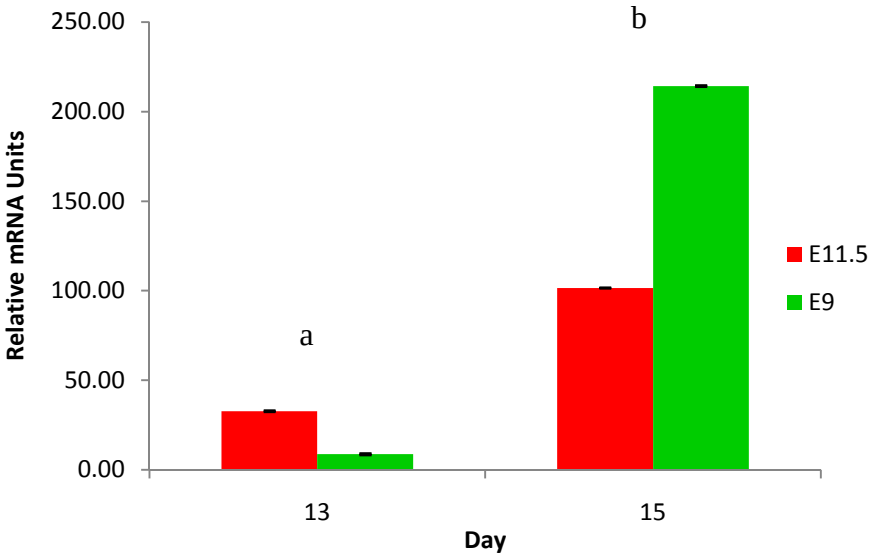

250.00
200.00
150.00
100.00
50.00
0.00
Relative mRNA Units
a
b
13
15
Day
E11.5
E9

Figure 4.5c. GPB1 was significantly affected by day ($P<0.004$) and treatment ($P<0.04$)in regards to relative mRNA units (mean ± SEM) was identified for ***GBP1***. Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference between E9 and E11.5.

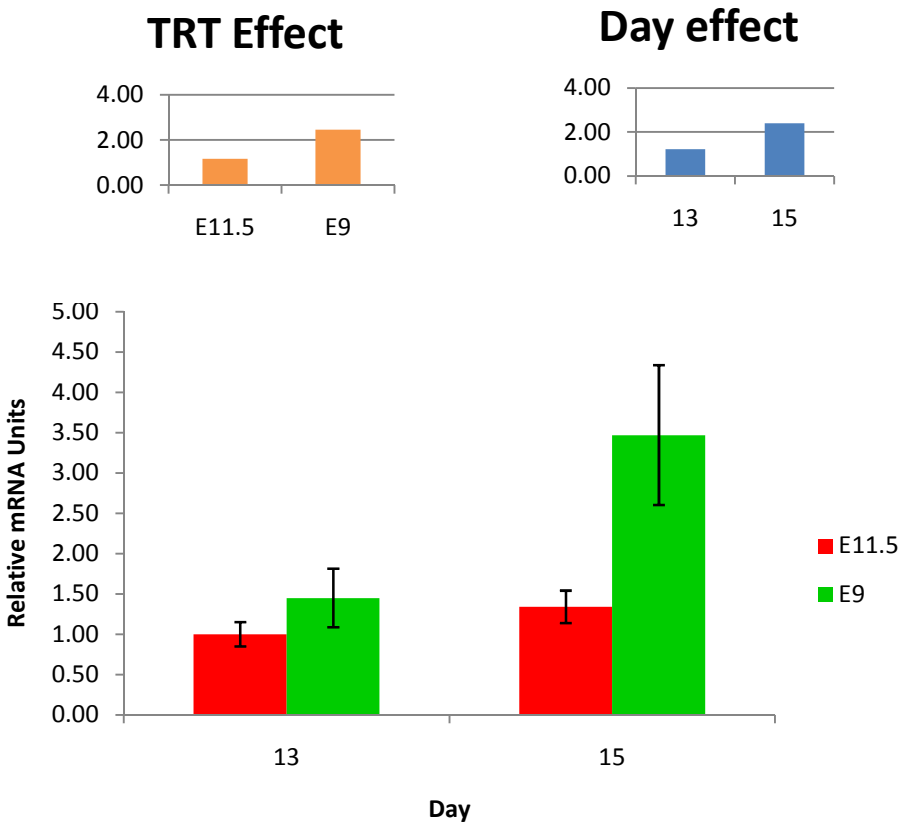

TRT Effect
4.00
2.00
0.00
E11.5
E9
Day effect
4.00
2.00
0.00
13
15
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Relative mRNA Units
13
15
Day
E11.5
E9

Figure 4.5d. A significant treatment effect of estrogen on relative mRNA units (mean ± SEM) was identified for ***CD 163*** ($P<0.04$) Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference ($P<0.05$) between endometrium that was exposed to early estrogen.

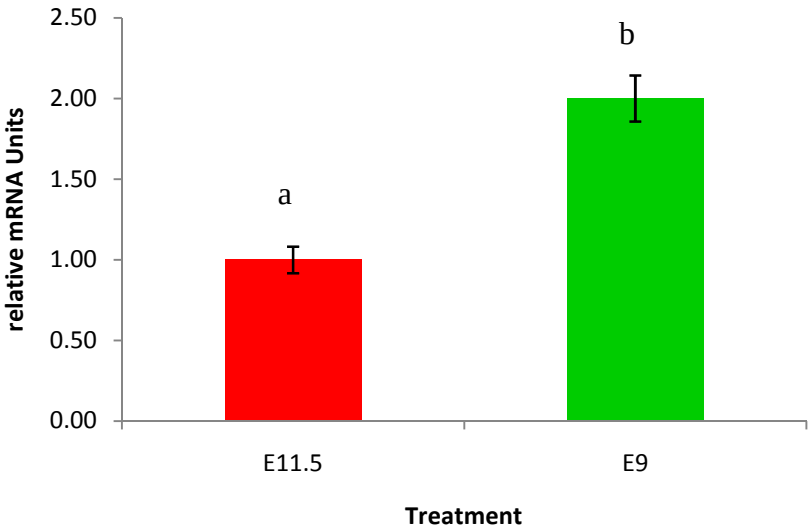

2.50
2.00
1.50
1.00
0.50
0.00
relative mRNA Units
a
b
E11.5
E9
Treatment

Figure 4.5e. A significant day by treatment interaction on relative mRNA units (mean ± SEM) was identified for ***CD 18*** ($P<0.01$). Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference ($P<0.05$) between endometrium that was exposed to early estrogen when comparing day 13 to day 15

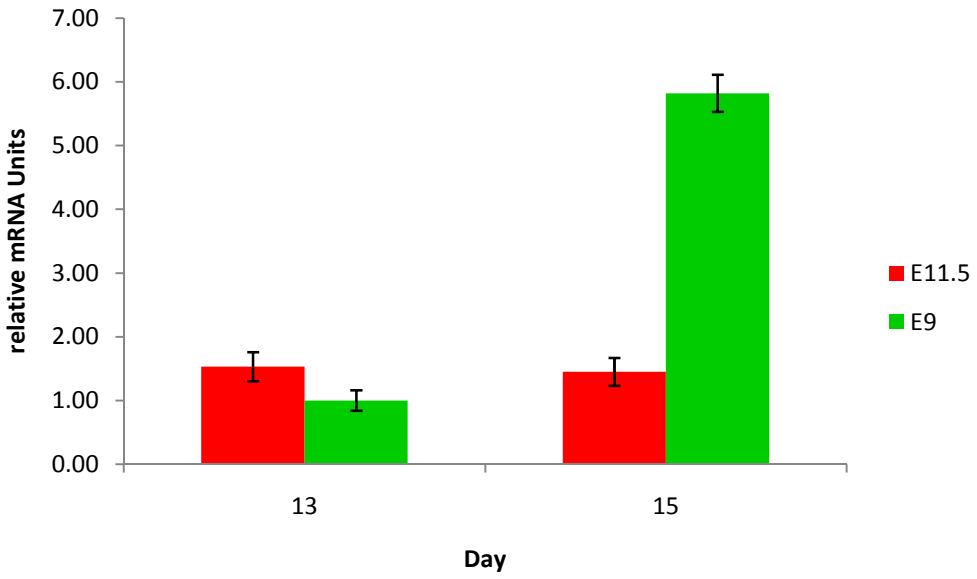

7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
relative mRNA Units
13
15
Day
E11.5
E9

Table 4.5. Results from quantitative RT-PCR confirming the changes observed with the Affymetrix Results. Represents validation of confirmed directional changes observed during the microarray analysis

Target Gene[¶]	AffyChip[§]	P-Value	QT-RT-PCR[#]	*p*-value
Chemokine Ligand 11				
13PP vs 15PPE	30.01	P<0.001	256	P<0.03
Chemokine Ligand 9				
13PP vs 15PP	6.41	P<0.001	2.36	P<0.04
13PPE vs 15PPE	5.31	P<0.001	28.0	P<0.04
G-Protein, IFN Inducible				
15PP vs 15PPE	6.40	P<0.001	1.80	P<0.004
CD 163				
13PP vs 15PP	1.94	P<0.001	2.30	P<0.01
CD 18, β2 Intergrin				
13PPE vs 15PPE	2.2	P<0.001	5.78	P<0.01

[¶]Primer sets were designed to specifically assay each of the targets using fluorescent (SYBR Green).
[#] Comparative C_T method was utilized for determining fold differences from the QT-RT-PCR.
[§]Fold differences were determined by using dChip for Affymetrix analysis

Table 4.6. Top 5 networks generated through the IPA analysis software. These networks had an IPA score of: 28, 8, 3, 2, and 2 respectively. These scores represent a numerical value of approximate fit for each network and determine focus genes based on relativity to the input data set.

ID	Molecules in Network	Score	Focus Molecu	Top Functions
1	Akt, ↑**BTK,** CCR3, ↑**CXCL9,** ↑**CXCL11,** CXCR3, DPP4, ERK, ↑**GBP1 (includes EG:2633),** ↑**HLA-DMA,** ↑**HLA-DMB,** HLA-DQA, IBTK (includes EG:25988), IFN Beta, Ifn gamma, ↑**IL2RG,** Interferon alpha, IRF5, KIR2DS2, KLRC2, ↑**LCP2,** MMP8, MMP12, MOG, NFkB, PI3K, PIK3R5, PLC gamma, ↓**RHOU,** RNASE2, ↑**TAP1,** TBK1, TICAM1, TNFSF12, ↑**TYROBP**	28	11	Antigen Presentation, Cell-mediated Immune Response, Humoral Immune Response
2	ACVR1, BIRC5, ↑**CD53,** CEBPA, DDX18, GGT1, HLA-C, HNF4A, IFNG, IL2, IL6, IL15, IL18, IL6ST, Integrin, ITGAL, Jnk, JUN, ↑**KLRF1,** MAPK8, NFkB, NR3C1, PIK3CG, ↑**PLEK,** PRKCA, PRKCB, PTPN11, PXR ligand-PXR-Retinoic acid-RXRα, retinoic acid, ↓**SLC38A1,** SLC5A3, STAT1, TGFBR1, TP53	8	4	Cell Death, Hematological Disease, Immunological Disease
3	BCL2, ↓**PCDHA1**	3	1	Cancer, Cardiovascular Disease, Cardiovascular System Development and Function
4	ATN1, HTT, MIR9-1 (includes EG:406910), ↓**PCTP,** PPARG	2	1	Skeletal and Muscular System Development and Function, Lipid Metabolism, Molecular Transport
5	COL1A1, COL2A1, FBLN2, FN1, heparan sulfate, heparin, HSPG2 (includes EG:3339), NID1, ↓**PRELP**	2	1	Cell-mediated Immune Response, Cellular Movement, Hematological System Development and Function

remaining days were all significantly similar regardless of early estrogen exposure or day.

Discussion

Estrogen is critical for the proper establishment of pregnancy in the pig (Bazer and Thatcher, 1977) however; it can be detrimental if uterine exposure to estrogen occurs prematurely during early gestation (Pope et al., 1986, Blair et al., 1991; Ashworth et al., 2006). With the delicate relationship between the optimal point of endometrial exposure to estrogen during early pregnancy and its affect on conceptus survivability, one can infer a spatiotemporal regulation of essential endometrial factors affecting pregnancy outcome occurs through aberrant estrogen exposure. Furthermore, although the endocrine-exocrine theory points to estrogen as the maternal recognition of pregnancy signal in the pig (Bazer and Thatcher, 1977), the molecular mechanisms behind estrogen's stimulation on the endometrium are not complete. Our approach isolated the direct effect of estrogen and its effects as an endocrine disruptor for the identification of endometrial genes altered by improper timing of estrogen stimulation without the presence of conceptus secreted factors.

Utilization of the Affymetrix GeneChip® Porcine Genome Array allowed the identification of a plethora of factors that are differentially expressed at the opening of the implantation window aberrantly due to early estrogen exposure. Furthermore, as determined though statistical analysis using dChip, 84 of the 117 genes that were differentially expressed were due to early estrogen exposure when comparing days 13 to 15. These data would suggest when aberrant estrogen exposure disrupts pregnancy the

window from days 13 to 15 is an important and critical time period for endometrial changes in gene expression and secretions essential for conceptus development and survival. Additionally, the lack of abundance in differential gene expression in endometrium between days 13 and 15 of gilts exposed to estrogen at the time that conceptus estrogen normally occurs (day 11-12) during early pregnancy indicates that early estrogen exposure selectively alters expression of endometrium genes which do not normally increase with conceptus estrogen secretion on day 12.

Cluster analysis was paramount in regards to identification of temporal expression patterns associated with estrogen regulation. When early estrogen disrupts pregnancy, the period between days 13 to 15 are the most critically affected with 84 genes found to be differentially regulated during this time period. Furthermore, the 84 differentially expressed genes were clustered into 3 groups of genes (Cluster 2, 6, and 7), and of these genes 47 were up regulated and 37 were downregulated due to the estrogen. Consequently, these genes might serve as an initial biomarker for possible identification of endocrine disruption of the early porcine implantation window.

Expression patterns involving multiple genes were analyzed using DAVID annotation cluster of interest. K-means clusters suggesting biological relevance in association of genes that are known to demonstrate either an estrogen response or an immunological function critical to pregnancy were picked for possible evaluation and quantified through qRT-PCR. Due to the roles in immunological regulation, chemokine ligand 11 (Hirota et al., 2006), chemokine ligand 9 (Imakawa et al., 2006), guanylate binding protein 1 (Kumar et al., 2001), cluster of differentiation 163 (Paidas et al., 2004) and cluster of differentiation 18 integrin β2 (Carty et al., 2008) were selected for closer

evaluation since they expression was found to be differentially effected by estrogen during the window of implantation.

Chemokines have been implicated with the enhancement of the biological immune response as well as having a key role in maintaining homeostasis of the immune system. Chemokines are classified into four distinct groups (CXC, CC, C and CX_3C) based on their inherent (amino acid sequence) and structural properties (Berger et al., 1999, Rollins et al., 1997). Early foundational studies limited chemokine expression to inflammatory and immune cells exclusively; however, subsequent studies have shown that to be false. Recently, chemokine expression was localized in the murine uterus at the time of peri-implantation (Nagaoka et al., 2003a). Furthermore, localization of chemokine receptors has been detected in the decidua and placenta of humans (Red-Horse et al., 2001). Chemokine ligand and receptor expression patterns are conspicuously expressed in uterus of the human, murine, and ovine during the window of implantation and/or attachment of the conceptus suggesting a key role for modulation of human endometrial erosion and attachment between the embryo and maternal tissues. Specifically, chemokine ligand 11 (CXCL-11) gene expression has been reported to increase during the window of implantation in the human (Hirota et al., 2006) and the ewe (Imakawa et al., 2006). Hirota et al., (2006) demonstrated exposure of uterine endometrial epithelial cells (EEC) in culture to IFN-γ stimulated secretion of CXCL-11, but not endometrial stromal cells (ESC). Furthermore, CXCL-11 stimulated proliferation in the ESC cells but inhibited proliferation in the EEC cells while concomitantly stimulating apoptosis. These data suggest IFN-γ working through CXCL-11 may play a regulatory role during implantation. Additionally, Hirota et al. (2006)

suggest a prejudice towards the stromal layer for survival conditions, while inducing negative conditions in the epithelial layer during endometrial invasion in the mouse. Hirota et al. (2006) also demonstrated that CXCR3, the receptor for CXCL-11, is present in the epithelial and stromal layers of the endometrium, T cells, and the conceptus trophoblastic cells. These data represent a useful model for motility and chemotaxis during the window of implantation in that upon secretion of CXCL-11 from the EEC layer, trophoblastic cells and T cells (T_h1) migrated accordingly. Hirota's data makes a nice complement to what our lab has already established to date in the pig. The appearance of CXCL-11 is closely associated with window of implantation in the pig. Additionally, our lab has previously characterized IL-18 secretion (Ashworth MD and Giesert RD, unpublished results) which originates from the uterine endometrium during the period of trophoblast attachment to the uterine surface from Day 13 to 18 of pregnancy. IL-18 must be processed from the latent form to the active molecule through processing by caspase-1, which our lab has also characterized during the porcine pregnancy (Ashworth MD and Geisert RD, unpublished results). One could infer based on CXCL-11 appearance during the window of implantation, that it may provide a pro-apoptotic signal to uterine epithelial cells, resulting in a release of caspase-1 from the uterine epithelial cells via CXCL-11's ability to induce apoptosis. Furthermore, this may be a mechanism allowing site specific areas of the endometrium to undergo apoptosis as opposed to the whole endometrium, while providing a receptive epithelial layer for proper attachment during this period.

Chemokine ligand 9 (CXCL-9) belongs to the same subfamily as CXCL-11, shares the common receptor (CXCR3) with CXCL-11 and has a similar temporal

expression pattern during the window of implantation in the sheep (Imakawa et al., 2006). Similar to CXCL-11, uterine CXCL-9 secretion is stimulated through progesterone and/or estrogen (Kitaya et al., 2004). Stark contrasts exist between CXCL-9 and CXCL-11 in that CXCL-9 is induced by the pleiotrophic cytokine IL-18, while CXCL-11 and CXCL-10, the only known chemokines controlled by estradiol and progesterone and are directly responsible for the migration of perhperial blood NK cells into the uterus and creating a residential NK cell then termed uNK cells (Sentman et al., 2004). The induction of CXCL-11 into the human endometrium is facilitated through the ovarian sex steroids estrogen and progesterone (Sentman et al., 2004); however, whether the steroid affects are direct or indirect have yet to be elucidated. Furthermore, the study also established chemokine receptor CXCR3 is expressed on the surface of uNK cells, which serves as ligands for CXCL-9, and CXCL-11.

Gyuanlate binding protein 1 (GPB1) belongs to the larger family of guanylate binding proteins that are characterized as GTPase proteins which contain a conserved domain possessing the capacity to undergo oligomerization under augmented GTPase activity (van der Bliek et al., 1999). Additionally, Cheng et al., (1991) characterized GPB1 as the most abundant IFN-γ inducible protein in the human body, and numerous studies have demonstrated proinflammatory cytokines (IL-1β, IFN-γ, and TNF-α) are all inducers of GPB1 (Lubeseser-Martellato et al., 2002). More importantly, recent studies demonstrated GPB1 has an inverse correlation to the expression of matrix metalloproteinase-1 (MMP-1) suggesting a possible role in controlling cellular invasion.

GPB1 has been localized to the human endometrium (epithelial and stromal layers) during early conceptus attachment to the uterine surface and invasion (Kumar et

al., 2001). Moreover, the expression exhibited was strongly localized to the endometrial glandular epithelial cells and the surrounding stromal cells during the mid secretory phase. However, expression was very weak in the glandular epithelial cells during the proliferative or late secretory phase of the pregnancy. Additionally, GBP1 is induced by IFN-γ and –α and the authors found that IFN-γ was a strong inducer of GBP1. During pregnancy in the human, IFN-γ is most abundant during this time frame (Kumar et al., 2001).

In the pig, GBP1 has only just recently just been sequenced (Ma et al., 2008). According to our data, it appears that estrogen administration can induce GBP1 gene expression either directly or indirectly. Our GBP1 expression data is concurrent with IFN-γ expression based on a study conducted during early pig pregnancy by Joyce et al. (2005). Although with our pseudopregnant model is devoid of any conceptus IFN-γ, data from Joyce et al., (2005) demonstrated that sparse immune cells such as uNK though out the uterus are carring out transcription for IFN-γ. Furthermore, the IFN-γ expression would temporally be expressed around the time of the second phase of estrogen.

The CD18 ligand is expressed on leukocytes and pairs with either CD11a, b, c, or d to form the complete β2 integrin unit. CD18 plays key roles in leukocyte activation, adhesion, and activation of the compliment as well. Pregnant women with severe preeclampsia show signs of elevated leukocyte activation within the fetal circulation (Mellembakken et al., 2001). Furthermore, these authors found that both infants and the mothers exhibited increased levels of surface expression of CD11c when the mother had preeclampsia. Mellembakken et al. (2001) attributed the origin of this phenomenon as an indicator of activated neutrophils. Moreover, Saini et al., (2004) repeated the experiment

but measured CD18 and CD11a, b, and c. Their study revealed that CD18 and CD11a were profusely upregulated in the severe preeclamsia women and it provided a feed forward mechanism to continue to activate neutrophils. Activated neutrophils can decrease blood flow, release proteases and reactive oxygen species that all lead to an increase in vasoreactivity. Exposure of gilts to estrogen on day 9 in the present study aberrantly elevated endometrial CD18 gene expression 6-fold on day 15 when vasodilation and blood flow would be critical during pregnancy. The increase observed in CD18 transcript may serve as a potential indicator of a dysfunctional window of attachment in the porcine pregnancy.

CD163 is a membrane bound glycoprotein and a member of the B group of the scavenger receptor cystein rich superfamily. CD163 appears to have two functions dependant on if it is secreted or bound to the cell membrane. In the cell bound form CD163 participates in hemoglobin clearance, and in its secreted soluble form it exhibits cytokine like functions such as anti-inflammatory properties. CD163 expression increases as monocytes mature, inferring it is probably associated with a distinct class of differentiated monocytes (Morganelli et al., 1988). During pregnancy the placenta contains more macrophages and dendritic cells that contain CD163 surface expression than anywhere else in the body (Hogger et al., 1998). In our study, timing of estrogen treatment did not affect CD 163 mRNA expression but CD163 did increase between days 13 to 15.

