EFFECT OF RU486, A PROGESTERONE ANTAGONIST, ON UTERINE PROGESTERONE RECEPTOR, EMBRYONIC DEVELOPMENT AND OVARIAN FUNCTION DURING EARLY PREGNANCY IN PIGS

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This thesis is dedicated to my family and friends who continuously support me with their patience, love and encouragement.

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3β-HSD	3beta-hydroxysteroid
AAG	alpha$_1$-acid glycoprotein
ACL	Accessory corpus luteum/corpora lutea
ACTB	Beta Actin
AF-1	Activation function domain-1
AF-2	Activation function domain-2
AF-3	Activation function domain-3
AIB1	Amplified in breast cancer 1
AP1	Activating protein 1
AR	Androgen receptor
bp	Base pair
C	Celsius
CA	Corpus albican/Corpora albicantia
CAT	Chloramphenicol acetyl transferase
CDK2	Cyclin-dependent kinase 2
cDNA	Cloned deoxyribonucleic acid
C-Fos	Cellular Finkel-Biskis-Jinkins murine osteogenic sacrcoma
ChIP	Chromatin immunoprecipitation
CL	Corpus luteum/Corpora lutea
cm	Centimeter
Co	Conceptus

CO	Corn oil
c-Src	Cellular sarcoma kinase
d	Day/Days
DBD	DNA binding domain
dd	Double distilled
DNA	Deoxyribonucleic acid
E2	Estradiol-17beta
EC	Estradiol cypionate
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
Elk-1	Ets-domain protein
ER	Estradiol receptor
ERα	Estradiol receptor alpha
F	Follicle
f-actin	Filamentous actin
FGF-7	Fibroblast growth factor 7
FGFR2	Fibroblast growth factor receptor 2IIIb
FSH	Follicle stimulating hormone
GE	Glandular epithelium/Glandular epithelia
GFP	Green fluorescent protein
GLM	General linear model
GnRH	Gonadotropin releasing hormone
GR	Glucocorticoid receptor

h	Hour
i.m.	Intramuscular
ID	Inhibitor domain
IGFBP-1	Insulin like growth factor binding protein-1
IHC	Immunohistochemistry
Ihh	Indian hedgehog
IKK	IκB kinase
IL-1	Interleukin-1
IL-1β	Interleukin-1 beta
INF-γ	Interferon-gamma
Irg1	Immunoresponsive gene 1
ITI	Inter-α-trypsin inhibitor
ITIH4	Inter-α-trypsin inhibitor heavy chain 4
IκB	Inhibitor of NF-κB
kDa	Kilodalton
KO	Knockout
L	Liter
LBD	Ligand binding domain
LE	Luminal epithelium/Luminal epithelia
LH	Luteinizing hormone
LHr	Luteinizing hormone receptor
lsmeans	Least squares means
MAPK	Mitogen-activated protein kinase

mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mRNA	Messenger ribonucleic acid
MR	Mineralocorticoid receptor
MUC-1	Mucin-1
MUC-4	Mucin-4
NF-κB	Nuclear factor-kappa B
ng	Nanogram
nm	Nanometer
NTC	No template control
Osf2	Osteoblast-specific transcription factor 2
OXT	Oxytocin
OXTR	Oxytocin receptor
P	Page
p21	Cyclin-dependent kinase inhibitor 1A
P4	Progesterone
P450arom	Cytochrome P450 aromatase
P450c17	Cytochrome P450 17alpha-hydroxylase
P450scc	Cytochrome P450 side-chain cleavage
PAT	PBS/Sodium azide/Tween-20 solution
PBS	Phosphate buffering solution

PCR	Polymerase chain reaction
pg	Picogram
PG	Prostaglandin
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGH_2	Progstaglandin H_2
PGR(s)	Progesterone receptor(s)
PGR-A	Progesterone receptor A
PGR-AB	Progesterone receptor A and progesterone receptor B
PGR-B	Progesterone receptor B
PGR-C	Progesterone receptor C
PKA	Phosphokinase A
PRE	Progesterone response element
PRL	Prolactin
PRLR	Prolactin receptor
PTGS2	Prostaglandin-endoperoxidase synthase 2
RANK	Receptor activator for nuclear factor-kappa B
RANKL	Receptor activator for nuclear factor-kappa B ligand
RIA	Radioimmunoassay
RT-PCR	Real time polymerase chain reaction
SAS	Statistical analysis system
SCF	Stem cell factor (kit ligand)
SEM	Standard error of the lsmean
Ser(345)	Serine 345

SF-1	Steroidogenic factor-1
Sp1	Stimulatory protein 1
SPP1	Phosphoprotein 1/osteopontin
StAR	Steroidogenic acute regulatory protein
STAT	Signal transducers and activation of transcription
SUMO	Small ubiquitin-like modifier
T1	Gilt treated with RU486 on d 3, 4 and 5 of pregnancy
T2	Gilt treated with RU486 on d 6 and d 7 of pregnancy
TDF	Thecal cell differentiation factor
TGF-β	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
US	United States
µg	Microgram
µL	Microliter
µm	Micrometer
µmol	Micromole

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Abstract

Progesterone (P4) has well documented functions in regulating uterine quiescence, uterine histotroph secretion, suppression of the immune response and steroid receptors. Establishment of pregnancy in the pig depends on down-regulation of the progesterone receptor (PGR) in the endometrial epithelium. In the pig, P4 causes down-regulation of the PGR in the luminal (LE) and glandular (GE) epithelium near d 8 of the estrous cycle and pregnancy. Full down-regulation of the PGR occurs by d 12. The mechanism through which P4 down-regulates PGR in the uterine epithelium but not within the myometrium or stroma is not understood. One hypothesis for cell specific down-regulation of the PGR is that P4 may act through the PGR to increase expression of receptor activator for nuclear factor-kappa B ligand (RANKL). In the pig uterine epithelium, RANKL may bind its receptor RANK and activate nuclear factor-kappa B (NF-κB). Able to bind up stream of the PGR gene, nuclear factor-kappa B is a transcription factor that can regulate expression of the PGR. Activation of NF-κB in uterine epithelium may result in down-regulation of the PGR exclusively within the LE

and GE of the pig. Using the P4 antagonist RU486, we evaluated the regulation of endometrial PGR by P4 and its spatial relationship to endometrial NF-κB activation and RANKL expression. Gilts were inseminated (d 0) and assigned to one of three treatments: RU486 (400 mg/d) on d 3, 4 and 5 of pregnancy (T1; n = 9); RU486 on d 6 and d 7 of pregnancy (T2; n = 9); or a non-treated control group (control; n = 9). Ovaries and uteri were harvested on d 8 or d 12 of pregnancy. Treatment of gilts with RU486 affected early conceptus development. Percent normal development was lowest in T1 gilts (chi-square = 7.00; $P < 0.05$). The endometrial PGR-B mRNA was more abundant in T1 and T2 gilts when compared with control gilts. There was a treatment effect ($P < 0.01$) on log-transformed endometrial RANKL mRNA expression as RANKL expression was greater in T1 (d 8 and d 12) and T2 gilts (d 12) when compared with controls. Activation of NF-κB (nuclear localization) in the uterine epithelium increased in T2 and control gilts but remained relatively unchanged in T1 gilts from d 8 to d 12. Inhibiting P4 action with RU486 during early pregnancy increased PGR-B mRNA expression, indicating that P4 is responsible for PGR down-regulation in the uterine epithelium. Our results do not support the hypothesis that RANKL mediates NF-κB inhibition of PGR. Activation of NF-κB on d 12 could be a result of conceptus elongation and secretion of interleukin-1 beta (IL-1β).

Progesterone may have a direct autocrine effect on the ovarian follicular development and growth during the estrous cycle and early pregnancy in the pig. While using RU486 to investigate the role of P4 in down-regulating the PGR in the uterine epithelium of the pig, gross anatomical observations of the ovaries revealed increased ovarian activity in response to RU486. Gilts treated with RU486, T1 and T2, had heavier

ovaries (16.1, 17.9 and 19.8 g [SEM = 1.1]; $P < 0.05$), greater mean follicular diameters (3.6, 5.6 and 4.9 mm [SEM = 0.5]; $P < 0.05$) and tended to have a greater number of corpora lutea (CL) (13.7, 16.8 and 15.0 [SEM = 1.0]; $P = 0.072$) when compared with control gilts (control, T1 and T2, respectively). Plasma P4 concentrations from d 8 to d 11 of pregnancy tended to be greater for RU486 treated gilts (21.9, 23.6 and 24.7 ng/mL; control, T1 and T2; $P = 0.064$). There was a treatment by day interaction for plasma E2 because T1 gilts had greater plasma E2 concentrations when compared with control and T2 gilts from d 2 to d 7 of pregnancy (2.4, 5.0 and 2.6 pg/mL; control, T1 and T2, respectively; $P < 0.001$). Gilts treated with RU486 had greater plasma E2 concentrations when compared with control gilts from d 8 to d 11 of pregnancy (1.9, 12.5, and 11.0 pg/mL; control, T1 and T2, respectively; $P < 0.05$). Uterine weight was reduced for T1 gilts compared with T2 or control gilts (785.3, 607.6, 780.9 g [SEM = 48.8]; control, T1 and T2, respectively; $P < 0.05$). In conclusion, treating gilts with RU486 during early pregnancy reduced uterine weight, stimulated ovarian follicular growth and accessory corpora lutea (ACL) formation.

CHAPTER ONE

INTRODUCTION

Pigs will ovulate 16 to18 oocytes during estrus, of which 95% are fertilized and initiate development. The United States (US) national agriculture statistics service (http://www.nass.usda.gov/) reports that on average, 9.5 piglets are born per litter, indicating drastic loss of conceptuses and less than optimal reproductive efficiency in the pig. Prenatal mortality in the pig is high, ranging from 20 to 46% (Pope et al., 1990). Although little embryonic loss occurs before day (d) 7 (Polge, 1982), the majority of prenatal mortality will take place before d 20 of gestation. These statistics indicate that embryonic loss occurs during the pre-implantation period. Investigators, therefore, have focused their resources on this specific time point within gestation.

Some breeds of pigs are more prolific than others but litter size can vary from sow to sow within a breed. Why some pigs produce smaller litters than others is poorly understood, however, the mechanisms that support early conceptus development may be to blame. The first 20 days of gestation are critical for development of the early conceptus as survival depends on it's ability to grow along with uterine receptivity. Establishment of pregnancy in the pig depends on the development of the conceptus and

the uterine environment, as asynchrony between the conceptus and uterus is detrimental to conceptus survival.

Progesterone (P4; the hormone of pregnancy) supports conceptus growth by stimulating the secretion of uterine proteins (histotroph) during gestation. Progesterone also controls expression of its own receptor within specific tissues of the uterus. For example, P4 down-regulates the progesterone receptor (PGR) in the uterine luminal epithelium (LE) and glandular epithelium (GE) by d 12 of the estrous cycle and pregnancy (Geisert et al., 1994). Down-regulation of PGR in the LE and GE is thought to be essential for allowing conceptus attachment to the uterine surface during the establishment of pregnancy. How P4 down-regulates PGR specifically in the uterine epithelium and not in the uterine stroma is not understood. Down-regulation of uterine PGR is not exclusive to the pig and occurs before implantation in a number of mammalian species including humans (Okulicz and Scarrell, 1998), cattle (Kimmins and MacLaren, 2001), sheep (Spencer and Bazer, 1995), western spotted skunk (Mead and Eroschenko, 1995), baboons (Hild-Petito et al., 1992), rhesus monkeys (Okulicz and Scarrell, 1998), and mice (Tan et al., 1999). Asynchrony between the development of the conceptus and PGR down-regulation (uterine receptivity) could be responsible, in part, for early embryonic loss in the pig and other species. Considering that the US has 6.06 million production sows (http://www.nass.usda.gov/), a better understanding of the mechanisms controlling early pregnancy in the pig could lead to greater pregnancy rates and profitability for US swine producers.

CHAPTER TWO

LITERATURE REVIEW

INTRODUCTION

The majority of pig conceptuses are lost between d 7 and d 20 of gestation (Polge, 1982; Pope, 1994), a time when conceptuses will release the maternal recognition of pregnancy signal (estradiol; E2) and attach to the uterus to establish pregnancy. Progesterone (P4) down-regulates the progesterone receptor (PGR) in the uterine epithelium but not within the stroma near d 8 of the estrous cycle and pregnancy in the pig and is thought to allow conceptus attachment to the uterus (Geisert et al., 1994). How P4 down-regulates the PGR in the uterine epithelium but not within the stroma is not understood. One theory suggests that P4 may increase expression of receptor activator for nuclear factor-kappa B ligand (RANKL) in the epithelium resulting in activation of nuclear factor kappa B (NF-κB), a transcription factor able to bind DNA and regulate gene expression. Activation of (NF-κB) in the uterine epithelium is thought to down-regulate PGR gene expression in the pig. The purpose of this chapter is to review literature that has contributed to the understanding of establishment of pregnancy in the

pig, how progesterone through PGRs can influence establishment of pregnancy and how nuclear factor-kappa B, a mediator of the inflammatory response, may be involved.

The Pig Estrous Cycle

The female pig will reach puberty at approximately six months of age. The steroid hormones P4 and E2 control luteal (d 3 to d 16) and follicular (d17 to d 2) phases of the estrous cycle, respectively. The pig estrous cycle can further be divided into four defined stages (proestrus, estrus, metestrus, and diestrus) and will divert into acyclic during pregnancy.

Proestrus (d 17 to d 20), beginning during the regression of corpora lutea (CL) and falling blood P4 concentrations, is characterized by recruitment of tertiary follicles that produce increasing concentrations of E2. The greater blood E2 concentrations and lower P4 cause a change in steroid negative feedback on the hypothalamus, and modify the release of gonadotrophin releasing hormone (GnRH) by basal medial nuclei into the median eminence. The hypophyseal portal system enables GnRH to reach the anterior pituitary, stimulating gonadotrophs to release follicle stimulating hormone (FSH) and luteinizing hormone (LH).

Before reaching 6 mm in diameter, most follicles undergo the process of atresia, degenerating and later disappearing from the ovary (Dailey et al., 1976; Grant et al., 1989). The remaining follicles reach approximately 8 to 12 mm in diameter and become preovulatory Graffian follicles. These large follicles are capable of producing high concentrations of E2 in the blood (~45 pg/mL), resulting in behavioral changes that are associated with signs of estrus.

During estrus (d 21 to d 2), the female pig will ovulate and remain receptive to the boar for 48 to 72 h. Ovulation typically occurs within 36 h of estrus. Peak E2 levels cause a surge release of LH from the anterior pituitary, resulting in ovulation of 16 to18 follicles. Although nearly 30 to 40% of recruited follicles are selected for maturation and ovulation, follicles selected represent only a fraction of approximately 80 to 85 tertiary follicles that are present on the ovary during the mid luteal phase. The later follicles fall victim to atresia (Guthrie et al., 1995; Schwarz et al., 2007). The LH surge not only causes lutenization of theca interna and granulosa cells in the follicle but also signals the oocyte to resume meiosis and release the first polar body. After follicles ovulate, the pig will experience a short period of reduced steroid production from the ovary, referred to as metestrus.

Metestrus (d 3 to d 4) is characterized by declining concentrations of E2 as well as maturation of corpora hemorragica to corpora lutea (CL), resulting in increasing P4 concentrations. During diestrus (d 5 to d 16), the uterine environment is completely dominated by P4. Production of P4 by the CL not only maintains uterine quiescence but also allows secretions of uterine proteins that support pregnancy.

The length of the pig estrous cycle is uterine-dependent and controlled by P4. Stimulation of uterine endometrium with P4 for 10 to 12 days leads to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production and secretion which causes luteolysis in the absence of the conceptus (Spencer et al., 2004). The cyclic pattern of CL P4 production and endometrial $PGF_{2\alpha}$ release allows the female to recycle and return to estrus for another attempt at conception.

Near d 12 of pregnancy the conceptus will release E2 as the maternal recognition of pregnancy signal. Conceptus release of E2 results in secretion of luteolytic $PGF_{2\alpha}$ into

the uterine lumen, protecting the CL and P4 production for the remainder of pregnancy. If conceptuses are not present, the uterus will release $PGF_{2\alpha}$ into the vasculature near d 15 of the estrous cycle, resulting in luteolysis and recruitment of follicles.

Early Pregnancy in the Pig

Both porcine ovaries will ovulate simultaneously, releasing as many as 16 to 18 oocytes. Fertilization takes place in the ampulla-isthmic junction of the oviduct. A single sperm penetrates the oocyte and releases the male pronuclei into the egg cytoplasm. After germinal vesicle breakdown, female and male pronuclei migrate to the center of the oocyte and the first mitotic division occurs, producing a two celled embryo. The two cells (blastomeres) are smaller than the initial single cell before cleavage as a result of cytoplasmic partitioning that takes place during the holoblastic cleavage. The blastomeres will undergo a second cleavage division within 24 h of fertilization, producing a four celled embryo. Although blastomeres continue to undergo mitosis, the rate of cleavage slows, occurring once every 24 to 26 h.

Cleavage between blastomeres may not be a synchronized event, as some cells divide faster than others. In accordance with the cleavage-driven model, blastomeres that divide more quickly may be positioned more toward the exterior of the cell mass, giving rise to the trophoblastic lineage (Piotrowska and Zernicka-Goetz, 2001). Cells that divide more slowly may be positioned toward the interior, giving rise to the inner cell mass.

The porcine embryo will enter the uterine horn approximately 5 days after ovulation, undergoing first morulation followed by blastulation. Blastomeres located on the surface of the morula produce tight cell junctions while pumping sodium ions into the

interior of the embryo, resulting in fluid accumulation and formation of the inner cell mass in the blastocoele (Senger, 2003).

Hatching occurs near d 8 of gestation, when thinning of the zona pellucida and cell expansion leads to expulsion of the blastocyst. Upon entering the uterus, porcine embryos will migrate through the uterine body and into the opposite uterine horn, intermingling with embryos fertilized in the opposite oviduct. Inadequate migration and uterine spacing of elongating conceptuses is detrimental to porcine embryonic survival in the uterus. Spherical conceptuses grow, by cell proliferation, from 4 to 9 mm in diameter between d 10 and d 12 of gestation (Geisert et al., 1982).

There are four developmentally different morphologies for porcine conceptuses on d 11 to d 12 of gestation; spherical, ovoid, tubular and elongated. The different conceptus morphologies are the result of changes in the trophectoderm. The 9 mm spherical conceptus will become ovoid and then tubular and then rapidly elongate to approximately 150 mm in as little as 2 to 3 h. Trophoblast elongation occurs at a rate of 35 to 40 mm/h, which is unique to the porcine conceptus. Trophoblast elongation is a result of cellular remodeling of the trophectoderm and endoderm rather than cellular hyperplasia (Geisert et al., 1982).

Formation of extra-embryonic membranes will begin after conceptus elongation and attachment to the uterine surface near d 13 of gestation (Friess et al., 1980). The allantois, arising from the embryonic hindgut, will continue to expand, making full contact with the meter long chorion by d 19 of gestation. The porcine chorion is completely vascularized by d 30 of gestation due to formation of allantoic blood vessels (Wislocky and Dempsy, 1946). Chorionic villi located throughout the chorion of the

placenta penetrate into the endometrium to form the fetal-maternal interface that is critical for embryonic oxygen and nutrient supply (Senger, 2003)

Maternal Recognition of Pregnancy

Porcine conceptuses will undergo rapid trophoblast elongation near d 12 of gestation. Spherical conceptuses, (approximately 10 mm in diameter) elongate to nearly 150 mm in less than 2 to 3 h. The unique cellular remodeling during elongation is thought to be dependent upon the uterine landscape as well as trophectoderm cytoskeletal rearrangement of filamentous actin (f-actin) (Mattson et al., 1990).

On d 15 and d 16 of the porcine estrous cycle, $PGF_{2\alpha}$ is released from the epithelia into the uterine vasculature. Prostaglandin $F_{2\alpha}$ can reach the ovaries by vascular countercurrent exchange between the closely associated uterine vein and ovarian artery, causing luteolysis. As conceptuses elongate through the uterus, they synthesize and secrete E2 as the maternal recognition of pregnancy signal. Secretion of E2 redirects luteolytic $PGF_{2\alpha}$ from the uterine vasculature to the uterine lumen (Bazer et al., 1982).

The uterus has an extensive epithelial surface and glandular architecture that is capable of producing $PGF_{2\alpha}$. A minimum of two embryos per uterine horn, therefore, must be present to release E2 as the maternal recognition of pregnancy signal (Dziuk, 1968). Only forty percent of $PGF_{2\alpha}$ released into the vasculature of the pig uterus is metabolized upon first pass through the lungs. Secretion of $PGF_{2\alpha}$ into the uterine lumen ensures complete protection for the CL. Removal of the luteolytic effect of $PGF_{2\alpha}$ is essential because P4 is required for conceptus survival and development during pregnancy (Spencer and Bazer, 2002). The term “maternal recognition of pregnancy”

was first coined by Short in 1969 when describing the process resulting in the extended life of the CL beyond which occurs during a normal estrous cycle. Today, the term maternal recognition of pregnancy refers to the process by which the developing conceptuses produce and release a chemical signal that indirectly prolongs the lifespan of the CL (Geisert et al., 1990), maintaining P4 production for an extended period of time.

Although it has been well established that $PGF_{2\alpha}$ is the uterine-produced luteolysin, the hormonal requirements for porcine luteolysis are not fully understood (Ziecik, 2002). After ovulation and establishment of CL, P4 concentrations in the blood begin to rise by d 3, reaching 20 to 40 ng/mL during the estrous cycle and pregnancy (Bazer et al., 1998). Stimulation of the porcine uterus with P4 for 7 to 8 days leads to loss of PGR within the LE and GE but not in the myometrium or stroma (Geisert et al., 1994). Progesterone causes down-regulation of PGR in the LE and GE near d 8 and is fully down-regulated by d 12 in both cyclic and pregnant pigs (Geisert et al., 1994; Persson et al., 1997; Sukjumlong et al., 2005). After binding PGR, P4 inhibits gene expression for estradiol receptor (ER) in the uterus. Down-regulation of PGR within the porcine uterine epithelium by d 10 of both the estrous cycle and pregnancy is followed by reestablishment of ER in these cell layers (Geisert et al., 1993).

Similar to a model described in sheep (Gray et al., 2000), porcine uterine $PGF_{2\alpha}$ is thought to be produced by the uterine LE and GE. Although not fully characterized in the pig, the McCracken model of luteolysis indicates that down-regulation of PGR in the LE and GE allows reestablishment of ER followed by oxytocin receptor (OXTR) within the LE and GE (McCracken et al., 1999). Oxytocin (OXT), when bound to OXTR, is thought to increase expression of enzymes involved in $PGF_{2\alpha}$ synthesis such as

prostaglandin-endoperoxide synthase 2 (PTGS2) as well as initiate pulsatile secretion of $PGF_{2\alpha}$ from the uterus. Prostaglandin-endoperoxidase synthase 2 is a rate-limiting enzyme in prostaglandin (PG) synthesis and catalyzes the conversion of arachidonic acid to prostaglandin H_2 (PGH_2), leading to production of $PGF_{2\alpha}$ in the LE and GE. Endometrial PTGS2 expression increases approximately 76-fold between d 5 and d 15 of the estrous cycle and pregnancy in the pig (Ashworth et al., 2006). In sheep, OXT secreted by the posterior pituitary and the ovary bind OXTR in the LE and GE and causes pulsatile release of $PGF_{2\alpha}$ from d 14 to d 16 of the estrous cycle (Spencer et al., 2004). Although the porcine uterus contains OXTR and concentrations of OXT increase in porcine blood during luteolysis, the source of OXT release in the pig has not been established. The porcine ovary contains low levels of OXT and OXT mRNA. The posterior pituitary, therefore, may be responsible for circulating levels of OXT (Bazer et al., 1998). The porcine uterus also produces OXT and it has been hypothesized that endometrial release of OXT may be involved in the pulsatile secretion of $PGF_{2\alpha}$ (Carnahan et al., 1996).

Conceptus release of E2 near d 12 of gestation serves as the initial maternal recognition of pregnancy signal in the pig. The E2 can redirect $PGF_{2\alpha}$ to the uterine lumen rather than the vasculature (Bazer and Thatcher, 1977; Bazer et al., 1982). Secretion of $PGF_{2\alpha}$ into the uterine lumen is referred to as the exocrine mechanism. A single pulse release of E2 from the pig conceptus will not maintain the CL. A second sustained release of E2 from the conceptus between d 14 to d 18 of gestation is essential to maintain CL function for the entire pregnancy. To induce pseudopregnancy, E2 must be administered on d 11 and again on d 14 to d 16 or daily from d 11 to d 15 of the

estrous cycle (Geisert et al., 1990). Single injections of E2 on either d 9.5, d 11, d 12.5, d 14, d 15.5, or d 14 to d 16 results in regression of CL and interestrous intervals of nearly 30 days (Geisert et al., 1987; Spencer et al., 2004).

The exocrine-endocrine theory for maintaining CL function in the pig was coined by Bazer and Thatcher in 1977. This model indicated that rerouting $PGF_{2\alpha}$ between the uterine vasculature and uterine lumen allowed maintenance of the CL rather than luteolysis and loss of P4 production. Although not fully understood, the mechanism of exocrine secretion is thought to be triggered by E2 induced expression of prolactin receptor (PRLR) in the uterine epithelia (Senger 2003; Young et al., 1990). Binding of prolactin (PRL) to PRLR is believed to cause an ion flux of calcium in the uterus, promoting secretion of $PGF_{2\alpha}$ into the uterine lumen (Gross et al., 1990; Senger, 2003). In both pregnant and pseudopregnant gilts, this calcium release and later uptake is closely associated with the exocrine secretion of $PGF_{2\alpha}$ into the uterine lumen (Spencer et al., 2004).

In addition to E2, the cytokine interleukin 1 beta (IL-1β) is also secreted by the porcine conceptus during trophoblastic elongation (Tou et al., 1996). Interleukin-1 beta is involved in many cellular activities including cell differentiation, proliferation and apoptosis. It is an important mediator of the inflammatory response and cell communication (pro-inflammatory cytokine). During early pregnancy in the human, IL-1β may initiate cross talk between the uterus and conceptus (Lindhard et al., 2002). Other studies in the mouse, report an increase in IL-1β expression by the peri-implantation blastocyst (Takacs and Kauma, 1996).

First identified by Tou et al. (1996), porcine conceptus expression of IL-1β is greatest on d 11, d 12 and d 13 of gestation. Compared with d 11 filamentous conceptuses, gene expression of IL-1β was reduced 2000-fold in d 15 conceptuses (Ross et al., 2003). Although absent in the uterine lumen of the cyclic pig on d 12 to d 15, IL-1β protein is greatest in the uterine lumen on d 12 of gestation, coincident with rapid trophoblastic elongation (Ross et al., 2003).

Conceptus attachment to the uterus

Synchrony between the developing conceptus and uterine environment is critical for survival. The conceptus must attach to the uterine surface in order to establish and maintain pregnancy. Porcine conceptuses will migrate through the uterine horns to establish adequate spacing before attaching to the uterine surface. Interuterine migration, which is completed by d 12 of pregnancy, is followed by rapid trophoblast elongation where the conceptuses grow to a meter in length between d 12 to d 16 of pregnancy (Bazer and Spencer, 2002). After trophoblast elongation, the conceptus trophectoderm attaches to the LE between d 13 and d 18 of gestation (Dantzer, 1985; Perry et al., 1981). Trophoblast attachment to the uterine LE in pigs is associated with the spacio-temporal loss of PGR in the uterine LE and GE (Geisert et al., 1994). After attachment, the conceptus can then establish a true epitheliochorial type placenta and consume maternal nutrients and oxygen needed to survive to term.

Numerous tissue adhesion and cell to cell communication molecules are present during placental attachment and establishment of pregnancy in mammals. Intergrins, cell transmembrane glycoproteins, are thought to be involved in both linkage and cell to cell

communication (Hynes, 1992; Lessey, 1995). Intergrins have well known roles in conceptus attachment in the pig. Intergrins can act as membrane bound cell surface receptors by forming α and β heterodimers that allow attachment and communication between the conceptus and uterine epithelium (Hynes, 1992; Jaeger et al., 2001; Lessey, 1995). Intergrins can also bind phosphoprotein 1 (SPP1; osteopontin), an E2-induced LE secreted protein that aids conceptus attachment to the uterine surface (White et al., 2005). The ligand specificity of the heterodimer is determined by α and β subunits that make up the surface receptor. At least three α (α_1, α_4, α_5) and two β (β_1, β_3) subunits exist in the porcine conceptus. The uterine epithelium contains at least five α (α_1, α_2, α_3, α_4, α_v) and three β (β_1, β_3, β_5) subunits (Bowen et al., 1996). Elevation of α_4, α_5 and β_1 subunits occurs in the uterine endometrium during conceptus elongation. The α_v and β_3 subunits remain constant during the estrous cycle and pregnancy (Bowen et al., 1996). These subunits are thought to dimerize and aid in binding of the conceptus to the uterine epithelium.

Stabilization of the extracellular matrix, a process that is critical during attachment and establishment of pregnancy, is thought to involve members of the inter-α-trypsin inhibitor (ITI) protein family and kallikrein. Inter-α-trypsin inhibitor heavy chain 4 (ITIH4), a member of the ITI protein family, is expressed in the endometrium during establishment of pregnancy (Geisert et al., 1998; Hettinger et al., 2001). Along the surface of the uterine epithelium, cleavage of ITIH4 by kallikrein, a plasma serine protease, is thought to expose hyaluronate and intergrin binding sites at the N-terminal end of the cleaved ITI (Geisert and Yelich, 1997). Exposure of the N terminus would allow conceptus hyaluronate and intergrins to associate with the uterine LE.

Steroid hormones are thought to be responsible for an environment conducive to conceptus attachment. Although PGR is maintained in the stroma and myometrium, P4 is thought to allow conceptus attachment to the uterine surface by down-regulating PGR in the LE and GE near d 10 of the estrous cycle and pregnancy (Geisert et al., 1994). Down-regulation of PGR in the uterine epithelium is followed by reestablishment of ER within these cell layers (Geisert et al., 1993). Kallikrein protease activity has been shown to increase during conceptus elongation and release of E2. It is possible that conceptus E2 acts on ER in the LE and GE to increase kallikrein protease activity subsequently modifying ITIH4.

Cell surface glycoproteins are also differentially expressed in the uterine epithelium during the estrous cycle and pregnancy and may be critical for conceptus attachment. Mucin-4 (MUC-4) is a large surface glycoprotein shown to increase expression during attachment in the pig (Ferrell et al., 2003). Mucin-1 (MUC-1), on the other hand, inhibits necessary intergrin binding between the conceptus trophectoderm and maternal LE in mice, indicating that loss of MUC-1 is important for conceptus attachment (Surveyor et al., 1995). In rodents and sheep, the implantation process is initiated after down-regulation of MUC-1 (Spencer and Bazer, 2002). MUC-1 expression is thought to be controlled by steroid hormones and is greatest following high E2 and low P4 plasma concentrations on d 0 to d 4 of the porcine estrous cycle (Bowen et al., 1996). Uterine expression of MUC-1 is undetectable by d 10 in both cyclic and pregnant gilts (Bowen et al., 1996) indicating that loss of MUC-1 from the porcine LE is temporally associated with high P4 plasma concentrations (Bowen et al., 1996) and loss of PGR from the LE and GE (Geisert et al., 1994).

Early Embryonic Loss in the Pig

Reducing the number of conceptuses lost during pregnancy would theoretically increase litter size and profitability for swine producers. Factors affecting litter size in the pig include; ovulation rate, fertilization rate, early embryonic loss, and uterine capacity. The female pig will ovulate 16 to 18 oocytes during estrus; however, approximately 30 to 50% of ova do not produce a piglet (Pope, 1994). Fertilization rate in the pig is greater than 95% (Polge, 1978). Much of this loss, therefore, is associated with prenatal mortality.

Early embryonic mortality between d 7 and d 20 of gestation (Polge, 1982; Pope, 1994) will account for the majority of loss and reproductive inefficiency in the pig. Early embryonic loss may occur at one of three developmental phases including: 1) pre-elongation development; 2) trophoblastic elongation; and 3) placental attachment (Geisert and Schmitt, 2001). Down-regulation of PGR in the LE and GE occurs near d 8 and completed by d 12 of both the estrous cycle and pregnancy in the pig. Down-regulation of uterine PGR is, therefore, temporally associated with early embryonic mortality. Inappropriate timing or malfunction of the PGR down-regulation mechanism could result in asynchrony between the conceptus and uterine environment, resulting in early embryonic loss.

Inadequate spacing as well as an insufficient number of conceptuses in the uterus during the time of maternal recognition of pregnancy may also lead to embryonic loss. Before d 18 of gestation, no fewer than two embryos per uterine horn must present in the uterus to block luteolysis and maintain pregnancy (Dziuk, 1968). When numerous

conceptuses are present, however, there is competition for uterine space. Prenatal mortality occurs because there is inadequate placenta blood flow and insufficient nutrients required for normal conceptus development.

Exogenous estrogens can cause changes in uterine protein secretions and embryonic loss (Gries et al., 1989; Long and Diekman, 1986; Pope et al., 1986). After treating pregnant gilts with E2 on d 9 and d 10 of pregnancy, Pope et al. (1986) reported complete embryonic loss by d 30 of gestation. Gries et al. (1989) also treated gilts with E2 on d 9 and d 10 and reported embryonic mortality as early as d 16 and d 18. Gries et al. (1989) also reported a reduction in endometrial peptide secretion that was temporally associated with E2 induced conceptus mortality.

Progesterone and Early Pregnancy

Progesterone is unequivocally required for the survival of the conceptus in most mammalian species. Actions of P4 are mediated through nuclear receptors located in the cells of target tissues. Progesterone receptor isoform B (PGR-B) and progesterone receptor isoform A (PGR-A) are the two most characterized PGR isoforms. Receptors are expressed within the mammalian reproductive tract. A spacio-temporal pattern of PGR expression exists in the mammalian uterus during the reproductive cycle and pregnancy. Progesterone modulates myometrial quiescence (Fomin et al., 1999), changes cervix and vaginal secretions (Ceric et al., 2005), maintains a state of immune suppression (Schust et al., 1996) and also promotes histotroph secretion to support conceptus growth and development (Spencer et al., 2002). Progesterone and other placental hormones also regulate uterine endometrial differentiation and function,

pregnancy recognition signaling, conceptus uterine interactions and uterine receptivity for blastocyst implantation (Spencer et al., 2004).

Progesterone Production by Corpora Lutea

Ovulation causes the formation of corpora hemorrhagica. These structures are short lived as theca and granulosa cells transform to luteal cells and the blood clot from ovulation is quickly absorbed. By d 3 of the estrous cycle or pregnancy, numerous CL have formed and mature luteal cells begin converting cholesterol to P4. Progesterone is composed of four hydrocarbon rings and two oxygenated functional groups and two methyl groups. Progesterone is synthesized from pregnenalone during steroidogenesis and can be produced in cells of the adrenal gland, brain, CL, and placenta.

Cholesterol can be obtained from the diet or can be synthesized within the endoplamic reticulum from acetyl CoA. Cholesterol from the diet can be transported through the blood by lipoproteins. Upon entering the luteal cell, free cholesterol is transported across the mitochondrial bilayer membrane by steroidogenic acute regulatory protein (StAR). In the mitochondria, cholesterol is converted to pregnenolone by the action of side-chain cleavage enzyme (P450scc). Pregnenolone is then transported out of the mitochondria and converted to P4 by 3-beta-hydroxysteroid dehydrogenase-isomerase (3β-HSD). Within CL, binding of LH to lutenizing hormone receptor (LHr) can induce activation of StAR and 3β-HSD. Progesterone can then leave the cell and enter the blood stream by free diffusion. Progesterone may bind proteins in the blood and become systemically transported to target tissues.

Progesterone Receptors

Progesterone modulates an assortment of biological processes through PGRs. After binding P4, the PGRs translocate to the nucleus and bind progesterone response elements (PRE) in the genome, recruiting co-activators and co-repressors that help regulate transciption (Conneely et al., 2003); this is considered a classical mechanism. Progesterone receptors can regulate biological process by non-classical mechanisms as well; activating kinase cascades and interacting with other transcription factors that act independent of PREs (Boonyaratanakornit et al., 2001; Daniel et al., 2009; Migliaccio et al., 1998).

The PGR is a part of the nuclear receptor superfamily including other steroid receptors such as, oxysterols, thyroid hormone, fat soluble vitamins as well as orphan receptors (Conneely et al., 2003). The PGR gene is located on porcine chromosome SSA 9p13-p11 (Hu et al., 1998). It is approximately 100 kilobases in length and contains eight exons. There are two alternative promoters (Kastner et al., 1990) and translation start sites with in the PGR gene (Conneely et al., 1989), resulting in two common PGR isoforms (PGR-B and PGR-A). A less common isoform, PGR-C has been reported in the mouse and human; the result of a third promoter and translation start site within the PGR gene.

Three conserved domains including a centrally located DNA binding domain (DBD), N terminal domain and a C terminal ligand binding domain (LBD) reside in PGR-A and PGR-B (Leonhardt and Edwards, 2002). Compared with PGR-A, the PGR-B isoform differs by an additional 164 amino acids at the N terminus of the protein (Li and O'Malley, 2003). The DBD is flanked at the C terminus by a hinge region containing the

LBD as well as elements involved in nuclear localization, receptor dimerization and heat shock protein interactions (Conneely et al., 2003; Vegeto et al., 1992). The DBD consists of approximately 68 amino acids for which two type II zinc fingers allow binding of the receptor to specific *cis*-acting DNA sequences (Conneely et al., 2003). An inhibitor domain (ID), responsible for recruitment of co-repressor proteins that inhibit transcription, is located at the N terminal end of PGR-B and in the first 140 amino acids of the PGR-A peptide. Evidence suggests that this domain may be more functional in the PGR-A, for which additional amino acids extending from the N terminus of PGR-B hinder the function of the ID located more centrally to the peptide (Leonhardt and Edwards, 2002). Indeed, PGR-A has been shown to act as a ligand dependent trans-dominate repressor of other steroid receptors including: ER, androgen receptor (AR), mineralocorticoid receptor (MR), glucocorticoid receptor (GR) and PGR-B (Leonhardt and Edwards, 2002).

A less characterized PGR isoform, PGR-C has a molecular weight of nearly 60 kDa and is a N-terminally truncated form of both PGR-B and PGR-A; arising from a third downstream start site within the PGR gene (Condon et al., 2006). Although lacking a functional DBD, PGR-C contains a functional LBD that can bind P4 and may act to sequester the steroid from other PGRs while residing in the cytoplasm (Condon et al., 2006). Containing both a nuclear localization and dimerization domain, PGR-C can also regulate transcription by directly binding other PGRs thereby altering there ability to bind PRE within DNA (Condon et al., 2006; Wei et al., 1997).

Wei et al. (1996) reported that PGR-C enhanced progestin induced transcriptional activity in the presence of PGR-B and PGR-A, yet PGR-C remained inactive in the

absence of PGR-B and PGR-A. Wei et al. (1996) hypothesize that: 1) PGR-C may recruit and sequester repressor proteins that would normally limit transcriptional activity of PGR-B and PGR-A; or 2) PGR-C can dimerize with PGR-B and/or PGR-A resulting in enhanced transcriptional activity.

Two activation function (AF) domains, AF-1 and AF-2, that flank the DBD at the N terminus and at the C terminus respectively, can be found in PGR-B and PGR-A (Li and O'Malley, 2003). These domains recruit co-activators to the receptor that modulate promoter specificity and the level of gene activation (Conneely et al., 2003; Meyer et al., 1992). The PGR-B isoform has a third AF domain, AF-3, located in the extended N terminal region (Sartorius et al., 1994; Xi and O'Malley, 2003). The AF-3 domain of PGR-B recruits a number of coactivators that are not recruited by ligand bound PGR-A (Giangrande et al., 2000).

When PGR-A and PGR-B are expressed individually in cultured cells, they maintain different trans-activator properties specific to both cell type and target gene promoter (Conneely et al., 2003; Meyer et al., 1992; Vegeto et al., 1993). Although PGR-B is a stronger activator of genes that are regulated by both isoforms, PGR-A was shown to dominate transcription regulation in specific cell types and gene targets (Leonhardt and Edwards, 2002).

A null mutation of the PGR gene in the female mouse results in numerous abnormalities including impaired sexual behavior, neuroendocrine gonadotrophin regulation, and mammary development as well as uterine dysfunction (Connelly et al., 2003). Selective ablation of PGR-A and PGR-B using knockout (KO) mice have given insight into individual isoform function and the distinct physiological roles contributed

by each. The PGR-A and PGR-B are expressed at approximately equal levels in the mouse uterus (Lydon et al., 1995). Conneely et al. (2002) reported that KO mice lacking PGR-A display severe uterine hyperplasia and ovarian abnormalities with no alterations in mammary and thymus development. Ablation of PGR-B resulted in reduced mammary and thymus development but did not affect uterine or ovarian responses to P4. According to Conneely et al. (2002), PGR-A is both necessary and sufficient to elicit ligand bound reproductive responses in the female whereas PGR-B is required for normal mammary and thymus development in response to P4.

Progesterone and Progesterone Receptor Interaction

Progesterone enters a target cell by passive transport after circulation through the blood. Progesterone associates with the LBD of the receptor and changes the receptor shape. The LBD consists of 12 α helices and 4 β sheets that fold into a three layer α helical structure containing a central hydrophobic core. Receptor activation requires numerous steps including binding of P4, a conformational change in the receptor and release of multi-protein complex consisting of heat shock proteins and immunophilins (Leonhardt and Edwards, 2002). Activated steroid receptors then enter the nucleus, dimerize and undergo receptor phosphorylation as they bind to specific PREs located in promoter regions of target genes. When PGR-A and PGR-B are expressed in equal ratios in the cell, they can form both homodimers and heterodimers before binding DNA (Conneely et al., 2003). During receptor DNA binding, a α-helix extending between the zinc fingers of the receptor DBD makes contact in the major grove of DNA. The classical PRE in DNA consist of inverted repeat hexanucleotide sequences separated by

three unspecified nucleotides (Conneely et al., 2003). The 15 basepair (bp) receptor recognition site allows a dimerized receptor to bind its own hexanucleotide sequence (Conneely et al., 2003). Recruitment of co-activators and transcription factors by the steroid receptor dimer results in formation of a large transcription initiation complex leading to expression of the target gene (Conneely et al., 2003).

Non-Classical Progesterone Receptor Gene Regulation

Ligand bound PGR may activate cytoplasmic kinases such as mitogen-activated protein kinase (MAPK) and cellular sarcoma kinase (c-Src) resulting in non-classical mechanisms of transcription modulation (Boonyaratanakornit et al., 2001; Daniel et al., 2009; Migliaccio et al., 1998). Non-classical mechanisms include activation of transcription factor effectors and PGR protein modifications that result in transcription modulation independent of PREs (Daniel et al., 2009; 2007).

Mitogenic protein kinases such as cyclin-dependent kinase 2 (CDK2), Casein Kinase II, and MAPK, activated by peptide growth factors or ligand bound PGR, have shown to differentially phosphorylate a total of 14 serine residues within PGRs (Daniel et al., 2009; Moore et al., 2007). Phosphorylation of serine residues may affect PGR localization, stability, transcription modulation and protein complex formation (Lange, 2004). After differential phosphorylation, PGR may interact with other transcription factors such as stimulatory protein 1 (Sp1) and modulate transcription of genes lacking PREs (Daniel et al., 2007, 2009). Other transcription factors that PGR may associate with to modulate non-classical gene regulation include activating protein 1 (AP1) and

members of the signal transducers and activation of transcription (STAT) family (Proietti et al., 2005; Tseng et al., 2003).

Challenging human ductal breast epithelial tumor (T47D) cell lines with a progestin, Faivre et al. (2008) demonstrated PGR induced MAPK activation followed by increased phosphorylation of the PGR-B isoform on a proline-directed MAPK consensus site at the N-terminus of receptor (Ser345). To demonstrate that binding of PGR-B to SP1 is regulated by Ser345 phosphorylation, Faivre et al. (2008) treated cancer cells expressing either wild type PGR-B or a mutant PGR-B, lacking the proline site, with a progestin. After immunoprecipitation, they found that T47D cells with wild type PGR-B retained SP1 bound PGR-B compared to mutant cells. When tested against a variety of progestin responsive promoters, both wild type PGR-B and mutant PGR-B increased expression of genes containing known PREs. Only wild type PGR-B, however, was able to increase expression of genes lacking PREs. Genes activated by wild type PGR-B that lacked PREs included cyclin-dependent kinase inhibitor 1A (p21) and epidermal growth factor receptor (EGFR), both containing numerous Sp1 promoter sites (Faivre et al., 2008; Hudson et al., 1990; Ishii et al., 1985; Owen et al., 1998).

Sumoylation and desumoylation in combination with phosphorylation of PGRs may function to differentially modulate transcription and promoter specificity (Daniel et al., 2009, 2007). Small ubiquitin-like modifier (SUMO) is a ~10kDa protein that can be reversibly attached to lysine residues of target proteins and modify protein-protein interaction, stability, sub-cellular localization, and transcription (Daniel et al., 2009; Geiss-Friedlander and Melchior, 2007). Sumoylation of a transcription factor often results in transcriptional repression of target genes. Many transcription factors are

subject to sumoylation including ets-domain protein (Elk-1), cellular Finkel-Biskis-Jinkins murine osteogenic sacrcoma (C-Fos), amplified in breast cancer 1 (AIB1) and PGR-B (Bossis et al., 2005; Daniel et al., 2009; Wu et al., 2006; Yang et al., 2003).

Cancer cells that are transfected mutant PGR-B, resulting in reduced sumoylation, become hypersensitive to low progestin and have increased transcriptional activity. Daniel et al. (2007) reported that phosphorylation antagonizes sumoylation of PGR-B and may function as one mechanism to de-repress PGR-B activity and increase transcriptional modulation on select PREs and endogenous promoters.

Progesterone Receptor Gene Expression during Early Pregnancy

Progesterone regulates gene expression through PGRs that are expressed in a spacio-temporal pattern in epithelial, stromal and myometrial cells of the uterus. Progesterone controls uterine receptivity for conceptus attachment, communication between cells types in the uterus, and maintains a uterine environment conducive for conceptus growth and development. Using PGR KO mice and mifepristone (RU486, a P4 antagonist) many groups have attempted to uncover actions of PGRs and mechanisms under PGR regulation.

Takamoto et al. (2002) used PGR KO mice to identify P4 regulated gene expression in the uterus. Following ovariectomy, PGR KO and wild type mice were injected with 1 mg of P4 and sacrificed 6 h later. After microarray analysis on uterine mRNA, they found significant differences in expression of the indian hedgehog (Ihh) gene in the mouse uterus. The Ihh gene is involved in cell proliferation and differentiation. An increase in Ihh expression occurs within 3 h following administration

of P4 to ovariectomized mice (Takamoto et al., 2002). Indian hedge hog regulates bone development (Vortkamp, 2002), gastrointestinal tract development (Ramalho-Santos et al., 2000) and embryonic vasculogenesis (Dyer et al., 2001). Progesterone's capacity to regulate expression of the Ihh gene is thought to play a pivotal role in early embryonic development.

Cheon et al. (2002) used both RU486 and PGR KO mice in combination with microarray analysis and real time polymerase chain reaction (RT-PCR) respectively to uncover genes that are regulated by the PGRs during implantation. Treating mice with RU486 on d 3 of pregnancy, near the time of conceptus attachment, changed expression of 148 known genes in the uterus when compared with controls. By d 4 of pregnancy, seventy-eight genes were up-regulated whereas seventy were down-regulated in response to RU486. Genes that were changed following administering of RU486 included those encoding growth factors, transcription factors, cell adhesion molecules, proteases, protease inhibitors, metabolic enzymes, and molecules involved in signal transduction, angiogenesis and immune function (Cheon et al., 2002). Following treatment with P4, specific transcripts that were largely down-regulated by RU486 were calcycli, follistatin, osteoblast-specific transcription factor 2 (Osf2) and immunoresponsive gene 1 (Irg1). These same transcripts were also undetectable in ovariectomized PGR KO mouse uterus when compared with wild type. This indicated that P4 positively regulated genes that were down-regulated by RU486.

Although the function of the these molecules in relation to establishment of pregnancy is not fully understood, transient expression of Irg 1 and Osf 2 on d 4 of

pregnancy may indicate involvement in conceptus attachment to the uterine surface (Cheon et al., 2002, 2003).

Calcitonin, amphiregulin, an epidermal growth factor expressed in the LE, and histidine decarboxylase are transiently regulated by P4 during implantation (Das et al., 1995; Ding et al., 1994; Paria et al., 1998). Using a cell line derived from human endometrial epithelial cells, Li et al. (2002) reported that calcitonin may regulate implantation by inhibiting expression of E-cadherin, a cell to cell adhesion molecule, in the LE.

Progesterone Receptor Down-Regulation in the Pig Uterus

Progesterone receptors mediate the actions of P4, by increasing gene expression (Spencer et al., 2004). Uterine PGRs maintain a spacio-temporal pattern of expression for which continuous exposure of P4 leads to down-regulation of PGRs in the epithelium (Spencer et al., 1995). Ligand-induced PGR down-regulation in the epithelium occurs before implantation in a number of mammalian species including humans (Okulicz and Scarrell, 1998), cattle (Kimmins and MacLaren, 2001), sheep (Spencer and Bazer, 1995), western spotted skunk (Mead and Eroschenko, 1995), baboons (Hild-Petito et al., 1992), rhesus monkeys (Okulicz and Scarrell, 1998), mice (Tan et al., 1999), and pigs (Geisert et al., 1994).

Down-regulation of PGR-A and PGR-B (PGR-AB) in the pig occurs in the uterine epithelium near d 8 of the estrous cycle and pregnancy. Full down-regulation of the PGRs occurs by d 12 (Geisert et al., 1994; Persson et al., 1997; Sukjumlong et al., 2005, Ka et al., 2007). Localizing PGR-AB using immunocytochemistry, Geisert et al.

(1994) reported intense staining of PGR protein in LE and GE during estrus (d 0) and on d 5 of the estrous cycle. Decreased staining of PGR protein was detected as early as d 7 and d 10 from the uterine epithelium during the estrous cycle and pregnancy, respectively. Although the concentration of PGR protein did not change in the myometrium or stroma, low concentrations were maintained in the porcine LE and GE from d 12 to d 18 of the estrous cycle and pregnancy. This period of low PGR coincided with uterine $PGF_{2\alpha}$ production and establishment of pregnancy in the pig. Using an enzyme immunoassay, Persson et al. (1997) also demonstrated greater concentrations of PGR protein in cyclic pig endometrium during estrus. Low concentrations of PGR protein were reported from d 14 to d 18 of the estrous cycle as well as d 25 to d 30 of pregnancy.

How P4 down-regulates PGRs in the LE and GE but not in the myometrium or stroma in the mammalian uterus is not understood. One model proposed by Geisert and others, stated that PGR down-regulation may be initiated through activation of nuclear factor-kappa B (NF-κB), a well-known transcription factor involved in the immune response. Interactions between the PGR and NF-κB in other tissues have been reported (Condon et al., 2006; McKay and Cidlowski, 1996, 1998). Activation of NF-κB is thought to occur in the LE and GE of the pig uterus during the establishment of pregnancy (Ashworth et al., 2006; Ross et al., 2003; White et al., 2005).

Reestablishment of Estradiol Receptor in the Pig Uterus

Activated PGR inhibits expression of ER in the uterus. Down-regulation of PGR in the porcine uterine epithelium during the estrous cycle and pregnancy is followed by

reestablishment of ER within these cell layers (Geisert et al., 1993). Reestablishment of ER leads to increased E2-induced gene expression and protein production by the epithelium (Geisert et al., 1993).

Fibroblast growth factor 7 (FGF-7) and uterine adhesion molecule, osteopontin (SPP1), are thought to be secreted by the LE in response to E2 (Ka et al., 2007; White et al., 2005). Fibroblast growth factor 7 stimulates cell proliferation, differentiation and migration in various organs. It is expressed in the LE from d 9 to d 12 of both the estrous cycle and pregnancy in the pig (Ka et al., 2007). Peak levels of FGF-7 expression, however, were reported on d 12 of pregnancy in the LE, coinciding with conceptus release of E2 (Ka et al., 2007). The fibroblast growth factor receptor 2IIIb (FGFR2; the receptor of FGF-7) is expressed by the conceptus and endometrial epithelium. Ka et al. (2007) suggested that uterine FGF-7 acts in a paracrine manner to stimulate trophectoderm proliferation and differentiation. After treating ovariectomized pigs with different combinations of steroids and steroid antagonist from d 4 to d 12 of the estrous cycle, Ka et al. (2007) found that: 1) P4 is permissive of FGF-7 expression by down-regulating PGR in the LE; 2) conceptus release of E2 is responsible for maximal expression of FGF-7 from the LE; and 3) PGR positive stroma cells release a progestamedian in response to P4 that induces FGF-7 expression in the LE.

Osteopontin, acting as an intergrin ligand, is an extracellular matrix glycoprotein involved in many biological processes including cell to cell adhesion. In all mammalian uteri studied thus far, osteopontin is up-regulated during pregnancy and is thought to aid in conceptus attachment to the uterine surface during the establishment of pregnancy. Although the direct mechanism is poorly understood, up-regulation of osteopontin in the

porcine LE is initiated during conceptus release of E2 near d 12 and is sustained for the remainder of pregnancy. White et al. (2005) administered 5 mg of E2 to cyclic gilts on d 11 to d 14 of the estrous cycle followed by hysterectomy on d 15. They reported that gilts injected with E2 had an 8-fold increase in endometrial osteopontin mRNA expression by d 15 of the estrous cycle compared with corn oil (CO)-treated gilts. When localizing osteopontin mRNA, White et al. (2005) reported that osteopontin was localized to the LE and that gilts treated with E2 retained a moderate but uniform hybridization that could not be detected in CO-treated animals. The mouse and rat osteopontin gene both contain E2 response elements that may allow the estrogen receptor α (ERα) to directly regulated expression. Following PGR down-regulation, the pig LE reestablishes ERα and initiates expression of osteopontin in response to conceptus release of E2 (Geisert et al., 1993, 1994; White et al., 2005).

Nuclear Factor-Kappa B

The NF-κB system can modulate gene expression in a number of tissues including bone, central nervous system, mammary gland, and uterus (Hayden and Ghosh, 2004; Page et al., 2002). Nuclear factor-kappa B was discovered in 1986 by Sen and Baltimore as an enhancer binding protein controlling gene expression in B cells. Nuclear factor-kappa B is a ubiquitously expressed transcription factor that controls gene expression in a wide variety of organisms including insects, sponges, yeast and mammals. Nuclear factor-kappa B can regulate gene expression of cytokines, adhesion molecules, anti-apoptotic factors, complement factors, immunoreceptors and growth factors (Hayden and Ghosh, 2004; McKay and Cidlowski, 1999).

Nuclear factor-kappa B exists as a dimerized DNA binding molecule consisting of two protein subunits from the Rel family of transcription factors. Proteins in the Rel family contain a conserved 300 amino acid sequence at the N-terminus, referred to as the Rel homology domain, which can mediate dimerization, nuclear localization and DNA binding of NF-κB (Hayden and Ghosh, 2004). Although the p65/p50 combination is the most common heterodimer in eukaryotes, the Rel family consists of mammalian proteins Rel B, c-Rel, p65(Rel A), p50(NF-κB1), and p52(NF-κB2) that can form both homodimers and heterodimers able to bind different DNA sequences known as κB-sites. (McKay and Cidlowski, 1999; Hayden and Ghosh, 2004).

Inactive NF-κB resides in the cytoplasm of the cell and is sequestered by a 60-70 kDa protein that associates specifically with the NF-κB dimer (McKay and Cidlowski, 1999). This family consists of various proteins (referred to as inhibitor of NF-κB; IκB) and includes IκBα, IκBβ, IκBγ, and IκB-R. These proteins can bind different NF-κB dimers in various cell types. The IκB proteins studied thus far contain three common domains that exist in the inhibitor peptide sequence. The IκB domains include multiple ankyrin repeated motifs that are thought to be involved in NF-κB and IκB binding, an acidic C-terminal domain believed to interact with the NF-κB nuclear localization/DNA binding regions and a second C-terminal domain that may regulate IκB degradation (McKay and Cidlowski, 1999). Release of NF-κB from IκB is initiated by phosphorylation of two lysine residues within the inhibitor protein by IκB kinase (IKK). Phosphorylation followed by polyubiquitination initiates degradation of IκB by the 26S proteasome (Ghosh et al., 1998). Zandi et al. (1998) reported that IKK may have greater phosphorylation affinity for IκB when bound to NF-κB, thus allowing free IκB to reside

in the cytoplasm with IKK. Activated NF-κB can increase expression of IκB, giving rise to a negative feed back mechanism in the cell (McKay and Cidlowski, 1999).