Croy et al., (1987) postulated that a thriving porcine pregnancy can be attributed to suppression of T cell responses and MHC molecules which allow semiallograft conceptus to establish pregnancy and escape immune rejection. In our microarray data,

network analysis of gene annotations revealed a distinct up regulation of $CD4^+$ T Cell signaling components, major histocampatibility complex II (MHC II), and transporter I, ATP-binding cassette, sub-family B (TAP I). At first glance these results appear confounding due in part to two major reasons: TAP proteins are normally associated with the loading of MHC I and not MHC II; however, studies involving conditions such as Sarcoidosis have demonstrated that TAP proteins can unusually aid in the loading of self antigens from the lysosome onto the MHC II cleft for antigen presentation and stability of the MHC itself (Xia et al., 2001). Second, these data are in disagreement to the observed increase in levels of $CD8^+$ T cells observed normally during the implantation window of days 10 through 14 of the porcine pregnancy (Bischoff et al., 1995). In the current study, estrogen disruption appears to aberrantly increase $CD4^+$ cell signaling and increase levels of MHC II expression.

In conclusion, over one hundred candidate genes were successfully identified as differentially expressed due to premature estrogen exposure through the use of Affymetrix GeneChip® Porcine Genome Array. The current study has provided insight in to the complexity and multiple immune factors associated with endocrine disruption of the endometrium during early porcine pregnancy. Furthermore, the analysis of associated pathways constructed from the raw microarray data provided valuable insight into the dysfunctional antigen presentation schemes due to early estrogen exposure.

Chapter V

Regulation and Expression of Uterine BIO-markers in the Porcine Endometrium Following Uterine Infusion of Estrogen and IL-1β.

Abstract

Embryonic loss during early gestation limits litter size in swine production. Failure of the conceptus to attach and implant into the uterus may contribute to the high rate of embryonic loss observed in swine. Implantation requires precise communication between the conceptus and embryo which includes up regulation of adhesion molecules at the maternal/fetal interface for embryo attachment involving pregnancy specific inflammatory responses. Trophinin and osteopontin are cell adhesion molecules expressed on pig and human endometrial epithelial surface which may function early for the attachment of the conceptus trophectoderm and uterine luminal epithelium. Leukocytes infiltrate endometrium during implantation, and the enymes cyclooxygenase (COX)-1 and COX-2 are expressed in human and pig endometrium during pregnancy, where they are proposed to regulate conceptus implantation and uterine angiogenesis. During implantation, increases in interleukin-1β occur in the mouse, human and pig which may regulate uterine pro-inflammatory cytokines. Studies have demonstrated estrogen to be obligatory for establishment of pregnancy in the pig. Furthermore, estrogen and interleukin-1β are concomitantly expressed during the window of the maternal recognition of pregnancy in the pig. The objective of the current study was to evaluate the hypotheses that estrogen plays a regulatory role in the uterine inflammatory and attachment window invoked by IL-1β secretions during the establishment of pregnancy in the pig. Cyclic gilts were treated with 5 mg corn oil (CO) or estradiol

cypionate (E) on Day 11 of the estrous cycle. On Day 12, gilts were randomly infused with either 15 ug of saline (S) or recombinant porcine IL-1β (rIL-1β). Uterine horns were removed at 4 h or 36 h post-infusion (5 gilts/trt/sampling periods) and endometrial mRNA was quantified using quantitative RT-PCR. Estrogen did not influence ($P > 0.1$) concentrations of endometrial COX-1 and COX-2 mRNA; however, rIL-1β increased ($P = 0.01$) endometrial COX-2 mRNA by 3.5 fold and up-regulated ($P = 0.05$) endometrial IL-1β expression by 3.5 fold. Moreover, increases observed in endometrial expression of osteopontin, trophinin, and interleukin 1 receptor accessory protein were increased ($P < 0.05$) at 36 h but not at 4 h post-E treatment. Estrogen-treated gilts exhibited a 13-fold increase in osteopontin and approximately a 4-fold increase in endometrial trophinin expression when compared to the gilts treated with CO. Furthermore, E-treated gilts demonstrated an increase in interleukin 1 receptor accessory protein (2.5 fold increase) inferring a possible regulatory relationship with the interleukin1β system. We hypothesize that interleukin-1β and estrogen secretion by the pig conceptus differentially modulates uterine expression of COX-1, COX-2, trophinin, osteopontin, and the interleukin-1 β system providing a regulated inflammatory environment that is essential to establishment of pregnancy.

Introduction

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 interleukin-1β (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).

During the maternal recognition of pregnancy and window of implantation, conceptus IL-1β expressed concomitantly with estrogen secretion may establish an immunological dialog between the foreign conceptus tissues and the maternal endometrium (Gesiert et al., 1982, Ross et al., 2003). Caspase-1, also refered to as IL-1β converting enzyme, cleaves the 31 kDa inactive form of IL-1β to its biologically active 18 kDa form. Although IL-1β and estrogen secretion are severely attenuated by day 15 of pregnancy, there is a uterine release of IL-18 (Ashworth and Geisert unpublished results) and a sustained second phase of conceptus estrogen secretion necessary to maintain CL function beyond 28 days of gestation (Geisert *et al*., 1987). This second phase of conceptus estrogen secretion is temporally associated with a pregnancy specific 8-fold increase in endometrial caspase-1 mRNA expression (Ashworth and Geisert unpublished results), that also activates IL-18 from the pro-form, and produces the signaling competent form of the mature IL-18. Increases in endometrial caspase-1 expression are not associated with endometrial release of IL-1β as luminal content is greatly diminished by days 15 and 18 (Ross et al., 2003b) of pregnancy suggesting based

on its appearance IL-18 may be an alternative cytokine substrate for caspase-1 cleavage during this time period.

Failure of the conceptus to attach to the uterine surface may contribute to the high rate of embryonic loss observed in swine. Implantation requires communication between the conceptus and maternal tissues which functions include up regulation of adhesion molecules at the maternal/fetal interface for embryo attachment involving pregnancy specific inflammatory responses. Trophinin and osteopontin are cell adhesion bio-markers expressed on pig (Nakano et al., 2003, White et al., 2006) and human endometrial epithelial surface (Sugihara et al., 2008).

In the pig, leukocytes infiltrate endometrium prior to and during the window of implantation (Bishof et al., 1995), and this event is auspiciously around the time an observed increase in the enzymes cyclooxygenase (COX)-1 and COX-2, which are responsible for the production of the obligatory prostaglandins (PG) that are expressed during human (Demers and Gabbe., 1976) and porcine (Ashworth et al., 2006) endometrium during early pregnancy. Prostaglandins are essential, and also postulated as a key regulator during conceptus attachment in the pig (Kraeling et al., 1985) and facilitate uterine angiogenesis (Ford et al., 1982). During early gestation, increases in IL-1β have been observed in several species including the mouse (Sato et al., 2001), human (Lappas et al., 2002) and pig (Ross et al., 2003) which may serve to stimulate uterine pro-inflammatory cytokines, and stimulate remodeling of the extracellular matrix (ECM) proteins and matrix metalloproteinases (MMP) through control of master transcription during early pregnancy. In addition to cytokine regulation, foundational studies have demonstrated estrogen to be essential for establishment of pregnancy in the pig (Bazer

and Thatcher 1977), and induce uterine secretions to aid in proper conceptus sustainment and development (Geisert et al., 1982). Furthermore, studies have demonstrated that estrogen and IL-1β are concomitantly expressed in vivo during the window of the maternal recognition of pregnancy in the pig.

Recently, studies have demonstrated that IL-1β stimulates the attachment factor trophinin in human endometrial cells (Sugihara et al., 2008), and estrogen stimulates the attachment factor osteopontin in porcine uterine endometrial cells in the mouse and pig (White et al., 2006). The objective of the current study was to evaluate how estrogen plays a regulatory role in the uterine inflammatory and attachment window invoked simultaneously with IL-1β secretions during the establishment 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.

Experimental Design

Cyclic gilts (5/trt) received either corn oil (CO) or 5 mg i.m. injection of estradiol cypionate (E) (A.J. Legere, Scottsdale, AZ) on day 11 of the estrous cycle. On day 12 (24 h post-treatment) of the estrous cycle, gilts were subjected to mid-ventral laparotomy.

A ligature was placed at the bottom of each uterine horn near the uterine bifurcation. One randomly selected uterine horn was infused with saline (2 ml) and the second uterine horn infused with 15 µg of recombinant porcine IL-1β (rIL-1β) (Cell Sciences Inc, Canton, MA). The amount of rIL-1β infused into the uterine horn is based on the total content of IL-1β in uterine flushing collected on day 13 of pregnancy measured in a previous study [Ross et al., 2003]. Because uterine flushings only reflect a single time point measurement, the amount of rIL-1β infused was increased by 3-fold. Uterine horns were removed at 4 and 36 h post-infusion (5 gilts/trt/sampling period).

Animal Surgeries and Endometrial Tissue Collection

Following induction of anaesthesia with 1.8 ml i.m. administration of a cocktail consisting of 2.5 ml cocktail (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 the uterus and ovaries 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. Supernatant collected and clarified 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/Storage

Gilts were unilaterally hysterectomized at 4 and 36 h post-infusion (5 gilts/trt/sampling period). Uterine horns were isolated and flushed with 20 ml sterile phosphate buffered saline (PBS; pH 7.4). Small 1 cm segments of the uterine endometrium from CO and rIL-1β treated uterine horn were fixed for subsequent immunocytochemistry with fresh 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7) for at least 24 h and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). Additional tissue was embedded in Tissue-Tek Optimal Cutting Temperature (OCT) Compound (Miles, Oneonta, NY), slow-frozen over liquid nitrogen, and stored at -80^{o}C until utilized for further evaluation. Endometrial tissues were dissected from the underlying myometrium and snap-frozen in liquid nitrogen.

Quantitative RT-PCR Gene Target Description.

Effects of IL-1β on endometrial gene expression of osteopontin (OPN), trophinin, tumor necrosis factor-α (TNF-α), interleukin-1 receptor-1 (IL-1 R1), interleukin-1 receptor accessery protein (IL-1RAP), IL-18, COX-1, and COX-2 were evaluated. Tissue 18S ribosomal RNA was determined and used to normalize gene expression. Primers for these target genes have been previously developed and validated in our laboratory (Ross et al., 2003, Ashworth et al., 2006).

Quantitative-RT-PCR Methods

Quantitative RT-PCR analysis of transcripts of interest was conducted as previously described by our laboratory (Ashworth et al., 2006). Endometrial total RNA was aliquoted to be used for PCR analysis. Genomic DNA removal and the synthesis of cDNA to be used for quantitative analysis were conducted by using the QuantiTect Reverse Transcription kit according to manufacturer's recommendations (Qiagen, Valencia, CA). Briefly, 0.5 μg of each endometrial sample (n = 5) was added to a genomic DNA wipeout buffer for 5 min at 37^{o}C, followed by a 2 min incubation on ice, then proceeding to the addition of reverse transcription buffer, RT primer mix and reverse transcriptase; and incubated at 42^{o}C for 30 min. Individual endometrial samples were taken at both 4 and 36 h from the steroid treated (CO and E) and infusion treated gilts (Saline and rIL-β). The PCR amplification was conducted using the ABI PRISM 7700 and Bio-Rad icycler Sequence Detection Systems (PE Applied Biosystems, Bio-rad Systems). The real-time detection during each amplification cycle was done by using either a sequence specific dual-labeled fluorescent probe designed to have a 5' reporter dye (6-FAM) and a 3' quenching dye (TAMRA) nested between the forward and reverse sequence specific primers or the intercalating dye, SYBR green. All primers and probes utilized for quantitative analysis for each target gene are presented in Tables 5.1 and 5.2. One hundred nanograms of synthesized cDNA were assayed for each sample in duplicate. Thermal cycling conditions using the dual labeled probe were 50^{o}C for 30 min, 95^{o}C for 15 min, followed by 40 repetitive cycles of 95^{o}C for 15 sec and a combined annealing/extension stage, 59^{o}C for 1 min. Fluorescent data acquisition was done during the annealing/extension phase when using the dual-labeled probes. Cycling conditions for SYBR green detection were 50^{o}C for 30 min and 95^{o}C for 15 min, followed by 40

repetitive cycles of 95 for 15^{o}C sec and variable annealing temperature for 30 sec, 72^{o}C for 33 sec and a variable temperature during fluorescent detection for 33 sec. Fluorescence detection temperature was determined by evaluating melting curve analysis for the samples and the no template control amplification plot. Detection temperatures were set at a temperature when the intended target was the only contributing factor to fluorescence. 18S ribosomal RNA was assayed as a normalization control to correct for loading discrepancies. Following RT-PCR, quantitation of gene amplifications were made by determining the cycle threshold (C_T) based on the fluorescence detected within the geometric region of the semilog view of the amplification plot. Relative quantitation of target gene expression was evaluated using the comparative C_T method as previously described [Hettinger et al. 2001; Ashworth et al., 2006]. The ΔC_T value was determined by subtracting the target C_T of each sample from its respective ribosomal 18S C_T value. Calculation of $\Delta\Delta C_T$ involves using the single greatest sample ΔC_T value (the sample with the lowest expression) as an arbitrary constant to subtract from all other sample ΔC_T values. Relative mRNA units for each sample were calculated assuming an amplification efficiency of 2 during the geometric region of amplification, and applying the equation, $2^{\Delta\Delta Ct}$. Relative units in figures are presented as mean ± SEM.

Table 5.1. Primer and probe sequence information for the quantitative amplification of each target gene.

NCBI Assession[a]	Target[b]	Primers (Forward/Reverse) and Probe[c]	Fluorescent Reporter[d]	Amplicon Size[e]
Ssc.30027.1.A1_at	**IL-1β**	(F) GCATGCTATAGTCTTGGCTGTCA (R) GCAAAGACACCTTCCCGCTTTGAA (P)TAGGCTGACCTGTTTCTCCCACT	5'Fam'3	174 bp
Ssc.26146.1.S1_at	**IL-1β RAP**	(F) TGCATCAACACCAGCCAAAGGATG (R) TTAGGCTGACCTGTTTCTCCCACT (P)GCATCAACACCAGACCTGTACCTGT	5'Fam'3	304 bp
Ssc.29054.1.A1_at	**IL-1β RI**	(F) TGCTTTGCTTCTGACTCGGCTCTA (R) TCTGTTTCACGGGAGGGTTTGACT (P)ATGCTATAGTCTTGGCTGTCACACCA	5'Fam'3	159 bp
Ssc.5053.1.S1_at	**COX 1**	(F) TACATGCCACAGGTCGCTCATCTT (R) TCAGGCAAGAATTCATCTCCCGGT (P) CAACACTTCACCCACCAGTTCTTC	5'Fam'3	132 bp
Ssc.14561.1.S1_at	**COX 2**	(F) AGGACAACAGCTCCATCATCTGCT (R) ACTCGCAGAACTGGCCGTAAATCT (P)AATACCGCAAGCGCTTTCTGCTGAAGCCCT	5'Fam'3	196 bp

[a]Gene bank Assession ID given by NCBI for which the probe set was designed.
[b]The target gene name was determined through manual annotation of the EST sequence used during the generation of the probe set.
[c]Forward and reverse primers for each target gene. The forward primer sequence is above the reverse for each target gene, and the probe is listed last below the primers. Forward and reverse do not necessarily indicate the *in vivo* direction of transcription.
[d]Florescent probe set was used to measure amount of amplified target during each cycle of quantitative RT-PCR.
[e]Amplicon size refers to the product size of the amplified PCR product.

Table 5.2 Primer and probe sequence information for the quantitative amplification of each target gene.

NCBI Assession[a]	Target[b]	Primers (Forward/Reverse) and Probe[c]	Fluorescent Reporter[d]	Amplicon Size[e]
Ssc.30027.1.A1_at	**IL-18**	(F) GCATGCTATAGTCTTGGCTGTCA (R) GCAAAGACACCTTCCCGCTTTGAA (P)CAAAGACACCTTCCCGCTTTGAA	5'Fam'3	152 bp
Ssc.26146.1.S1_at	**Osteopontin**	(F) TGCATCAACACCAGCCAAAGGATG (R) TTAGGCTGACCTGTTTCTCCCACT (P)CAAAGACACCTTCCCGCTTTGAA	5'Fam'3	148 bp
Ssc.29054.1.A1_at	**TNF-α**	(F) TGCTTTGCTTCTGACTCGGCTCTA (R) TCTGTTTCACGGGAGGGTTTGACT (P)CAAAGACACCTTCCCGCTTTGAA	5'Fam'3	121 bp
Ssc.5053.1.S1_at	**Trophinin**	(F) TACATGCCACAGGTCGCTCATCTT (R) TCAGGCAAGAATTCATCTCCCGGT (P) CAAAGACACCTTCCCGCTTTGAA	5'Fam'3	174 bp

[a]The gene bank Assession ID given by NCBI for which the probe set was designed.
[b]The target gene name was determined through manual annotation of the EST sequence used during the generation of the probe set.
[c]Forward and reverse primers for each target gene. The forward primer sequence is above the reverse for each target gene, and the probe is listed last below the primers. Forward and reverse do not necessarily indicate the *in vivo* direction of transcription.
[d]The Florescent probe set, was used to measure amount of amplified target during each cycle of quantitative RT-PCR.
[e]Amplicon size refers to the product size of the amplified PCR product.

Uterine Luminal Flushing TNF-α Content

Total luminal content of TNF- α in the uterine flushings were quantified using a commercial ELISA (R and D Systems, NJ) in accordance with manufacturer's specifications. Samples were analyzed in duplicate with a single assay. The intra-assay coefficient of variation for the assay was 4.6%.

Uterine Luminal Flushing IL-1β Content

Total luminal content of IL-1β in the uterine flushings were quantified using a commercial ELISA (R and D Systems, NJ) in accordance with manufacturer's specifications. Samples were analyzed in duplicate with a single assay. The intra-assay coefficient of variation for the assay was 3.9%.

Quantitation and Statistical Analysis

Normalized QT-RT-PCR ΔC_T values and uterine luminal content of IL-1β and TNF-α were analyzed using the PROC MIXED of the Statistical Analysis System. Due to considerable differences in endometrial immunological state, analysis was performed separately by sampling times (4 or 36 h). The statistical model was factorial in nature, and the analysis tested the fixed effects of steroid (E or CO), infusion (IL-1β or Saline), and the interaction on the targeted endometrial mRNA expression. Significance ($P < 0.05$) was determined by probability differences of least squares means between steroid treatment and infusion on endometrium mRNA expression at either 4 or 36 h. If the

treatment effect was significant, ($P < 0.05$), treatment means were separated by using the PDIFF option of SAS.

Results

Endometrial IL-1β mRNA Expression

IL-1β mRNA expression at 4 or 36 h was unaffected by estrogen-treatment. A significant infusion effect ($P < 0.05$) was observed for endometrial IL-1β mRNA abundance at 4 h post-infusion. At 4 h, endometrial gene expression for IL-1β was 3.5-fold greater (Figure 5.1a) in the uterine horn of gilts infused with rIL-1β compared to the saline infused horn. Endometrial IL-1β mRNA expression not significantly different ($P > 0.05$) between infusion treatments at 36 h (Figure 5.1b).

Endometrial IL-1β RAP Expression

IL-1β RAP mRNA expression was unaffected due to infusion of rIL-1β at the 4 or 36 h A significant steroid effect ($P < 0.05$) was observed for IL-1β RAP endometrial mRNA expression at 4 and 36 h post-infusion. At 4 h, there was a 5.5-fold elevation in endometrial IL-1β RAP mRNA expression in gilts treated with E compared to CO (Figure 5.2 a,b). At 36 h there was a 2.5-fold increase in endometrial IL-1β RAP mRNA expression compareed to CO-treated gilts.

Endometrial IL-1β Receptor Type 1 Expression

IL-1 R1 endometrial mRNA expression was not effected by estrogen treatment at 4 or 36 h. A significant infusion effect ($P < 0.05$) was observed in endometrial IL-1β R1 mRNA expression at 4 h (Figure 5.3a). There was a 4-fold increase in endometrial IL-1 R1mRNA expression in the uterine horn infused with rIL-1β compared to uterine horn that received saline. Although rIL-1β had a significant effect on endometrial IL-1 R1 mRNA expression at 4h, it was not significantly different at 36 h (Figure 5.3b)

IL-1β Protein in Uterine Flushing

A significant infusion ($P < 0.05$) and steroid effect ($P < 0.05$) was observed for content of IL-1β protein in uterine flushings (Figure 5.4a). Infusion of rIL-1β increased the uterine luminal content of IL-1β from 8000 to 11,000 pg compared to the uterine horn receiving saline. At 36 h, IL-1β and estrogen exposure did not affect uterine secretion of IL-1β (Figure 5.4b).

Endometrial COX-1 mRNA Expression

No significant infusion or steroid effect ($P > 0.05$) was observed in endometrial COX-1 mRNA abundances at 4 h (Figure 5.5a) or 36 h (Figure 5.5a). Endometrial mRNA expression of COX-1 did exhibit a tendency to increase due to estrogen-treatment ($P = 0.06$), however, at 36 h was similar between E and CO gilts.

Endometrial COX-2 mRNA Expression

COX-2 mRNA expression at 4 or 36 h was not significantly affected by treatment of gilts with estrogen. A significant infusion effect ($P < 0.05$) was observed for

endometrial COX-2 mRNA abundances at 4 h (Figure 5.6a). Endometrial mRNA expression of COX-2 was increased by approximately 4-fold in the uterine horn infused with rIL-1β compared to the horn receiving saline. Although rIL-1β had a significant effect on endometrial COX-2 mRNA expression at 4 h, expression was not different at the 36 h (Figure 5.6b)

Endometrial COX-2 Immunohistochemistry (IHC)

COX-2 protein expression was unaffected due to estrogen at 4 or 36 h. There was an increase in localization for endometrial COX-2 protein at 4 h post-rIL-1β infusion (Figure 5.7a). Localization of COX-2 protein was observed primarily in uterine luminal epithelium, and to a lesser degree the glandular epithelium. Infusion of saline into the contralateral horn failed to induce COX-2 protein expression in uterine luminal and glandular epithelium.

Endometrial Osteopontin mRNA Expression

Osteopontin mRNA expression was unaffected by infusion with rIL-1β. Although estrogen increased endometrial OPN mRNA expression by 3-fold at 4 h, the increase was not statistically significant (Figure 5.a). A significant steroid effect ($P < 0.05$) was detected for endometrial OPN expression at 36 h (Figure 5.8b). Gilts treated with estrogen exhibited a 13-fold increase in the endometrial OPN mRNA expression compared to gilts that received CO.

Endometrial TNF-Protein Concentration in Uterine Flushings

A significant steroid effect ($P < 0.05$) was detected for TNF-α protein content in the uterine flushings at 4 h (Figure 5.9a), however estrogen had no effect at the 36 h (Figure 5.9a). Estrogen significantly increased the uterine luminal content of TNF-α protein at 4 h compared to saline treated gilts, 10,756 and 8,680 pg respectively. TNF-α protein in the uterine lumen was increased by infusion of rIL-1β ($P <0.05$) at 36 h but not at 4h. The infusion of rIL-1β increased TNF-α protein in the uterine lumen compared to the saline treated horn (36,988 and 9,725 pg respectively).