After activation of IKK by various pro-inflammatory signals, phosphorylation of IκB results in release of NF-κB from the inhibitor protein (Ghosh et al., 1998). Exposure of nuclear localization sequences in the transcription factor results in translocation of NF-κB into the nucleus. The IκB and NF-κB complex may also be associated with phosphokinase A (PKA) which is believed to enhance the transcriptional regulation of NF-κB following phosphorylation of the dimerized Rel subunits (McKay and Cidlowski, 1999).

NF-κB Activation and Gene Expression during Early Pregnancy

Activation of the most common NF-κB heterodimer, p65/p50, can occur following exposure of the cell to viruses, viral proteins, lipopolysaccharides, oxidative stressors and inflammatory cytokines such as receptor activator for NF-κB ligand (RANKL) and IL-1 (Cao and Karin, 2003; Cao et al., 2001; McKay and Cidlowski, 1999). After activation, NF-κB can translocate to the nucleus and bind DNA at specific κB-sites in the promoter region of target genes. Binding to the promoter enables recruitment of transcription factors and co-factors that aid in modulation of gene expression.

Over 150 genes including interferon-gamma (INF-γ), tumor necrosis factor alpha (TNFα), insulin like growth factor binding protein-1 (IGFBP-1), IL-1β and PTGS2, are transcriptionally regulated by NF-κB (Heike, 1999; Hiscott et al., 1993; Lang et al., 1999; Shakhov et al., 1990; Sica et al., 1997; Yamamoto et al., 1995). Prostaglandin-

endoperoxide synthase 2 is a rate limiting enzyme in PG synthesis. Expression of endometrial PTGS2 is necessary for successful implantation in the mouse and establishment of pregnancy in the pig (Kraeling et al., 1985; Lim et al., 1997).

During conceptus implantation in the human and mouse, NF-κB is activated in the endometrium and is temporally associated with increased expression of IL-1β by the blastocyst (Lindhard et al., 2002; Nakamura et al., 2004; Page et al., 2002; Takacs and Kauma, 1996). Enhanced endometrial PTGS2 expression is temporally associated with conceptus release of IL-1β and activation of NF-κB in the human and mouse endometrium (Chakraborty et al., 1996; Marions et al., 1999). Conceptus release IL-1β during trophoblast elongation is thought to stimulate the porcine uterus in preparation for conceptus attachment. Secretion of IL-1β by the porcine conceptus coincides with increased endometrial PTGS2 expression perhaps enhanced by NF-κB activation within the LE and GE (Ashworth et al., 2006; Ross et al., 2003).

The cytokine RANKL is regulated by P4 in the mammary gland and also stimulates activation of NF-κB (Brisken et al., 2002; Fata et al., 2000). Inhibitory effects of NF-κB on PGRs by physical and non-physical interaction have been reported (Condon et al., 2006; McKay and Cidlowski, 1998). Cytokines that active NF-κB in the uterus, may indirectly down-regulate PGRs (McKay et al., 1999), a mechanism that may exist in the pig (Ashworth et al, 2006). Endometrial RANKL expression increases on d 10 of the pig estrous cycle, and is temporally associated with PGR down-regulation (Ross and Geisert, unpublished results). Expression of the RANKL receptor (receptor activator for NF-κB; RANK), is localized in the LE throughout the estrous cycle and pregnancy (Ross and Geisert, unpublished results). Activation of NF-κB by P4-induced RANKL may

serve as one mechanism controlling PGR down-regulation in the pig uterus (Ashworth et al., 2006; Ka et al., 2007). Existence of a putative NF-κB response element, located at 1330 bp upstream of the human PGR gene (Condon et al., 2006), suggests that NF-κB directly binds DNA to regulate expression of PGR within LE and GE cells of the uterus.

Progesterone Receptor and NF-κB Interactions

Inhibitory effects of NF-κB on steroid receptors including AR, GR, ER and PGRs by physical and non-physical interaction have been reported (Condon et al., 2006; Kalkhoven et al., 1995; McKay and Cidlowski, 1999, 1998). McKay et al. (1999) suggested that cytokine receptors that active NF-κB in the uterus, may be involved in PGR down-regulation.

McKay and Cidlowski (1998) reported inhibition of PGR-B function by NF-κB following co-transfection of African Green Monkey kidney (COS-1) cells with human PGR-B, p65, and the chloramphenicol acetyl transferase (CAT) reporter gene. Transfection of more PGR-B expression vector could not antagonize actions of the p65 subunit, but transfection of more p65 expression vector led to a decrease in PGR-B reporter gene expression. The results indicated that the p65 subunit of NF-κB can repress PGR-B activity in a dose-dependent manner.

McKay and Cidlowski (1998) suggested that antagonism of NF-κB on PGR-B could occur by two mechanisms. First, NF-κB and PGR-B may compete for co-factors that aid in transcription, resulting in altered PGR-B modulated expression. Second, that p65 and PGR-B may physically bind rendering PGR-B nonfunctional. Studies using *in*

vitro binding assays have reported a direct physical interaction between PGR and the p65 subunit of NF-κB (Kalkhoven et al., 1995).

A study by Condon et al. (2006) reported a direct effect on PGR-C gene expression in uterine myometrium, possibly through NF-κB, resulting in reduced PGR-B function and loss of myometrial quiescence associated with labor in the human. Loss of myometrial PGR-B function, in the midst of sustained PGR-B gene expression and increased P4 concentrations, is thought to initiate labor (Condon et al., 2006). An increase in NF-κB activation, associated with an increase in PGR-B and PGR-C gene expression, reportedly occurs during parturition in the mouse (Condon et al., 2006; Condon et al., 2004).

Condon et al. (2006) reported a 200-fold increase of PGR-C mRNA in the presence of transcriptionally active PGR-B in contracting human myometrium during parturition. An increase in NF-κB activation in parallel with greater levels of PGR-B and PGR-C gene expression was also observed in human myometrium during labor (Condon et al., 2006). To better understand PGR-C action on PGR-B, a line of cultured human myometrial cells, that exclusively express PGR-B, were transfected with increasing amounts of a PGR-C expression vector (Condon et al., 2006). Transfection of PGR-C resulted in decreased PGR-B gene expression in a dose dependent manner, indicating that PGR-C may act to block PGR-B expression.

After analysis of the human PGR gene by Condon et al. (2006), a putative NF-κB response element was located 1330 bp up-stream of the 5′ flanking sequence. Nuclear factor-kappa B may regulate expression of PGR isoforms through this response element (Condon et al., 2006). After chromatin immunoprecipitation (ChIP) analysis, it was

determined that NF-κB binds this response element in IL-1β challenged cultured human myometrial cells (Condon et al., 2006).

Condon et al. (2006) suggest that NF-κB induced expression of PGR-C may function to sequester P4 from PGR-B. The loss of PGR-B function as a result of increased PGR-C expression may lead to myometrial contractions and the onset of labor in the human.

Progesterone Receptor Antagonist RU486

Mifepristone (RU486) can effectively antagonize the actions of P4 through competitive binding of PGRs (Fiala and Gamzell-Danielsson, 2006; Leonhardt and Edwards, 2002). Developed in 1982 by the Roussel Uclaf pharmaceutical company in France, mifepristone is commonly used in clinical studies involving pregnancy termination, expulsion after fetal death, cervical ripening before surgical abortion, labor induction and emergency postcoital contraception (Fiala and Gamzell-Danielsson, 2006; Leonhardt and Edwards, 2002). Mifepristone can also bind GR and AR with a binding affinity three times that of dexamethasone and one third that of testosterone, respectively. Therefore, loss of steroid action in glucocorticoid and testosterone target tissues is possible as well (Fiala and Gamzell-Danielsson, 2006).

Mifepristone is a 19-norsteroid consisting of a hydrophobic 1-propynyl substituent located at the 17α-position as well as a p-(dimethylamino) phenyl group at the 11β-position thought to be involved in binding and stabilizing the inactive PGR, respectively (Fiala and Gamzell-Danielsson, 2006). Although RU486 does not make the same interaction with the receptor LBD, the antagonist can bind PGRs with 2.5 to 5 times

greater affinity than P4, effectively competing with the steroid hormone (Fiala and Gamzell-Danielsson, 2006). Antagonist bound PGRs are able to dimerize and bind PRE within DNA, but are considered transcriptionally inactive (Fiala and Gamzell-Danielsson, 2006; Leonhardt and Edwards, 2002). Antagonistic effects of RU486 are thought to involve a conformational change in the C-terminal tail and inactivation of the AF-2 domain of PGRs (Leonhardt and Edwards, 2002). This results in an inability to recruit co-activators that promote transcription. In some tissues, a slight agonistic affect of RU486 has also been reported, for which RU486 may increase PGR mediated transcription of specific genes (Fiala and Gamzell-Danielsson, 2006; Terakawa et al., 1988).

Mifepristone is orally active and rapidly absorbed, resulting in peak concentrations within 1 to 3 h (Fiala and Gamzell-Danielsson, 2006; Sarkar, 2002). After a single oral administration of 600 mg (a dose widely used in clinical abortions), RU486 can reach a peak plasma concentration of 2.5 μmol/L within 90 minutes (Fiala and Gamzell-Danielsson, 2006). Although RU486 maintains a 70% absorption rate in the gut, its bioavailability is reduced to nearly 40% following first pass through the liver (Fiala and Gamzell-Danielsson, 2006; Sarkar, 2002). After entering the blood, RU486 can bind α_1-acid glycoprotein (AAG), and has a half life of 20 to 40 h (Fiala and Gamzell-Danielsson, 2006; Heikinheimo et al., 2003). Binding of RU486 to AAG limits the tissue availability of the antagonist resulting in a low metabolic clearance rate of 0.55 L/kg per day (Heikinheimo et al., 2003). At concentrations greater than 2.5 μmol/L, RU 486 will exceed the saturation level of AAG in the blood, resulting in metabolism of free RU486 (Fiala and Gamzell-Danielsson, 2006). As RU486 is being metabolized it

undergoes demethylation and hydroxylation giving rise to three metabolites (Heikinheimo et al., 2003; Leonhardt and Edwards, 2002). These metabolites have a lower affinity for the receptor, however, can still bind PGRs and may contribute as much as 23 to 33% of the anti-progestagenic effects of RU486 (Fiala and Gamzell-Danielsson, 2006). Mifepristone and its metabolites may remain biologically active in the body for 6 to 7 days (Sarkar, 2002; Sartor and Figg, 1996).

It has been suggested that decreased P4 concentrations during early pregnancy, as a result of a high plane of nutrition, leads to embryonic loss in the pig. In an attempt to investigate the influence of P4 on day 2 and 3 of pregnancy in relation to conceptus development and uterine capacity in the pig, Vallet and Christenson (2004) injected white crossbred pregnant gilts with different combinations of P4, E2 or RU486. In the first experiment, unilaterally hysterectomized-ovariectomized gilts received either no treatment, 5 mg of E2 on d 11 and d 12 of gestation, or 200 mg of P4 on d 2 and d 3 after mating. Although no differences were observed between the E2-treated and control gilts, they found that gilts treated with P4 during early pregnancy had increased fetal weights and a reduced litter size when compared with controls. Vallet and Christenson (2004) later treated gilts with P4 on d 2 and d 3 of pregnancy but allowed them to farrow. Treating pigs with P4 resulted in reduced pregnancy rates and a shorter gestation of 0.5 days when compared with controls. In another experiment, Vallet and Christenson (2004) randomly assigned gilts to receive 1) no treatment 2) 100 mg, 3) 200 mg, or 4) 400 mg of RU486 in corn oil (CO) on d 2 of pregnancy. After flushing the uterine horns on d 11 of pregnancy with 20 mL of saline, the number and diameter of the conceptuses were recorded as uterine flushings were assayed for total protein and total acid

phosphatase. Compared with controls, the 400 mg dose of RU486 reduced both uterine protein secretion and conceptus diameter by 50% as well as reduced uterine capacity. These experiments indicated that both too little or too early secretion of P4 during pregnancy results in reduced uterine capacity in the pig (Vallet and Christenson, 2004).

The Nuclear Factor-Kappa B and Progesterone Receptor Down-Regulation Hypothesis

Early embryonic mortality is extremely high in mammalian species, and occurs in parallel with establishment of pregnancy and PGR down-regulation. The pig experiences significant conceptus mortality (20 to 46%) before term (Pope et al., 1990) with the majority of loss occurring between d 7 and d 20 of gestation (Polge, 1982). In the pig, PGR down-regulation in the LE and GE begins near d 8 of both the estrous cycle and pregnancy where full down-regulation occurs by d 12 (Geisert et al., 1994). Early embryonic mortality in the pig results in drastic reproductive inefficiency and reduced profitability in the swine industry. A better understanding of the mechanisms controlling PGR down-regulation in pigs as well as other mammals could result in methods to reduce early embryonic mortality.

One theory proposed by Geisert and others is that PGR down-regulation is through P4-induced uterine LE and GE RANKL expression and the activation of NF-κB within the LE and GE. The transcription factor may then enter the nucleus, bind a NF-κB response element upstream of the PGR gene, block PGR expression, and down-regulate PGR within the LE and GE. We plan to test this hypothesis using RU486, a P4 antagonist that can block P4 action and block PGR down-regulation. If the NF-κB hypothesis is correct, RU486 will bind PGRs in the uterine epithelium and block P4

induced RANKL expression. A decrease in RANKL and NF-κB activation would theoretically result in continued PGR expression within the LE and GE and conceptus mortality during the time of establishment of pregnancy in the pig.

Endometrium, removed from both control and RU486 treated pigs, will be assayed for endometrial PGR and RANKL gene expression using RT-PCR. Uterine sections will also be collected and assayed for activation of NF-κB using IHC. According to the hypothesis, RU486 treated pigs should maintain increased endometrial PGR gene expression, decreased RANKL gene expression and decreased NF-κB activation when compared with control pigs.

CHAPTER THREE

EFFECT OF RU486, A PROGESTERONE ANTAGONIST, ON UTERINE PROGESTERONE RECEPTOR B, ACTIVATION OF NUCLEAR FACTOR-KAPPA B AND EMBRYONIC DEVELOPMENT DURING EARLY PREGNANCY IN PIGS

Abstract

Early development and establishment of pregnancy in the pig depends on down-regulation of progesterone receptor (PGR) in the endometrial epithelium. This study evaluated the regulation of endometrial PGR by progesterone (P4) and its spatial relationship to endometrial nuclear factor-kappa B (NF-κB) activation and receptor activator for nuclear factor-kappa B ligand (RANKL) expression. Gilts were inseminated (d 0) and assigned to one of three treatments: RU486 (400 mg/d) on d 3, 4 and 5 of pregnancy (T1; n = 9); RU486 on d 6 and d 7 of pregnancy (T2; n = 9); or a non-treated control group (control; n = 9). Uteri were harvested on d 8 or d 12. Treatment of gilts with RU486 affected early conceptus development. Percent normal development was lowest in T1 pigs (chi-square = 7.00; $P < 0.05$). The endometrial PGR-B mRNA was

more abundant in T1 and T2 pigs when compared with control pigs. There was a treatment effect ($P < 0.01$) on log-transformed endometrial RANKL mRNA expression as RANKL expression was greater in T1 (d 8 and d 12) and T2 pigs (d 12) when compared with controls. Activation of NF-κB (nuclear localization) in the uterine epithelium increased in T2 and control pigs but remained relatively unchanged in T1 pigs from d 8 to d 12. Inhibiting P4 action with RU486 during early pregnancy increased PGR-B mRNA expression, indicating that P4 is responsible for PGR down-regulation in the uterine epithelium. Our results do not support the hypothesis that RANKL mediates NF-κB inhibition of PGR. Activation of NF-κB on d 12 could be a result of conceptus elongation and secretion of interleukin-1 beta (IL-1β).

INTRODUCTION

Progesterone is unequivocally required during the establishment of pregnancy in mammals and has numerous functions including uterine quiescence, uterine histotroph secretion, and suppression of the immune response (Fomin et al., 1999; Schust et al., 1996; Spencer et al., 2002). Progesterone also controls expression of the progesterone receptor (PGR) in the uterus and down-regulates PGR exclusively within endometrial epithelium but not within the myometrium or stroma before implantation. Progesterone receptor down-regulation is thought to enable conceptus attachment to the uterine surface and establishment of pregnancy. Progesterone induced PGR down-regulation within the epithelium occurs before implantation in a number of mammalian species including humans (Okulicz and Scarrell, 1998), cattle (Kimmins and MacLaren, 2001), sheep

(Spencer and Bazer, 1995), western spotted skunk (Mead and Eroschenko, 1995), baboons (Hild-Petito et al., 1992), rhesus monkeys (Okulicz and Scarrell, 1998), mice (Tan et al., 1999), and pigs (Geisert et al., 1994). In the pig, P4 causes down-regulation of PGR-A and PGR-B in the luminal (LE) and glandular (GE) epithelium near d 8 of the estrous cycle and pregnancy (Geisert et al., 1994). Full down-regulation occurs by d 12 (Geisert et al., 1994; Persson et al., 1997; Sukjumlong et al., 2005). The mechanism through which P4 down-regulates PGRs in the uterine epithelium but not within the myometrium or stroma is not understood.

In the mammary gland, P4 increases expression of receptor activator for nuclear factor-kappa B ligand (RANKL), which can activate nuclear factor-kappa B (NF-κB). Nuclear factor-kappa B is a transcription factor able to bind DNA and regulate gene expression (Fata et al., 2000). In the human, Condon et al. (2006) reported inhibition between NF-κB and PGRs and that NF-κB binds a response element upstream of the human PGR gene, thereby regulating transcription of PGRs. Endometrial RANKL expression has been detected on d 10 of the pig estrous cycle, and is temporally associated with PGR down-regulation (Ross and Geisert, unpublished results). Expression of the RANKL receptor (receptor activator for NF-κB; RANK), is localized in the LE throughout the estrous cycle and pregnancy in the pig (Ross and Geisert, unpublished results). In the pig uterine epithelium RANKL may bind its receptor RANK and activate NF-κB, resulting in the down-regulation of PGR expression within the LE and GE. Expression of prostaglandin-endoperoxidase synthase 2 (PTGS2), a gene regulated by NF-κB, increases within the uterine epithelium of the pig and is temporally

associated with PGR down-regulation and conceptus attachment during establishment of pregnancy (Ashworth et al., 2006).

To test the hypothesis that P4 induces RANKL expression within the LE and GE resulting in activation of NF-κB and PGR down-regulation, we used RU486 (a P4 antagonist) to block P4 action during early pregnancy in the pig. Decreased staining of PGR protein was detected as early as d 7 in the uterine epithelium during the estrous cycle, indicating that PGR down-regulation (at the transcriptional level) occurs before d 7 (Geisert et al., 1994). The RU486 was administered to gilts on d 3, 4 and 5 to block PGR down-regulation before it begins (T1) and on d 6 and d 7 to block PGR down-regulation following initiation of the mechanism (T2). Gilts were then sacrificed on d 8 and d 12 of pregnancy to assay for mRNA and protein thought to be involved in PGR down-regulation and to observe conceptus development. Endometrium from non-treated control and RU486 treated gilts was assayed for PGR-B, RANKL, and RANK mRNA abundance as well as PGR-B protein and NF-κB activation. Endometrial PTGS2 and fibroblast growth factor 7 (FGF-7) mRNA abundance was measured to determine if expression was enhanced in the presence of the conceptus on d 12 of pregnancy.

MATERIALS AND METHODS

Experimental Animals and Management

The project was conducted in accordance with the Guide for Care and Use of Animals and approved by the University of Missouri Institutional Animal Care and Use Committee. Twenty-seven mature, cycling Large White Landrace crossbred gilts were

checked for estrus behavior twice daily in the presence of an intact boar. Gilts were artificially inseminated at the onset of estrus (d 0 of pregnancy) and 24 h later (d 1) with fresh semen collected from a single Large White Landrace crossbred boar of proven fertility.

Inseminated gilts were randomly selected to one of the following three treatment groups; 1) 400 mg (i.m.) of RU486 (D. Philibert, Roussel-UCLAF, Paris, France) on d 3, 4 and 5 of pregnancy (T1; n = 9); 2) 400 mg of RU486 on d 6 and d 7 of pregnancy (T2; n = 9); or 3) a non-treated control group (control; n = 9). In preparation of treatment, 5 g of RU486 was mixed with 50 mL of corn oil (vehicle) to make a 100 mg per mL suspension in multiple glass vials. The corn oil and RU486 were thoroughly mixed before injections into the neck were given.

Tissue Collection

The reproductive tract was removed by midventral laparotomy on either d 8 or d 12 of pregnancy following euthanasia with Euthasol (Virbac AH; Fort Worth, TX). Upon arrival to the lab, ovaries were removed and the broad ligament was dissected free from the uterine horns. The uterus was washed with 0.9% sodium chloride (Baxter; Deer Field, IL) and placed on an ice cold dissection tray. Multiple sections of uterine horn (approximately 1 cm) near the uterine bifurcation were removed and fixed by placing in 10% buffered formalin phosphate (Fisher Scientific; Fair Lawn, NJ). After 48 hours sections were imbedded in paraffin in preparation of immunohistochemistry (IHC). Uterine horns were flushed twice with 20 mL of 1 X phosphate buffering solution (PBS) to remove conceptuses. Conceptuses were examined under a light microscope and the

number as well as morphology of conceptuses was recorded. Diameter of each conceptus was measured by using a calibrated eye-piece micrometer. After conceptus removal, one uterine horn was cut along its anti-mesometrial border, and endometrium (5 to 10 g) was removed with scissors. Endometrium was snap frozen in liquid nitrogen and stored at -80° C until extraction of RNA.

Immunohistochemistry (immunofluorescence) for NF-κB

Uterine tissue sections (5 μm) were mounted onto frosted white microscope slides, deparaffinized and rehydrated for immunohistochemistry. Epitope retrieval was achieved by boiling slides for 6 min in 0.01M sodium citrate buffer. Slides were allowed to cool for 45 min followed by room temperature incubation overnight with 500 μL of a rabbit polyclonal antibody, directed against the p65 subunit of NF-κB, (sc-372, Santa Cruz Biotechnology; Santa Cruz, CA; 200 μg per mL) and PAT [0.001% Tween-20 and 0.001% sodium azide (1.0%) in 1 X PBS] solution at a ratio of 1:100. Following the 24 h incubation, slides were placed in 1 X PBS for 30 min before adding 500 μL of a secondary donkey anti-rabbit monoclonal antibody, containing a Cy2 conjugate (711-225-152, Jackson ImmunoResearch Laboratories; West Grove, PA; 1.5 mg/mL), and PAT solution (1:100). Slides were allowed to incubate at room temperature with the secondary antibody for 1 h and 10 min before re-submerging slides in 1 X PBS. As a technique to reduce background fluorescence, the secondary antibody/PAT solution was allowed to incubate with a previously washed bovine liver powder for 1 h at 37°C before being added directly to the slides. Cover slips were then mounted over the uterine tissue with Flouromount-G™ (SouthernBiotech; Birmingham, AL) and the slides were then

refrigerated at 8°C over night in the dark. A negative control slide was made without a primary antibody.

Scoring of NF-κB Activation

Nuclear factor-kappa B activation in uterine epithelial cells was blindly scored by three independent investigators using a Leica light microscope with a green fluorescent protein filter (GFP) at 400 X magnification. Two tissue sections per pig were scored for NF-κB activation (nuclear localization) in the luminal epithelial (LE), glandular epithelial near the lumen (surface GE) and glandular epithelial distal to the lumen (deep GE) on a 0 to 10 scale (0 indicating no nuclear localization and 10 indicating complete nuclear localization; Figure 3.1, p. 72).

Immunohistochemistry for PGR-B

Tissue sections (5 μm) were deparaffinized and rehydrated in a series of graded alcohol solutions. As an epitope retrieval technique, slides were boiled for 6 min in 0.01M sodium citrate buffer. Sections were then allowed to cool for 45 min, incubated in 0.3% H_2O_2 in tap water for 30 min (to quench endogenous peroxidase activity) and then submersed in 1 X PBS for 5 min.

Following 1 X PBS submersion, a normal blocking serum, supplied by a Vectastain *elite* ABC kit (6101, Vector Laboratories, Inc. Burlington, CA), was used to sequester endogenous peroxidase activity. A primary monoclonal rabbit mAb anti-progesterone receptor antibody (C142, Cell Signaling Technology, Inc, Danvers, MA), was diluted in 1 X PBS (1:200) and 250 μL of solution was added to each slide. Slides

were allowed to incubate with the primary antibody over night at room temperature. The next day, a biotinylated secondary antibody, also supplied by the Vectastain *elite* ABC kit, was added to each slide at the manufacture's recommendations. Following incubation with the ABC kit reagents, tissue sections were incubated with the ImmPACT DAB peroxidase substrate (SK-4105, Vector Laboratories) for 5 min. Afterwords, sections were gently rinsed in double distilled (dd) water and submerged for 40 sec in hematoxylin (GH5216, Sigma Aldrich, St. Louis, MO) diluted with dd water (1:3). Following hematoxylin staining, slides were gently rinsed with tap water and allowed to incubate for 1 min in Scotts Bluing Reagent (Statlab Medical Products, Lewisville, TX). After the 1 min incubation, slides were rinsed for 2 min in tap water and dehydrated in graded alcohol followed by two rounds of xylene. Afterwords, cover slips were mounted over tissue sections with Permount (Fisher Scientific). A negative control slide was made without a primary antibody.

Scoring of PGR staining

Two sections per pig were blindly scored for PGR-B protein within nuclei of LE, surface GE, deep GE, myometrium and stromal cells by two independent investigators using a Leica light microscope at 400 X magnification. Because cell types differed by percentage of nuclei stained rather than intensity of staining, each cell type was scored on a 0 to 4 scale were 0 indicated 0% of nuclei and 4 indicated 100% of nuclei stained for PGR protein.

RNA Isolation and Reverse Transcription

Total cellular RNA was isolated from 100 mg of endometrium with 1 mL of TRIzol reagent (Invitrogen; Carlsbad, CA) following the manufacture's recommendations. The integrity of RNA was determined by calculating the ratio of absorbance at 260 nm and 280 nm (NanoDrop ND-1000, NanoDrop Technologies; Wilmington, DE), followed by gel electrophoresis (0.8% agarose gel in 0.09 M Tris-borate and 0.002 M EDTA buffer with 0.5 μg/mL ethidium bromide). The RNA was stored at -80°C before reverse transcription of 5 μg of total cellular RNA to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystem; Foster City, CA).

Primer sets and RT-PCR

Primer sets for PGR-B, RANKL, receptor activator for nuclear factor kappa B (RANK), prostaglandin-endoperoxidase synthase 2 (PTGS2), fibroblast growth factor 7 (FGF-7), and beta actin (ACTB) were designed based on porcine nucleotide sequences (Table 3.1, p. 70). To avoid amplification of contaminating genomic DNA, primers were designed to anneal in different exons if possible. The PCR products produced from primer sets were sequenced at the University of Missouri DNA Core to verify amplification of sequence of interest. The 25 μL real time polymerase chain reactions (RT-PCR) were prepared with 1 μM concentrations of both forward and reverse primers and 1 μL (4,500 ng) of the cDNA sample using Power SYBR® Green (Applied Biosystems). One plate per primer set was used (all 27 cDNA samples were ran in triplicate on a single plate). Within each plate, a high, medium and low concentration

(sequential 1:4 dilutions) of pooled cDNA was ran in triplicate and used as a standard. A no template control (NTC) was also ran in triplicate and included with each plate. The PCR reactions were performed and SYBR green fluorescence quantified by the ABI Prism 7500 Sequence Detector (Applied Biosystems). Machine settings consisted of an initial temperature of 50.0°C of 2 min, a polymerase activation temperature of 95.0°C for 10 min followed by 40 PCR cycles consisting of 2 stages: 1) melting at 95°C for 15 sec and 2) annealing and extension at 60.0°C for 1 min. Analysis of amplification plots were performed by the Sequence Detection Software (Applied Biosystems).

RT-PCR Calculations

The equation: efficiency = $10^{(-1/slope)}$ was used to calculate the amplification efficiencies of all six RT-PCR assays. The slope refers to the slope of a linear plot of C_T values achieved by the high, medium and low pooled cDNA standards versus log copies of amplified transcript (dose in ng). With each additional PCR cycle, doubling of the transcript leads to an efficiency of 100% and a 2 fold increase in product. The typical PCR reaction will amplify the transcript at slightly less than twice the amount and will maintain an efficiency that is slightly less than 100%. Using the efficiency and mean C_T value of the medium control for each RT-PCR assay, fold change differences in PCR product between the samples were calculated using the equation: fold change = efficiency $^{(\text{mean medium control } C_T - \text{mean sample } C_T)}$.