Endometrial IL-18 mRNA Expression

Infusion or steroid treatment did not significantly ($P > 0.05$) effect endometrial IL-18 mRNA abundance at either 4 h or 36 h (Figure 5.10a,b).

Endometrial Trophinin mRNA Expression

Trophinin endometrial mRNA expression was not affected by uterine rIL-1β infusion at 4 or 36 h. A significant steroid effect ($P < 0.05$) was detected for endometrial trophinin mRNA expression at 36 h (Figure 5.11a). Treatment of gilts with estrogen increased the mRNA expression of trophinin by 3.5-fold compared to gilts that received CO. Although estrogen had a significant effect on trophinin mRNA expression at 36 h, there was no statistical difference at the 4 h (Figure 5.11b)

Figure 5.1. IL-1β mRNA expression was unaffected due to estrogen at 4 or 36 h. A significant infusion effect ($P < 0.05$) was observed for endometrial IL-1β mRNA abundance at 4 h post-infusion. A) At 4 h, endometrial gene expression for IL-1β was 3.5-fold greater in the uterine horn infused rIL-1β compared to saline (SAL). B) Endometrial gene expression for IL-1β at 36 h was not significantly different ($P > 0.05$) between rIL-1β and SAL infused horns. Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference ($P<0.05$) between infusion and steroid endometrium comparing time 4 h and 36 hpost-treatment (n = 5/time /status)

Figure 5.1a

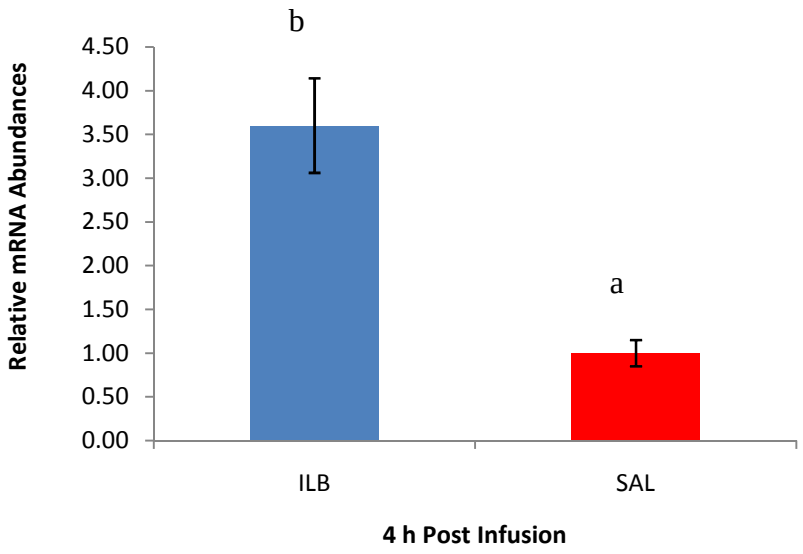


Figure 5.1b

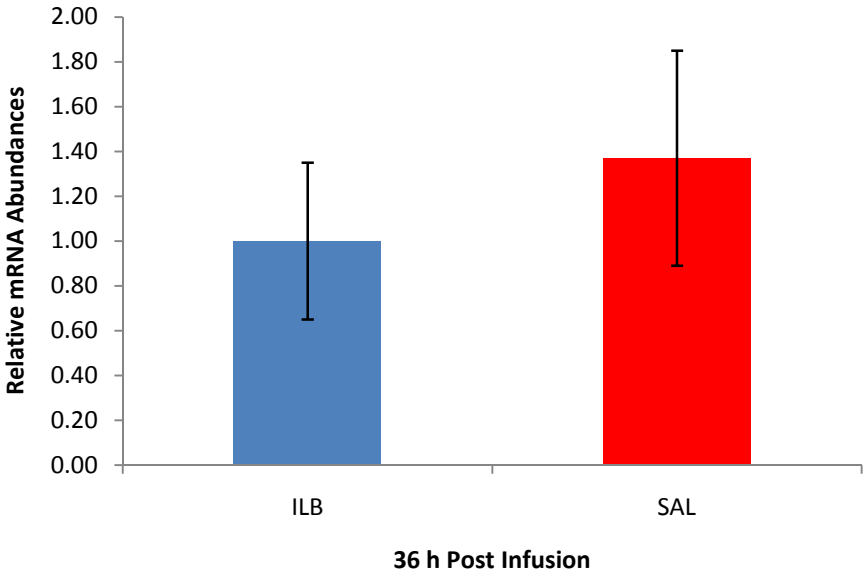


Figure 5.2. *IL-1β RAP* mRNA expression was unaffected due to infusion of rIL-1β at 4 or 36 h. A significant steroid effect was observed (P > 0.05) for endometrial *IL-1β RAP* mRNA abundance at 4 h (a) and 36 h (b) post-treatment. Estrogen increased *IL-1β RAP* at 4 h approximately 5.5-fold, and over 2.5-fold at 36 h. Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference (P<0.05) between infusion and steroid endometrium comparing 4 hand 36 h post-treatment (n = 5/time/status)

Figure 5.2a

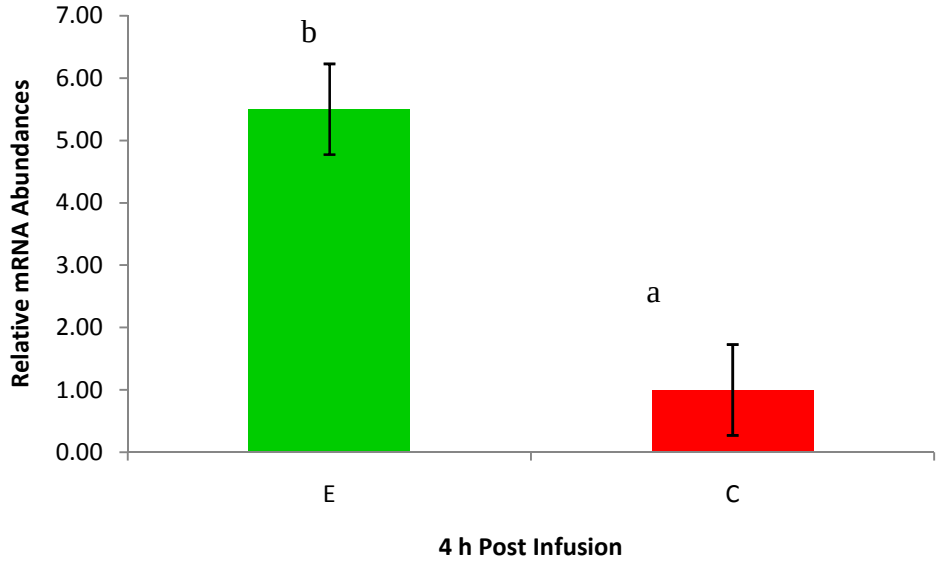


Figure 5.2b

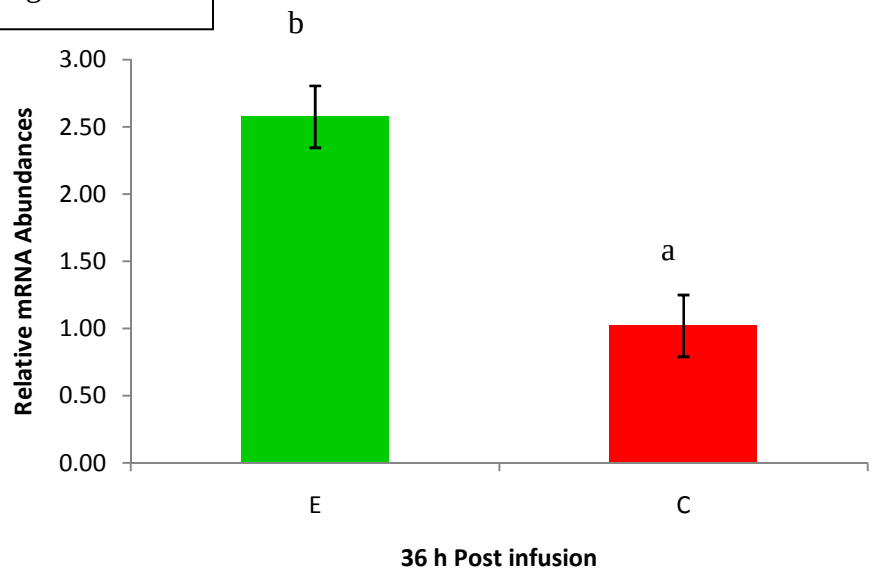


Figure 5.3. *IL-1R1* mRNA expression was unaffected due to estrogen of at 4 or 36 h A significant infusion effect ($P < 0.05$) was observed for endometrial *IL-1β R1*mRNA abundance at 4 h post-infusion. A) At 4 h, endometrial mRNA abundances for *IL-1β R1* was approximately 4-fold greater in the uterine horn infused with rIL-1β compared with saline (SAL). B) Endometrial gene expression for *IL-1β R1* at 36 h was not significantly different ($P > 0.05$). Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference ($P<0.05$) between infusion and steroid endometrium comparing 4 and 36 h post-treatment ($n = 5$/time /status).

Figure 5.3a

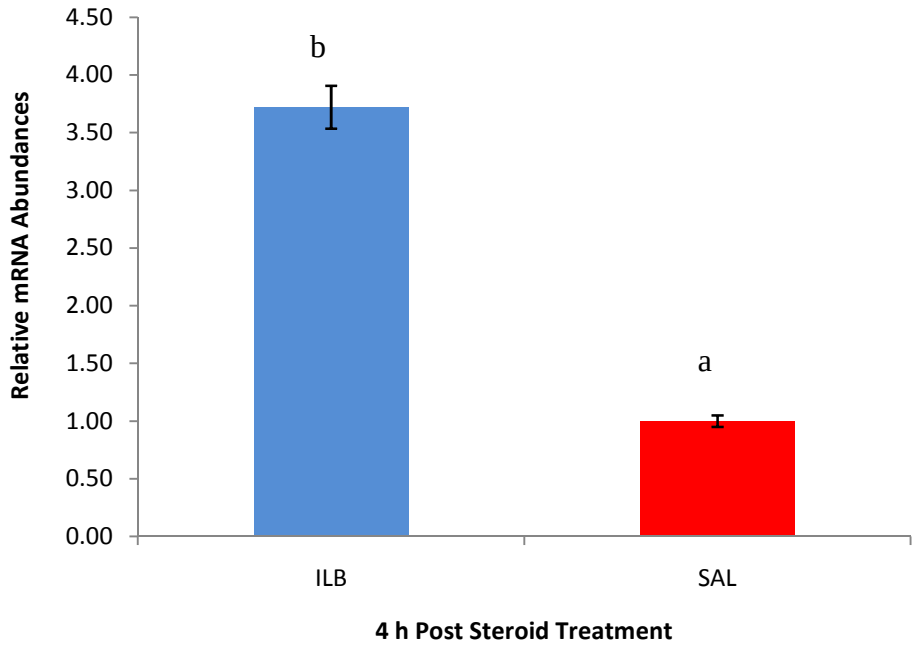


Figure 5.3b

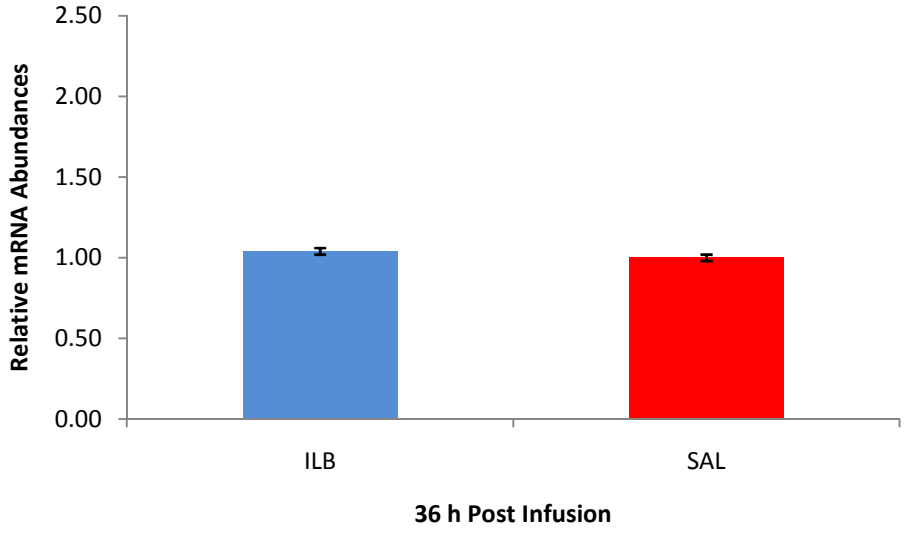


Figure 5.4. IL-1β assay to determine relative amounts of total protein collected from gilts at 4 and 36 h post-infusion and/or steroid treatment (n = 5/time/status). A significant steroid effect was observed for IL-1β for 4 and 36 h post-steroid treatment ($P < 0.05$), and at 36 h there no significant IL-1β effect ($P > 0.05$). Relative optical density for each sample was corrected for total protein concentration in the sample. Data is presented as relative pg mean ± SEM.

Figure 5.4a

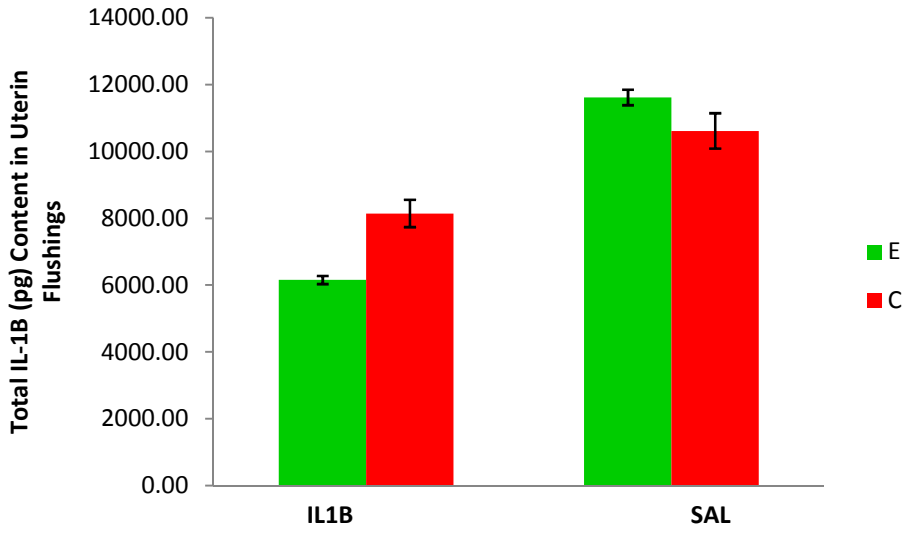


Figure 5.4b

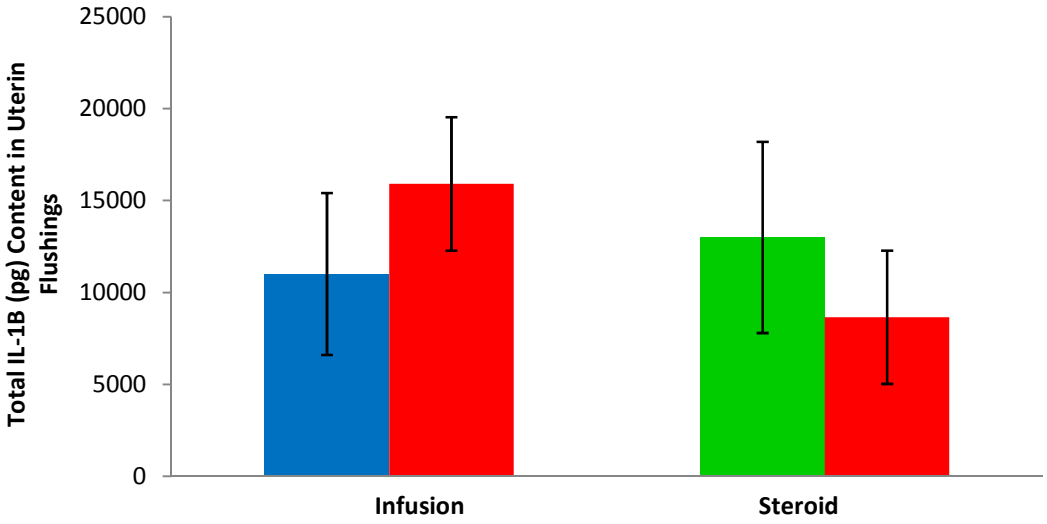


Figure 5.5. *COX-1* mRNA expression was unaffected due to estrogen-treatment or infusion of rIL-1β at 4 or 36 h. A tendency was observed (P = 0.06) for endometrial *COX-1* mRNA abundance at 4 h post-estrogen exposure (figure 5.5a), however, there was no estrogen effect on *COX-1* mRNA expression at 36 h (figure 5.5b). Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference (P<0.05) between infusion and steroid at 4 h and 36 h post-treatment (n = 4/time/status)

Figure 5.5a

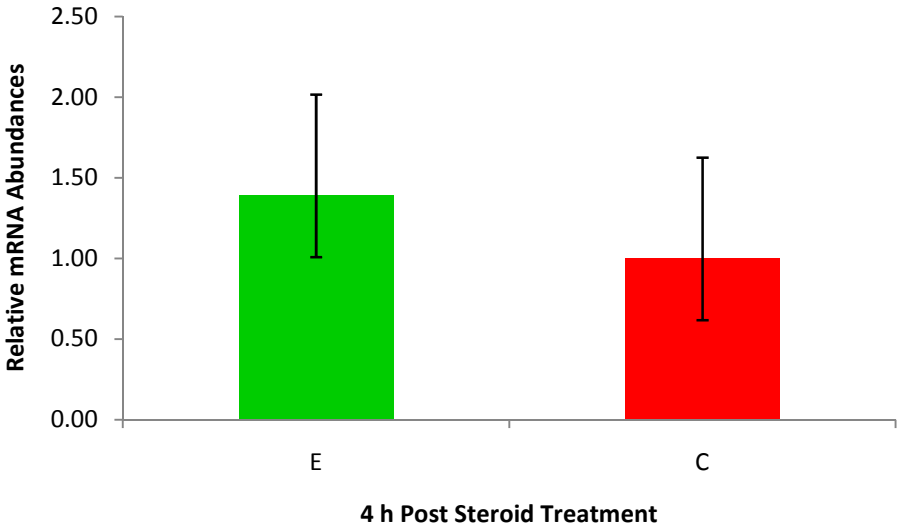


Figure 5.5b

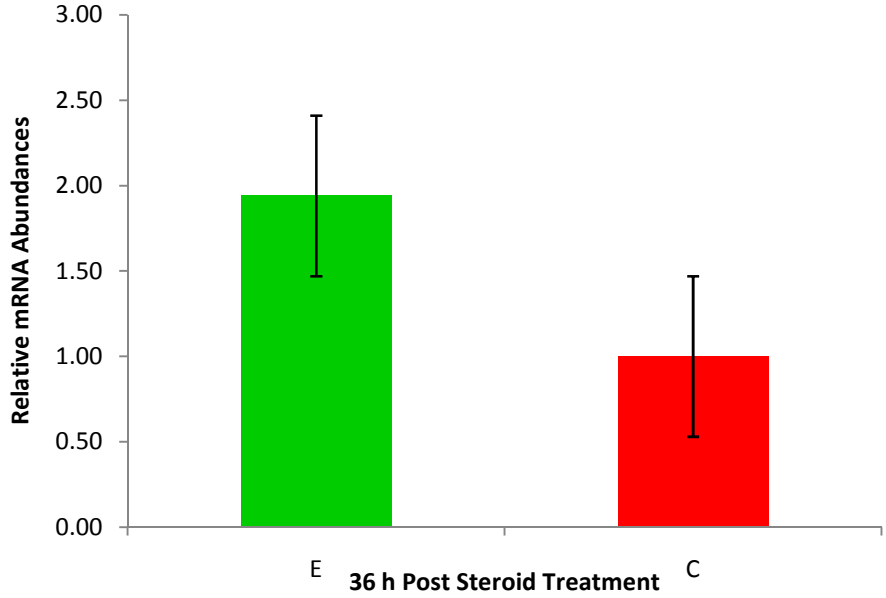


Figure 5.6. COX-2 mRNA expression was unaffected due to estrogen at 4 or 36 h. A significant infusion effect ($P < 0.05$) was observed for endometrial COX-2 mRNA abundance at 4 h post-infusion. A) Endometrial mRNA abundance for COX-2 was approximately 4-fold greater in the uterine horn infused with rIL-1β compared to saline (SAL). B) Endometrial gene expression for COX-2 was not significantly different ($P > 0.05$) between infused horns. Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference ($P<0.05$) between infusion and steroid endometrium comparing 4 h and 36 h post-treatment (n = 4/time/status).

Figure 5.6a

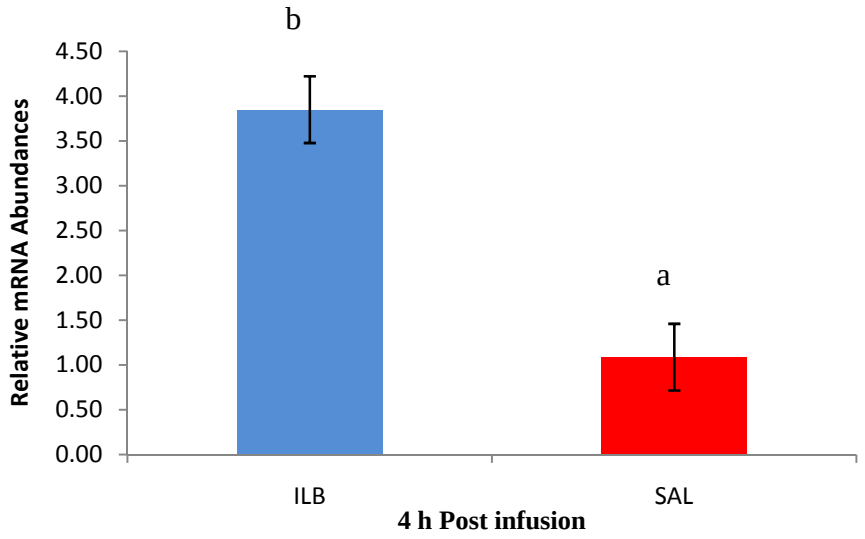


Figure 5.6b

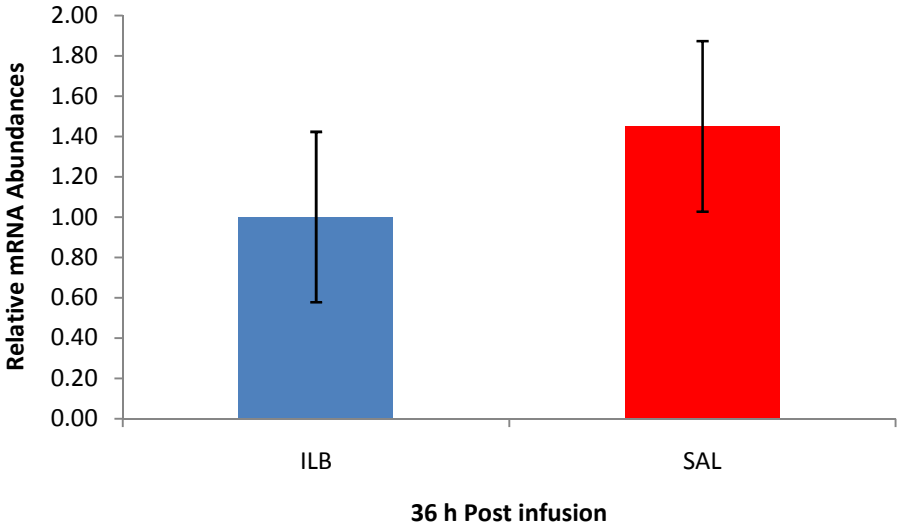


Figure 5.7. Localization for endometrial COX-2 protein at 4 h post-saline and rIL-1β infusion. Localization of COX-2 protein was localized primarily to uterine luminal epithelium, and to a lesser degree the glandular epithelium. Infusion of saline into the contra-lateral horn failed to induce COX-2 protein expression in uterine luminal and glandular epithelium. Image was optimized using 10x objective. Tissue-bound primary antibody was detected with fluorescein-conjugated secondary antibody (Chemicon International, Temecula, CA). Slides were overlaid with Prolong antifade mounting reagent (Molecular Probes, Eugene, OR) and a cover glass added. Tissues were fixed in -20°C methanol, washed in PBS containing 0.3% Tween 20 in PBS, blocked in 10% normal goat serum, and incubated overnight at 4°C with the primary antibody or IgG (negative control).

Figure 5.7 a,b

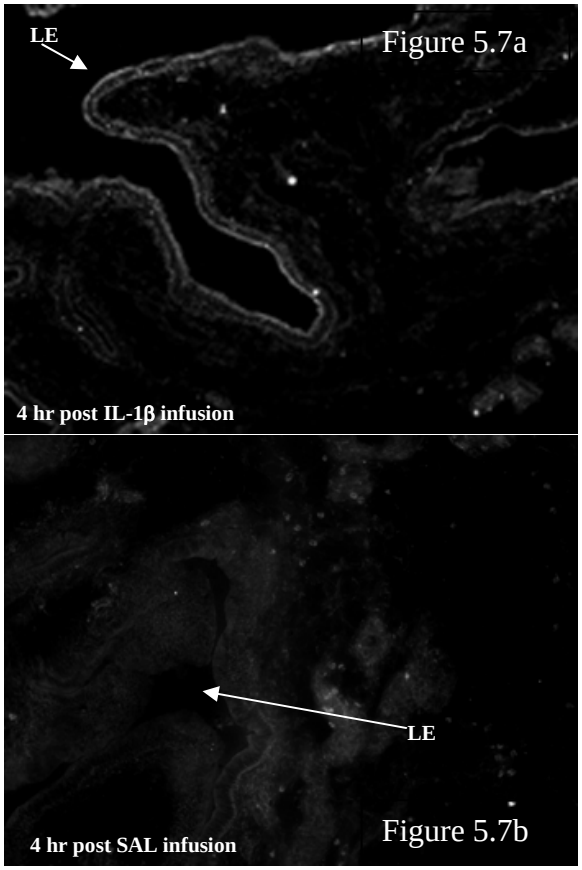

LE
Figure 5.7a
4 hr post IL-1β infusion
LE
4 hr post SAL infusion
Figure 5.7b

Figure 5.8. Osteopontin mRNA expression was unaffected by infusion with rIL-1β at 4 or 36 h. A significant steroid effect ($P < 0.05$) was observed for endometrial OPN mRNA abundance at 36 h post-infusion. A) Endometrial gene expression for OPN at 4 h was not significantly different ($P > 0.05$) between estrogen and corn oil treated gilts. B) Endometrial mRNA abundances for OPN was approximately 14-fold greater at 36 h in gilts treated with estrogen compared with corn oil. Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference ($P<0.05$) between infusion and steroid endometrium comparing time 4 h and 36 h post-treatment ($n = 5$/time/status).

Figure 5.8a

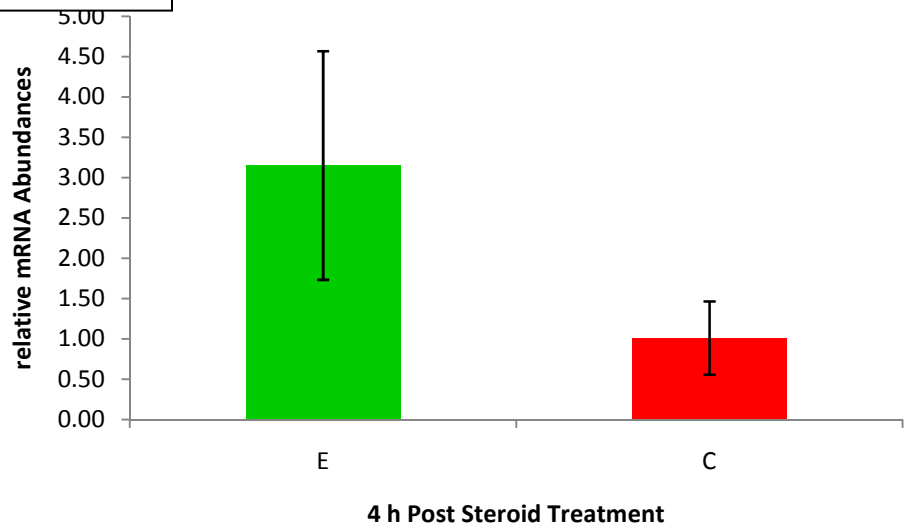


Figure 5.8b

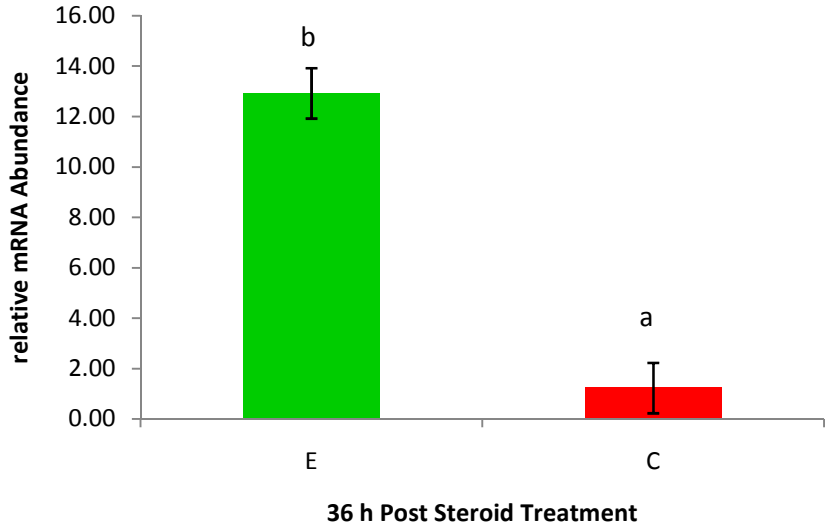


Figure 5.9. TNF-α assay to determine relative amounts of total protein collected from gilts at 4 h and 36 h post-infusion and/or steroid treatment(n = 5/time/status). A significant steroid effect was observed for TNF-α for 4 h post-estrogen treated gilts ($P < 0.05$), and significant rIL-1β effect ($P < 0.05$) was detected at 36 h. Relative optical density for each sample was corrected for total protein concentration in the sample. Data is presented as relative pg mean ± SEM.

Figure 5.9a

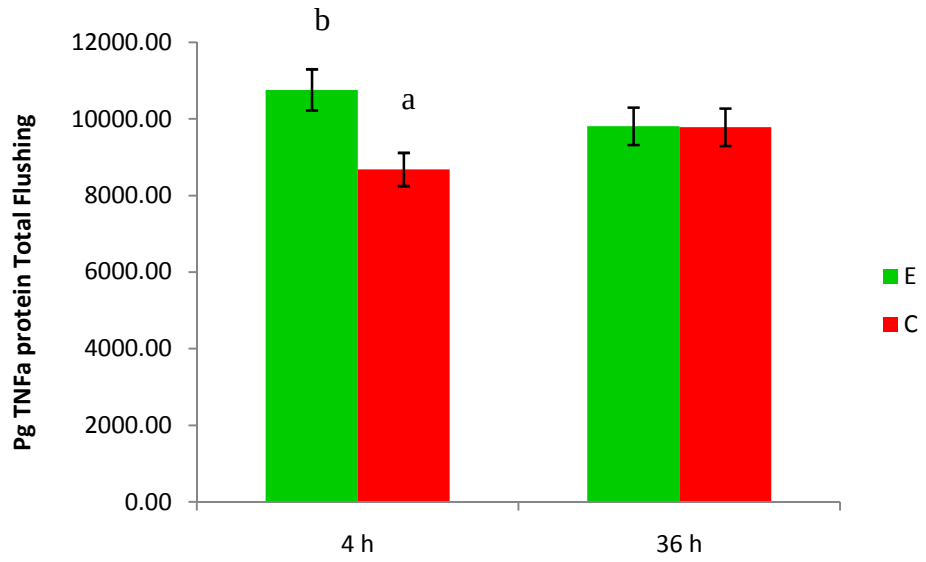


Figure 5.9b

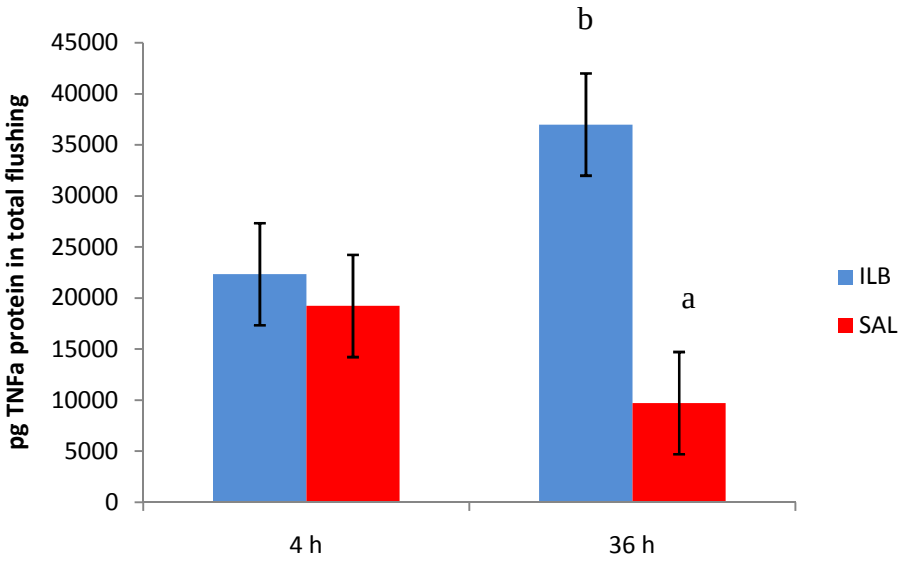


Figure 5.10. No statistically significant steroid or infusion effect ($P > 0.05$) was observed for endometrial IL-18 mRNA abundance at 4 h (a) or 36 h (b) post-treatment. Abundance of mRNA was calculated from the real-time PCR analysis as described in *Methods and Materials*. Relative mRNA abundance is presented as mean ± SEM.

Figure 5.10a

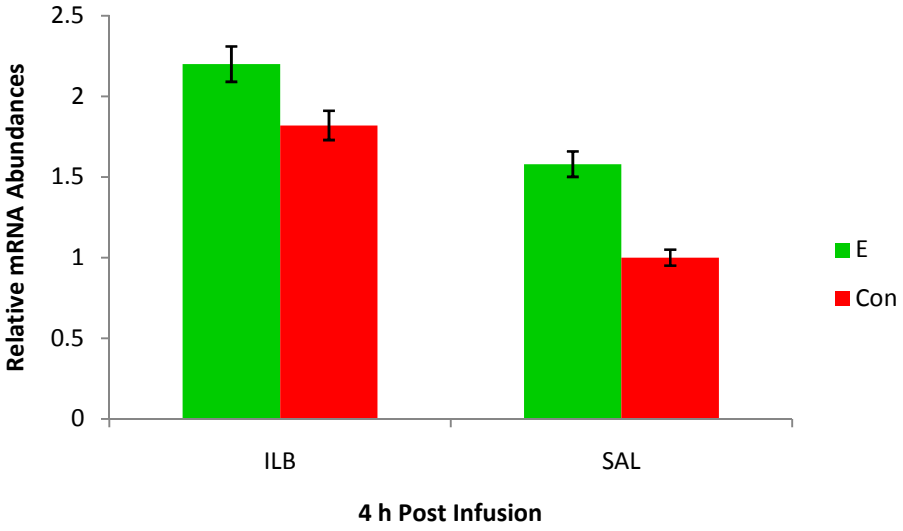


Figure 5.10b

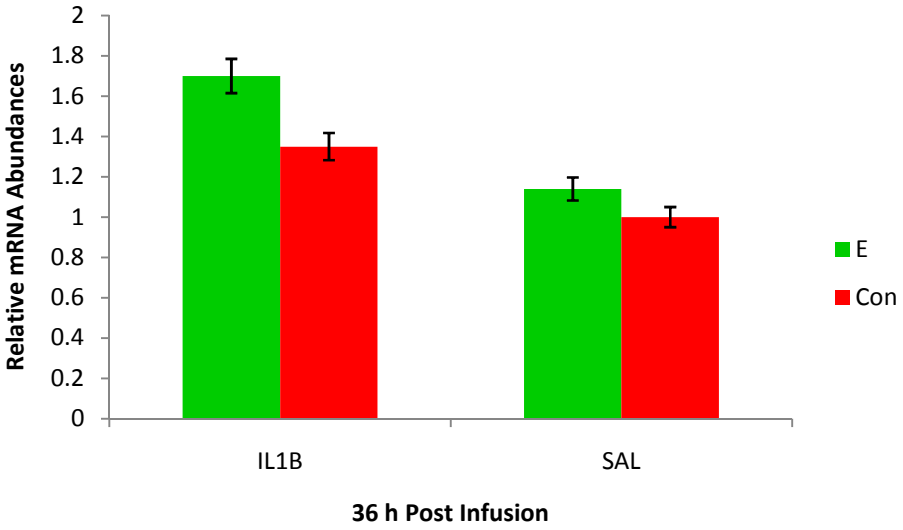


Figure 5.11. Trophinin mRNA expression was not affected uterine infusion with rIL-1β at 4 or 36 h A significant steroid effect ($P < 0.05$) was observed for endometrial trophinin mRNA abundance at 36 h post-infusion. Endometrial gene expression for trophinin at 4 h (Figure 5.10a) was not significantly different ($P > 0.05$) between infusion horns. Endometrial mRNA abundances for trophinin at 36 h (Figure 5.10b) were approximately 3.5-fold greater ($P < 0.05$) in the uterine horn infused with rIL-1β compared with saline. Relative mRNA abundance is presented as mean ± SEM. Bars without a common superscript represent a statistical difference ($P<0.05$) between infusion and steroid endometrium comparing time 4 hand 36 h post-treatment (n = 5/time/status).

Figure 5.11a

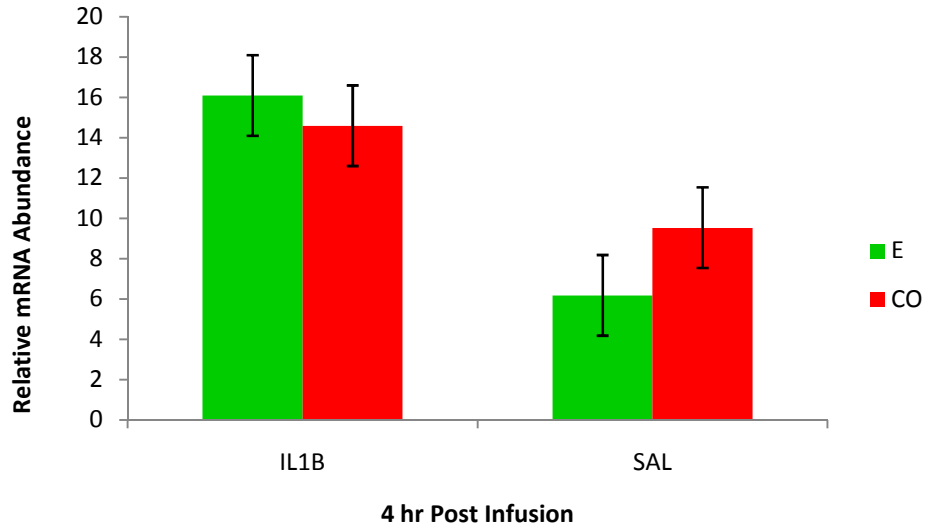
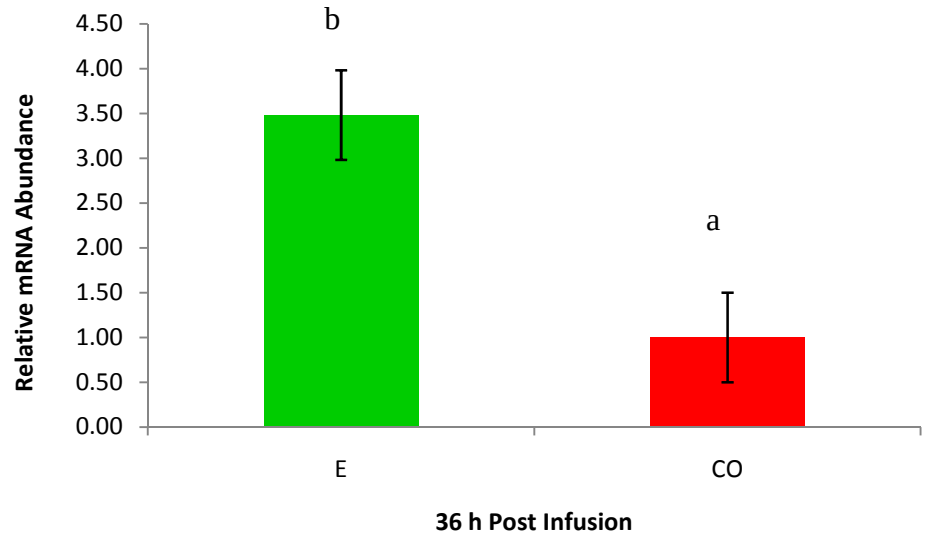


Figure 5.11b



Discussion

During early pregnancy in the pig, the induction of obligatory prostaglandin synthesis may be related to the conceptus synthesis and production of IL-1β which is transiently expressed with estrogen during establishment of pregnancy and rapid trophoblastic elongation (Tou et al., 1996b; Ross et al., 2003a). Interleukin-1β is capable of inducing phospholipase A2 mRNA expression (Kol et al., 2002), and cycoloxygenase-2 mRNA expression (Huang et al., 1998) in reproductive tissues of various species. The presence of both IL-1β receptors (IL-1β R1, -2) required for proper IL-1β signaling in the elongating pig conceptuses (Ross et al., 2003a) suggest that this may be a valid role of conceptus synthesized and released IL-1β. However, the transient, pregnancy specific up-regulation of both receptors in the uterine endometrium suggests a role for the cytokine with respect to induction of a uterine response as well. Interleukin-1 receptor type 1 (IL-1R1) and IL-1 receptor accessory protein (IL-1β RAP) are both required for IL-1β signaling to occur. The present study indicated that IL-1β acts to increase IL-1β and IL-1β R1 mRNA, while estrogen stimulates an increase in IL-1β RAP mRNA. The expression of IL-1R1 in the conceptus and endometrium in the presence of elevated IL-1β levels suggests that the secretion of IL-1β may have both an autocrine and paracrine effect on the endometrium (Ross et al., 2003a). Ross et al. (2003a) hypothesized that due to the potential autocrine signaling, conceptus synthesis and release of IL-1β may be a potential contributor to trophoblastic elongation. Moreover, the sharp decline in IL-1β protein in the porcine uterine flushings observed by Ross (2003) and Ashworth (2006) may be the result of an increase in IFN-γ. The extremely sharp decline in protein around day 15 of pregnancy in the pig is conspicuously around the time of a copious increase in

conceptus originated IFN-γ, which has been demonstrated to attenuate IL-1β protein expression levels in vivo in the mouse (Xia et., 2007). These results may provide a possible mechanism by which the porcine embryo can rapidly turn off its own production of IL-1β protein in the uterine environment, and in part may need to move to a more productive cytokine during the time of attachment to the uterine surface.

The inflammatory response invoked by the conceptus, most likely through the actions of IL-1β, would be one of the fundamental mechanisms created by the conceptus for crosstalk with the endometrium. Moreover, studies in the mouse have demonstrated that TH2 proliferation requires IL-1β expression by antigen presenting cells. These data suggest a possible role of IL-1β in regulating the magnitude of the maternal immune response which is permissive to the allograft conceptus antigens in the pig. Thus, the precise actions of IL-1β have not been completely elucidated, however, it appears that it has an exerted effect on the TH1 and TH2 repertoire at the site of attachment, which are both obligatory for early establishment of pregnancy and acceptance of paternal alloantigens (Mellor and Munn, 2000).

Uterine immunological stimulation is followed by the formation of a conceptus-endometrium extracellular matrix (ECM). IL-1β has been shown in human studies to promote the formation of the ECM through the induction of cell surface integrin binding factors (Yang et al., 1999). The current study demonstrated a need for both IL-1β and estrogen for proper production and maintenance of the ECM through matrix metalloproteinase (MMP) regulation (Salamonsen and Wolley 1999). Additionally, the current study provides evidence that estrogen acutely increased the content of TNF-α in the uterine luminal fluid 4 h post-estrogen exposure. Given these data, recent studies

have demonstrated that the ECM linker molecule tumor necrosis factor α stimulated gene 6 (TSG-6), which is stimulated by TNF-α, is found in the uterine luminal fluid of the pregnant gilt, complexed with key ECM components during pregnancy (Ashworth and Geisert, unpublished results). This may be one pathway for estrogen to begin to stimulate ECM production. Also, the current study demonstrated that the key attachment factors OPN and trophinin are exclusively up-regulated through estrogen exposure in the porcine endometrium at 36 h post-estrogen treatment and not through IL-1β secretion. These data are congruent with studies demonstrating trophinin is regulated at some level through estrogen exposure in the mouse (Suzuki et al., 2007), and OPN is regulated through estrogen in the porcine uterus (White et al., 2006). Cyclooxygenase -1 and -2 are the major source for the production of the required prostaglandins needed during early pregnancy. During ECM stability, PG's have been shown to induce TSG-6 to aid in ECM integrity. Furthermore, the current study provided evidence that IL-1β increases endometrial COX-2 mRNA abundance and induced expression in COX-2 protein in the uterine luminal epithelium. These data would be consistent with changes in COX-2 expression during early pregnancy in the pig reported by Ashworth et al. (2006). Therefore, conceptus IL-1β secretion may help maintain ECM intergrity indirectly through induction of endometrial COX-2.