Statistical Analysis for Conceptuses

The difference between treatment groups for the percentage of gilts containing normal conceptuses was assessed by using a chi-square test. The level of significance was set at $\alpha = 0.05$ with 2 degrees of freedom, so that the critical value X^2 was 5.991. A chi-square statistic greater than 5.991 indicated a significant difference between treatments at $\alpha = 0.05$.

Statistical Analysis for RT-PCR, immunofluorescents and IHC

The experiment was conducted as a completely randomized design with two factors (treatment and day of pregnancy). Fold change over medium control endometrial gene expression, immunoflourescents score, and IHC score were analyzed using the general linear models (GLM) procedure of the Statistical Analysis System (SAS institute Inc. Cary, NC, USA). The dependent variables in the model statement included treatment, day and their interaction. Least squares means (lsmean) and standard errors were generated using the LSMeans statement of SAS. All reported means are the adjusted least squares means ± standard error of the lsmean (SEM) and the significance was declared when $P < 0.05$. To test for significance, natural log transformed data for endometrial RANKL expression (fold change over medium control) and NF-κB activation score were also analyzed.

RESULTS

Conceptus Development

Conceptuses collected on d 8 were considered normal if hatched from the zona pellucida. Spherical conceptuses, less than 11 mm in diameter, were considered normal as well as ovoid, tubular, and elongated on d 12. The RU486 affected early conceptus development (Figure 3.2, p. 73). Normal conceptus development was lowest in T1 gilts (chi-square = 7.00; $P < 0.05$; Table 3.2, p. 71).

On d 8 of pregnancy, 100% of control and T2 gilts had conceptuses that appeared developmentally normal (hatched blastocysts) (Table 3.2, p. 71; Figure 3.2 A, p. 73). This percentage was 60% on d 8 for T1 gilts. One T1 gilt produced numerous two-celled embryos (Figure 3.2 B, p. 73) and another T1 gilt had non-fertilized oocytes. Although three out of five T1 gilts produced morphologically normal conceptuses on d 8 (hatched blastocysts), conceptuses were slightly reduced in size (averaging 0.29 mm) when compared with hatched blastocysts from control (0.61 mm) and T2 (0.53 mm) gilts. This may indicate retardation of conceptus growth in T1 gilts by d 8 of pregnancy.

By d 12 of pregnancy, elongated conceptuses were flushed from 75% (3 of 4) of control gilts (Table 3.2, p. 71). Elongated conceptuses were considered a normal morphology on d 12. One control gilt had two spherical conceptuses (averaging 2.3 mm). Elongated conceptuses were flushed from 60% of T2 gilts on d 12 of pregnancy. No conceptuses were recovered from one gilt and a second gilt produced two large conceptuses [hatched blastocyst averaging approximately 12.5 mm in diameter (Figure 3.2 C, p. 73)]. There were no pregnancies in T1 gilts by d 12 of gestation (Table 3.2, p.

71). Neither blastocysts nor elongated conceptuses were found but three gilts did contain what appeared to be non-fertilized oocytes (Figure 3.2 D, p. 73).

Endometrial PGR-B mRNA Expression

Expression of ACTB (control gene) was similar between days and treatments (Figure 3.3, p. 74). Treatment did affect endometrial expression of PGR-B ($P < 0.01$). Fold change expression of PGR-B mRNA was greater in T1 (9.1 ± 1.0) gilts when compared with control (3.2 ± 1.0) gilts (d 8 and d 12 combined; Figure 3.4, p. 75). The PGR-B mRNA expression was intermediate for T2 (5.9 ± 1.0) gilts (d 8 and d 12 combined; lsmeans ± SEM indicate fold change over medium control).

PGR-B Protein in the Luminal Epithelium (LE)

There was a treatment by day interaction ($P < 0.001$) for PGR-B protein score within the LE. PGR-B protein in control gilts decreased from d 8 (3.5 ± 0.3) to d 12 (0.9 ± 0.4) when compared with T1 gilts (Figure 3.5, p. 76 and Figure 3.6, p. 77). Although T1 (2.4 ± 0.3) and T2 (2.9 ± 0.4) gilts contained reduced PGR-B protein when compared with controls on d 8, protein levels for T1 (3.1 ± 0.4) and T2 (0.0 ± 0.3) gilts were greater and less than that of controls, respectively, by d 12 (lsmeans ± SEM; 0 indicates 0% of nuclei and 4 indicates 100% of nuclei containing PGR protein).

PGR-B Protein in the Surface Glandular Epithelium (GE)

There was a treatment by day interaction ($P < 0.01$) for PGR-B protein score within surface GE. Protein levels were similar between treatments on d 8. The PGR-B

protein decreased in control gilts from d 8 (3.8 ± 0.2) to d 12 (1.9 ± 0.3) when compared with T1 gilts (Figure 3.7, p. 78 and Figure 3.8, p. 79). Protein levels for T1 (3.4 ± 0.3) and T2 (1.5 ± 0.2) gilts were greater and less than controls, respectively, by d 12.

PGR-B Protein in the Deep GE

There was no effect of day or treatment for PGR-B protein within the deep GE. The amount of PGR-B protein remained high for all treatments on d 8 and d 12 of pregnancy (Figure 3.9, p. 80 and Figure 3.10, p. 81).

PGR-B Protein in the Stroma

Although there was no effect of day for PGR-B protein in the stroma, there was a tendency for an effect of treatment ($P = 0.06$). Protein levels in control gilts (1.9 ± 0.3) tended to be less than T2 gilts (2.9 ± 0.3) while T1 gilts (2.3 ± 0.3) were intermediate (d 8 and d 12 combined; Figure 3.11, p. 82 and Figure 3.12, p. 83).

PGR-B Protein in the Myometrium

A treatment by day interaction did exist for PGR-B protein ($P < 0.05$) within the myometrium. PGR-B protein decreased from d 8 (3.5 ± 0.2) to d 12 (2.8 ± 0.2) in control gilts when compared with RU486 treated (T1 and T2) gilts (Figure 3.13, p. 84 and Figure 3.14, p. 85). Protein levels were greater in T2 (4.0 ± 0.2) gilts when compared with both T1 (3.5 ± 0.2) and control gilts (3.5 ± 0.2) on d 8 of pregnancy, however, protein levels in T1 (3.8 ± 0.2) gilts slightly increased and were similar to T2 (3.9 ± 0.2) gilts by d 12.

Uterine NF-κB Activation

The NF-κB activation data were highly variable. The data were log transformed, therefore, to reduce variance. There was an effect of day ($P < 0.05$) on NF-κB activation within the LE for raw (Figure 3.15, p. 86) and log transformed data (Figure 3.16, p. 87). Nuclear factor-kappa B activation (nuclear localization) increased from d 8 (0.8 ± 0.1) to d 12 (1.2 ± 0.1) of pregnancy (log transformed data; Figure 3.16, p. 87 and Figure 3.17, p. 88). There was a tendency for a treatment by day interaction of NF-κB activation score within the LE (log transformed data; $P = 0.062$). NF-κB activation tended to be greater in control (1.4 ± 0.2) and T2 (1.3 ± 0.2) gilts when compared with T1 (0.7 ± 0.2) gilts on d 12 (Figure 3.18, p. 89) (lsmeans ± SEM after log transformation; 0 indicated no nuclear localization and 10 complete nuclear localization).

There was a tendency for an effect of day on NF-κB activation within the surface GE for raw ($P = 0.098$; Figure 3.19, p. 90) and log transformed ($P = 0.085$; Figure 3.20, p. 91) data. Nuclear factor-kappa B activation tended to increase from d 8 (0.36 ± 0.08) to d 12 (0.57 ± 0.08) of pregnancy (log transformed data; Figure 3.21, p. 92).

There was a tendency for an effect of day ($P = 0.058$) on NF-κB activation in deep GE for raw data (Figure 3.22, p. 93) and an effect of day ($P < 0.05$) on NF-κB activation with in the deep GE for log transformed data (Figure 3.23, p. 94). Activation increased from d 8 (0.24 ± 0.09) to d 12 (0.54 ± 0.09) of pregnancy (log transformed data; Figure 3.24, p. 95).

Endometrial RANKL and RANK mRNA Expression

The RANKL expression data was highly variable (Figure 3.25, p. 96). The data were log transformed, therefore, to reduce variance. Treatment affected RANKL mRNA expression within total endometrium (log transformed data; $P < 0.01$). The pattern of RANKL expression was similar to endometrial PGR mRNA expression. RANKL expression was greater in T1 (2.7 ± 0.4) gilts when compared with control (0.6 ± 0.4) gilts and intermediate in T2 (1.5 ± 0.4) gilts [d 8 and d 12 combined; lsmeans ± SEM indicate fold change over medium control after log transformation; (Figure 3.26, p. 97)]. There was no effect of treatment or day on fold change mRNA expression of RANK, the receptor for RANKL, in the endometrium (Figure 3.27, p. 98).

Endometrial PTGS2 mRNA Expression

A treatment by day interaction existed for endometrial PTGS2 mRNA expression ($P < 0.01$). A trend similar to NF-κB activation, expression was greatest in control (3.4 ± 0.6) and T2 (4.4 ± 0.6) gilts when compared with T1 (0.3 ± 0.6) gilts on d 12 of pregnancy (lsmeans ± SEM indicate fold change over medium control; Figure 3.28, p. 99).

Endometrial FGF-7 mRNA Expression

There was an effect of day ($P < 0.05$) on endometrial FGF-7 mRNA expression. Expression of FGF-7 increased from d 8 (1.5 ± 3.5) to d 12 (14.0 ± 3.6) of pregnancy (Figure 3.29, p. 100). There was a tendency for a treatment by day interaction for FGF-7 expression ($P = 0.063$). Endometrial FGF-7 expression tended to be greater in control

(15.5 ± 6.5) and T2 (26.4 ± 5.8) gilts when compared with T1 (0.1 ± 6.5) gilts on d 12 of pregnancy (lsmeans ± SEM indicate fold change over medium control).

DISCUSSION

Progesterone causes down-regulation of the PGR in the uterine epithelium before implantation in a number of mammalian species (Hild-Petito et al., 1992; Kimmins and MacLaren, 2001; Mead and Eroschenko, 1995; Okulicz and Scarrell, 1998; Spencer and Bazer, 1995; Tan et al., 1999). In the pig, P4 causes down-regulation of PGR-A and PGR-B in the LE and GE near d 8 of the estrous cycle and pregnancy (Geisert et al., 1994). Full down-regulation of PGRs occurs by d 12 (Geisert et al., 1994; Persson et al., 1997; Sukjumlong et al., 2005). Progesterone receptor down-regulation is thought to allow conceptus attachment to the uterine surface and establishment of pregnancy. How P4 causes down-regulation of its own receptor is not understood.

It has been suggested that PGR down-regulation is caused by P4-induced uterine epithelial expression of RANKL, acting in an autocrine fashion to activate NF-κB within the LE and GE. Nuclear factor-kappa B may then enter the nucleus, bind a response element upstream of the PGR gene, and block PGR expression resulting in PGR down-regulation within the LE and GE. Gilts were treated with RU486, a P4 antagonist, to test the hypothesis that P4 induces RANKL expression within the LE and GE resulting in activation of NF-κB and PGR down-regulation. Although RU486 is a well know glucocorticoid antagonist, it can effectively be used to block P4 action during early pregnancy in the pig.

Decreased staining of PGR-A and PGR-B protein has been detected as early as d 7 from the uterine epithelium during the estrous cycle, indicating that PGR down-regulation (at the transcriptional level) occurs before d 7 (Geisert et al., 1994). The RU486 was administered to gilts on d 3, 4 and 5 to block PGR down-regulation before it begins (T1) or on d 6 and 7 to block PGR down-regulation following initiation of the mechanism (T2). Gilts were then sacrificed on d 8 and d 12 of pregnancy to measure endometrial PGR-B, RANKL, and RANK mRNA abundance. Progesterone receptor protein and NF-κB activation was also measured within the endometrium. Endometrial PTGS2 and FGF-7 mRNA was also measured to assess expression in the presence of the conceptus on d 12 of pregnancy.

According to the NF-κB and PGR down-regulation hypothesis, RU486 will bind PGRs in the uterine epithelium and theoretically block P4-induced RANKL expression. A decrease in epithelial RANKL protein and NF-κB activation would result in maintained PGR mRNA expression during the time of establishment of pregnancy.

Vallet and Christenson (2004) reported a 50% reduction in conceptus diameter by d 11 after treating gilts with 400 mg of RU486 on d 2 of pregnancy. We found a similar effect by d 8 when treating gilts with 400 mg of RU486 on d 3, 4 and 5 (T1) of pregnancy. Conceptus diameter in T1 gilts was nearly half that of controls by d 8 and pregnancy was completely inhibited by d 12. Blocking P4 action shortly after insemination, therefore, is incompatible with conceptus survival. Although treating gilts with RU486 on d 6 and d 7 (T2) slightly reduced conceptus diameter by d 8 of pregnancy when compared with controls, the majority of T2 gilts produced conceptuses that

elongated by d 12. Progesterone action during the first 6 days of pregnancy may be adequate to support gestation until d 12 in gilts.

Uterine PGR maintains a spacio-temporal pattern of expression in the pig for which continuous exposure of P4 leads to down-regulation of PGR exclusively within the epithelium (Geisert et al., 1994; Sukjumlong et al., 2005; Ka et al., 2007). Down-regulation of PGR gene expression is thought to occur before d 7 because down-regulation of PGR protein occurs near d 8 of the estrous cycle and pregnancy in the pig (Geisert et al., 1994). Full down-regulation of the receptor occurs by d 12 (Geisert et al., 1994; Ka et al., 2007; Persson et al., 1997; Sukjumlong et al., 2005).

Blocking P4 action with RU486 on d 3, 4 and 5 (T1) or on d 6 and d 7 (T2) of pregnancy maintained endometrial expression of PGR-B on d 8 and d 12 when compared with control gilts. This supports the general concept that P4 causes down-regulation of PGR in the pig uterine epithelium near d 8 of pregnancy where full down-regulation occurs by d 12 (Geisert et al., 1994). Increased expression of PGR-B in T1 and T2 gilts would indicate that RU486 can effectively block PGR down-regulation before and during the mechanism respectively.

Earlier treatments of RU486 (T1; d 3, 4 and 5 of pregnancy), a time when PGR down-regulation begins, in combination with a greater number of RU486 injections may account for greater expression of PGR-B in T1 gilts when compared with T2 gilts on d 8. Although a slight non-significant decrease in PGR-B expression occurred, T1 gilts maintained greater expression of PGR-B when compared with both control and T2 gilts through d 12 of pregnancy. Both RU486 (half-life; approximately 20 to 40 h) and its metabolites are capable of antagonizing P4 action and can remain active for 6 to 7 days

(Fiala and Gamzell-Danielsson, 2006; Heikinheimo et al., 2003; Sarkar, 2002; Sartor and Figg, 1996). A final injection of RU486 on d 5 of pregnancy (T1) followed by reduced RU486 activity may explain the slight reduction in endometrial PGR-B expression in T1 gilts. The RU486 may be losing its antagonistic effect on P4 action (PGR down-regulation) near d 12.

Gilts treated with RU486 on d 6 and d 7 of pregnancy (T2) maintained intermediate endometrial PGR-B expression when compared with both control and T1 gilts. This may indicate that PGR expression resumed within the epithelium, in response to RU486, following an initial P4-induced down-regulation. Endometrial PGR-B expression levels within T2 gilts did not reach that of T1 gilts by d 12 of pregnancy. This may indicate that timing (interruption of PGR down-regulation) and/or number of RU486 injections were not sufficient to increase expression of PGR-B within T2 gilts to levels similar to T1 gilts by d 12.

There was not a significant decrease in endometrial PGR-B expression from d 8 to d 12 of pregnancy in control gilts; indicating that PGR down-regulation at the transcriptional level had already occurred before d 8 in control animals. These results are consistent with those reported by Geisert et al. (1994) where staining of PGR protein had decreased in the pig epithelium by d 7 of the estrous cycle. Although expression of PGR-B slightly increased in control animals from d 8 to d 12, this increase was not significant and levels remained low when compared with T1 and T2 gilts.

Compared with T1 gilts, PGR-B protein decreased within the LE of control gilts from d 8 to d 12 of pregnancy, consistent with observations made by Geisert et al. (1994). The loss of PGR-B protein within the LE of control gilts coincides with the reduced

endometrial PGR-B mRNA expression observed on d 8 and d 12 of pregnancy (PGR down-regulation). The loss of PGR protein occurred in a cell by cell manner as adjacent epithelial cells may be stained either positive or negative for PGR protein. Although PGR-B protein also decreased from d 8 to d 12 in the deep GE, the loss of protein was less prominent, indicating that PGR down-regulation decreases in strength from the LE to the deep GE.

Compared with control gilts, treating gilts with RU486 (T1 and T2) resulted in reduced PGR-B protein within LE on d 8 of pregnancy. Zaytseva et al. (1993) reported decreased decidual PGR protein within 12 h after administering 600 mg of the antagonist RU486 to terminate pregnancy in the human. Lange et al. (1999) demonstrated that ligand bound PGR degradation is initiated by phosphorylation of a serine residue within PGRs by mitogen-activated protein kinases (MAPKs). After phosphorlyation, PGRs are then targeted for degradation by the 26S proteasome (Lange et al., 1999). Binding of RU486 to PGRs may have enhanced PGR phosphorylation and degradation by the 26S proteasome resulting in reduced PGR-B protein within the LE of treated gilts on d 8.

Although PGR-B protein in the LE was reduced on d 8, protein levels increased from d 8 to d 12 in T1 gilts, coinciding with greater endometrial PGR-B gene expression. The increase in PGR-B protein may have resulted from blocking P4 action and PGR down-regulation at the transcriptional level. Maintaining PGR-B expression within the LE, therefore, resulted in greater PGR-B protein synthesis and protein concentration observed within the LE of T1 gilts on d 12. This trend was not observed in T2 gilts were although endometrial expression of PGR-B was greater than control gilts during early pregnancy (d 8 and d 12), PGR-B protein within the LE continued to decrease, reaching

levels similar to controls by d 12. The presence of residual RU486 (a result of later injections) may account for continued PGR protein clearance and reduced levels on d 12.

The PGR-B protein decreased from d 8 to d 12 in the surface GE of controls gilts in parallel with reduced endometrial PGR-B expression. These results are consistent with the initial observation made by Geisert et al. (1994) where full down-regulation of PGR protein occurred exclusively within the LE and GE by d 12 of pregnancy in the pig. Much like the LE, T1 and T2 gilts had greater and less PGR-B protein, respectively, when compared to control gilts by d 12.

The PGR down-regulation mechanism does not occur in the uterine myometrium and stroma. Stromal PGR-B protein did not change during early pregnancy (d 8 to d 12) in control gilts; however, there was a tendency for an effect of treatment. Control gilts tended to have fewer PGR-B positive stromal cells when compared with T2 gilts (T1 gilts being intermediate) on both d 8 and d 12 of pregnancy. The effect that RU486 may have on stroma PGR-B is not understood and may involve either a slight negative affect that P4 has on PGRs within the stroma or possibly reestablishment of ER within this tissue. After binding PGR, P4 inhibits gene expression for estradiol receptor (ER) in the uterus (McCracken, 1980), however ligand bound ER increases expression of PGR. Loss of P4 action in RU486 treated gilts may have allowed for a greater concentration of ER within stromal cells resulting in an increase in PGR-B expression and protein synthesis. Increased plasma E2 concentrations were observed in RU486 treated gilts (see Chapter Four). This may have been enough to drive ER induced PGR expression within the stroma. On the other hand, not all stroma cells were stained positive for PGR-B protein,

indicating this mechanism alone may not account for greater PGR protein within in the stroma of RU486 treated gilts.

Although not as extensive as the LE, a slight decrease in myometrial PGR-B protein was observed in control gilts from d 8 to d 12 of pregnancy. When localizing PGR-A and PGR-B simultaneously in the pig uterus, Sukjumlong et al. (2005) reported decreased staining intensity for PGR protein as well as number of PGR positive myometrial cells from estrus to d 11 of pregnancy. This may indicate that fluctuations in myometrial PGR are a common feature in the pig uterus. Similar to the stroma, treating gilts with RU486 (T1 and T2) resulted in slightly more PGR-B positive myometrial cells on both d 8 and d 12 of pregnancy when compared with control gilts. Like the stroma, increases in myometrial PGR protein in RU486 treated gilts may indicate that P4 has a slight negative effect on myometrial PGR-B or that reestablishment of ER lead to increased PGR expression and protein synthesis.

It has been suggested that NF-κB, induced by P4-driven RANKL expression may bind DNA and down-regulate expression of PGRs within the pig uterine epithelium. Nuclear factor-kappa B is a transcription factor that controls expression of many different genes including interferon-gamma (INF-γ), tumor necrosis factor alpha (TNFα), insulin like growth factor binding protein-1 (IGFBP-1), interleukin-1 beta (IL-1β) and prostaglandin-endoperoxidase synthase 2 (PTGS2) (Heike, 1999; Hiscott et al., 1993; Lang et al., 1999; Shakhov et al., 1990; Sica et al., 1997; Yamamoto et al., 1995). In some tissues, NF-κB antagonizes the actions of PGRs (Condon et al., 2006; McKay and Cidlowski, 1998). Condon et al. (2006) located a putative NF-κB response element 1330

bp upstream of the human PGR gene, indicating that NF-κB directly binds DNA to regulate expression of PGRs.

In this study, there were no differences in NF-κB activation on d 8 of pregnancy between treated (T1 and T2) and control gilts, a time when RU486 treated animals maintained greater endometrial PGR-B and RANKL expression when compared with control animals. This would indicate that NF-κB is not directly involved in PGR down-regulation. Activation of NF-κB tended to be greater in LE of control and T2 gilts when compared with T1 gilts on d 12 of pregnancy. Considering that pregnancy was completely ablated in T1 gilts and that both control and T2 gilts contained elongated conceptuses on d 12, release of IL-1β by the elongating conceptus may account for increases in NF-κB activation.

Interluekin-1 beta is a pro-inflammatory cytokine involved in many cellular activities including cell differentiation, proliferation and apoptosis. Interluekin-1 beta is an important mediator of the inflammatory response and can activate NF-κB (Shirakawa et al., 1989; Soloff et al., 2004). Studies suggest IL-1β may initiate cross talk between the uterus and conceptus and may play an important role during pregnancy in the human and mouse (Lindhard et al., 2002; Takacs and Kauma, 1996). The pig conceptus will also release IL-1β during early pregnancy (Tou et al., 1996). On d 11 of gestation, the pig conceptus increases gene expression for IL-1β. This increase is followed by a sharp decline on d 15 and d 18 of gestation (Ross et al., 2003). Compared with the day 11 filamentous conceptus, gene expression of IL-1β is reduced 2000-fold in the d 15 conceptus (Ross et al., 2003). Figure 3.30 (B) (p. 101) displays a T2 conceptus on d 12 of pregnancy adjacent to activated NF-κB within the LE of the pig uterus. Activation of

NF-κB by the porcine conceptus appears to be a localized event, where activation is prevalent within LE cells near the conceptus, but absent within LE cells distal to the conceptus (Figure 3.30 C, p. 101).

Other cytokines such as receptor activator for NF-κB ligand (RANKL) can activate NF-κB and are expressed by the pig uterus (Geisert and Ross, unpublished results). Within the mouse mammary gland, receptor activator for NF-κB (RANK; the receptor for RANKL) is exclusively expressed in the mammary epithelium and stimulated by RANKL to promote mammary growth (Fata et al., 2000). In studies using RANK and RANKL knockout (KO) mice, reduced mammary gland development is observed and supplementation of RANK and RANKL can result in recovery of the normal phenotype (Fata et al., 2000). Steroid hormones such as P4 drive RANKL expression within the mammary gland that can act on RANK-expressing epithelium to promote growth (Fata et al., 2000). The pig uterus expresses RANK exclusively within the LE and GE throughout the estrous cycle (Geisert and Ross, unpublished results). Endometrial expression of RANKL increases by d 10 of the estrous cycle (Geisert and Ross, unpublished results). Progesterone is thought to drive RANKL expression in the pig uterine epithelium, binding its receptor RANK and inducing NF-κB activation within the LE and GE. The NF-κB and PGR down-regulation hypothesis states that activation of NF-κB blocks PGR expression in the uterine epithelium and down-regulates PGR.

In this study, treating gilts with RU486 (T1 and T2) resulted in greater endometrial RANKL gene expression when compared with control gilts on both d 8 and d 12 of pregnancy. This would indicate that P4 does not increase epithelial RANKL expression to induce NF-κB activation and therefore, PGR down-regulation within the

pig uterus. Endometrial RANKL expression within T1 gilts was approximately 16-fold greater on d 8 and 23-fold greater on d 12 when compared with control gilts. Endometrial RANKL expression in T2 gilts increased to nearly 22-fold over controls by d 12. The increase in RANKL expression within treated gilts may indicate that P4 can inhibit RANKL expression within the pig endometrium during early pregnancy.

Increased endometrial RANKL expression on d 10 of the pig estrous cycle has been reported and is temporally associated with PGR down-regulation (Geisert and Ross, unpublished results). There was a slight increase in RANKL expression from d 8 to d 12 in control gilts. Considering that RU486 led to an increase in RANKL expression within treated gilts, increases in endometrial RANKL expression near d 10 of the estrous cycle and pregnancy may result from PGR down-regulation and loss of PGR function within the LE and GE that occurs near d 8. The P4 driven RANKL expression reported within the mammary gland of the mouse may be tissue specific and absent from the pig uterus.

Geisert and Ross (unpublished data) reported uniform RANK (the receptor for RANKL) expression exclusively within the LE and GE through all days of the estrous cycle. In this study, no significant differences were observed for endometrial RANK expression between days or treatments, consistent observations made by Geisert and Ross (unpublished results). Considering the uterine epithelium increases expression of RANKL near d 10 of the pig estrous cycle, and that RANK is uniformly expressed within LE and GE, this cytokine may coordinate a biological response within the uterus near the time of establishment of pregnancy in the pig.

Prostaglandin-endoperoxide synthase 2 (PTGS2) is a rate limiting enzyme in PG synthesis and expression of PTGS2 during implantation is temporally associated with

increased NF-κB activation within the uterus (Chakraborty et al., 1996; Marions et al., 1999). During implantation in the human and mouse, the conceptus will secrete IL-1β and induce activation of NF-κB, resulting in greater endometrial PTGS2 expression (Chakraborty et al., 1996; Marions et al., 1999). Endometrial PTGS2 expression increases approximately 76-fold between d 5 and d 15 of the estrous cycle and pregnancy in the pig and is necessary for successful establishment of pregnancy (Ashworth et al., 2006; Kraeling et al., 1985). Conceptus release of IL-1β during trophoblast elongation is thought to enhance endometrial PTGS2 expression by activating NF-κB within the pig uterus (Ashworth et al., 2006; Ross et al., 2003). Conceptus release of IL-1β near d 12 of pregnancy, increasing endometrial PTGS2 expression, is thought to stimulate the pig uterus in preparation for conceptus attachment (Ashworth et al., 2006; Ross et al., 2003).

In this study, endometrial PTGS2 mRNA abundance was measured to determine if the presence of the conceptus enhanced PTGS2 expression within the pig uterus. Although only slight differences were observed between treatments on d 8 of pregnancy, endometrial expression of PTGS2 was approximately 2 and 3-fold greater in control and T2 gilts, respectively, when compared with T1 gilts on d 12. Greater expression of PTGS2 in control and T2 gilts is likely the result of conceptus induced NF-κB activation. This trend was not observed in T1 gilts where loss of pregnancy and loss conceptus IL-1β secretion, may have resulted in decreased NF-κB activation and decreased endometrial PTGS2 expression by d 12.

Fibroblast growth factor 7 (FGF-7) stimulates cell proliferation, differentiation and migration in various organs. Fibroblast growth factor 7 is expressed by the pig LE from d 9 to d 12 of the estrous cycle and pregnancy (Ka et al., 2007). Peak expression of

FGF-7 was reported within the LE on d 12 of gestation, in the presence of the conceptus (Ka et al., 2007).

In this study, endometrial FGF-7 mRNA abundance was measured to determine if elongated conceptus in RU486-treated gilts could enhance FGF-7 expression within the uterus. Expression of FGF-7 was approximately 15 and 26-fold greater in control and T2 pig, respectively, than T1 gilts on d 12. Elongated conceptuses recovered exclusively from control and T2 gilts may have increased endometrial FGF-7 expression. The increase in FGF-7 may have been caused by conceptus release of E2 near d 12 (maternal recognition of pregnancy signal).

The fibroblast growth factor receptor 2IIIb (FGFR2; the receptor of FGF-7) is expressed by the conceptus and endometrial epithelium (Ka et al., 2000; 2007). Ka et al. (2007) suggested that epithelial increases in FGF-7 expression in response to conceptus E2 could stimulate trophectoderm differentiation and proliferation during pregnancy. They found that P4 is permissive of FGF-7 expression by down-regulating PGR with the LE and that conceptus release of E2 is responsible for maximal expression of FGF-7 within LE. Although T2 gilts were treated with RU486 on d 6 and d 7, blocking P4 action during early pregnancy, 60% of T2 gilts produced what appeared to be normal conceptuses on d 12, as well as greater endometrial expression of FGF-7 when compared with T1 gilts. This may indicate that loss of P4 action after d 6 of pregnancy may not be detrimental to conceptus elongation and release of E2 near d 12.

CONCLUSIONS

The P4 antagonist, RU486, can effectively block P4 action during early pregnancy. Treating gilts with RU486 after insemination affected early conceptus development, uterine gene expression, and uterine PGR protein. Pregnancy was compromised by d 12 for gilts treated with RU486 on d 3, 4 and 5 of pregnancy (T1). Inhibition of P4 action shortly after insemination, therefore, is incompatible with conceptus survival. There was a slight numeric reduction in the percentage of gilts that produced normal conceptuses when treated with RU486 on d 6 and d 7 of pregnancy (T2). Blocking P4 action with RU486 during early pregnancy (T1 and T2) increased endometrial PGR-B mRNA expression on d 8 and d 12 when compared with controls. This supports the general concept that P4 is responsible for PGR down-regulation in the uterine epithelium. Our results do not support the hypothesis that RANKL mediates NF-κB inhibition of PGR. Both RANKL and PGR-B expression were clearly elevated in T1 gilts on d 8; a time when activated NF-κB was low. Greater activation of NF-κB was only detected on d 12 in treatments that were conducive to early conceptus development (T2 and control). Activation of NF-κB, therefore, is temporally associated with the secretion of IL-1β by the elongating porcine conceptus on d 12 of pregnancy (Ross et al., 2003). The activation of NF-κB on d 12 coincided with greater PTGS2 expression; a response observed previously in the pig (Ashworth et al., 2005). Endometrial expression of FGF-7 was also greatest in T2 and control gilts on d 12. Within the LE, maximal expression of FGF-7 occurs on d 12 of pregnancy in response to conceptus (Ka et al., 2007).

Loss of PGR from the uterine epithelium occurs before implantation in a number of mammalian species. The loss of PGR from the LE and GE during the estrous cycle and pregnancy in the pig is associated with loss of MUC-1 (Bowen et al., 1996) and secretion of SPP1 (osteopontin) from the LE surface (White et al., 2005), mechanisms that are necessary for conceptus attachment and establishment of pregnancy. How P4 causes down-regulation of its own receptor is not understood. An alternative hypothesis, is that PGR positive stroma cells may release a progestamedin in response to P4 that may act on LE and GE, either down-regulating PGR and (or) allowing the epithelia to respond to P4 following PGR down-regulation (Ka et al., 2007). Indeed, P4 does cause down-regulation of its own receptor within the epithelium and the underlying stroma cells maintain PGR in the pig uterus (Geisert et al., 1994; Ka et al., 2007), phenomena also observed in this study. Clearly, uncovering the mechanism controlling PGR down-regulation in the uterine epithelium would allow for greater understanding of uterine mechanisms responsible for establishment of pregnancy and increase reproductive efficiency in the pig.

Table 3.1 Accession number, gene, primer sequences (forward and reverse primers; 5' to 3') and location of the primers within the GenBank sequence for PCR amplifications.

Accession Number	Gene	Primer	Primer sequence	Primer location
XM_001925795.1	RANK	Forward	taacttgtggcactgggtca	390 to 409
		Reverse	caccgtctggacagcacata	552 to 571
XM_001925694.1	RANKL	Forward	acacggatttgcaagacaca	293 to 312
		Reverse	accttccaccatggcttttt	434 to 453
NM 214321	PTGS2	Forward	tcgaccagagcagagagatgagat	1433 to 1456
		Reverse	accatagagcgcttctaactctgc	1543 to 1566
AJ245447	FGF-7	Forward	ctgccaagtttgctctacag	1 to 20
		Reverse	tccaactgccacggtcctgat	271 to 291
U07786	ACTB	Forward	acatcaaggagaagctctgctacg	266 to 289
		Reverse	gaggggcgatgatcttgatcttca	608 to 631
GQ903679	PGR-B	Forward	tcagactgaagtcggggaac	692 to 711
		Reverse	gggtgaaatctccacctcct	870 to 889

Table 3.2 Conceptus flushing results for control gilts (n = 9), gilts treated with RU486 on d 3, 4 and 5 of pregnancy (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 of pregnancy (T2; n = 9). Conceptuses were flushed on d 8 and d 12. Conceptus status (normal) was based on morphology. Conceptuses collected on d 8 were considered normal if hatched from the zona pellucida. Spherical conceptuses, less than 11 mm in diameter, were considered normal as well as ovoid, tubular, and elongated on d 12. Percentage of gilts with normal conceptuses was lowest in T1 gilts (chi-square = 7.00; $P < 0.05$).

Day of Pregnancy	d 8			d 12		
Treatment	Control	T1	T2	Control	T1	T2
Morphology[1] and Average Diameter[2] (mm)	HB (0.63) HB (0.59) HB (0.43) HB (0.94) HB (0.46)	HB (0.24) HB (0.45) HB (0.18) 2C (0.15) O (0.13)	HB (0.50) HB (0.85) HB (0.50) HB (0.27)	HB (2.30) E E E	O (0.10) O (NR) O (NR) NF	T (110.0) HB (12.5) E E NF
Gilts with Normal Conceptuses	5/5	3/5	4/4	3/4	0/4	3/5
(%)	(100)	(60)	(100)	(75)	(0)	(60)

[1] Morphologies collected from each gilt: (HB) hatched blastocyst, (2C) 2-celled, (O) oocyte, (E) elongated, (T) tubular or (NF) not found

[2] Average diameters (mm) of conceptuses or oocytes collected. Diameters of oocytes collected on d 12 from two T1 gilts were not recorded (NR)

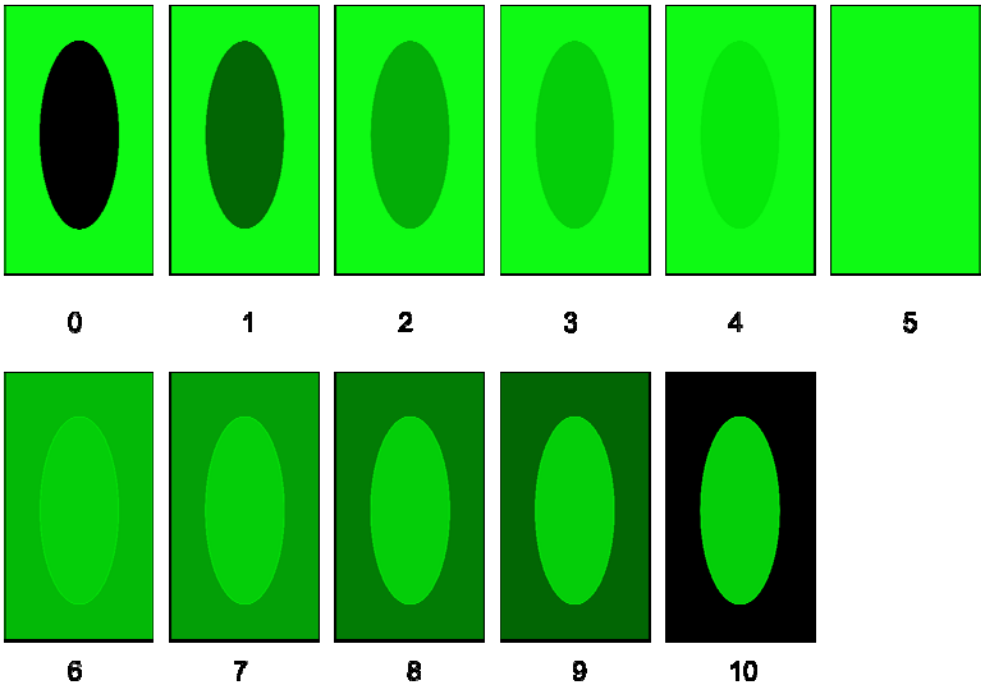
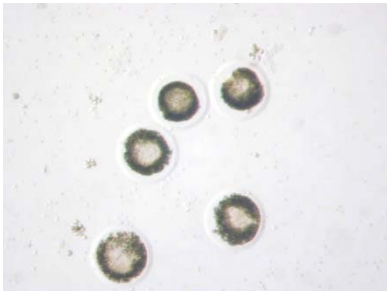


Figure 3.1 Scale used by investigators to score activation of NF-κB in the gilt uterine epithelia. Activation (nuclear localization) was scored on a 0 to 10 scale where 0 indicated no nuclear localization and 10 indicated complete nuclear localization.

A

B

C



D

Figure 3.2 (A) Conceptuses of hatched blastocyst morphology collected from a control gilt on d 8 of pregnancy. (B) A two-celled conceptus collected on d 8 of pregnancy from a gilt treated with RU486 on d 3, 4 and 5 (T1) of pregnancy. (C) One of two spherical conceptuses (averaging 12.5 mm) collected on d 12 of pregnancy from a gilt treated with RU486 on d 6 and d 7 (T2) of pregnancy. (D) Numerous non-fertilized oocytes collect on d 12 of pregnancy from a T1 gilt.

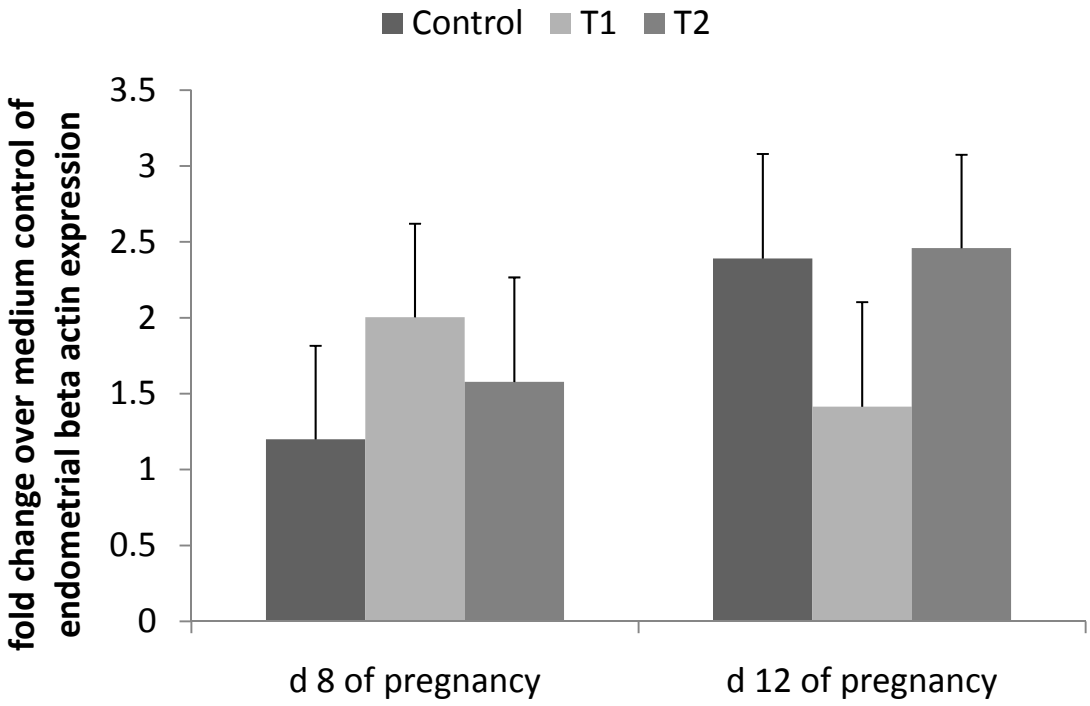


Figure 3.3 Least squares means for fold change over medium control of beta actin (ACTB) expression in endometrium of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. Endometrium was removed on d 8 and d 12 of pregnancy (n = 4 to 5). No significant differences in ACTB expression were reported between days or treatments.

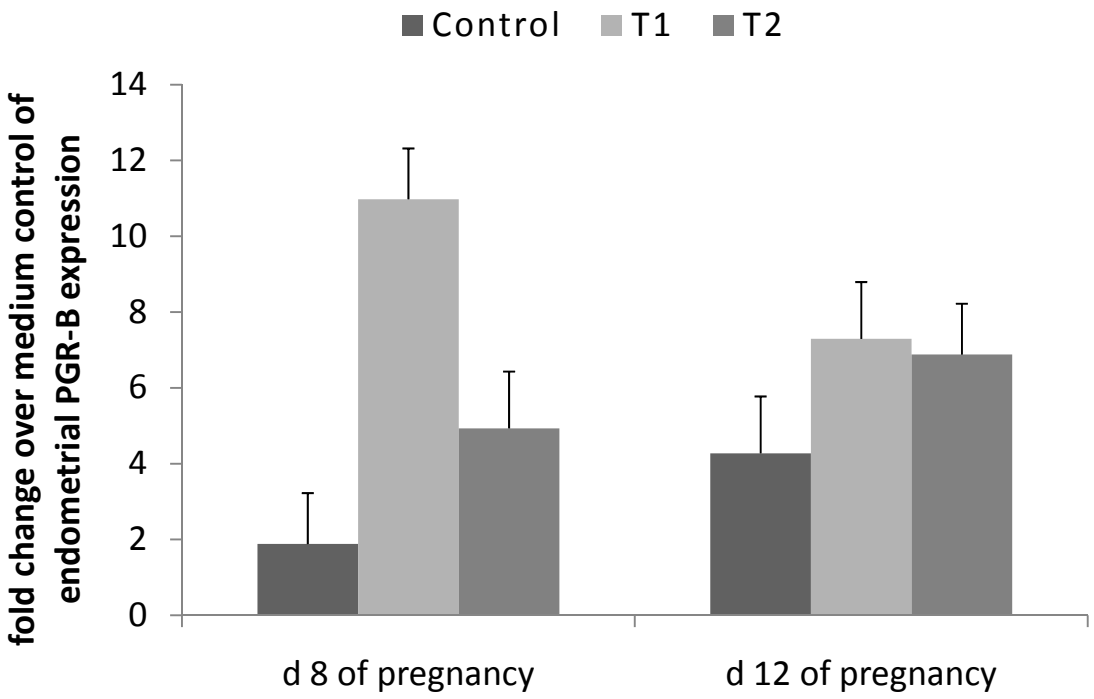


Figure 3.4 Least squares means for fold change over medium control of progesterone receptor B (PGR-B) expression in endometrium of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. Endometrium was removed on d 8 and d 12 of pregnancy (n = 4 to 5). Treatment affected endometrial PGR-B expression ($P < 0.01$). Endometrial PGR-B expression was greater in T1 and T2 gilts compared with control gilts during early pregnancy.

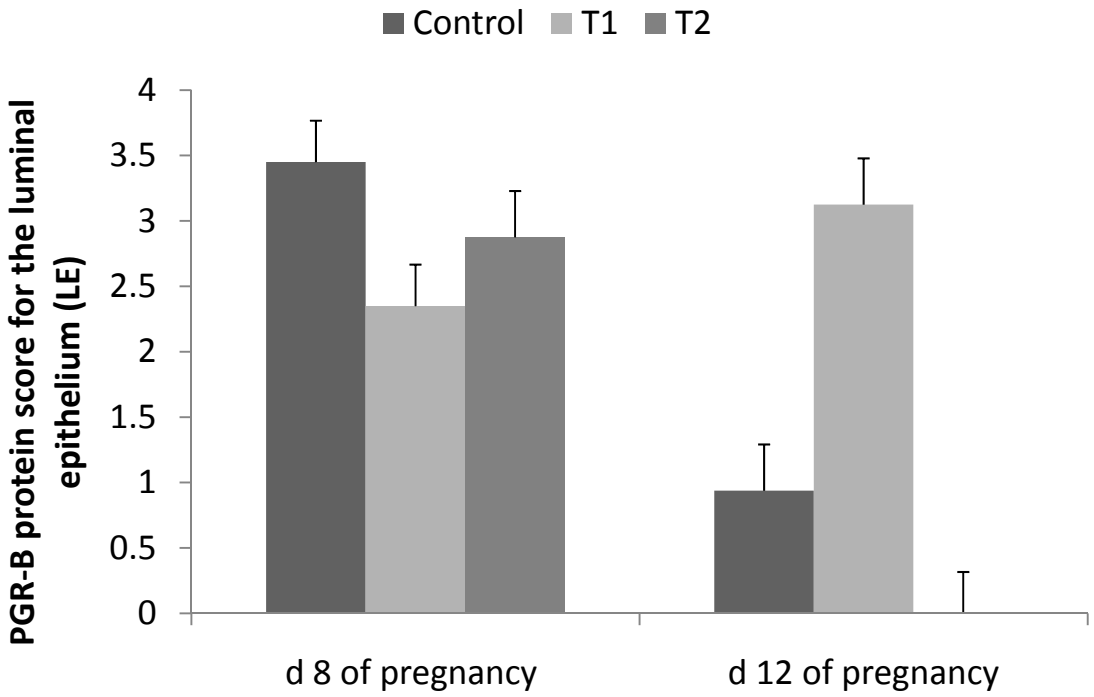


Figure 3.5 Least squares means for progesterone receptor B (PGR-B) protein score for the LE of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy (n = 4 to 5). Nuclei were scored on a 0 to 4 scale where 0 indicated 0% and 4 indicated 100% of nuclei containing PGR protein. There was a treatment by day interaction ($P < 0.001$). Luminal epithelial PGR-B protein in control gilts decreased from d 8 to d 12 when compared with T1 gilts. Treated gilts, T1 and T2, had reduced PGR-B protein on d 8 when compared with control gilts, however, protein levels for T1 and T2 were greater and less than controls, respectively, by d 12.

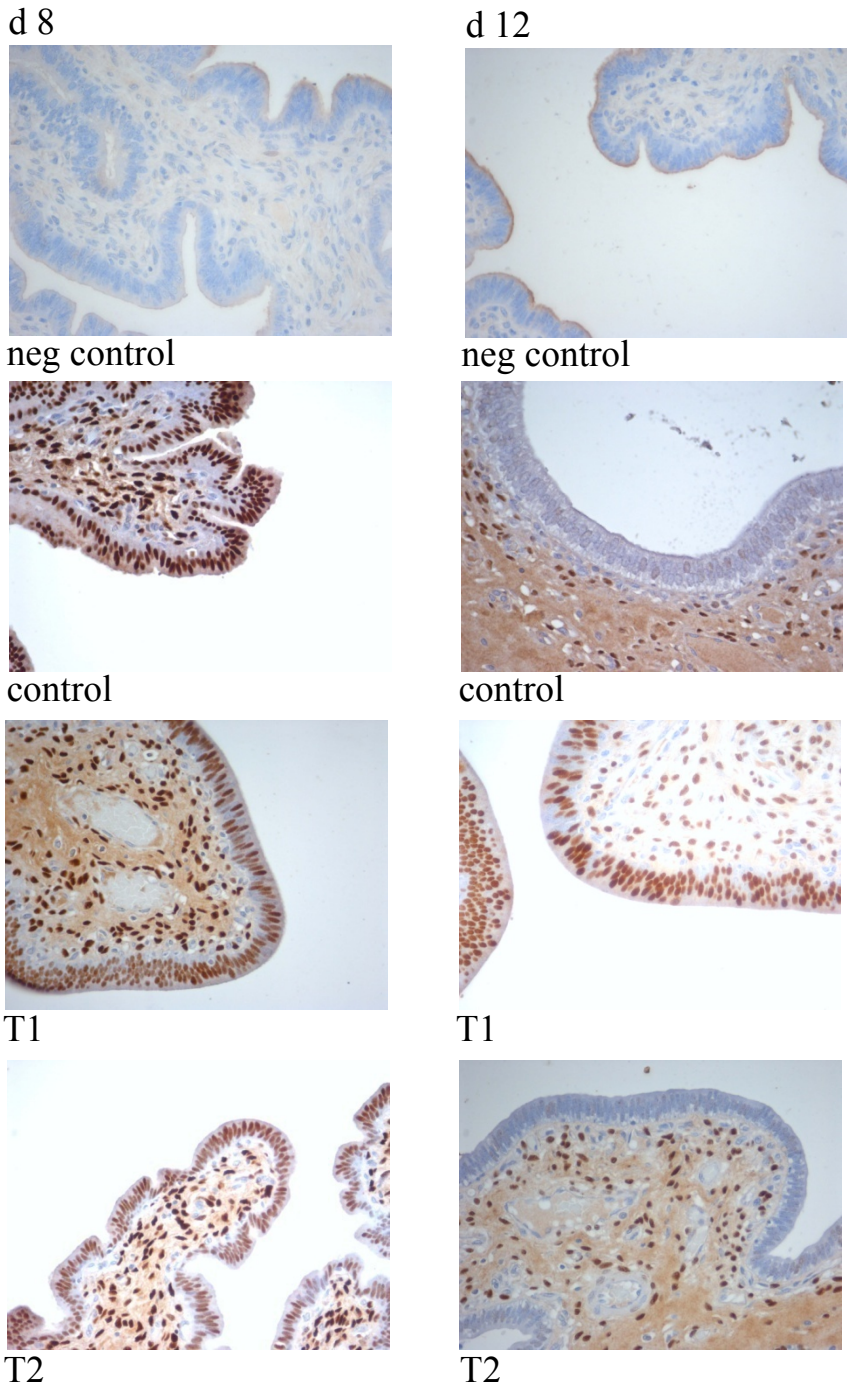


Figure 3.6 Progesterone receptor B (PGR-B) protein in the luminal epithelium (LE) of control gilts, gilts treated with RU486 on d 3, 4 and 5 (T1) and gilts treated with RU486 on d 6 and d 7 (T2) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy. Images were taken with a Leica light microscope at 400 X magnification.

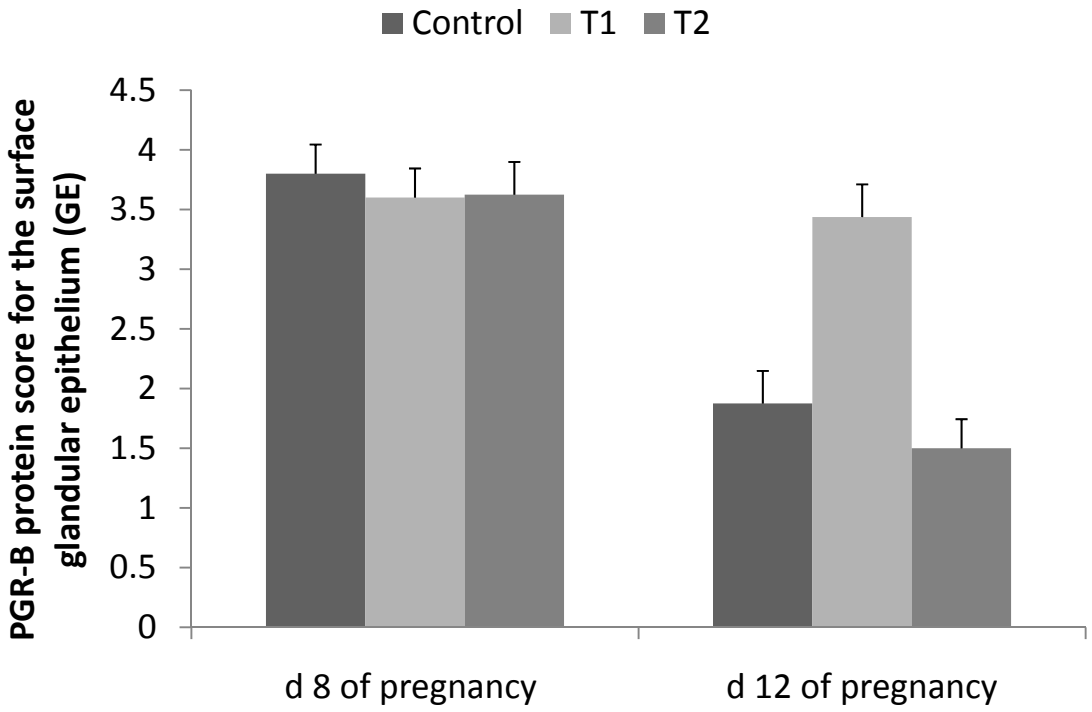


Figure 3.7 Least squares means for progesterone receptor B (PGR-B) protein score for the surface GE of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy (n = 4 to 5). Nuclei were scored on a 0 to 4 scale where 0 indicated 0% and 4 indicated 100% of nuclei containing PGR protein. There was a treatment by day interaction ($P < 0.01$) for PGR-B protein in the surface GE. Progesterone receptor B protein decreased from d 8 to d 12 in control gilts when compared with T1 gilts. Protein levels were similar between treatments on d 8, however, protein levels for T1 and T2 gilts were greater and less than controls, respectively, by d 12.

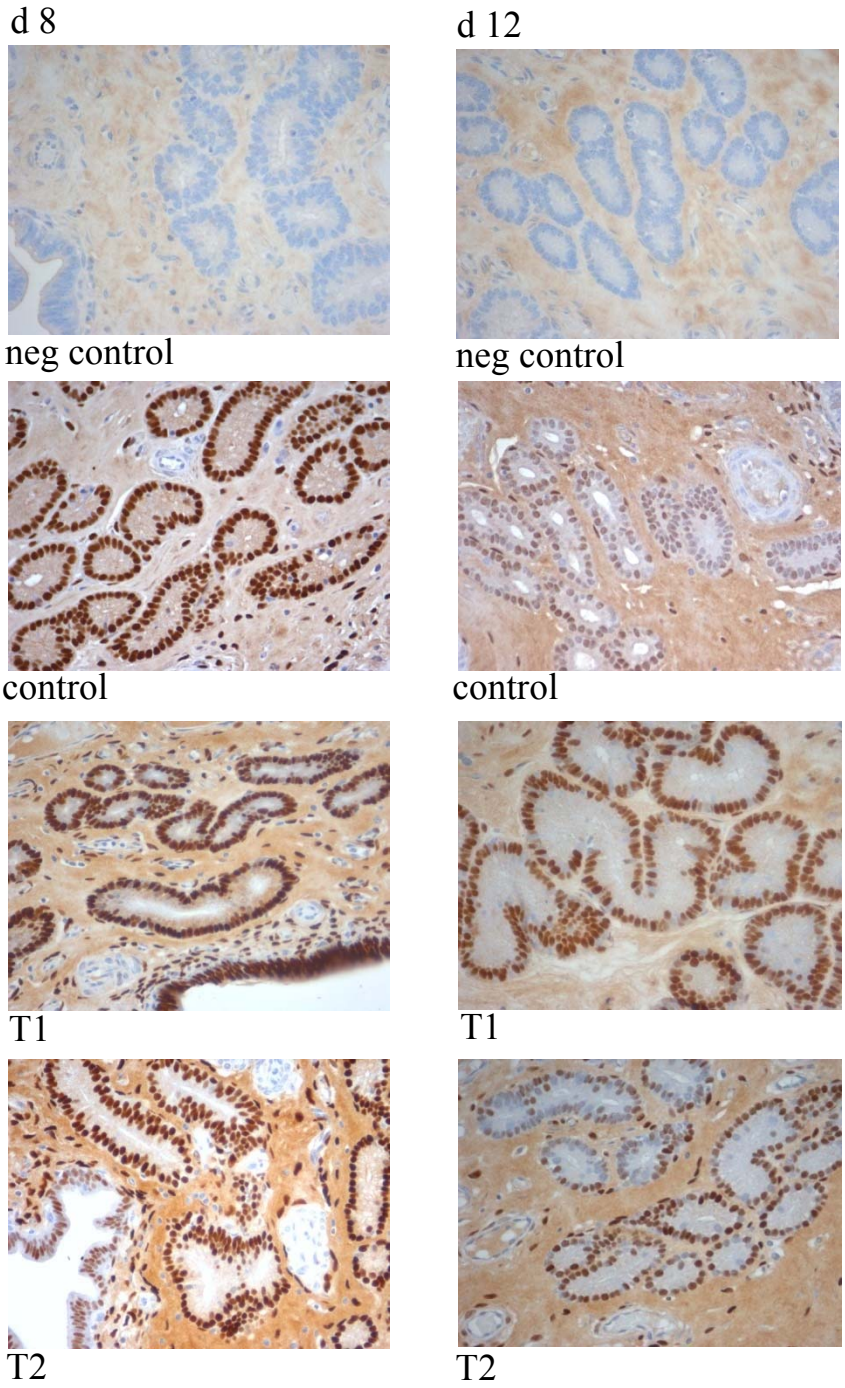


Figure 3.8 Progesterone receptor B (PGR-B) protein in the surface glandular epithelium (GE) of control gilts, gilts treated with RU486 on d 3, 4 and 5 (T1) and gilts treated with RU486 on d 6 and d 7 (T2) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy. Images were taken with a Leica light microscope at 400 X magnification.