Data from the current study suggests that IL-1β acts predominantly as an acute inflammatory response and up-regulation of IL-1β's own mRNA and protein, and its ancillary signaling appendages such as IL-β R1. This activation is rapid and appears to be lost quickly without continual stimulation. Furthermore, other up-regulated genes from the infusion of IL-1β are acute inducible products of the NF-kB pathway,

suggesting IL-1β inducing the NF-kB works through the IL-1β R1. These data would be in concurrence with data from Strakova et al., (2005), where the baboon psuedopregnant female do not express COX-2 protein in the endometrium but in the presence of the conceptus IL-1β secretions COX-2 is induced. In contrast, with exception of IL-1β RAP, estrogen activation of several genes occurs after 24 h. The estrogen induction of trophinin and OPN further dictates the role of estrogen as ECM supportive and facilitates attachment based on its timing and appearance during the window of implantation.

Chapter VI

Porcine Endometrial Expression of Acute Phase Extraceullar Matrix Components (ECM) Following Estrogen Disruption of Pregnancy.

Abstract

In the pig, administration of estrogen to gilts on days 9 and 10 of pregnancy causes conceptus fragmentation and death between days 15 to 18 of gestation. Conceptus degeneration is associated with breakdown of the microvilli surface glycocalyx on the lumenal epithelium (LE). We previously identified endometrial expression of inter-alpha-trypsin inhibitor (ITI) and hyaluronic acid (HA), which are key components of extracellular matrix (ECM). Tumor necrosis factor stimulated gene-6 (TSG-6) serves as a linker for ECM expansion and is stimulated by prostaglandin E (PGE). We hypothesized that early estrogen administration alters the normal expression of ECM components forming LE glycocalyx. Bred gilts (4gilts/trt/day) were treated with either 5 mg estradiol cypionate (E) or corn oil (CO) on days 9 and 10 of gestation. Uterus was surgically removed on either day 10, 12, 13, 15 and 17 of gestation and endometrial tissue snap frozen in liquid nitrogen. Endometrial TNF-α, kallikrein 4, TSG-6, IL-6, and ITIH expression was detected throughout normal pregnancy. TSG-6 protein was detected across all days of gestation and was not affected by E. Uterine content of PGE increased across the days of pregnancy ($P<0.001$). Present study demonstrates that E-treatment does not alter the pattern of endometrial ITIH and TSG-6 gene expression which may play an important role in maintenance of LE glycocalyx during implantation in the pig.

Introduction

Porcine conceptuses attach to the glycocalyx present on the apical border of the surface epithelium occuring during the window of implantation on days 13 through 16 which is preceeded by rapid expansion of the trophoblastic membrane on day 12 of pregnancy (King *et al*., 1982; Stroband and Van der Lende, 1990; Geisert and Yelich, 1997). Elongation of the trophoblastic membrane involves changes in cellular morphology through activation of a plethora of embryonic factors (Ross *et al*., 2008) as well as the presentation of uterine factors associated with adhesion and attachment to the epithelial surface that will allow incessant attachment of the placenta throughout gestation (Bowen *et al*., 1996; Jaeger et al., 2001). Apposition and adhesion of the trophectoderm to the uterine epithelial apical surface provides a localized increase in transcapillary transport (Keys and King, 1988) as well as an increase in uterine blood flow (Ford and Christensen, 1979). The increase in vascular flow occurrs concomitantly with estrogen synthesis and secretion by the elongating conceptuses (Ford *et al*., 1982; Geisert *et al*., 1982), which is essential for the maintenance of the corpora lutea and establishment of pregnancy in the pig.

Many of the endometrial responses evoked by the developing porcine conceptuses during this period of early development and uterine attachment bear a resemblance to the acute phase response induced during tissue inflammation (Salier *et al*, 1996; Geisert and Yelich, 1997). A glycoprotein member of the acute phase proteins, inter-α-trypsin inhibitor heavy chain 4 (ITIH4), has previously been isolated and characterized in the porcine endometrium (Geisert *et al*., 1998). ITIH4 is one of four members of the inter-α-

trypsin inhibitor heavy chain family of protease inhibitors, which is composed of three other heavy chains (ITIH1, ITIH2 and ITIH3) and one light chain, termed bikunin (Salier *et al*., 1996). Stabilization of the extracellular matrix occurs via the linkage of the various family members (Bost *et al*., 1998). Binding of bikunin to ITIH occurs in three forms, pre-α-inhibitor (PαI), inter-α-like inhibitor (IαLI) and inter-α-inhibitor (IαI). PαI is a complex of bikunin bound to a single heavy chain of ITIH3, IαLI is comprised of bikunin and ITIH2 (Enghild *et al*., 1991) while covalent binding of bikunin to ITIH1 and ITIH2, forms IαI (Bost *et al*., 1998). Bikunin attaches to the heavy chains through binding to a chondroitin sulfate chain. The serine protease inhibitory activity of IαI complex originates from bikunin, a 30 kDa serine protease inhibitor containing two kunitz-type inhibitory domains (Xu *et al*., 1998), as the heavy chains are devoid of protease inhibitory activity. ITIH4 is exclusive from other inter-α-trypsin inhibitor heavy chains as the Asp-Pro-His-Phe-Ile-Ile sequence for binding to bikunin is absent (Enghild *et al*., 1989; Salier *et al*., 1996; Bost *et al*., 1998;) and, in contrast to the other ITIH, is a substrate for the plasma serine protease kallikrein (Nishimura *et al*., 1995). Since ITIH function as hyaluronic-binding proteins, so ITI has been proposed to serve an essential role with stabilization of the extracellular matrix (Bost *et al*., 1998). Evidence for a role of the ITI family in expansion of the cumulus oocyte complex (Chen *et al*., 1994; Chen *et al*., 1996) supports a principal function of the ITI family in matrix stabilization through interaction with hyaluronic acid. The noninvasive, superficial epitheliochorial placentation in the pig is associated with an attenuation of the uterine epithelial glycocalyx as well as interdigitation of the trophoblast to the uterine microvilli (Dantzer, 1985). Although there is limited information concerning the apical interactions between

the trophoblast and uterine surface epithelium, results suggest that changes in the glycocalyx play a major role in initial trophoblastic attachment (Bowen *et al*., 1996; Jaeger et al., 2001). Detection and characterization of ITIH4 (Geisert *et al*., 1998) and bikunin (Hettinger *et al*., 2001) in the porcine endometrium suggests that the ITI family may be associated with constituentive maintenance of the uterine apical glycocalyx necessary for trophoblastic attachment in the pig (Stroband and Van der Lende, 1990). Furthermore, 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 extracellular matrix (ECM) (Chen et al., 1994). Applying Chen's ovarian ECM model for oocyte ovulation, Geisert and coworkers (1998) suggested a similar role for ITIH4 stabilization of the uterine epithelial surface glycocalyx to prevent conceptus cellular erosion and invasion into the uterine stroma.

Rheumatoid arthritis studies have shown that patients with increased concentrations of ITI-HA in the synovial fluids are closely associated with chronic inflammation (Hutadilok et al., 1988). During inflammation tumor necrosis factor-

stimulated gene 6 (TSG-6) has been 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 linker module between different serum derived hyaluronin associated protein-hyaluronic acid complex (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, ITIH1 and ITIH2 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).

There are two decisive time periods during early pregnancy which most embryonic mortality occurs in the pig. The periimplantation period (Days 10 to 15), and post-placental attachment and expansion of the trophoblast (Days 18 to 40) have been shown to be the periods of greatest loss in regards to litter size. Pope et al (1994) estimated 30% of conceptus death occurs in early gestation (Days 10-18). Furthermore, Pope et al. (1986) demonstrated that administration of estradiol-17β (5 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 brake down in the uterine glycocalyx occurring approximately day 14 of gestation (Blair et al., 1991).

The objective of the present study was to evaluate if endocrine disruption of pregnancy alters ITIH 1, 2, 3, 4, TNF-α, kallikrein 4, TSG-6, and bikunin gene expression in the endometrium through alterations in PGE_2 concentrations in the uterus during early pregnancy of the pig.

MATERIALS AND METHODS

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 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.

Early Exposure of Pregnant Gilts to Estrogen

Bred gilts were randomly assigned one of the following treatment groups: Control (n = 20), i.m. injection of corn oil (2.5 ml) on days 9 and 10 of gestation or E (n = 20), 5 mg i.m. injection of estradiol cypionate (A.J. Legere, Scottsdale, AZ) on Days 9 and 10 of gestation. Gilts were hysterectomized on either day 10, 12, 13, 15 and 17 of

pregnancy. Following induction of anaesthesia with 1.8 ml im 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 flushed with 20 ml PBS, conceptuses were collected, flushings centrifuged to remove cellular debris, and the superatnant stored at -20°C. Endometrial tissue was harvested from the uterine horn and snap frozen in liquid nitrogen for subsequent RNA analysis both as previously described (Ross et al., 2006).

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 nm and purity was verified using the 260/280 ratio.

Quantitative 1-Step RT-PCR

Quantitative analysis of endometrial (ITIH 1, 2, 3, 4, TNF-α, kallikrein 4, IL-6, TSG-6, and bikunin) mRNA were analyzed 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 7500 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 (ITIH 1, 2, 3, 4, TNF-α, IL-6, TSG-6, kallikrein 4, and bikunin) (Table 1) were generated from porcine sequences previously established in our lab (Geisert et al., 2003). 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. Using the comparative C_T method (Ashworth et al., 2006), relative quantification and fold gene expression difference between treatment and day were determined for the endometrial samples. Differences in mRNA expression of (ITIH 1, 2, 3, 4, TNF-α, IL-6, TSG-6, kallikrein 4, and bikunin) 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

Table 6.1. PCR primer and probe sequences used for quantitative RT-PCR.

Gene	Forward Primer/Reverse Primer/Probe		GenBank Accession
ITIH1	Forward	5'-TGACTGGCGTGGACACTGA-3'	Y11546
	Reverse	5'-GCACAGGGTGTCCTCTTTCTG-3'	
	Probe	5'-CCCCACTTCATCATCCGCGTGC-3'	
ITIH2	Forward	5'-CGCGGTAGGACCTTCAGTGA-3'	Y11545
	Reverse	5'-GAAGTGCGGGTCATTTTCGA-3'	
	Probe	5'-AGGTAATGCCTCCTCCACACGTGATGA-3'	
ITIH3	Forward	5'-TGGTTTGTTCACGACAATGG-3'	D84320
	Reverse	5'-AGGACGACTCTGTCGATCGG-3'	
	Probe	5'-TGTCCCCGACCTGTTCTGAGTGG-3'	
ITIH4	Forward	5'-TGCGTACAAGTGGAAGGAAACA-3'	U43164
	Reverse	5'-CCCGCCTTGTCCATGGT-3'	
	Probe	5'-TCTACTCAGTGATGCCCGGCCTCAA-3'	
Bikunin	Forward	5'-TGTGGAGGCCTGCAGTCTC-3'	X53685
	Reverse	5'-CATCAAACGCCCAGAGCTG -3'	
	Probe	5'-CATCGTCTCCGGCCCCTTGCC-3'	
Kallikrien 4	Forward	5'-ATGTTCCTCCTGCTGACAGC-3'	X54374
	Reverse	5'- CCCTGGACACCTCTGTCACC-3'	
	Probe	5'- ATTCCTACACCATCGGGCTGGGTCT-3	
TNF-α	Forward	5'- CAAGGACTCAGATCATCGTCTCA-3'	X45736
	Reverse	5'- CGGCTTTGACATTGGCTACAA-3'	
	Probe	5'- ACCTCAGATAAGCCCGTCGCCCAC	
TSG-6	Forward	5'- TACAAGCTTACCTACGCAGAGGC-3'	X84554
	Reverse	5'- TTTCTGGCTGCCTCTAACTGCT-3'	
	Probe	5'- AGCGGTGTGTGAATACGAAGGTGGCCGTCT-3'	
IL-6	Forward	5'- AGCTATGAACTCCCTCTCCACAAG-3'	X64354
	Reverse	5'- TGCTTCTGGTGATGGCTACTGCCTTCCCT3'	
	Probe	5'- GGAAGAAGATGCCAAAGGTGATGC-3'	

as an arbitrary constant to subtract from all other ΔCT sample values. Fold differences in gene expression of the target gene are equivalent to $X^{-\Delta\Delta CT}$, where X is the efficiency of the amplification reaction.

Western Blot analysis of TSG-6

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 was assayed by Bradford Assay. Samples 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 was separated electrophoritcally in a 10% 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, 150mM 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 rat anti-human TSG-6 monoclonal antibody (1:10,000) (Gift from Dr Day, UK). After washing 3X with TTBS for 10 min each, the membrane was incubated with goat anti-rat secondary antibody (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).

Enzyme-Linked PGE_2 Competitive Binding Assays

Total content of prostaglandin E_2 (PGE_2) in the uterine flushings collected were quantified using a commercial ELISA (R&D Systems, Minneapolis, MN) in accordance with manufacturer's specifications. All standards and samples were assayed in duplicate. The intra-assay coefficient of variation for the PGE_2 assay was 5.6 %

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 evaluate endometrial ITIH 1, 2, 3, 4, TNF-α, TSG-6, IL-6, kallikrein 4, and bikunin mRNA expression and uterine flushing PGE_2 content included the effects of day, treatment (Con, E), and the day x treatment interaction. If the treatment effect was significant, ($P < 0.05$), treatment means were separated and compared by t-test using the PDIFF option of SAS. Additionally, due to unequal variances of PGE_2 the original data was transformed using log and square root transformations for the statistical analysis.

Results

Conceptus Development and Viability

Normal spherical conceptuses were recovered from the flushing media of Con and E-treated gilts on day 10 of pregnancy. On day 12 of pregnancy, a select few pregnancies

contained spherical conceptuses, but filamentous concetuses were recovered in the majority of the gilts. Viable peri-attachment conceptuses were recovered in both Con and E-treated gilts on day 13 of pregnancy; however, conceptuses collected on days 15 and 17 of the E-treated gilts were fragmented and degenerated.

ITIH 1, 2, 3, and 4, mRNA Expression

Endometrial mRNA expression for ITIH 1 and 3 (Figures 6.1 and 6.3) were unaffected by day of pregnancy or E treatment ($P > 0.05$). Endometrial mRNA expression for ITIH 2 was significantly affected by treatment (Figure 6.2). Endometrial gene expression for ITIH 4 was significantly affected by day of pregnancy (Figure 6.4).

Bikunin mRNA Expression

Endometrial gene expression for bikunin was not altered due to E-treatment, however there was a significant effect on day of pregnancy ($P < 0.05$). Although bikunin expression was similar on days 10, 12, and 13 of pregnancy, expression increased by 3-fold on day 15 and 5-fold on day 17

Kallikrein 4 mRNA Expression

Endometrial mRNA expression for kallikrein 4 exhibited a significant treatment by day interaction ($P < 0.05$). Kallikrein 4 mRNA expression was the highest in Con gilts on day 12 but E-Treatment severely attenuated kallikrein 4 mRNA expression by 8.5-fold on day 12 (Figure 6.6).

TNF-α Gene Expression

Endometrial gene expression for TNF-α (Figure 6.7) demonstrated a significant day effect ($P < 0.05$). TNF-α gene expression was greatest on day 15 of pregnancy and lowest on days 10, 12, and 13. Days 10, 12, and 13 were statistically the same, while day 17 was different from days 10, 12, 13, and 15.

IL-6 Gene Expression

Endometrial gene expression for IL-6 exhibited a significant day effect ($P < 0.05$). Gene expression was the lowest in Con gilts on day 12 of pregnancy (Figure 6.8).

TSG-6 Gene Expression

Endometrial gene expression for TSG-6 (Figure 6.9) was unaffected by day of pregnancy or E-treatment ($P > 0.05$).

TSG-6 Western Blot analysis

Western blot analysis of endometrial cytoplasmic protein detected a 36-kDa immunoreactive product to the TSG-6 antiserum free and TSG-6 ITIH complex controls (Figure 6.10A). Expression of the free (36-kDa) and complexed (139 kDa) products

Figure 6.1 Relative changes in mRNA abundances in endometrial ITIH 1 gene expression on days 10, 12, 13, 15, and 17 following either control animals (Orange bars) or E-treated animals (Black bars). No differences were detected between means for ITIH 1 gene expression due to estrogen treatment or day of pregnancy ($P > 0.05$).

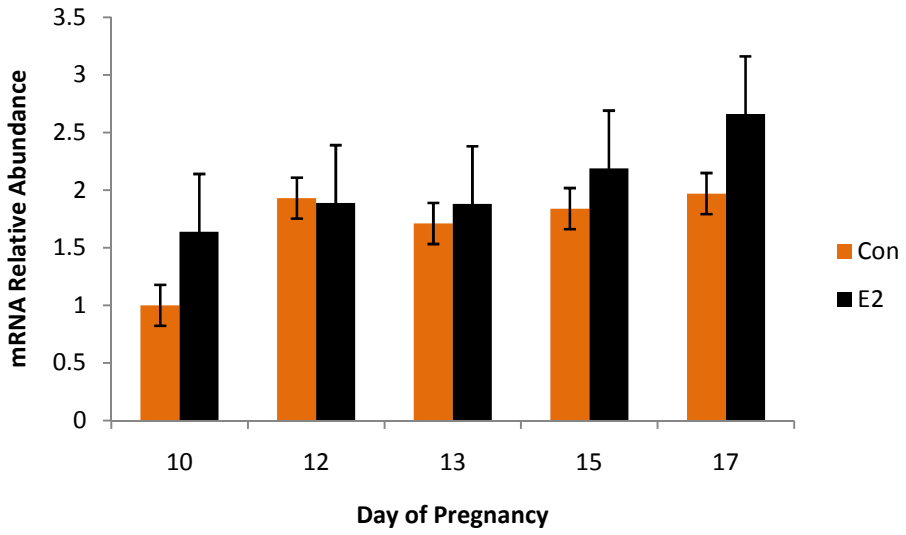

3.5
3
2.5
2
1.5
1
0.5
0
mRNA Relative Abundance
10
12
13
15
17
Day of Pregnancy
Con
E2

Figure 6.2 Relative changes in mRNA abundances in endometrial ITIH 2 gene expression on days 10, 12, 13, 15, and 17 following either control animals (Orange bars) or E-treated animals (Black bars). A significant treatment effect was detected between means for ITIH 2 gene expression due to estrogen treatment ($P > 0.05$).

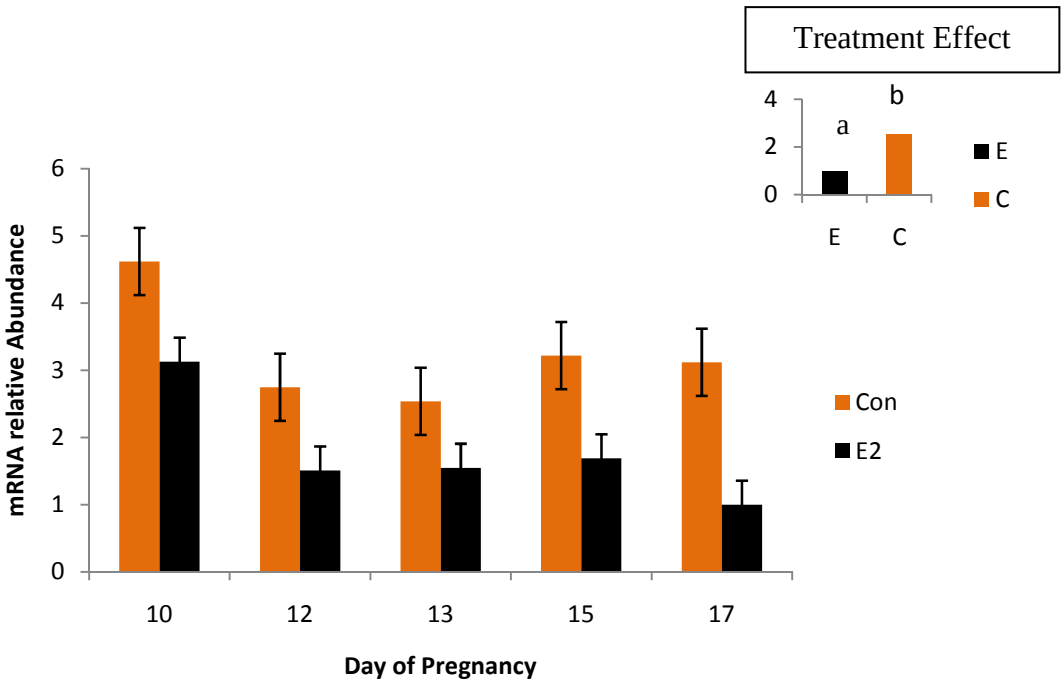

Treatment Effect
4
2
0
a
b
E
C
E
C
mRNA relative Abundance
6
5
4
3
2
1
0
10
12
13
15
17
Day of Pregnancy
Con
E2

Figure 6.3 Relative changes in mRNA abundances in endometrial ITIH 3 gene expression on days 10, 12, 13, 15, and 17 following either control animals (Orange bars) or E-treated animals (Black bars). No differences were detected between means for ITIH 3 gene expression due to estrogen treatment or day of pregnancy ($P > 0.05$).

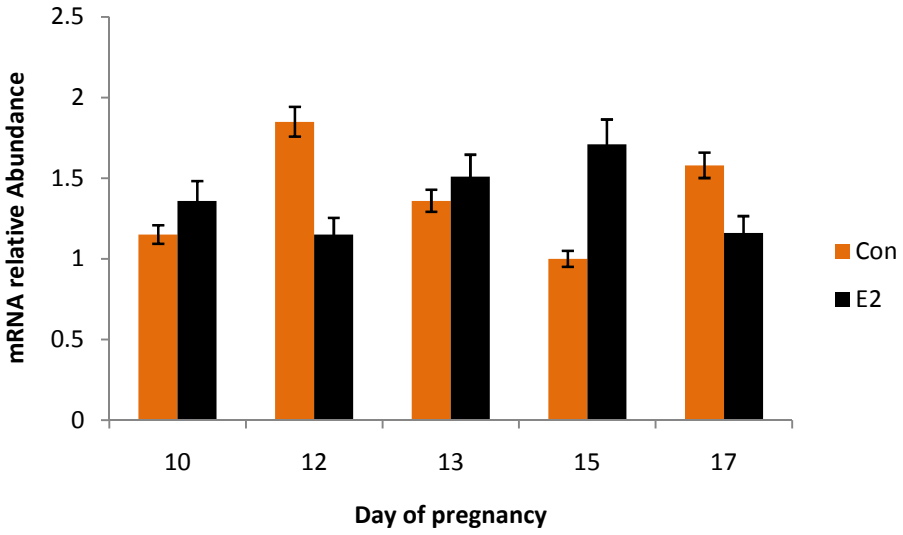

2.5
2
1.5
1
0.5
0
mRNA relative Abundance
10
12
13
15
17
Day of pregnancy
Con
E2

Figure 6.4 Relative changes in mRNA abundances in endometrial ITIH 4 gene expression on days 10, 12, 13, 15, and 17 following either control animals (Orange bars) or E-treated animals (Black bars). A significant day effect was detected between means for ITIH 4 gene expression due to day of pregnancy ($P > 0.05$).