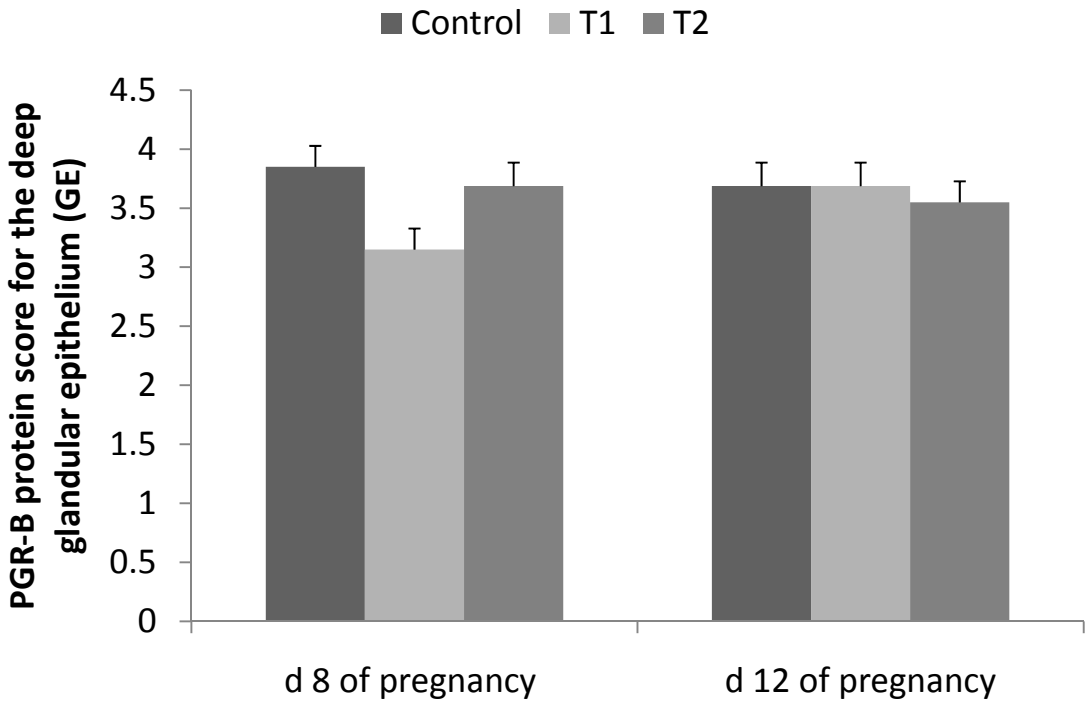


Figure 3.9 Least squares means for progesterone receptor B (PGR-B) protein score for the deep glandular epithelium (GE) of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy (n = 4 to 5). Nuclei were scored on a 0 to 4 scale where 0 indicated 0% and 4 indicated 100% of nuclei containing PGR protein. There was no effect of day or treatment on PGR-B protein in deep GE. Protein levels remained high for all treatments on d 8 and d 12 of pregnancy.

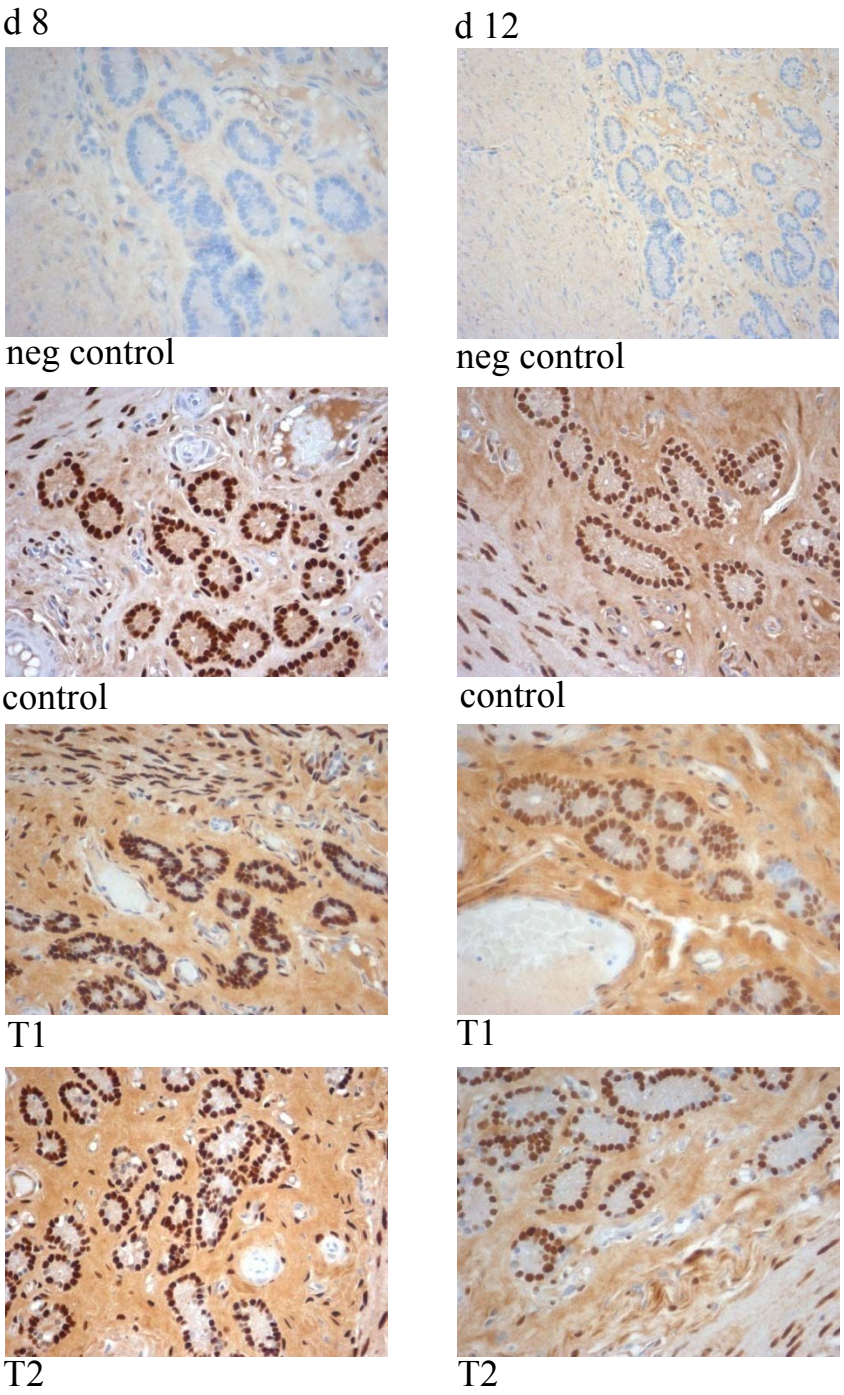


Figure 3.10 Progesterone receptor B (PGR-B) protein in the deep glandular epithelium (GE) of control gilts, gilts treated with RU486 on d 3, 4 and 5 (T1) and gilts treated with RU486 on d 6 and d 7 (T2) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy. Images were taken with a Leica light microscope at 400 X magnification.

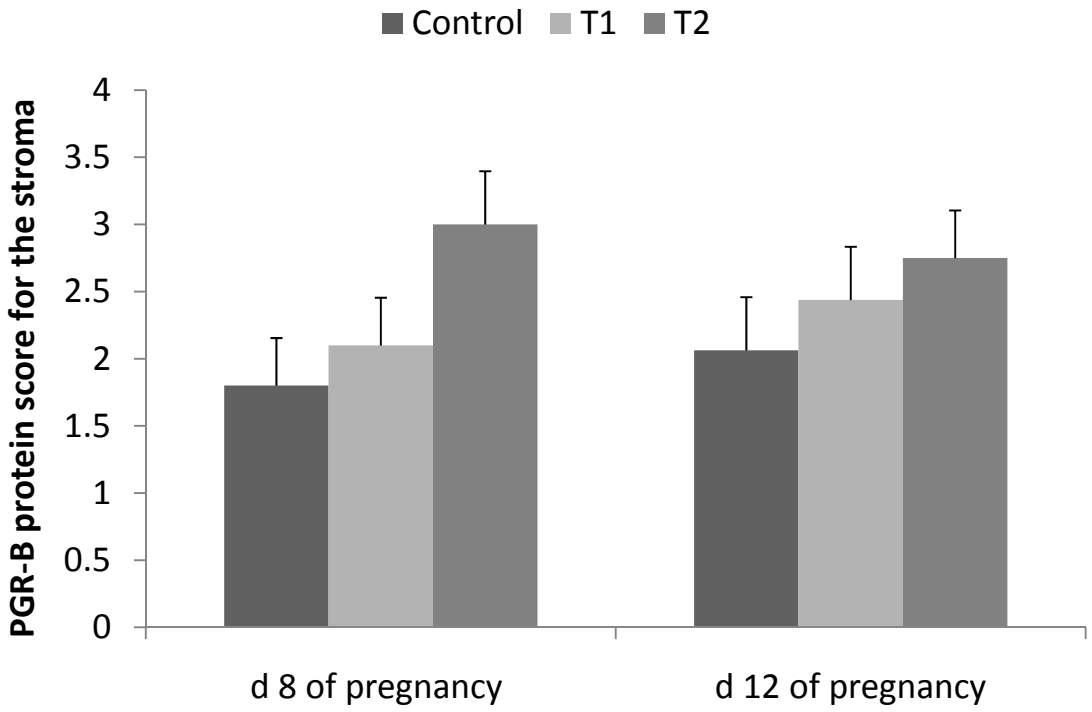


Figure 3.11 Least squares means for progesterone receptor B (PGR-B) protein score for the stroma of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy (n = 4 to 5). Nuclei were scored on a 0 to 4 scale where 0 indicated 0% and 4 indicated 100% of nuclei containing PGR protein. There was a tendency for an effect of treatment (P = 0.06) for PGR-B protein in the stroma. Protein levels in control gilts tended to be less than T2 gilts as T1 gilts were intermediate.

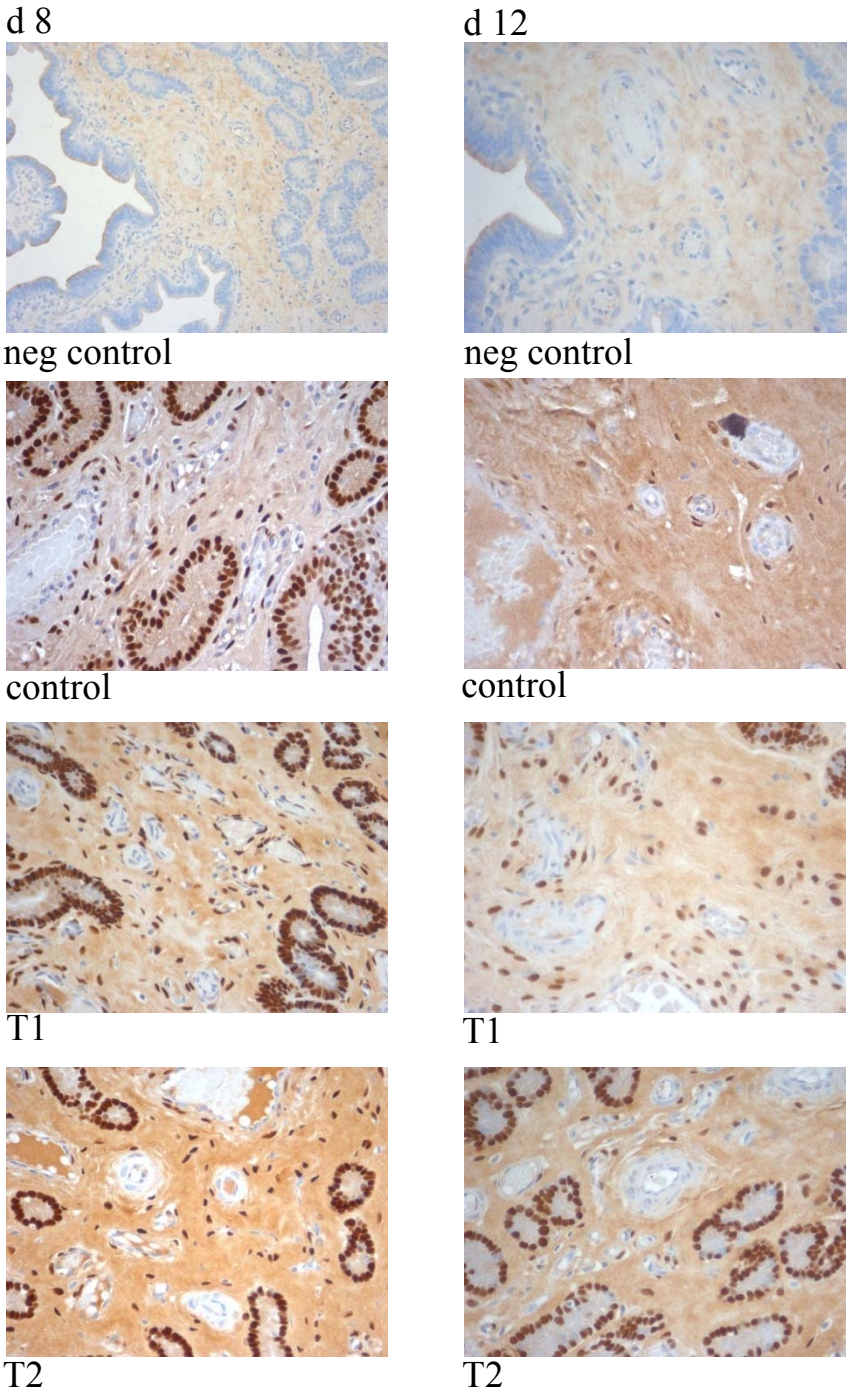


Figure 3.12 Progesterone receptor B (PGR-B) protein in the stroma of control gilts, gilts treated with RU486 on d 3, 4 and 5 (T1) and gilts treated with RU486 on d 6 and d 7 (T2) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy. Images were taken with a Leica light microscope at 400 X magnification.

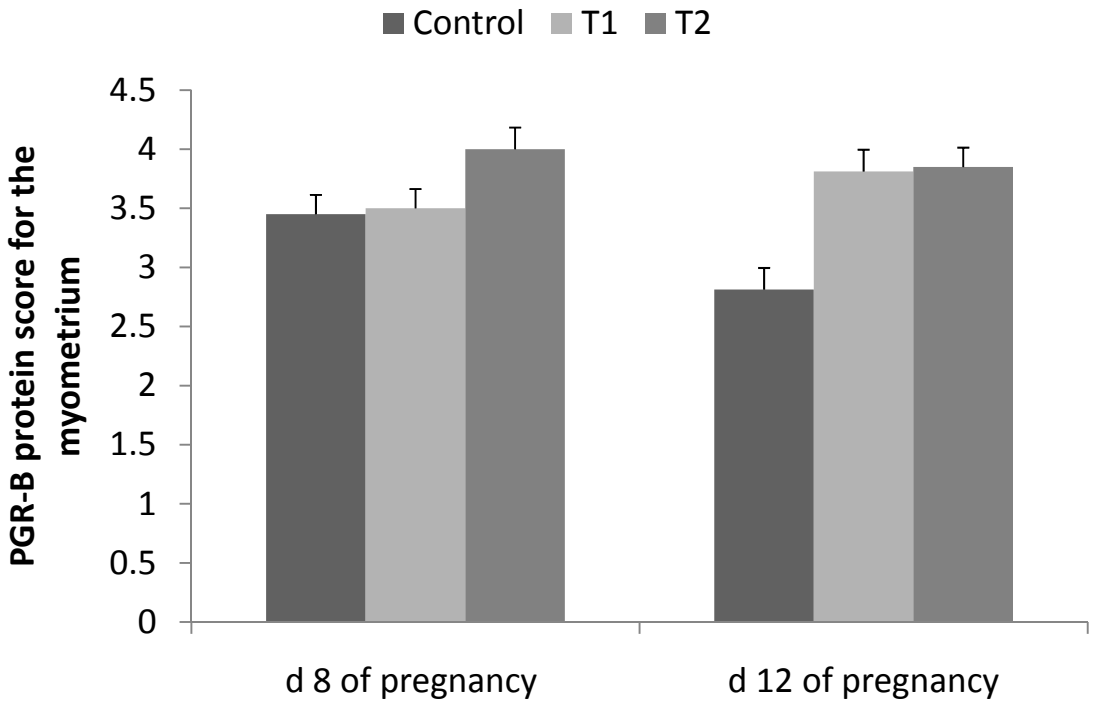


Figure 3.13 Least squares means for progesterone receptor B (PGR-B) protein score for the myometrium of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy (n = 4 to 5). Nuclei were scored on a 0 to 4 scale where 0 indicated 0% and 4 indicated 100% of nuclei containing PGR protein. There was a treatment by day interaction ($P < 0.05$) for myometrial PGR-B protein. Progesterone receptor B protein decreased from d 8 to d 12 in control gilts when compared with both T1 and T2 gilts. Protein levels were greater in T2 gilts when compared to both T1 and control gilts on d 8 of pregnancy, however, protein levels in T1 gilts slightly increased and were similar to T2 gilts by d 12.

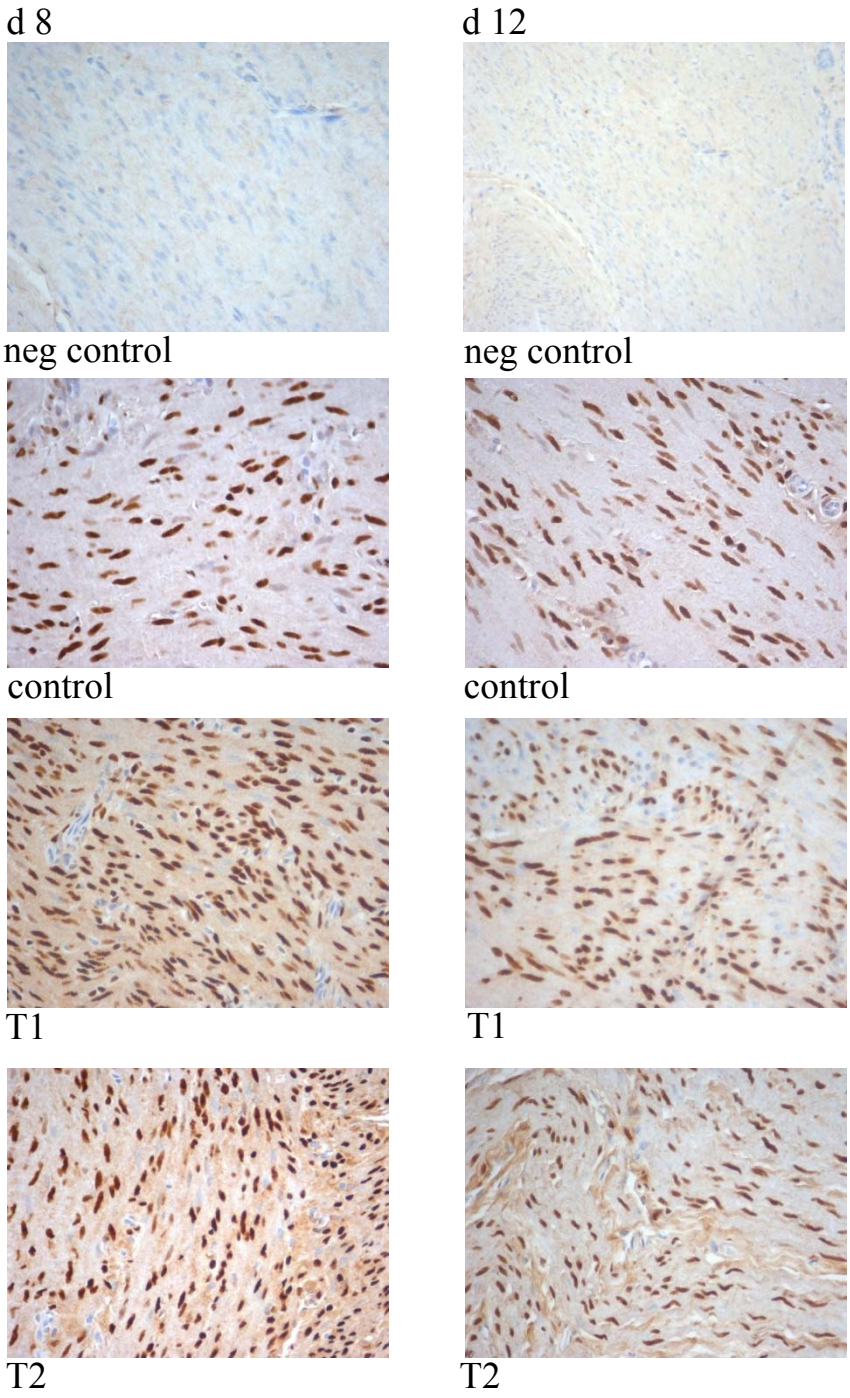


Figure 3.14 Progesterone receptor B (PGR-B) protein in the myometrium of control gilts, gilts treated with RU486 on d 3, 4 and 5 (T1) and gilts treated with RU486 on d 6 and d 7 (T2) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy. Images were taken with a Leica light microscope at 400 X magnification.

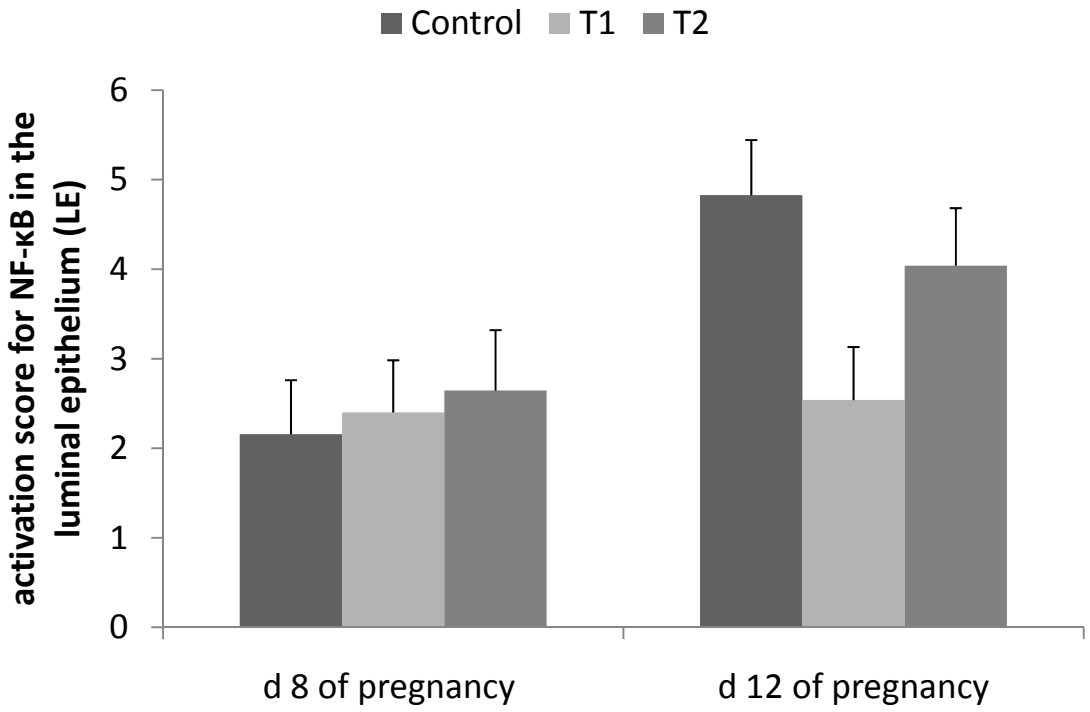


Figure 3.15 Least squares means for nuclear factor-kappa B (NF-κB) activation (nuclear localization) score for the luminal epithelium (LE) of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy (n = 4 to 5). Activation was scored on a 0 to 10 scale where 0 indicated no nuclear localization and 10 indicated complete nuclear localization. There was an effect of day ($P < 0.05$) on NF-κB activation in the LE. Activation increased from d 8 to d 12 of pregnancy.

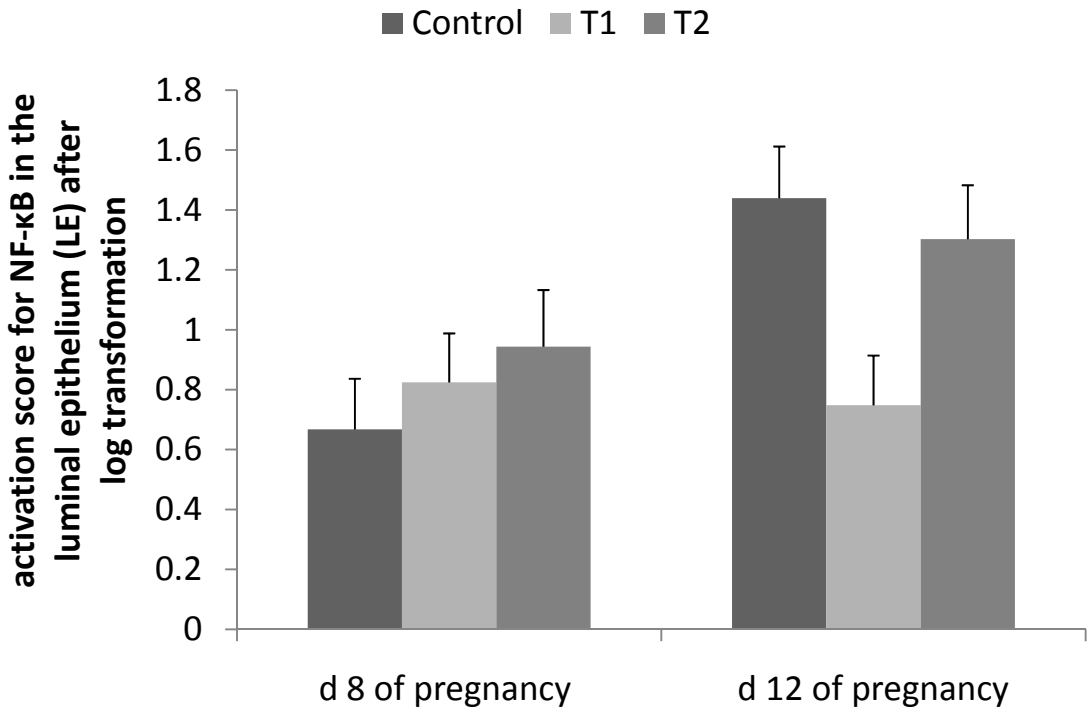


Figure 3.16 Least squares means for log transformed nuclear factor-kappa B (NF-κB) activation (nuclear localization) score for the luminal epithelium (LE) of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy (n = 4 to 5). Activation was scored on a 0 to 10 scale where 0 indicated no nuclear localization and 10 indicated complete nuclear localization. There was an effect of day ($P < 0.05$) on log transformed NF-κB activation in the LE. Activation increased from d 8 to d 12 of pregnancy. There was a tendency for a treatment by day interaction ($P = 0.062$) for log transformed NF-κB activation. The LE of control and T2 gilts had greater NF-κB activation when compared with T1 gilts on d 12 of pregnancy.