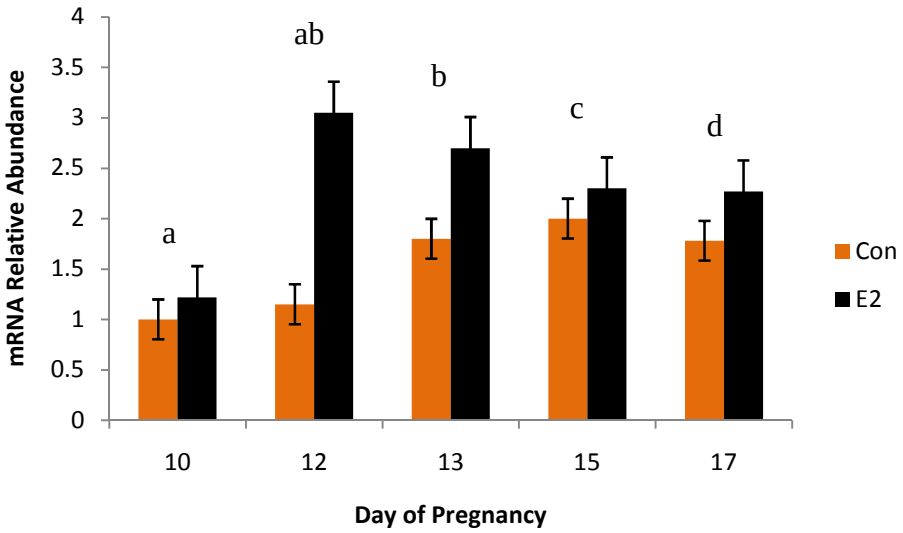
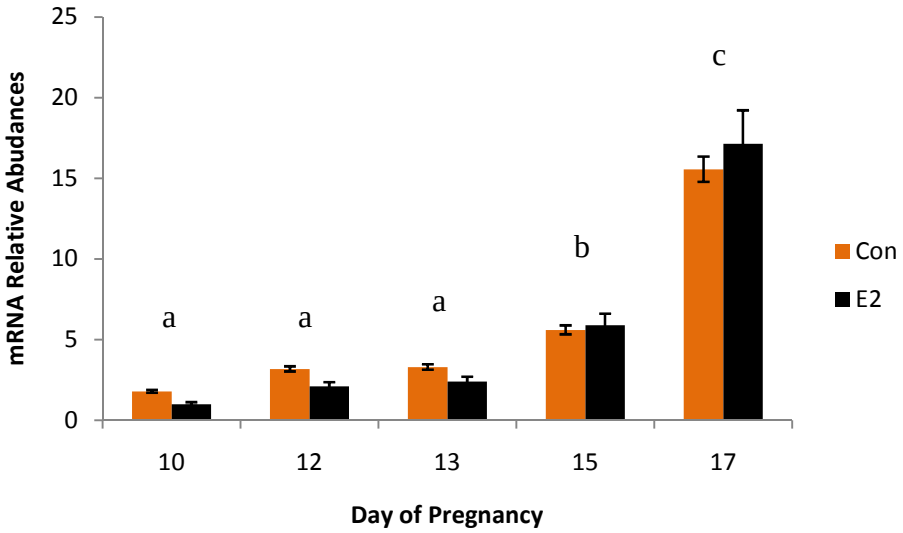

mRNA Relative Abundance
4
3.5
3
2.5
2
1.5
1
0.5
0
a
ab
b
c
d
10
12
13
15
17
Day of Pregnancy
Con
E2

Figure 6.5 Relative changes in mRNA abundances in endometrial Bikunin gene expression on days 10, 12, 13, 15, and 17 of either control animals (Orange bars) or E-treated animals (Black bars). No differences were detected between means for bikunin gene expression due to estrogen treatment, however there was a significant effect on the specificity of day of pregnancy ($P < 0.05$). Days with differing superscripts are statistically different.


25
20
15
10
5
0
mRNA Relative Abudances
a
a
a
b
c
10
12
13
15
17
Day of Pregnancy
Con
E2

ELISA Assay of Prostaglandins in Uterine Flushings

No treatment effect was detected for total uterine content of PGE_2. Content of PGE_2 in the uterine flushings was effected by day ($P<0.001$). Uterine PGE_2 content was lowest (6.23 ng) on day 10 of gestation (Figure 6.11), increasing 100-fold after conceptus elongation (Day 13). Uterine content of PGE_2 reached a maximum (1700 ng) by day 17 of gestation. No treatment effect was detected for total uterine content of PGE_2 in uterine flushings expressed in the uterine luminal flushings (Figure 6.10B) were unaffected by ay and endocrine disruption of pregnancy.

Discussion

The noninvasive form of implantation in the pig results from endometrial secretion of protease inhibitors that regulate the highly invasive activity of the trophoblast (Roberts et al., 1993). Furthermore, although pig conceptuses are extremely proteolytic and invasive outside the uterine environment (Samuel and Perry, 1972), endometrial secretion of protease inhibitors permits trophoblastic attachment to the glycocalyx on the apical uterine surface epithelium (Stroband and Van der Lende, 1990). Placentation in the pig is associated with a reduction of the uterine epithelial glycocalyx as well as interdigitation of the trophoblastic and uterine microvilli (Danzter, 1985). Although there is limited information concerning the apical interactions between the trophoblast and uterine surface epithelium, results suggest that change in the glycocalyx plays a major role in porcine placental attachment (Bowen et al., 1996; Jaeger et al., 2001).

Figure 6.6. Relative changes in mRNA abundances in endometrial kallikrein 4 gene expression on days 10, 12, 13, 15, and 17 of either control animals (Orange bars) or E-treated animals (Black bars). A significant treatment * day interaction was detected between means for kallikrein 4 gene expression ($P < 0.05$). Days with differing superscripts are statistically different.

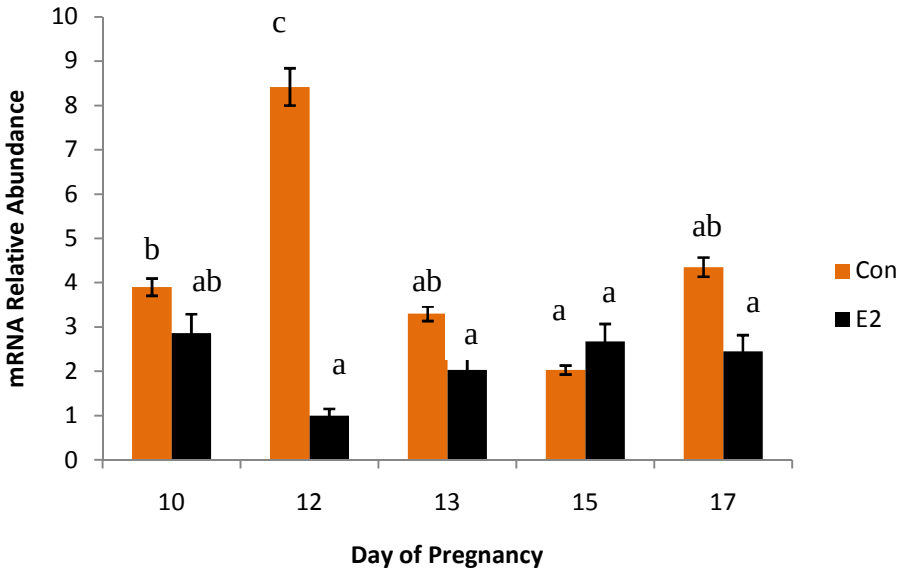

mRNA Relative Abundance
10
9
8
7
6
5
4
3
2
1
0
b
ab
c
a
ab
a
a
a
ab
a
10
12
13
15
17
Day of Pregnancy
Con
E2

Figure 6.7. Relative changes in mRNA abundances in endometrial TNF-α gene expression on days 10, 12, 13, 15, and 17 of either control animals (Orange bars) or E-treated animals (Black bars). No differences were detected between means for TNF-α gene expression due to estrogen treatment, however there was a significant effect on the specificity of day of pregnancy ($P < 0.05$). Days with differing superscripts are statistically different.

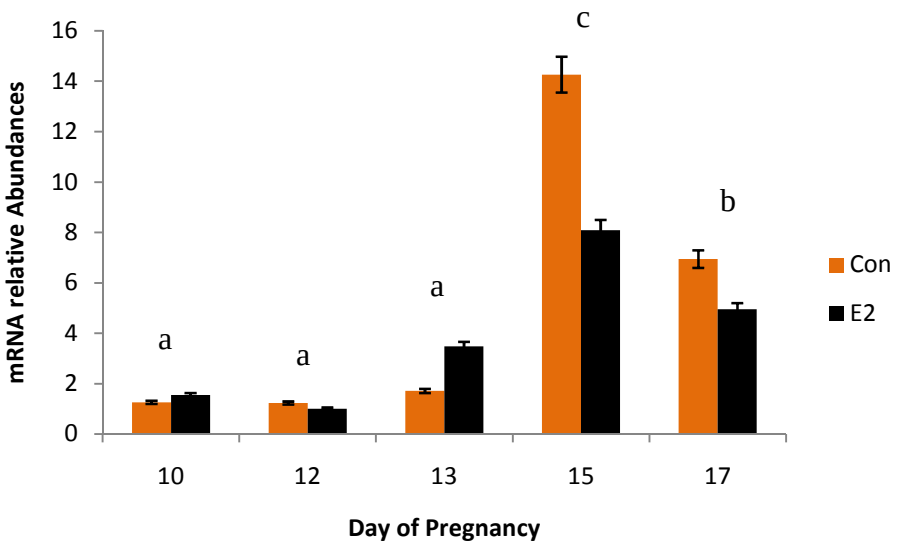

16
14
12
10
8
6
4
2
0
mRNA relative Abundances
a
a
a
c
b
10
12
13
15
17
Day of Pregnancy
Con
E2

Figure 6.8. Relative changes in mRNA abundances in endometrial IL-6 gene expression on days 10, 12, 13, 15, and 17 of either control animals (Orange bars) or E-treated animals (Black bars). A significant day effect was detected between means for IL-6 gene expression due to specificity of day of pregnancy ($P < 0.05$). Days with differing superscripts are statistically different.

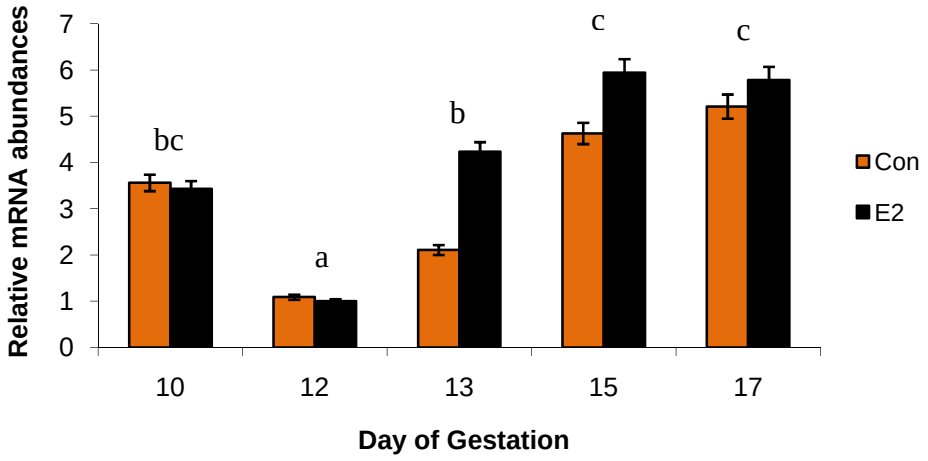

Relative mRNA abundances
7
6
5
4
3
2
1
0
bc
a
b
c
c
10
12
13
15
17
Day of Gestation
Con
E2

Figure 6.9. Relative changes in mRNA abundances in endometrial TSG-6 gene expression on days 10, 12, 13, 15, and 17 following either control animals (Orange bars) or E-treated animals (Black bars). No differences were detected between means for TSG-6 gene expression due to estrogen treatment or day of pregnancy ($P > 0.05$).

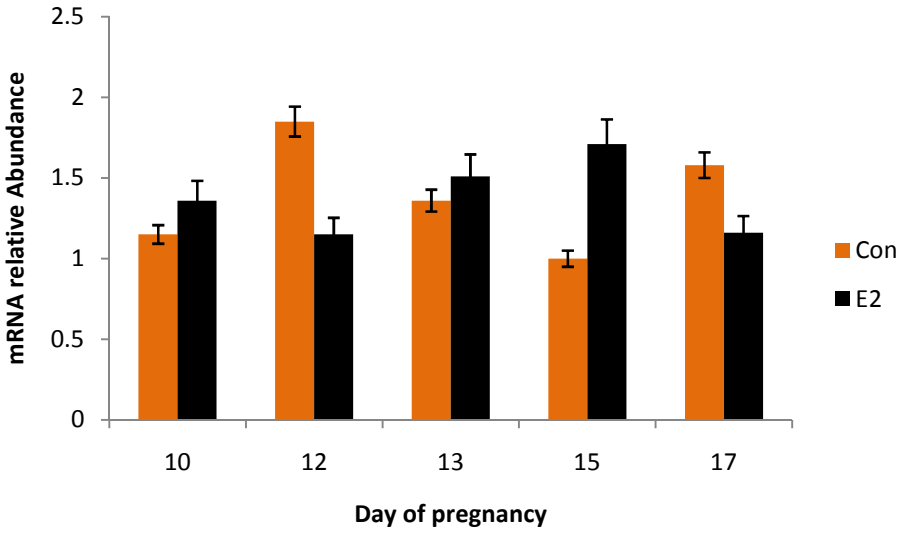
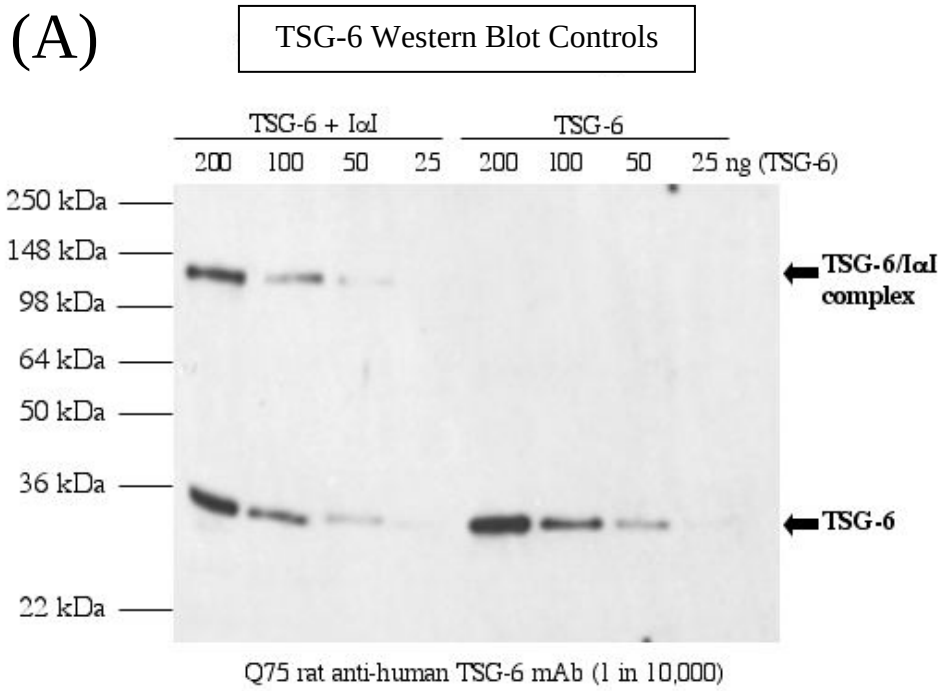
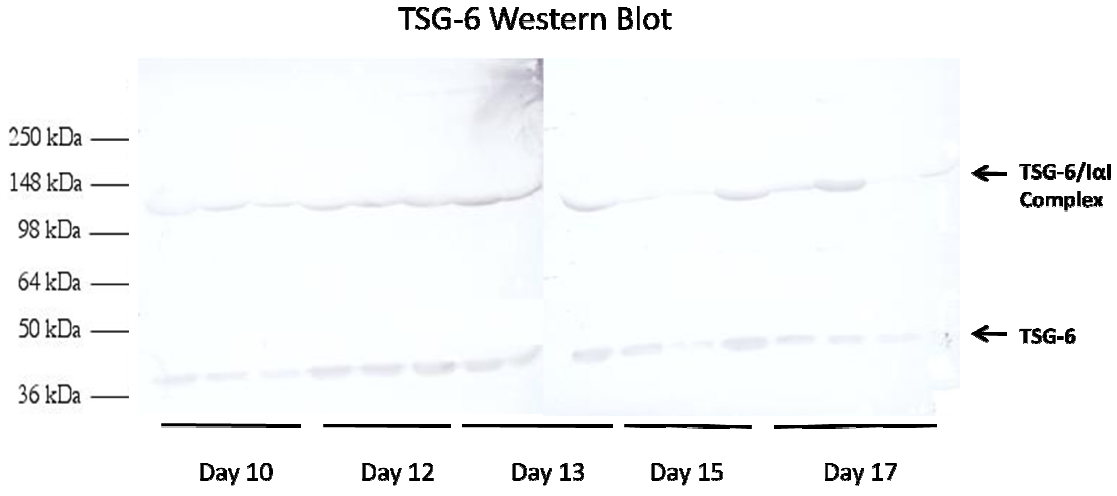

2.5
2
1.5
1
0.5
0
mRNA relative Abundance
10
12
13
15
17
Day of pregnancy
Con
E2

Figure 6.10. A) Western blot analysis of endometrial TSG-6 expression of free TSG-6 (36 kDa) Free TSG-6 and TSG-6 complexed with ITIH (~139 kDa). **B)** Free TSG-6 and TSG-6 complexed with ITIH expressed in the uterine luminal fluid of the control animals during early porcine pregnancy.

(A)

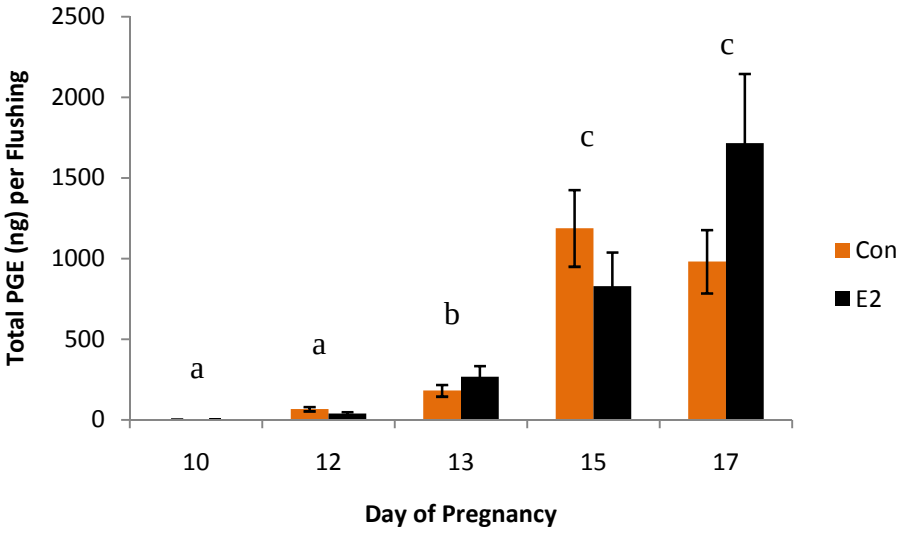


(B)



Q75 rat anti-human TSG-6 (200 ng) mAB (1 in 10,000)

Figure 6.11. Total uterine luminal content of PGE_2 in the uterine luminal flushings on days 10, 12, 13, 15, and 17 following either control animals (Orange bars) or E-treated animals (Black bars). Days with different superscripts represents significant differences ($P < 0.05$).


2500
2000
1500
1000
500
0
Total PGE (ng) per Flushing
a
a
b
c
c
10
12
13
15
17
Day of Pregnancy
Con
E2

We have previously demonstrated that estrogen administration causes disruption of the apical uterine surface glycocalyx (Blair et al., 1991). Breakdown of the epithelium glycocalyx was associated with the loss of an endometrial 30 kDa glycoprotein which was determined to be a cleavage product of ITIH4 (Geisert et al., 1998). All ITIH's possess a von Willebrand type-A domain which functions as a target for adhesion molecules such as integrins, collagen, proteoglycans and heparin. Previously, our lab demonstrated through western blot analysis of uterine flushings and endometrial cultures established that associations between the ITIH's and bikunin (light chain) occur in the pig (Geisert et al., 2003). Since the release of bikunin may be necessary for the association of hyaluronic acid with ITIH 1, 2, 3 (Jessen et al., 1994), and loss of bikunin gene expression causes infertility in mice through disruption of the cumulus oophorus (Zhuo et al., 2001), it is possible that disruption of ITIH and/or bikunin might occur with estrogen treatment. However, our results indicate that estrogen treatment on day 9 does not alter endometrial gene expression of bikunin or ITIH1, 3, and 4.

TSG-6, which is a secreted product, has been demonstrated to be involved with extracellular matrix formation through forming complexes with the ITIH's (Carrette et al., 2001). Our study demonstrated the presence of TSG-6 and that TSG-6 protein forms a complex with the ITIH's as found in the uterine flushings of the pregnant gilt. Therefore, the all components for forming the apical glycocalyx in the pig LE is present. However, estrogen treatment of gilts did not alter TSG-6 mRNA expression.

Kallikrein 4 gene expression was significantly depressed on day 12 of pregnancy in gilts treated with estrogen on day 9. There is a large increase in IGF-1 and -2 in the uterine lumen on day 12 of pregnancy in the pig (Geisert et al., 2001). IGF binding

proteins (IGFBP) are a substrate for kallikrein 4 which is a member of the serine protease family. The proteolysis of IGFBP's around day 12 of pregnancy would be coincidental with the observed increase in bioavailible IGF's within the uterine luminal fluid (Geisert et al., 2001) and the increase in kallikrein 4 mRNA expression making kallikrein 4 an excellent candidate for IGFBP proteolysis. However, recent studies conducted by Ashworth et al (2005) demonstrated that estrogen disruption of pregnancy resulted in early proteolysis of IGFBP's on day 10 of porcine pregnancy. Albeit, we did observe a severe attenuation in kallikrein 4 due to estrogen treatment on day 12, kallikrein 4 mRNA was not increased on day 10. Thus, kallikrein 4 expression does not have a temporal relationship with the premature release of IGFBP's observed by Ashworth et al (2005). Given these results, kallikrein 4 appears to be regulated by estrogen at some level, but the mechanism and target of the endocrine dysfunction still remains unclear.

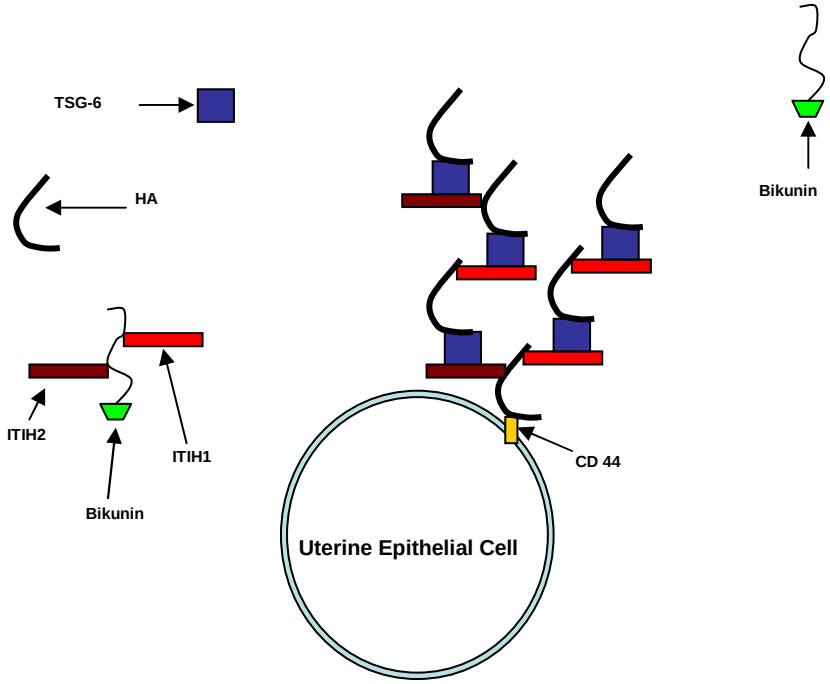
TNF-α was unaffected by estrogen treatment but was affected by day of pregnancy. Consequently, the TNF-α increase we observed in PGE_2 production would provide a possible means for the expression levels observed in TSG-6 protein. Although TSG-6 gene expression was unaffected by estrogen treatment, it is the first time TSG-6 protein has been localized to the porcine uterus complexed with the ITIH's during inflammation 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 cross linker 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, ITIH1 and ITIH2 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 6.12).

Studies have demonstrated that TSG-6 gene expression can be induced by TNF-α, IL-1β, and PGE_2 (Fujimoto et al., 2002). Furthermore, the current study has demonstrated a linked product containing TSG-6 and ITIH's through western blotting. Therefore, ITIH 2 appeared to be the only ITIH altered by the early estrogen exposure and this becomes paramount in regards to the ECM stabilization. ITIH2 has a direct involvement in the formation of the required heterodimerization with ITIH1, and the attenuated gene expression levels may be a persistent problem leading to decreased levels of ITIH2 in the uterine surface creating a dimer deficiency for ITIH1. Additionally, future studies will be required to clarify if the gene expression exhibiting ITIH2 deficiency is translated deficiency in the ITIH2 protein product in the uterine lumen. . Future studies will be needed to understand the role of bikunin, ITIH and TSG-6 plays together in stabilization of the ECM during early porcine pregnancy.

Figure 6.12. Postulated role of ITIH and TSG-6 involvement with stabilization of the hyaluronan-containing extracellular matrix (ECM) of the complex between CD 44 anchored to the uterine epithelial cells.

Figure 6.12


TSG-6
HA
ITIH2
ITIH1
Bikunin
Bikunin
CD 44
Uterine Epithelial Cell

Endometrial IL-6 mRNA expression was found to be significantly decreased on day 12 of pregnancy compared to the other days measured (10, 13, 15, and 17). This is in agreement with the results of Anegon et al., (1994) who demonstrated that conceptus mRNA expression of IL-6 was lowest around day 11 to 12 during conceptus development. Our data and Anegon's data suggest there is a temporal down regulation of IL-6 gene expression in both conceptus and maternal tissues during the critical period of maternal recognition of pregnancy; the reason has yet to be elucidated

In the current study, we have shown that the transcriptome for ITIH 1,-3, and-4 appear to be controlled through aspects other than estrogen regulation. Conversely, ITIH 2 appears to be regulated at some level through the appearance of estrogen, and ITIH 4 appears to be day dependent based on its appearance during pregnancy. However, for all the ITIH's there may be an additional layer of regulation at the regulatory RNA level, which may be regulated through estrogen either directly or indirectly but this remains unknown at the present.

Chapter VII

SUMMARY AND CONCLUSION

The establishment of pregnancy in any mammalian species requires specific timed molecular events at the conceptus uterine interface during days 11 to 15 of gestation. It is during this period that the conceptuses produce both IL-1β and estadiol-17β concomitant with the rapid elongation of the conceptus trophectoderm. Sufficient secretory capacity of the pig conceptus (Polge et al., 1966; Dziuk, 1968) and an adequate extent of trophoblastic elongation (Stroband and Van der Lende, 1990) are requirements for the establishment of pregnancy and conceptus survival in the pig. Acquiring these major conceptus developmental landmarks requires the temporal and spatial expression of the appropriate mRNA transcripts to generate a phenotype suitable for continued growth and development. However, little information has been isolated specifically identifying endometrial transcripts devoid of any conceptus contributions that are critical for this stage of endometrial development.

Not only is appropriate conceptus development critical for the establishment of pregnancy in the pig, uterine endometrial function regulating uterine receptivity and conceptus attachment are necessary components for pregnancy establishment. Two major factors regulating endometrial function during the period of conceptus attachment and implantation are conceptus synthesis and release of estradiol-17β and conceptus release of IL-1β.

Cooperatively, the research presented in this dissertation uses separate approaches to better understand the biology of pregnancy establishment in pigs with respect to uterine dynamic changes during days 10 to 18 of gestation. This diminutive period of development regarding the length of gestation is critical due to the numeruous physiological events occurring in both the conceptus and the endometrium required for pregnancy establishment in the pig. Conceptus trophoblastic elongation is simultaneous with endometrial programming that allows uterine receptivity, and uterine receptivity can be modified in response to conceptus secretory factors, such as estradiol-17β (Geisert et al., 1982b) and IL-1β (Ross et al., 2003b).

Estrogen is critical for the proper establishment of pregnancy in the pig (Bazer and Thatcher, 1977) however; it can be detrimental if uterine exposure to estrogen occurs prematurely during early gestation (Pope et al., 1986, Blair et al., 1991; Ashworth et al., 2006). With the delicate relationship between the optimal point of endometrial exposure to estrogen during early pregnancy and its affect on conceptus survivability, one can infer a spatiotemporal regulation of essential endometrial factors affecting pregnancy outcome occurs through aberrant estrogen exposure. Furthermore, although the endocrine-exocrine theory points to estrogen as the maternal recognition of pregnancy signal in the pig (Bazer and Thatcher, 1977), the molecular mechanisms behind estrogen's stimulation on the endometrium are not complete. Our approach isolated the direct effect of estrogen and its effects as an endocrine disruptor for the identification of endometrial genes altered by improper timing of estrogen stimulation without the presence of conceptus secreted factors.

During the microarray study of this thesis, utilization of the Affymetrix GeneChip® Porcine Genome Array allowed the identification of a plethora of factors that are differentially expressed at the opening of the implantation window aberrantly due to early estrogen exposure. Furthermore, as determined though statistical analysis using dChip, 84 of the 117 genes that were differentially expressed were due to early estrogen exposure when comparing days 13 to 15. These data would suggest when aberrant estrogen exposure disrupts pregnancy; the window from days 13 to 15 is as an important and critical time period for endometrial changes in gene expression and secretions essential for conceptus development and survival. Additionally, the lack of abundance in differential gene expression in endometrium between days 13 and 15 of gilts exposed to estrogen at the time that conceptus estrogen normally occurs (Day 11-12) during early pregnancy indicates that early estrogen exposure selectively alters expression of endometrial genes which do not normally increase with conceptus estrogen secretion on day 12.

Therefore, cluster analysis was paramount in regards to identification of temporal expression patterns associated with estrogen regulation. When early estrogen disrupts pregnancy, the period between days 13 to 15 are the most critically affected with 84 genes found to be differentially regulated during this time period. Furthermore, the 84 differentially expressed genes were clustered into 3 groups of genes (Cluster 2, 6, and 7), and of these genes 47 were up regulated and 37 were downregulated due to the estrogen. Consequently, these genes might serve as an initial biomarker for possible identification of endocrine disruption of the early porcine implantation window.

Chemokines have been implicated with the enhancement of the biological immune response as well as having a key role in maintaining homeostasis of the immune system. Chemokines are classified into four distinct groups (CXC, CC, C and CX_3C) based on their inherent (amino acid sequence) and structural properties (Berger et al., 1999, Rollins et al., 1997). Early foundational studies limited chemokine expression to inflammatory and immune cells exclusively; however, subsequent studies have shown that to be false. Recently, chemokine expression was localized in the murine uterus at the time of peri-implantation (Nagaoka et al., 2003a). Furthermore, localization of chemokine receptors has been detected in the decidua and placenta of humans (Red-Horse et al., 2001). Chemokine ligand and receptor expression patterns are conspicuously expressed in uterus of the human, murine, and ovine during the window of implantation and/or attachment of the conceptus suggesting a key role for modulation of endometrial erosion and attachment between the embryo and maternal tissues. Specifically, chemokine ligand 11 (CXCL-11) gene expression has been reported to increase during the window of implantation in the human (Hirota et al., 2006) and the ewe (Imakawa et al., 2006). Hirota et al., (2006) demonstrated exposure of uterine endometrial epithelial cells (EEC) in culture to IFN-γ stimulated secretion of CXCL-11, but not the uterine endometrial stromal cells (ESC). Furthermore, CXCL-11 stimulated proliferation in the ESC cells but inhibited proliferation in the EEC cells while concomitantly stimulating apoptosis. These data suggest IFN-γ working through CXCL-11 may play a regulatory role during implantation. Additionally, Hirota et al. (2006) suggest a prejudice towards the stromal layer for survival conditions, while inducing negative conditions in the epithelial layer during endometrial invasion in the mouse.

Hirota et al., (2006) also demonstrated that CXCR3, the receptor for CXCL-11, is also present in the epithelial and stromal layers of the endometrium, T cells, and the conceptus trophoblastic cells. These data represent a useful model for motility and chemotaxis during the window of implantation in that upon secretion of CXCL-11 from the EEC layer, trophoblastic cells and T cells (TH1) migrated accordingly. Hirota's data makes a nice complement to what our lab has already established to date in the pig. The appearance of CXCL-11 is closely associated with window of implantation in the pig. Additionally, the current study characterized IL-18 secretion which originates from the uterine endometrium during the period of trophoblast attachment to the uterine surface from day 13 to 18 of pregnancy. IL-18 must be processed from the latent form to the active molecule through processing by caspase-1. One could infer based on CXCL-11 appearance during the window of implantation, that it may provide a pro-apoptotic signal to uterine epithelial cells, resulting in a release of caspase-1 from the uterine epithelial cells via CXCL-11's ability to induce apoptosis. Furthermore, this may be a mechanism allowing site specific areas of the endometrium to undergo apoptosis as opposed to the whole endometrium, while providing a receptive epithelial layer for proper attachment during this period. Similar to CXCL-11, uterine CXCL-9 secretion is stimulated through progesterone and/or estrogen (Kitaya et al., 2004). Stark contrasts exist between CXCL-9 and CXCL-11 is that CXCL-9 is induced by the pleiotrophic cytokine IL-18, while CXCL-11 and CXCL-10 and the only known chemokines controlled by estradiol and progesterone and are directly responsible for the migration of perhperial blood NK cells into the uterus and creating a residential NK cell then termed uNK cells (Sentman et al., 2004). The induction of CXCL-11 into the human endometrium is facilitated through the

ovarian sex steroids estrogen or progesterone (Sentman et al., 2004); however, whether the affects are direct or indirect from the steroids have yet to be elucidated. Furthermore, the study also established chemokine receptor CXCR3 is expressed on the surface of uNK cells, which services the ligands CXCL-9 and -11. According to data in the study, the overall chemokine theme suggests clearly that CXCL11 is estrogen regulated, while based on temporal expression CXCL9 is IL-18 induced. These alterations in gene expression demonstrated in the uterine endometrium are similar to the asynchrony between the endometrium and conceptus described by Polge (1982) where embryos transferred greater than 48 h out of synchrony with the uterine endometrium fail to establish pregnancy. These data suggest that exogenous estrogen administration causes significant alterations in endometrial gene expression on days 12 and 13 of gestation and may prevent attachment and promote immunological rejection of the conceptus resulting in its deterioration by day 15 of gestation. However, the underlying implication is that during normal gestation, conceptus induced changes through endogenous estradiol-17β secretion occur synchronously to the dramatic expression and phenotypic changes that enable the conceptus to survive in a uterine environment following estrogen stimulation.

Estrogen is a critical component for the establishment of pregnancy in the pig (Bazer and Thatcher, 1977) but can also be detrimental if uterine endometrial exposure to estrogen occurs at an abnormal time during early gestation (Pope et al., 1986, Blair et al., 1991; Ashworth et al., 2006). The critical relationship between the temporally acceptable point of endometrial exposure to estrogen during pregnancy and its affect on conceptus survivability suggests that spatiotemporal regulation of key factors affecting pregnancy outcome occur through estrogen. Also, while it is widely accepted that estrogen is the

maternal recognition of pregnancy signal, the molecular mechanisms behind estrogen stimulation are not well described. Our approach has allowed the identification of endometrial genes affected by estrogen stimulation in the presence of IL-1β

The opening of the implantation window is a critical process which occurs during peak progesterone production by the corpora lutea, is characterized by uterine receptivity to conceptus attachment, and occurs in all reproductively capable gilts, regardless if mating, fertilization and early conceptus development have transpired. Endometrial phenotype during uterine receptivity is poised to secrete prostaglandins in an endocrine fashion, resulting in luteolysis and a return to estrus or release prostaglandins in and exocrine fashion, sustaining the corpora lutea; and establish a communication network with the developing conceptus, thereby commencing gestation. The downregulation of progesterone receptor (Fazleabas et al., 1999; Spencer and Bazer, 1995; Meikle et al., 2001; Hartt et al., 2005) and the activation of NFκB (Nakamura et al., 2004a) during pregnancy establishment are not unique to the pig (Lessey et al., 1988, 1996). We were interested in the molecular factors that contribute to this endometrial phenotype.

This study evaluated potential mechanisms by which endometrial modifications can occur through the interplay between estrogen around the time of maternal recognition, and the conceptus secretions of IL-1β into the uterine lumen. In the current study, the data suggest that IL-1β actions are predominantly that of acute inflammatory responses and up regulation of IL-1β's own mRNA and protein, and its ancillary signaling appendages such as IL-R1. Additionally, of the genes observed in this study, IL-1β regulation of the mRNA expression was limited to the 4 h sampling point post-infusion. Furthermore, genes in the current study up regulated from the infusion of IL-1β

are acute inducible products of the NF-κB pathway, suggesting IL-1β inducing the NF-κB working through the IL-1β R1. These data would be in concurrence with data observed from Strakova et al., (2005), where the authors observed that the baboon psuedopregnant animal did not express COX-2 protein in the endometrium; however, in the presence of the conceptus IL-1β secretions COX-2 was induced. In contrast, gene induced through the actions of estrogen for the observed mRNAs were induced at the 36 h sampling point except for IL-1β RAP, which was induced at the 4 and 36 h sampling periods by estrogen. The estrogen induction of trophinin and OPN further dictates the role of estrogen as ECM supportive and facilitates attachment based on its timing and appearance during the window of implantation. The involvement of steroid hormones, as well as their receptors, has in many studies been shown to affect NFκB activity in a variety of tissues. Estrogen, progesterone, and their receptors have been shown in multiple tissue types to regulate NFκB activity (Kalkoven et al., 1996; Ghilsetti et al., 2005, Hardy et al., 2006) and as described, these mechanisms associate well with the steroid hormone regulation of the estrous cycle and early pregnancy in the pig.

While the IL-1β study provided delightful insight into cytokine control during days 12 through 15 of the porcine pregnancy, the IL-18 study conducted in this thesis provided some very insightful data by which these days were under the control of IL-18. Ross et al. (2003) demonstrated that the peri-implantation porcine conceptuses secrete large amounts of IL-1β into the uterine lumen during the transition to 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 IFN-γ. In the

elongating porcine conceptuses on day 12, 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. Moreover, Joyce et al., (2006) demonstrated that greatest levels of IFN-γ occur from the trophoblast during days 15 through 18 of the porcine pregnancy, which is clearly complimentary to our IL-18 increase during days 15 through 18 of pregnancy. Furthermore, both the mRNA and protein expression patterns observed in caspase-1are temporally aligned for the proper processing of both IL-1β and IL-18 during early pregnancy. Conceptus caspase-1 mRNA expression is greatest in abundance during days 13 and 17. Furthermore, endometrial caspase-1mRNA expression was markedly elevated during days 15 and 17 of pregnancy. These data would infer IL-1β processing upon or during liberation from conceptus origin during the initial release of IL1β around day 12 of pregnancy, based on an absence of caspase-1 protein found in the uterine luminal flushings. Additionally, the increased expression of caspase-1 mRNA levels observed in the endometrium with an elevation of caspase-1 protein in uterine flushings would be consistent with our increase in observed secretion of IL-18 during days 15 to 18. Given these data, we have clearly established evidence for cytokine crosstalk between the developing conceptuses and endometrium based on gene expression of IL-18 exclusively expressed in endometrium, while completely devoid in all days of conceptus during early pregnancy.

The noninvasive form of implantation in the pig results from endometrial secretion of protease inhibitors that regulate the highly invasive activity of the trophoblast (Roberts et al., 1993). Furthermore, although pig conceptuses are extremely proteolytic and invasive outside the uterine environment (Samuel and Perry, 1972), endometrial secretion of

protease inhibitors permits trophoblastic attachment to the glycocalyx on the apical uterine surface epithelium (Stroband and Van der Lende, 1990). Placentation in the pig is associated with a reduction of the uterine epithelial glycocalyx as well as interdigitation of the trophoblastic and uterine microvilli (Danzter, 1985). Although there is limited information concerning the apical interactions between the trophoblast and uterine surface epithelium, past results suggest that change in the glycocalyx plays a major role in porcine placental attachment (Bowen et al., 1996; Jaeger et al., 2001). All ITIH's possess a von Willebrand type-A domain which functions as a target for adhesion molecules such as integrins, collagen, proteoglycans and heparin. Previously, our lab demonstrated through western blot analysis of uterine flushings and endometrial cultures established that associations between the ITIH's and bikunin (light chain) occur in the pig (Geisert et al., 2003). Since the release of bikunin may be necessary for the association of hyaluronic acid with ITIH 1, 2, 3 (Jessen et al., 1994), and loss of bikunin gene expression causes infertility in mice through disruption of the cumulus oophorus (Zhuo et al., 2001), it is possible that disruption of ITIH and/or bikunin might occur with estrogen treatment. However, our results indicate that estrogen treatment on day 9 does not alter endometrial gene expression of bikunin or ITIH1, 3, and 4. TSG-6, which is a secreted product, has been demonstrated to be involved with extracellular matrix formation through forming complexes with the ITIH's (Carrette et al., 2001). Our study demonstrated the presence of TSG-6 and that TSG-6 protein forms a complex with the ITIH's as found in the uterine flushings of the pregnant gilt. Therefore, the all components for forming the apical glycocalyx in the pig LE is present. However, this study determined estrogen treatment of gilts did not alter TSG-6 mRNA or protein

expression. Nevertheless, the current study was the first to determine that TSG-6 does form a complex with ITIH 1 and 2 in the uterine flushings of the pregnant gilt.

The current dissertation has supplied a wealth of information that could be used for the generation of a plethora of hypothesis' to describe the specific functions of genes that regulate conceptus trophoblastic elongation, estrogen's molecular role as the maternal recognition of pregnancy signal and chemokine activation and function as it relates to the obligatory chemotaxis during pregnancy establishment in the pig.

Current technologies, such as RNA interference, regulatory RNAs, and use of asymmetrical models such as psuedopregnancy to answer critical biological conditions observed during pregnancy, and disruption of pregnancy. Through miRNA arrays and independant studies, this may allows pecific targets through which modifications can be determined to improve pig reproductive efficiency.