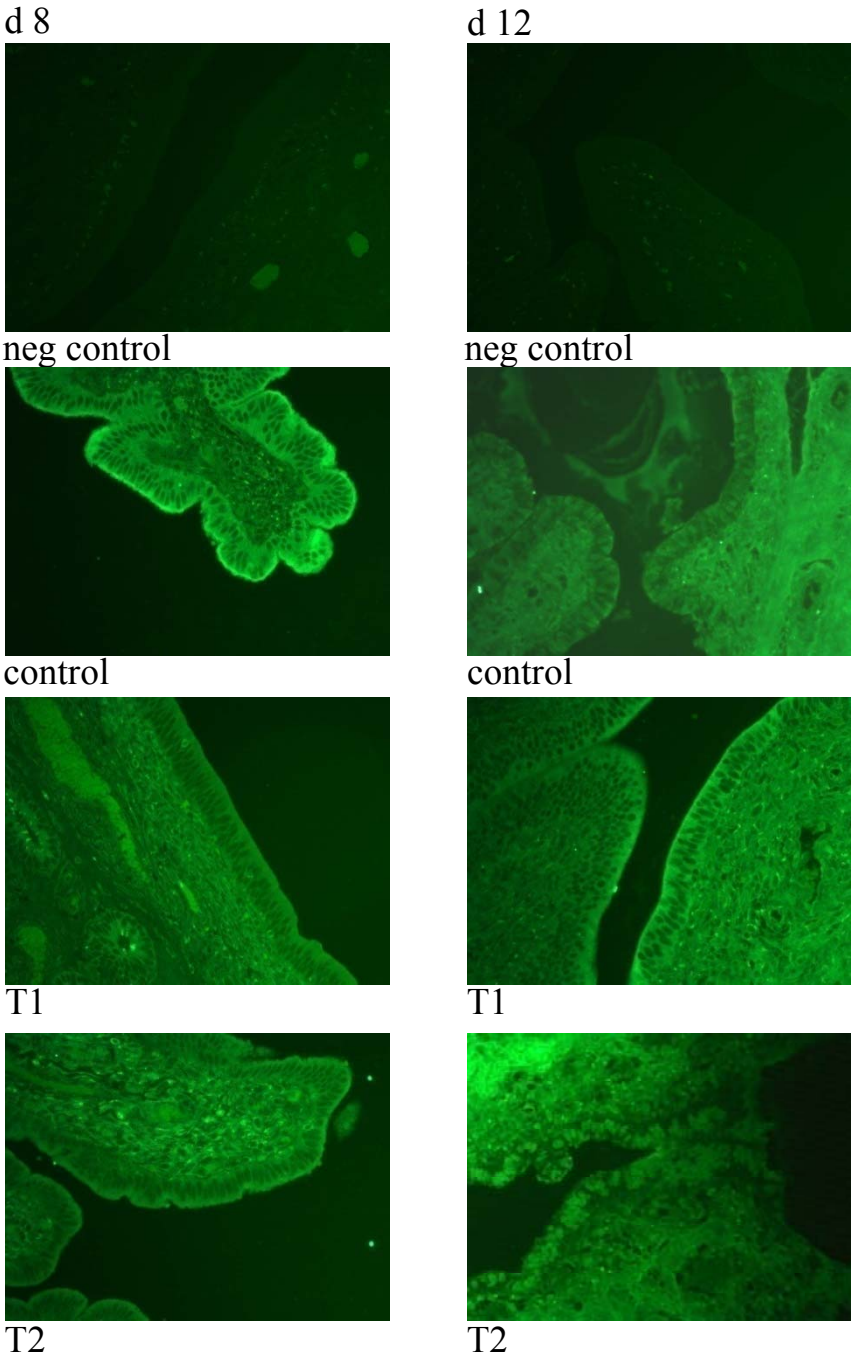
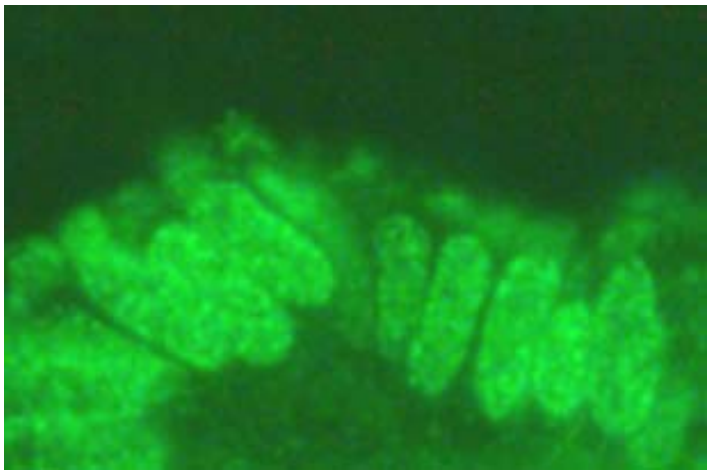


Figure 3.17 Immunofluorescence for NF-κB in the luminal epithelium (LE) of control gilts, gilts treated with RU486 on d 3, 4 and 5 (T1) and gilts treated with RU486 on d 6 and d 7 (T2) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy. Images were taken with a Leica light microscope with a green fluorescent protein (GFP) filter at 400 X magnification.

A



B

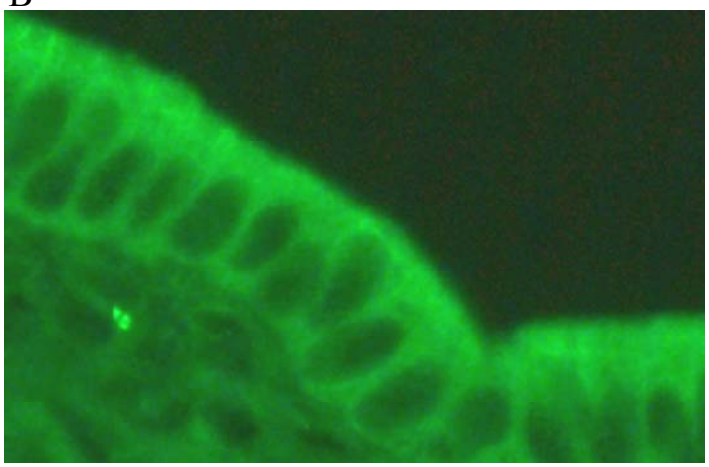


Figure 3.18 Immunofluorescents for nuclear factor-kappa B (NF-κB) in the uterine luminal epithelium (LE) at 400 X magnification plus a zoom. (A) Complete nuclear localization (activation) of NF-κB in the uterine LE of a gilt treated with RU486 on d 6 and d 7 (T2) of pregnancy. The uterus was removed on d 12 of pregnancy. (B) Cytoplasmic localization of NF-κB in the uterine LE of a gilt treated with RU486 on d 3, 4 and 5 of pregnancy. The uterus was removed on d 12 of pregnancy. Cytoplasmic localization indicates that NF-κB is not activated.

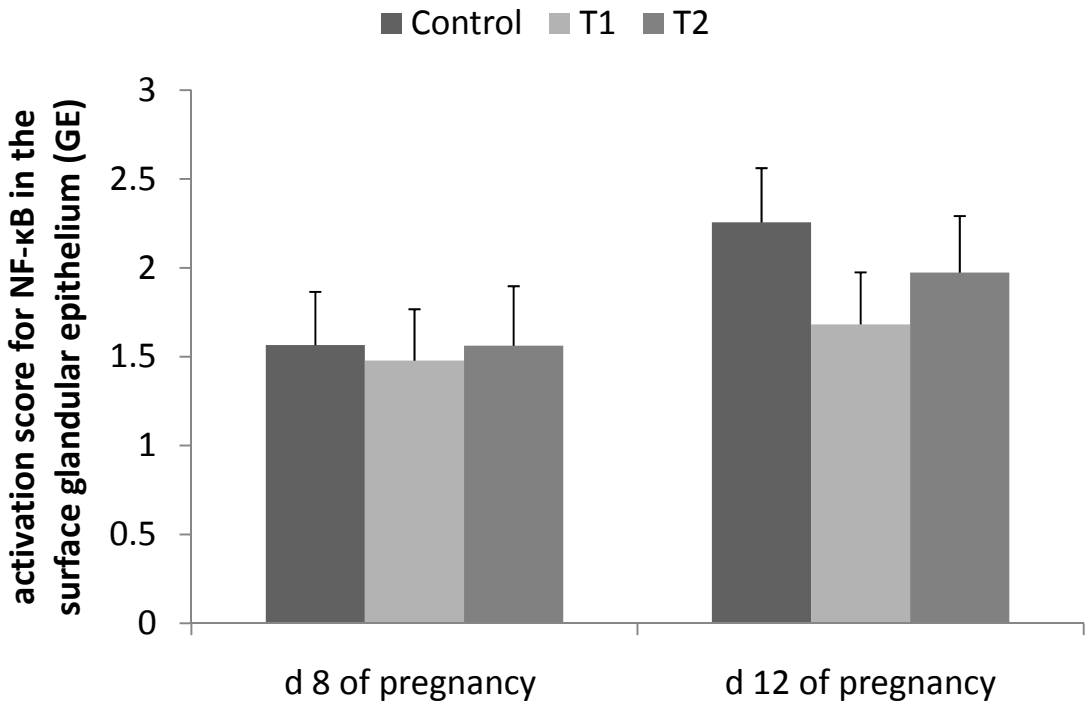


Figure 3.19 Least squares means for nuclear factor-kappa B (NF-κB) activation (nuclear localization) score for the surface glandular epithelium (GE) of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy (n = 4 to 5). Activation was scored on a 0 to 10 scale where 0 indicated no nuclear localization and 10 indicated complete nuclear localization. There was a tendency for an effect of day (P = 0.098) as NF-κB activation increased in the surface GE from d 8 to d 12 of pregnancy.

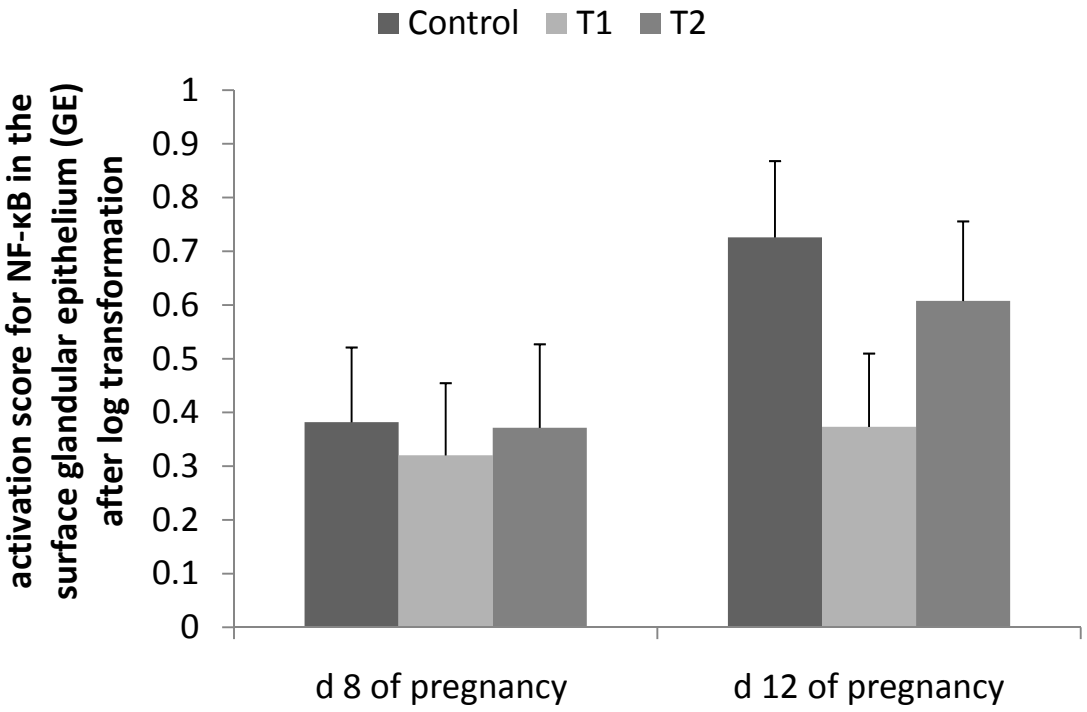


Figure 3.20 Least squares means for log transformed nuclear factor-kappa B (NF-κB) activation (nuclear localization) score for the surface glandular epithelium (GE) of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy (n = 4 to 5). Activation was scored on a 0 to 10 scale where 0 indicated no nuclear localization and 10 indicated complete nuclear localization. There was a tendency for an effect of day (P = 0.085) on log transformed NF-κB activation in the surface GE. Activation of NF-κB tended to increase from d 8 to d 12 of pregnancy.

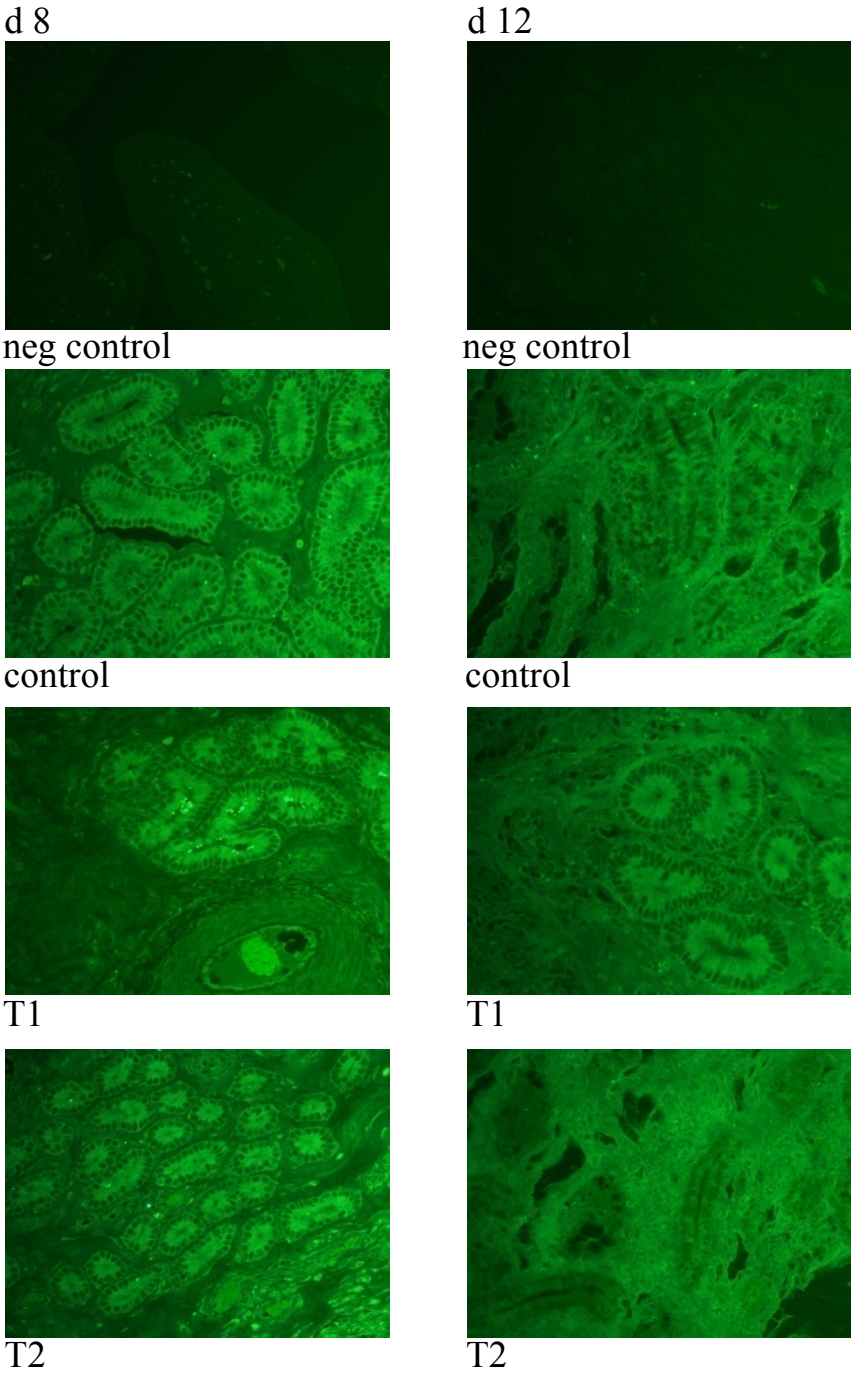


Figure 3.21 Immunofluorescence for NF-κB in the surface glandular epithelium (GE) of control gilts, gilts treated with RU486 on d 3, 4 and 5 (T1) and gilts treated with RU486 on d 6 and d 7 (T2) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy. Images were taken with a Leica light microscope with a green fluorescent protein (GFP) filter at 400 X magnification.

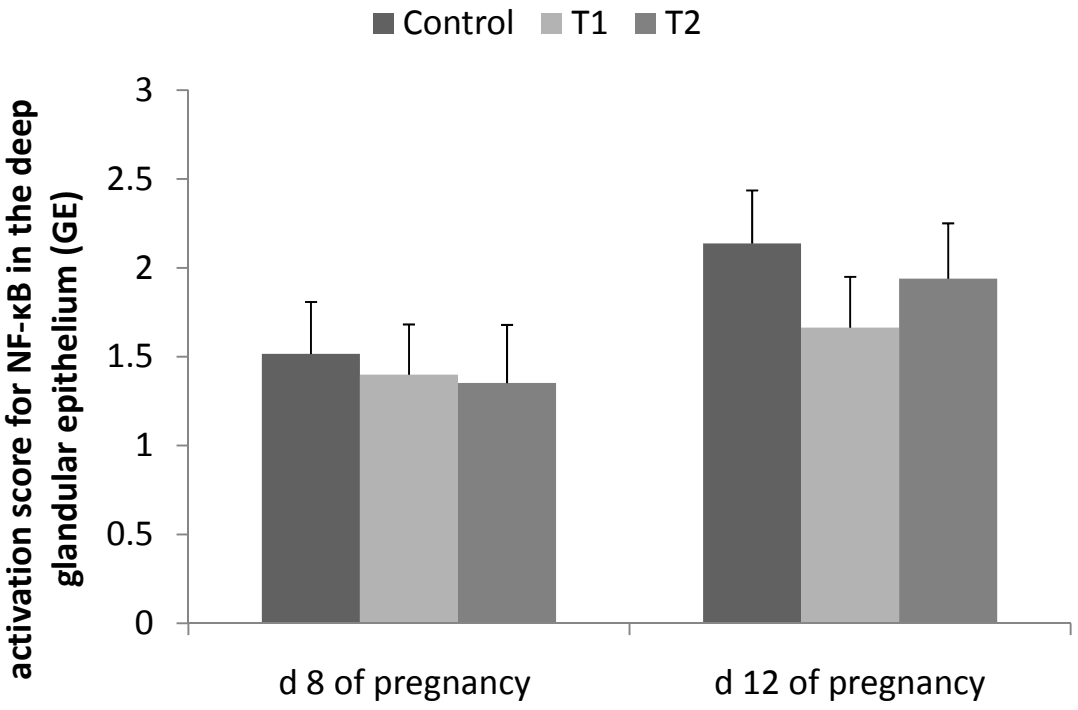


Figure 3.22 Least squares means for nuclear factor-kappa B (NF-κB) activation (nuclear localization) score for the deep glandular epithelium (GE) of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy (n = 4 to 5). Activation was scored on a 0 to 10 scale where 0 indicated no nuclear localization and 10 indicated complete nuclear localization. There was a tendency for an effect of day (P = 0.058) as activation of NF-κB increased in the deep GE from d 8 to d 12 of pregnancy.

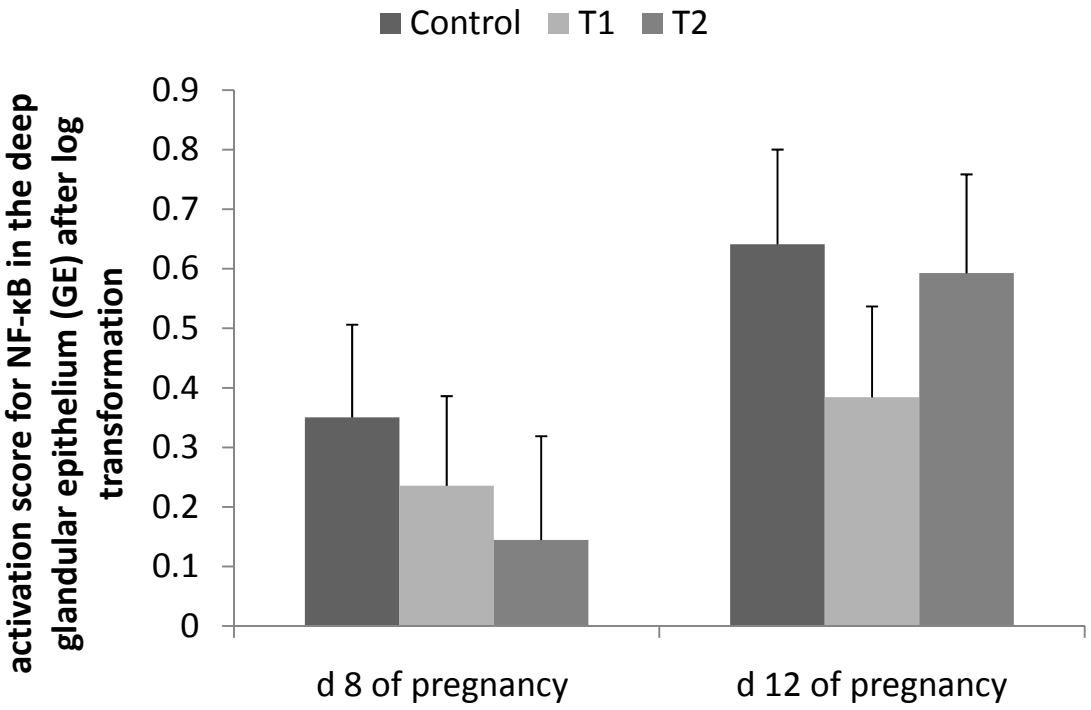


Figure 3.23 Least squares means for log transformed nuclear factor-kappa B (NF-κB) activation (nuclear localization) score for the deep glandular epithelium (GE) of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy (n = 4 to 5). Activation was scored on a 0 to 10 scale where 0 indicated no nuclear localization and 10 indicated complete nuclear localization. There was an effect of day ($P < 0.05$) on log transformed activation of NF-κB. Activation increased from d 8 to d 12 of pregnancy in the deep GE.

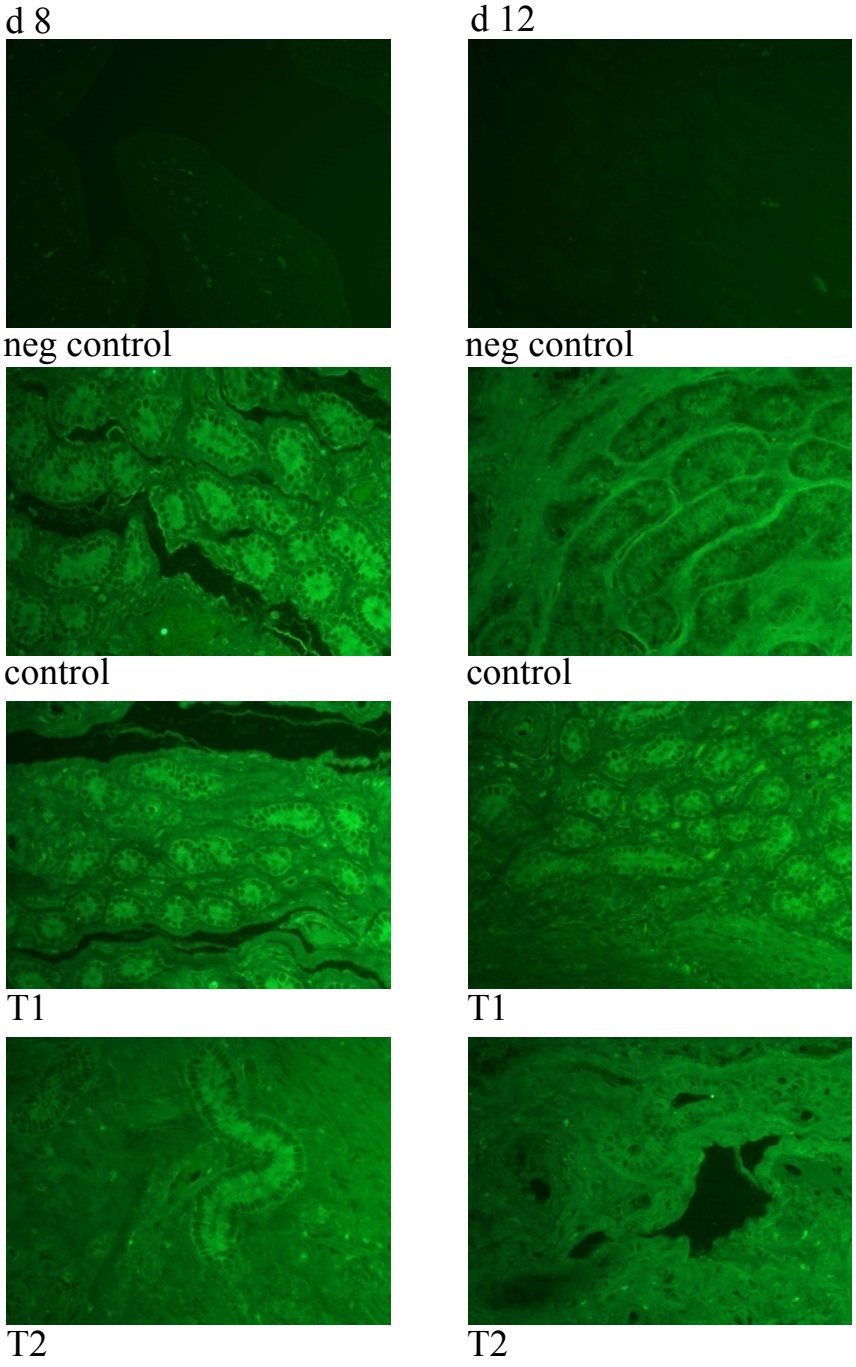


Figure 3.24 Immunofluorescence for NF-κB in the deep glandular epithelium (GE) of control gilts, gilts treated with RU486 on d 3, 4 and 5 (T1) and gilts treated with RU486 on d 6 and d 7 (T2) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy. Images were taken with a Leica light microscope with a green fluorescent protein (GFP) filter at 400 X magnification.

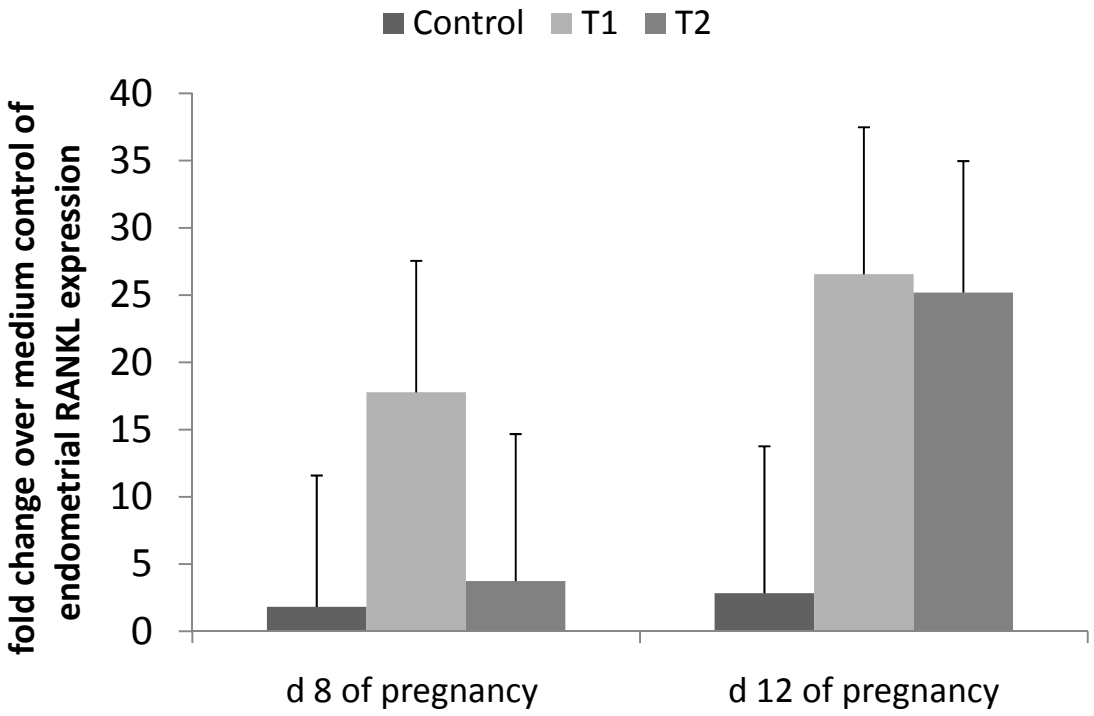


Figure 3.25 Least squares means for fold change over medium control of receptor activation for NF-κB ligand (RANKL) expression in endometrium of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. Endometrium was removed on d 8 and d 12 of pregnancy (n = 4 to 5). No significant differences in receptor activator for NF-κB ligand (RANKL) expression were reported between days or treatments.