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Appendix Ia

TABLES CHARACTERIZING COMPLETE ENDOMETRIAL GENE EXPRESSION DATA FOR ALL COMPARISONS DURING ENDOCRINE DISRUPTION OF THE PSEUDOPREGNANT PIG

Table A.1a. Differentially expressed genes *UP-REGULATED* during endocrine disruption of 13PP compared to 13PPE

Probeset	GENE	P value	Fold Change	ACCESSION	Human GENEID
Ssc.23629.1.A1_at	Predicted Soluble carrier family 34 (Sodium phosphate) Transcript Variant 2	0.0009	3.18	CO942177	114593382
Ssc.11858.1.S1_at	FMOD: fibromodulin	0.0003	2.64	CN163410	397329
Ssc.23305.1.S1_at	Bovine Transmembrane Protein 144	0.0010	2.31	NM_001101989	
Ssc.14436.1.S1_at	Macaca mulatta ubiquitin specific protease 14 (tRNA-guanine Transglycosylase)	0.0006	1.98	XM_001090667	
Ssc.24625.1.A1_at	MRNA, clone:AMP010061G05, expressed in alveolar macrophage	0.0008	1.9	CK461494	
Ssc.21253.1.S1_at	strongly similar to NP_038919.1 SAM pointed domain containing ets transcription factor [Mus musculus]	0.0001	1.82	BX667251	
Ssc.7139.1.S1_at	MRNA, clone:LVRM10061G07, expressed in liver	0.0004	1.79	BX925457	
Ssc.5282.1.S1_at	moderately similar to NP_570968.1 cAMP responsive element binding protein 3-like 4 [Homo sapiens]	0.0001	1.77	BF077968	
Ssc.19509.1.A1_at	MRNA, clone:AMP010057H06, expressed in alveolar macrophage	0.0008	1.64	CF362615	
Ssc.5041.1.A1_at	Transcribed locus, moderately similar to NP_001028886.1 nucleolar protein 1, 120kDa [Homo sapiens]	0.0005	1.63	CN154405	
Ssc.9986.1.A1_at	Transcribed locus	0.0008	1.45	CN157453	
Ssc.2542.1.S1_at	MRNA, clone:TES010023G04, expressed in testis	0.0000	1.42	BQ603949	
Ssc.12977.1.A1_at	MRNA, clone:OVRM10041E01, expressed in ovary	0.0001	1.33	BI404425	
Ssc.8630.1.A1_at	Transcribed locus	0.0009	1.28	BF713211	
Ssc.23810.1.S1_at	MRNA, clone:OVRM10040C07, expressed in ovary	0.0005	1.26	CK453668	
Ssc.9353.1.A1_at	Transcribed locus	0.0007	1.25	BF710330	
Ssc.16669.1.S1_at	MRNA, clone:AMP010044G02, expressed in alveolar macrophage	0.0007	1.19	BG610589	

	Ssc.12686.1.A1_at	Transcribed locus	0.0008	1.11	BI403284	

Table A.1b. Differentially expressed genes *DOWN-REGULATED* during endocrine disruption of 13PP compared to 13PPE

PROBESET	GENE	P-value	Fold Change	ACCESSION	Human
Ssc.13262.1.S1_at	EIF4EBP1: eukaryotic translation initiation factor 4E binding protein 1	0.0004	-1.27	BX924410	397633

Table A.1a. Differentially expressed genes *DOWN-REGULATED* during endocrine disruption of 13PP compared to 15PP

PROBESET	GENE	ACCESSION	GENE ID	P-Value	Fold Change	Human
Ssc.5053.1.S1_at	CD163: CD163 molecule	NM_213976.1		0.0008	1.94	397031

Table A.2b. Differentially expressed genes *DOWN-REGULATED* during endocrine disruption of 13PP compared to 15PP

PROBESET	GENE	ACCESSION	GENE ID	P-Value	Fold Change	Human
Ssc.25116.1.A1_at	Transcribed locus	CK463855		0.0001	-1.37	
Ssc.6526.1.S1_a_at	MRNA, clone:LVRM10138D07, expressed in liver	BI359943		0.0009	-1.4	
Ssc.6526.3.A1_a_at	similar to LPS-responsive vesicle trafficking, beach and anchor containing	CN160927		0.0001	-1.45	

Table A.3a Differentially expressed *UPREGULATED* genes during endocrine disruption of 13PP compared to 15PP

PROBESET	GENE	Fold Change	P-Value	ACCESSION	Human GENEID
Ssc.30027.1.A1_at	CXCL-11	30.08	0.0004	NM_001128491	5689773
Ssc.29054.1.A1_at	guanylate binding protein 1, interferon-inducible,	6.41	0.0009	NM_001128473.1	166706902
Ssc.26146.1.S1_at	(C-X-C motif) ligand 9	5.35	0.0000	NM_001114289	4505186
Ssc.17821.1.A1_at	Pleckstrin	5.19	0.0007	NP_062422	11464971
Ssc.204.1.S1_at	CYP3A29: cytochrome P450 3A29	4.64	0.0004	NM_214423.1	403324
Ssc.507.1.A1_at	TYROBP: TYRO protein tyrosine kinase binding protein	4.51	0.0005	AF152021.1	38157998
Ssc.12825.1.A1_at	NKp80 NK receptor	4.05	0.0006	NM_001101276.1	13016700
Ssc.3879.1.S1_at	strongly similar to plexin C1 [Mus musculus]	3.82	0.0009	NP_061267.1	54712
Ssc.9229.1.S1_at	MRC1: mannose receptor, C type 1	3.7	0.0001	AY368183.1	38569736
Ssc.548.1.S1_a_at	MMP7: matrix metallopeptidase 7 (matrilysin, uterine)	3.38	0.0003	NM_214207.1	397411
Ssc.15565.1.S1_at	lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa)	3.3	0.0002	NM_001076844.1	47078282
Ssc.9778.1.S1_at	proteoglycan 1 precursor-like, Transcript Variant 1	3.18	0.0002	XM_001928855.1	100049692
Ssc.11073.1.S1_at	LMP7: proteasome subunit LMP7	3.09	0.0009	NM_213935.1	396975
Ssc.19943.2.S1_at	Ssc.19943.2	2.88	0.0006	BX675463	
Ssc.15370.1.S1_at	MRNA, clone:AMP010, expressed in alveolar macrophage	2.68	0.0005	BX666577	
Ssc.15419.1.S1_at	Bruton agammaglobulinemia tyrosine kinase (BTK)	2.4	0.0002	NM_001034589.1	77736124
Ssc.13777.1.S1_at	SLA-DMA alpha 2 domain	2.35	0.0009	AB117618.1	36062
Ssc.27863.1.S1_at	TAP1: Transporter 1, ATP-binding cassette, sub-family B	2.33	0.0002	BI337485	53759115
Ssc.14561.1.S1_at	CD18: CD18 leukocyte adhesion molecule	2.2	0.0009	NM_213908.1	47522671
Ssc.15296.1.S1_at	Similar to CD53 (Bovine)	2.2	0.0007	NM_001034232	61553526
Ssc.1177.1.S1_at	C1S: complement component 1, s subcomponent	2.14	0.0006	NM_001005349.1	52694688
Ssc.15739.1.S1_at	IL2R-G: interleukin 2 receptor, gamma	2.09	0.0010	EU026383.1	4557881
Ssc.16012.1.S1_at	Caspase-1 (ICE)	2	0.0006	NM_214162.1	47523411
Ssc.19101.1.A1_at	CD1 Cluster gene	1.93	0.0004	AB221038.1	118764585
Ssc.12882.1.A1_at	MHC class II, DM beta	1.79	0.0006	BI404058	47115148

Ssc.16038.1.S1_at	MHC class II antigen	1.79	0.0003	AB012858.1	194040228
Ssc.19778.1.S1_at	MRNA, clone:THY010107H05, expressed in thymus	1.65	0.0003	BX675283	
Ssc.11243.1.A1_at	Transcribed locus	1.64	0.0006	BI181152	
Ssc.16008.1.S1_at	FCNB: ficolin	1.64	0.0002	NM_214160.1	
Ssc.26300.2.S1_at	Transcribed locus, moderately similar to XP_947303.1 PREDICTED: similar to unc-93 homolog B1 isoform 1 [Homo sapiens]	1.6	0.0007	BX918761	
Ssc.28997.1.S1_at	Transcribed locus	1.6	0.0008	CA781087	
Ssc.19364.1.S1_at	SBAB-707F1.3: complement component 2	1.58	0.0002	BI360075	
Ssc.5489.3.S1_at	MRNA, clone:SPL010046C07, expressed in spleen	1.58	0.0004	BE663177	
Ssc.27070.1.A1_at	Transcribed locus	1.57	0.0000	CN166386	
Ssc.6433.1.S1_at	Transcribed locus, strongly similar to NP_066363.1 hypothetical protein LOC57169 [Homo sapiens]	1.56	0.0003	BX672562	
Ssc.13354.1.A1_at	Transcribed locus	1.49	0.0008	CN166737	
Ssc.1403.1.S1_at	Clone rcad21b_e4.y1.abd, mRNA sequence	1.45	0.0007	CB478967	
Ssc.16529.2.S1_at	MRNA, clone:AMP010064D06, expressed in alveolar macrophage	1.4	0.0002	CB288184	
Ssc.22354.1.A1_at	Transcribed locus, strongly similar to NP_061134.1 Jun dimerization protein p21SNFT [Homo sapiens]	1.36	0.0002	CK461037	
Ssc.2064.1.A1_at	Transcribed locus, moderately similar to XP_946275.1 PREDICTED: similar to Transmembrane 7 superfamily member 1 [Homo sapiens]	1.32	0.0006	CK458372	
Ssc.18934.1.S1_a	Transcribed locus, strongly similar to XP_991749.1]	1.32	0.0004	CN159172	
Ssc.3848.1.S1_at	MRNA, clone:OVRM10058D03, expressed in ovary	1.32	0.0003	CN166777	
Ssc.14480.1.S1_at	CAMK2D: calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	1.3	0.0009	NM_214381.1	
Ssc.1853.1.S1_at	MRNA, clone:LNG010060B03, expressed in lung	1.23	0.0010	CN157731	
Ssc.19154.1.S1_at	Transcribed locus, strongly similar to NP_059089.1 caseinolytic protease, ATP-dependent, proteolytic subunit homolog [Mus musculus]	1.23	0.0003	CN163324	

Ssc.5096.2.S1_at	Transcribed locus	1.19	0.0009	CO953965		

Table A.3 Differentially expressed *DOWN-REGULATED* genes during endocrine disruption of 13PP compared to 15PP

Ssc.19550.1.S1_at	MRNA, clone:SPL010050C11, expressed in spleen	1.17	0.0004	CN162516	
Ssc.12303.1.S1_at	Transcribed locus, moderately similar to XP_947327.1 PREDICTED: similar to Tripartite motif protein 16 (Estrogen-responsive B box protein) [Homo sapiens]	-1.22	0.0005	BQ604664	
Ssc.6310.1.A1_at	Antigen NY-BR-84-like protein mRNA, partial sequence	-1.26	0.0007	CN154592	
Ssc.10843.1.A1_at	EIF4A2: eukaryotic translation initiation factor 4A isoform 2	-1.27	0.0000	BM083158	
Ssc.6526.3.A1_a_at	Similar to LPS Trafficiking Rheus macca	-1.31	0.0009	CN160927	
Ssc.10991.2.S1_at	strongly similar to NP_997696.1 HLA-B associated transcript 5 [Rattus norvegicus]	-1.31	0.0007	AW312320	
Ssc.16363.1.S2_at	TMOD3: tropomodulin 3 (ubiquitous)	-1.32	0.0010	CN153516	
Ssc.7939.2.A1_at	Transcribed locus	-1.32	0.0003	BF710416	
Ssc.11803.1.A1_at	Transcribed locus, strongly similar to NP_060531.1 PRP38 pre-mRNA processing factor 38 (yeast) domain containing B [Homo sapiens]	-1.34	0.0009	BI185841	
Ssc.21990.1.S1_at	Transcribed locus	-1.34	0.0008	BX666595	
Ssc.14107.1.S1_at	Transcribed locus	-1.35	0.0009	CK449583	
Ssc.27826.1.S1_at	Transcribed locus	-1.35	0.0004	BG834080	
Ssc.10991.1.A1_at	Transcribed locus, strongly similar to NP_997696.1 HLA-B associated transcript 5 [Rattus norvegicus]	-1.43	0.0007	BI182984	
Ssc.5503.2.S1_at	Transcribed locus	-1.43	0.0005	BF193281	
Ssc.6526.1.S1_a_at	MRNA, clone:LVRM10138D07, expressed in liver	-1.5	0.0001	BI359943	
Ssc.29458.1.A1_at	Transcribed locus	-1.51	0.0009	CO956431	
Ssc.9822.1.A1_at	Transcribed locus	-1.51	0.0002	BI399019	
Ssc.8161.2.A1_at	Transcribed locus	-1.56	0.0009	BQ601084	
Ssc.18021.1.A1_at	Transcribed locus	-1.58	0.0006	CF180456	
Ssc.18397.1.A1_at	Transcribed locus	-1.69	0.0005	CF178770	

Ssc.1377.3.S1_at	MRNA, clone:LNG010080E11, expressed in lung	-1.7	0.0008	BP157471	
Ssc.25082.1.S1_at	Transcribed locus	-1.76	0.0003	CN160088	
Ssc.7954.1.A1_at	Transcribed locus	-1.8	0.0008	BQ599859	
Ssc.24625.1.A1_at	MRNA, clone:AMP010061G05, expressed in alveolar macrophage	-1.81	0.0006	CK461494	
Ssc.9003.1.A1_at	MRNA, clone:OVRM10211E06, expressed in ovary	-1.87	0.0002	CO992161	
Ssc.9039.1.A1_at	Transcribed locus	-1.87	0.0009	CF366256	
Ssc.8331.1.S1_at	Transcribed locus	-1.9	0.0008	BF713029	
Ssc.14436.1.S1_at	Macaca mulatta ubiquitin specific protease 14, Transcript Variant 1	-1.92	0.0004	XM_001090667	
Ssc.5082.1.A1_at	Transcribed locus	-1.96	0.0003	BI233968	
Ssc.11801.3.A1_at	asparagine-linked glycosylation 9 homolog	-1.99	0.0004	NM_001077691	118026936
Ssc.7451.1.S1_at	Bos taurus ras homolog gene family, member U	-2.14	0.0001	NM_001098147	45827773
Ssc.9039.2.S1_at	protein tyrosine phosphatase, receptor	-2.16	0.0002	XM_001174413.1	5788
Ssc.5372.1.S1_at	Similar to KIAA0644 Gene product	-2.53	0.0005	NM_014817.3	9865
Ssc.23305.1.S1_at	Similar to Transmembrane protein 144	-2.65	0.0009	NM_001101989	156120626
Ssc.30424.1.A1_at	solute carrier family 38, member 1 [Homo sapiens]	-2.75	0.0006	NM_001077484	117168276
Ssc.18135.1.S1_at	proline/arginine-rich end leucine-rich repeat protein	-3.04	0.0006	NM_054077	41349453
Ssc.22988.1.S1_at	phosphatidylcholine transfer protein	-3.12	0.0007	NM_174835	156151416
Ssc.30534.1.A1_at	protocadherin transcript variant 3	-4.31	0.0004	NM_001098171.1	14165401

Appendix Ib

METHODOLOGY FOR SELECTED PROTOCOLS

InSitu hybridization methods

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 (w/v) 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 permanently stored at RT.

Solution preparations

4% Paraformaldehyde

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 solution)
3. When solution is clear, remove from heat
4. Add 1X PBS to make final desired volume
5. After solution is cool, adjust pH to 7.2

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

Add components to 800 mL of ddH20, adjust pH to 7.6, bring to 1000 mL final volume.

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 block 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 (with small amount of gelatin dissolved) to expand sections in the ribbons.
    d. Use positively charged slides to hold sections to slide.

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

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

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

2. Extract once with an equal volume of PCI (Phenol:Chloroform:Isoamly alcohol) 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 μl 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/μl

Note: About which enzyme to use with the promoter.

Preparation of Probe

1. Set up probe synthesis reaction (enough for about 10 slides) in a 1.5 mL tube. Use RNAse free tubes and pipet tips.

Order	Solution	10 Slides	20 Slides	40 Slides
1	Water (Ambion DEPC)	4.0 µL	8.0	16.00
2	5X Transfer Buffer (Vortex)	2.5 µL	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 µγ	2.00	4.0
6	Rnasin (Keep Cold)	0.5 µL	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 Separations, Inc-CAT# CS-90)
   -Need two columns for 4X reaction. The column can only handle 100 uL of fluid. Need to rehydrate the column at least 1 h 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 at least 30 min –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 reaction (40 slides) double the volume-6uL RQ-1-DNase, 1.0 uL RNasin. Vortex, centrifuge briefly and incubate for 15 min at 37^{o}C.

5. After incubation add 20 mL yeast tRNA (10mg/ml) and 40 uL (1-2 X reaction)
   -Phenol:Chlorofrom:Isoamyl alcohol (Ratio 25:24:1; light sensitive, store at –4^{o}C).
   -For 3-4X Rxn 40 mL 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****

   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)

6. Add a volume of chloroform to match PCI
   1X=40uL
   3X=80uL

a. Vortex and spin at full speed for 5 min
   -Complete SEP column preparation: Take top and bottom cap off. Place in tube 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). Centrifuge at 750 x g for 2 min. Use column immediately- do not let it dry out.
b. Add PCI:CI purified probe to Centrisep column (place in middle of matrix and don't touch matrix with pipet tip).
c. Centrifuge column for at 750 x g for 2 min. then discard the column. Purified probe is the flow through remaining in the tube.

7. Precipitate with 60 uL 3M NaOAc (pH 5), 1 uL yeast tRNA, 5uL Dextran T500 (10mg/ml) and 300uL EtOH for 1-2X reactions. Double for 3-4X reactions.

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

9. Wash pellets with 70% EtOH

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

11. Count in β-counter (1-2 uL).

Calculation for probe Hybridization

Antisense -20 slides: 5 X 10^6 cpm/slide
20 slides X 70 uL hybridization soln/slide=1400 uL (may add for 1-2 more slides) + Volume of probe (Need 5 million cpm/slide)

Final solution should be 10% DTT (100 mM DTT) so, divide the amount of hybridization solution + amount of probe and divide the sum by 9 to determine amount of 1M DTT to add.

Need only 1-2 slides for sense Probe.
Vortex, quick spin and incubate at 70 C for 10 min to denature probe.

Summary
cRNA probes (5 X 106 cpm/slide) with hybridization Solution containing 100 mM DTT at 70 C for 10 min. then place in 55^{o}C incubator before until applied to slide.

Preparation of Slides

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 min and agitate every 1-2 min "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
6. Fresh 4% paraformaldhyde (PAF) 20 min-1X
   - 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 min, then add enough 10X PBS to make final concentration 1X. pH to 7.2 with 12N NaOH "slowly" and bring to volume with ddH_20
7. PBS for 5 min-2X
8. Proteinase K for 7.5 min-1X

   For 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_2O
9. 4% PAF for 15 min 1X(Can reuse the PAF from first wash in step 6)-Dispose PAF in waste bottle
10. ddH2O 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.

Probe Hybridizations

1. Following the denaturation of the probe (70 C), add the Hyb soln to the middle of the slide. Set pipetman at 75 μL, but when adding don't blowout last fluid to avoid air bubbles.
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. Formamide 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 min</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. 1X TEN 10 min 37 C. Repeat twice.
12. 1X 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. 1X TEN 15 Min at 37 C
14. 50 % Formamide/2X SSC/50 mM BME at 65^{o}C for 20 min.
15. 2X SSC at RT for 15 min
16. 0.1X SSC RT for 12 min
17. 70% EtOH/0.3 M Ammonium Actetate at RT for 5 min. Repeat once.
18. 95% EtOH/0.3 M Ammonium Acetate for RT for 1min.
19. 100% EtOH at RT for 1 min. Repeat once.
20. Air dry and expose to Kodak Biomax Film overnight. Develop 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. Use Kodak BioMax MR film-packaged in single sheets. Make sure emulsion side down (Non-shiny side)-notched should be in upper left corner. Expose at least 16 hours.

Autoradiography

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

   -Emulsion is aliquoted 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 alternative, 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 holes slides following covering with emulsion. Place a slide about 3-4 notches 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 conical 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 with ddh20 in the conical 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_2O bath.
4. Dip each slide to the bottom of the slide dipper. Wipe off excess, polish back to remove film from edge.
5. After dipping , place towels in light tight box. Leave lids off during this time. Allow to dry for 6 to 8 hours. Rotate slides 3-6 hours.
6. After 3-6 hours drying go to dark room and place lids on slide boxes. Wrap in foil, store at 4C for number of days or weeks based on exposure to X-ray film.
7. To develop allow slides to reach RT
8. Kodak D-19 developer (78.3g / 500 mL) for 4 min-1x
9. ddH2O- 0.5 min-1X
10. Fixer for 5 min-1X

Fixer (Kodak Rapid Fixer with Hardener):

171 mL water
59.5 mL Solution A (Fixer)
6.5 mL Solution B (Hardener)

11. Water for 5 min
12. Hematoxylin (diluted with ddH2O 1:3) for 30 seconds
13. Water for 30 sec
14. Water for 5 min. Repeat until hematoxylin quits leaching into water.
15. 70 EtOH-3 min
16. 95 EtOH-1 min 2X
17. 100 EtOH-1 min 2X
18. Xylene for 3 min 3X
19. Permount and coverslip

Other *In Situ* Hybridization Solutions:

5X SSC/10 mM BME (500mL)
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 sterilize. DEPC treat and autoclave.

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

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

Hybridization Solution (100 mL)

50 mL Formamide – (50%)
6 mL 5M NaCL – (0.3 M)
2 mL 1M Tris-HCl pH 8.0 - (20mM)
1 mL 0.5 M EDTA pH 8.0 – (5mM)
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. H_2O to 20 mL

Store Frozen in -20C

Before use add 1M DTT to a final concentration of 100 mM DTT for probes during hybridization.

VITA

Morgan Dean Ashworth

Candidate for the Degree of

Doctor of Philosophy

Thesis: TRANSCRIPTIONAL AND TRANSLATIONAL FACTORS CONTRIBUTING TO ENDOCRINE DISRUPTION OF THE PREGNANT AND PSUEDOPREGNANT MODEL PHENOTYPE IN PIGS

Major Field: Molecular Reproduction

Minor Field: Biochemistry

Biographical:

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

Education: Graduated from Lone Grove High School, Lone Grove, Oklahoma in May of 1987. Earned Bachelor of Science degree from Oklahoma State University, Stillwater, Oklahoma in August 2001. Earned a Master of Science Degree at Oklahoma State University, Stillwater, Oklahoma in May 2005. Completed the requirements for Doctor of Philosophy degree at Oklahoma State University, Stillwater, Oklahoma, in May, 2009.

Experience: Lab Technician, Oklahoma State University, 2001-2009.

Professional Organizations: Society for the Study of Reproduction, Sigma Xi Scientific Research Society, Animal Science Graduate Student Association.

Name: Morgan Dean Ashworth Date of Degree: May, 2009*

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: IDENTIFICATION AND CHARACTERIZATION OF UTERINE TRANSCRIPTIONAL AND TRANSLATIONAL FACTORS CONTRIBUTING TO ENDOCRINE DISRUPTION OF THE PREGNANT AND PSEUDOPREGNANT MODEL PHENOTYPE IN PIGS

Pages in Study: 312 Candidate for the Degree of Doctor of Philosophy

Major Field: Animal Science

Scope and Methods of Study: Embryonic mortality has been demonstrated to occur through endocrine disruption of the uterine environment in the pig. Exogenous estrogen administration to pregnant gilts on days 9 and 10 of pregnancy results in complete embryonic mortality by day 16 of gestation. The current investigation discovered uterine dysfunction caused by early estrogen exposure in pigs through removing conceptus contributions via construction of an endocrine disrupted psuedopregnant pig model. The following study focused on the window of attachment during early gestation in which a significant amount of embryonic mortality occurs. This investigation examined potential mechanisms involved during the window of attachment, and how endocrine disruption within those mechanisms may lead to reduced litter size in swine production.

Findings and Conclusions: Successful pregnancy in the pig appears to center around a robust and well defined immune response at the proper time. The current dissertation established four distinct studies which all culminated around proper timing of the vital immune response during days 10 through 18 of early pregnancy in the pig. Additionally, the current study used microarray analysis to demonstrate potential roles estrogen may play in regulating the immune system during this critical time of pregnancy in the pig. Furthermore, all four studies confirmed estrogen's ability to craft mistiming during the uterine window of attachment through transcriptional and translational regulation. These studies have provided a list of potential candidate genes and proteins to further elucidate the mechanism by which early exposure of estrogen terminates pregnancy in the pig.

ADVISER'S APPROVAL: Udaya E Desilva