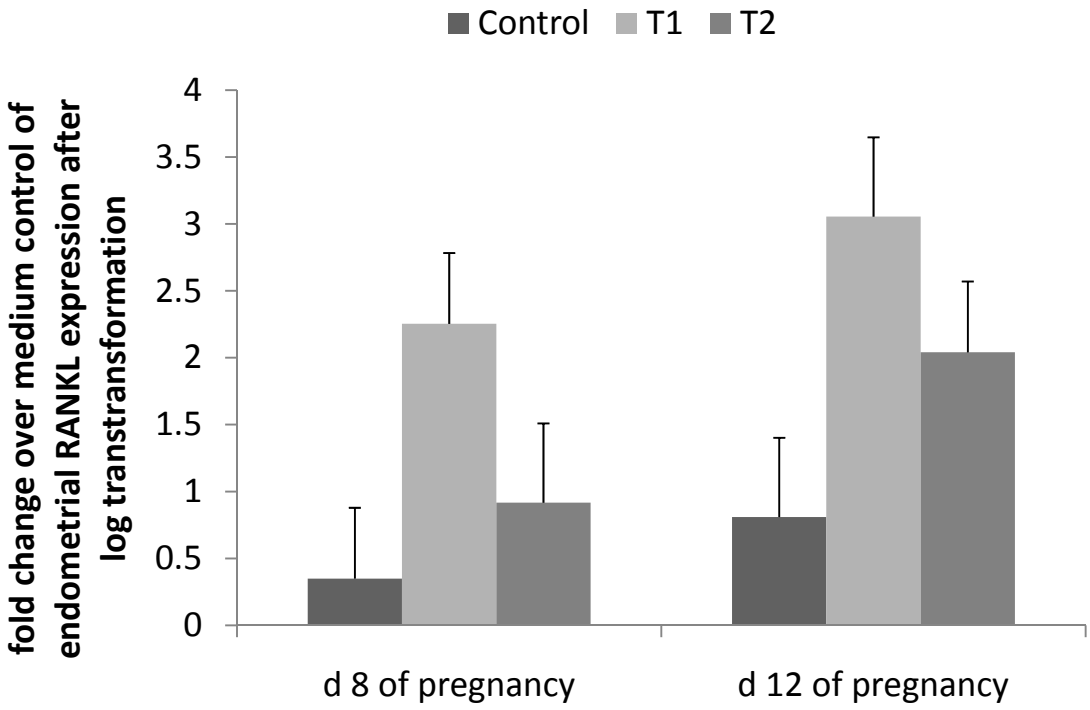


Figure 3.26 Least squares means for fold change over medium control after log transformation of receptor activator for NF-κB ligand (RANKL) expression in endometrium of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. Endometrium was removed on d 8 and d 12 of pregnancy (n = 4 to 5). Treatment affected endometrial RANKL expression ($P < 0.01$). Receptor activator for NF-κB ligand (RANKL) expression was greater in T1 and T2 gilts when compared with control gilts.

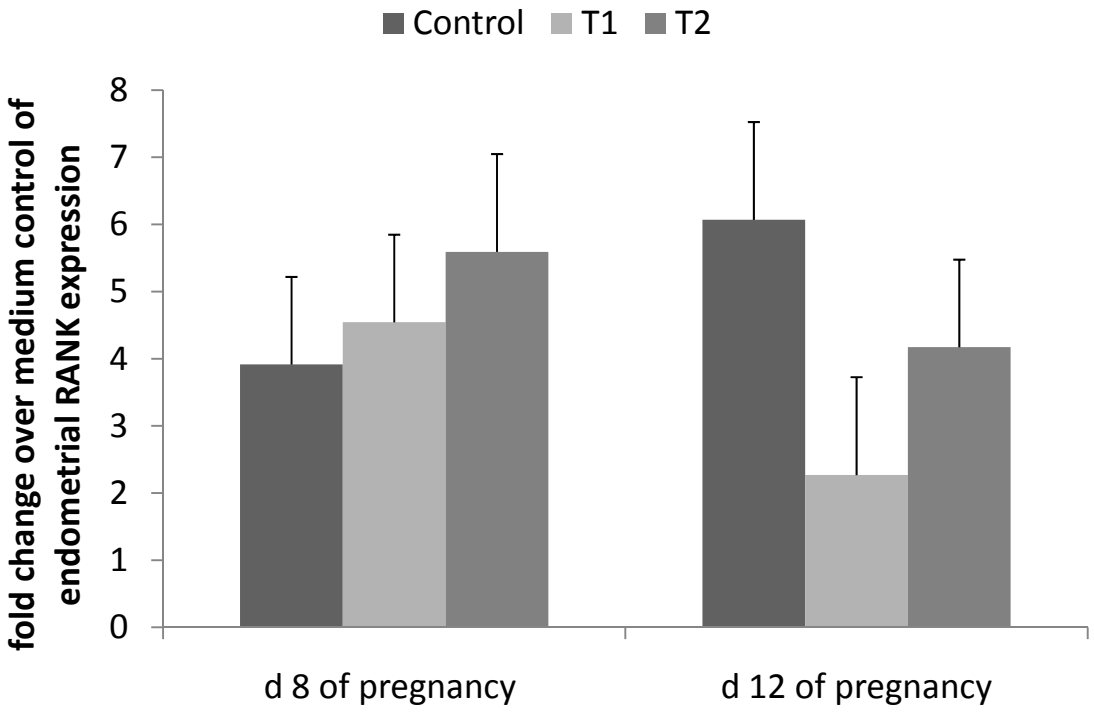


Figure 3.27 Least squares means for fold change over medium control of receptor activator for NF-κB (RANK) expression in endometrium of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. Endometrium was removed on d 8 and d 12 of pregnancy (n = 4 to 5). No significant differences were reported for endometrial RANK expression between days or treatments.

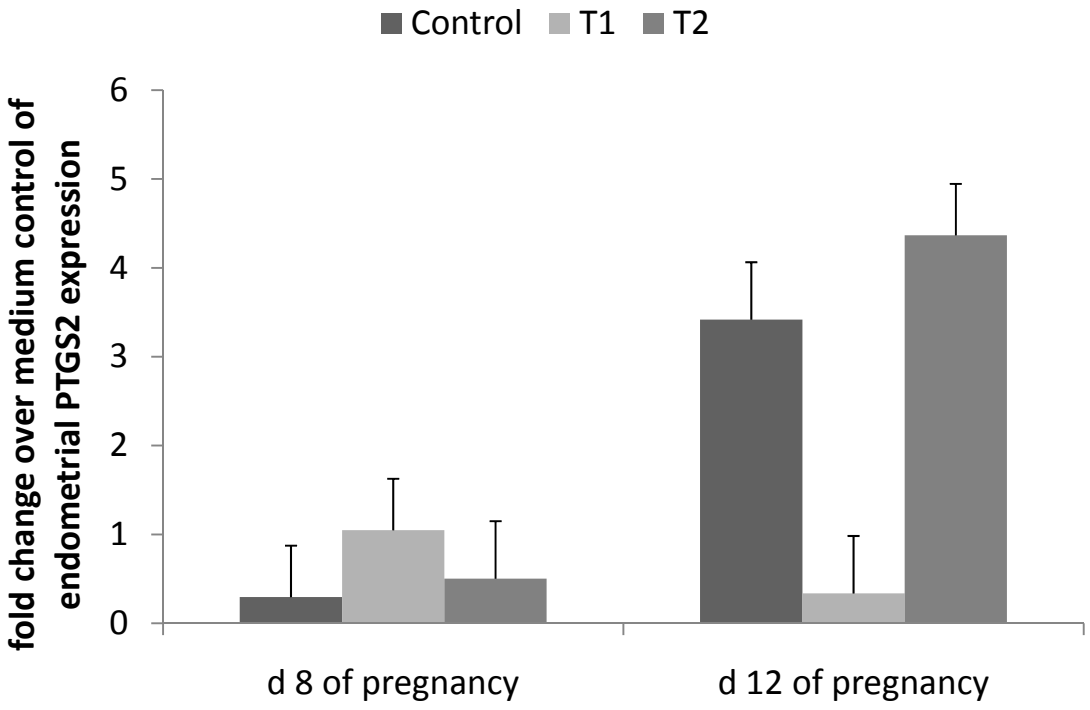


Figure 3.28 Least squares means for fold change over medium control of endometrial prostaglandin-endoperoxidase synthase 2 (PTGS2) expression in endometrium of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. Endometrium was removed on d 8 and d 12 of pregnancy (n = 4 to 5). There was a treatment by day interaction ($P < 0.01$) for PTGS2 expression. Expression of PTGS2 was greater in control and T2 gilts when compared with T1 gilts on d 12 of pregnancy.

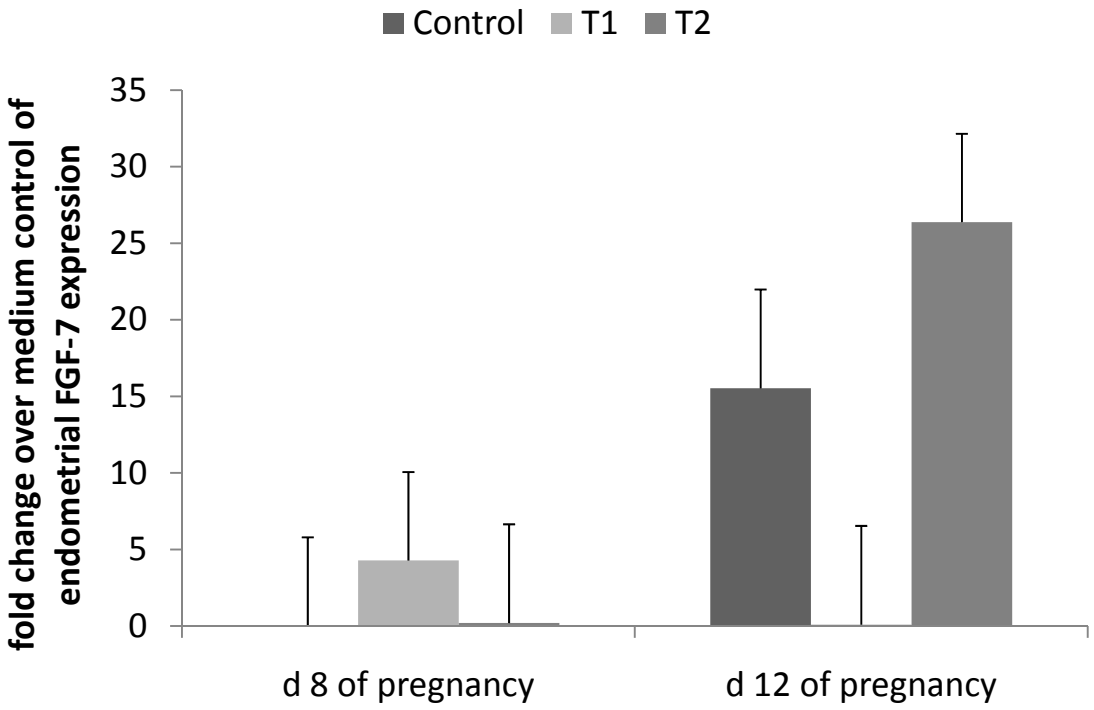


Figure 3.29 Least squares means for fold change over medium control of fibroblast growth factor 7 (FGF-7) expression in endometrium of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy (n = 4 to 5). There was an effect of day ($P < 0.05$) as expression of FGF-7 increased from d 8 to d 12 of pregnancy. There was a tendency for a treatment by day interaction ($P = 0.063$). Fibroblast growth factor 7 expression tended to be greater in the endometrium of control and T2 gilts when compared with T1 gilts on d 12 of pregnancy.

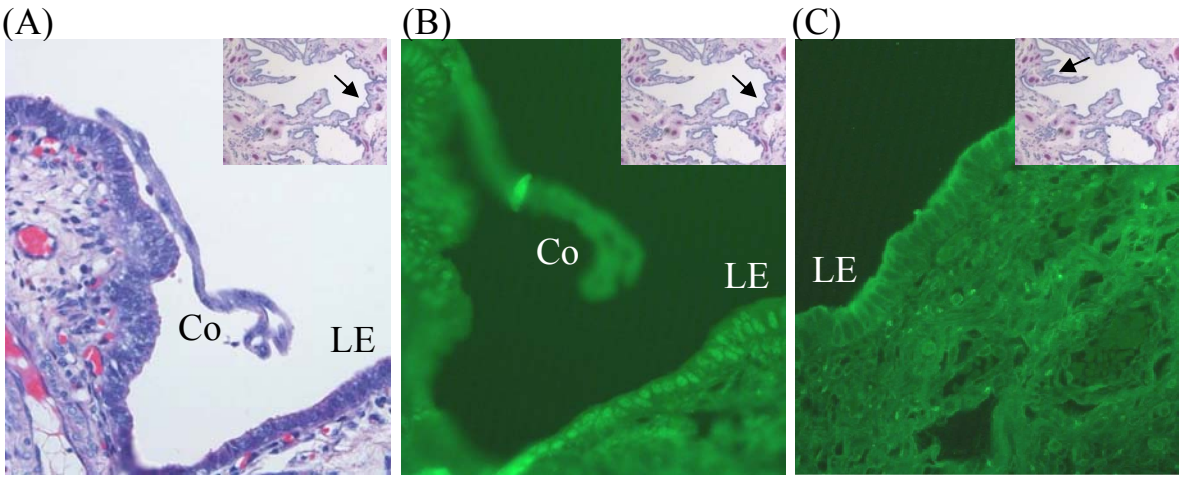


Figure 3.30 (A) Hematoxylin-stained image of a conceptus (Co) attaching to the uterine luminal epithelium (LE) of a T2 gilt on d 12 of pregnancy. (B) Immunofluorescence for NF-κB in the LE near the conceptus from image A. (C) Immunofluorescence for NF-κB in the LE, however, distal to the conceptus in image B. The absence of NF-κB nuclear localization in the LE of image C may indicate that NF-κB activation is locally-induced by the conceptus. Images were taken using a Leica light microscope at 400 X magnification plus a zoom.

CHAPTER FOUR

EFFECT OF RU486, A PROGESTERONE ANTAGONIST, ON OVARIAN FUNCTION DURING EARLY PREGNANCY IN PIGS

Abstract

The pig estrous cycle is controlled by ovarian secretion of steroid hormones progesterone (P4) and estradiol (E2). Progesterone has well documented functions in regulating uterine quiescence, uterine histotroph secretion, suppression of the immune response, steroid receptors, and controlling the hypothalamic-pituitary-gonadal axis. Synthesis of P4 from corpora lutea (CL) may have a direct autocrine effect on the ovarian follicular development and growth during the estrous cycle and early pregnancy. The P4 antagonist, RU486, can be used to explore how P4 controls ovarian follicular and uterine growth in the pig. Gilts were artificially inseminated (d 0) and assigned to one of three treatments: RU486 (400 mg/d) on d 3, 4 and 5 of pregnancy (T1; n = 9); RU486 on d 6 and d 7 of pregnancy (T2; n = 9); or non-treated control (control; n = 9). The ovaries and uterus were collected on either d 8 or d 12. Treating gilts with RU486 affected ovarian function. Gilts treated with RU486, T1 and T2, had heavier ovaries (16.1, 17.9 and 19.8

g [SEM = 1.1]; P < 0.05), greater mean follicular diameters (3.6, 5.6 and 4.9 mm [SEM = 0.5]; P < 0.05), and tended to have a greater number of CL (13.7, 16.8 and 15.0 [SEM = 1.0]; P = 0.072) when compared with control gilts (control, T1 and T2, respectively). Plasma P4 concentrations from d 8 to d 11 of pregnancy tended to be greater in RU486 treated gilts (21.9, 23.6 and 24.7 ng/mL; control, T1 and T2; P = 0.064). There was a treatment by day interaction for plasma E2 because T1 gilts had greater plasma E2 concentrations when compared with control and T2 gilts from d 2 to d 7 of pregnancy (2.4, 5.0 and 2.6 pg/mL; control, T1 and T2, respectively; P < 0.001). Gilts treated with RU486 (T1 and T2) had greater plasma E2 concentrations when compared with control gilts from d 8 to d 11 of pregnancy (1.9, 12.5, and 11.0 pg/mL; control, T1 and T2, respectively; P < 0.05). Uterine weight was reduced for T1 gilts when compared with T2 or control gilts (785.3, 607.6, 780.9 g [SEM = 48.8]; control, T1 and T2, respectively; P < 0.05). In conclusion, treating gilts with RU486 during early pregnancy stimulated ovarian follicular growth, accessory corpora lutea (ACL) formation and reduced uterine weight.

INTRODUCTION

Progesterone secretion from corpora lutea (CL) regulates multiple biological functions through progesterone receptors (PGRs) located in many cell types, including those found within the mammary gland, uterus, ovary and hypothalamus. Progesterone blocks gonadotrophin releasing hormone (GnRH) release by nuclei located in the hypothalamus and causes a decrease in follicle stimulating hormone (FSH) and lutenizing

hormone (LH) secretion from the anterior pituitary (Chabbert-Buffeta et al., 2000; Skinner et al., 1998). The release of FSH and LH from the anterior pituitary controls follicular growth and ovulation of follicles during the estrous cycle. During the luteal phase of the estrous cycle and early pregnancy in the pig, P4 mediates a suppression of FSH and LH release that effectively inhibits final maturation and growth of antral follicles (Guthrie, 2005).

Local ovarian modulators such as growth factors and steroid hormones may also regulate follicular development by regulating expression of gonadotropin receptors and/or by modulating other related processes in the pig (Cardenas and Pope, 2001; Flowers et al., 1991). Flowers et al. (1991) indicted that the initial increase in ovarian follicular E2 secretion following luteolysis, is independent of increased secretion of FSH and LH from pituitary. This would suggest that factors other than FSH and LH are involved in the initial growth of follicles or that declining P4 concentrations may sensitize the ovary to FSH or LH during the estrous cycle (Flowers et al., 1991). The results from many studies indicate that P4 may locally regulate ovarian function in the pig. During the luteal phase of the pig estrous cycle (high blood P4 concentrations), large follicles do not grow and steroidogenesis is suppressed in healthy follicles (Guthrie, 1995; Guthrie and Copper, 1996). Chan and Tan (1986) reported that P4 antagonized the stimulatory effect of gonadotrophins on estrogen production in cultured pig granulosa cells. Slomczynska et al. (2000) localized PGRs within granulosa cells of the pig ovarian follicle wall by using immunohistochemistry (IHC). This indicates that P4 may act locally to regulate ovarian function in the pig.

The collective interpretation of these studies is that P4 may regulate follicular growth by acting on both the hypothalamus and ovary in the pig. We used RU486 to investigate the role of P4 and PGR in regulating uterine-conceptus development during early pregnancy in the gilt (see Chapter Three). In this chapter, the results on ovarian, uterine and endocrine responses to RU486 administration are presented.

MATERIAL AND METHODS

Experimental Animals and Management

The project was conducted in accordance with the Guide for Care and Use of Animals and approved by the University of Missouri Institutional Animal Care and Use Committee. Twenty-seven mature, cycling Large White Landrace crossbred gilts were checked for estrus behavior twice daily in the presence of an intact boar. Gilts were artificially inseminated at the onset of estrus (d 0 of pregnancy) and 24 h later (d 1) with fresh semen collected from a single Large White Landrace crossbred boar of proven fertility.

Inseminated gilts were randomly selected to one of the following three treatment groups; 1) 400 mg (i.m.) of RU486 (D. Philibert, Roussel-UCLAF, Paris, France) on d 3, 4 and 5 of pregnancy (T1; n = 9); 2) 400 mg of RU486 on d 6 and d 7 of pregnancy (T2; n = 9); or 3) a non-treated control group (control; n = 9). In preparation of treatment, 5 g of RU486 was mixed with 50 mL of corn oil (vehicle) to make a 100 mg per mL suspension in multiple glass vials. The corn oil and RU486 were thoroughly mixed before injections into the neck were given.

Blood samples (10 mL) were collected daily from the jugular vein and centrifuged at 2500 x g for 15 min. After centrifugation, blood plasma was decanted and stored at -20°C until radioimmunoassay (RIA).

Following euthanasia with Euthasol (Virbac AH; Fort Worth, TX) the reproductive tract was removed by midventral laparotomy on either d 8 or d 12 of pregnancy. Upon arrival to the lab, the ovaries were removed and the uterine and ovarian weights were recorded (Figure 4.1, p. 117). The number of corpora lutea (CL) on each ovary was recorded and the follicular diameters (mm) were measured.

Progesterone Assay

Plasma P4 concentrations were measured by a Coat-A-Count RIA kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA). A dilution series and added mass assay was performed to verify linearity and recovery. The intraassay and interassay CV were 6.0% and 16.8%, respectively. The assay sensitivity was 0.02 ng/mL while the cross reactivity for RU486 was 0.0005%.

Estradiol Assay

Plasma E2 concentrations were measured using a RIA protocol previously described for the bovine (Kirby et al., 1997) and validated for the pig by Liu et al. (2000). The intraassay and interassay CV were 14.0% and 2.3% respectively. The assay sensitivity was 0.25 pg/mL.

Statistics

The experiment was conducted as a completely randomized design with two factors (treatment and day of pregnancy). Uterine and ovarian weight (g) as well as mean follicular diameter (mm) and number of corporal lutea (CL) on each ovary were analyzed using the general linear models (GLM) procedure of the Statistical Analysis System (SAS institute Inc. Cary, NC, USA). The dependent variables in the model statement included treatment, day and treatment by day. Preplanned contrasts were analyzed for control and RU486 treated (T1 and T2) gilts (C1) as well as between T1 and T2 gilts (C2). Least squares means and standard errors were generated using the LSmeans statement of SAS. All reported means are the adjusted least squares means ± standard error of the lsmean (SEM). Significance was declared when $P < 0.05$.

Plasma P4 and E2 concentrations were analyzed and standard errors were obtained using the PROC MIXED procedure of the Statistical Analysis System (SAS institute Inc. Cary, NC, USA) with the restricted maximum likelihood (REML) estimation method. The variables in the model statement included treatment, day and a treatment by day interaction. All reported means were obtained by PROC MIXED estimates ± standard error and significance was declared when $P < 0.05$. Plasma E2 and P4 concentration estimates between d 2 and d 7 include all gilts. Estimates between d 8 and d 11 include only those gilts that were sacrificed on d 12 (those sacrificed on d 8 of pregnancy were deceased at that time).

RESULTS

Plasma Progesterone Concentrations

There was a day effect ($P < 0.001$) and a tendency ($P = 0.064$) for a treatment by day interaction for plasma P4 concentrations between d 8 to d 11 of pregnancy. Gilts treated with RU486, T1 and T2, tended to have greater plasma P4 concentrations compared with control gilts (23.6 ± 2.1, 24.7 ± 1.9 and 21.9 ± 2.1 ng/mL for T1, T2 and control, respectively) (Figure 4.2, p. 118).

Plasma Estradiol-17β Concentrations

Plasma estradiol-17β concentrations increased in RU486 treated gilts when compared with control gilts during early pregnancy (Figure 4.3, p 119). There was a treatment by day interaction ($P < 0.001$) for plasma E2 concentrations from d 2 to d 7 of pregnancy. Plasma E2 concentrations (pg/mL) were greater in T1 gilts when compared with control and T2 gilts from d 2 to d 7 (5.0 ± 0.6, 2.6 ± 0.6 and 2.4 ± 0.6 for T1, T2 and control, respectively; $P < 0.001$). There was an effect of treatment on plasma E2 concentrations between d 8 to d 11 of pregnancy. Gilts treated with RU486, T1 and T2, had greater plasma E2 concentrations when compared with control gilts from d 8 to d 11 (12.5 ± 2.6, 11.0 ± 2.5 and 1.9 ± 2.7 pg/mL for T1, T2 and control, respectively; $P < 0.05$).

Ovarian Weight

Treatment of gilts with RU486 affected ovarian weight (Table 4.1, p. 116; Figure 4.4, p. 120). Gilts treated with RU486, T1 (17.9 ± 1.0 g) and T2 (19.8 ± 1.1 g), had heavier ovaries than control (16.1 ± 1.0 g) gilts ($P < 0.05$). There was a tendency for a treatment by day interaction ($P = 0.087$), as ovarian weight tended to be greater in T1 (19.1 ± 1.5 g) and T2 (22.5 ± 1.5 g) gilts compared with control (15.3 ± 1.3 g) gilts on d 12 of pregnancy.

Follicular Diameter

Treatment of gilts with RU486 increased ovarian follicular growth (Table 4.1, p. 116; Figure 4.5, p. 121). Gilts treated with RU486, T1 and T2, had greater mean follicular diameters when compared with control gilts (5.6 ± 0.5, 4.9 ± 0.5 and 3.6 ± 0.5 mm for T1, T2 and control, respectively; $P < 0.05$).

Number of Corpora Lutea

There was an effect of day on the number of CL in gilt ovaries ($P < 0.01$). The number of CL increased from d 8 (13.2 ± 0.8) to d 12 (17.0 ± 0.8). There was a tendency for an effect of treatment on the number of CL ($P = 0.072$), because gilts treated with RU486, T1 and T2, tended to have more CL than control gilts (16.8 ± 1.0, 15.0 ± 1.0 and 13.7 ± 1.0 for T1, T2 and control, respectively) (Table 4.1, p. 116; Figure 4.6, p. 122).

Uterine Weight

Uterine weight (Table 4.1, p. 116) was affected by day ($P < 0.01$) and treatment ($P < 0.05$). Gilts had a greater uterine weight on d 12 (799.5 ± 36.5 g) when compared with d 8 (649.6 ± 39.1 g) of pregnancy. Uterine weight for T1 gilts was less than T2 and control gilts (607.6 ± 46.0, 780.9 ± 48.8 and 785.3 ± 44.0 g for T1, T2 and control, respectively; $P < 0.05$).

DISCUSSION

Mifepristone or RU486 is typically used as an abortifacent in women and is administered in combination with a prostaglandin (PG). The RU486 binds PGRs within the reproductive tract and blocks progesterone's supportive effect on the pregnancy. The prostaglandin increases myometrial contractions and expulsion of the fetus (Fiala et al., 2006; Leonhardt and Edwards, 2002; Radestad and Bygdeman, 1992). RU486 has been exploited as a contraceptive agent by blocking ovulation at the ovarian level in the mouse and at the hypothalamic level in human and primate (Ledger et al., 1992; Loutradis et al., 1991; van Uem et al., 1989). When administered to the rat, however, RU486 reportedly increases blood E2 concentrations, initiates ovulation and shortens the estrous cycle (Tebar et al., 1998). In response to RU486, Forcelledo et al. (1994) observed greater ovarian weight, greater blood P4 concentrations, an increase in immunoreactive and bioactive blood LH concentrations and an increase in the number of oocytes collected from pre-pubertal rats. Schreiber et al. (1982) found that P4 inhibits FSH-stimulated

estrogen production and induction of LH receptors (LHR) in rat granulosa cells and decreases follicular growth.

To our knowledge, the effects of RU486 on ovarian function in the pig are unknown. Progesterone controls ovarian function by regulating GnRH release from the hypothalamus (Chabbert-Buffeta et al., 2000; Skinner et al., 1998), however, pig ovarian follicles reportedly contain PGR within granulosa cells, indicating that P4 may act locally to regulate ovarian function in the pig (Slomczynska et al., 2000). Our laboratory used RU486 to investigate the role of P4 and PGR in regulating uterine-conceptus development during early pregnancy in the gilt. The present study presents results on ovarian, uterine and endocrine responses to RU486 administration during early pregnancy.

Treatment of gilts with RU486 (T1 and T2) resulted in greater plasma P4 concentrations from d 8 to d 11 of pregnancy when compared with non-treated controls. The increased concentration of P4 in plasma of RU486 treated gilts could be the result of removing P4's negative effect on GnRH release from the hypothalamus and greater secretion of LH from the anterior pituitary. Forcelledo et al. (1994) reported similar results in that RU486 increased ovarian and circulating P4 concentrations as well as immunoreactive and bioactive blood LH concentrations in rats. The increase in the number of CL (d 8 to d 12) found in the ovaries of RU486 treated gilts, may contribute to greater plasma P4 concentrations in T1 and T2 gilts from d 8 to d 11 of pregnancy. The presence of unfertilized oocytes within the uteri of gilts treated with RU486 from d 3 to d 5 (T1) is suggested of ovulation of follicles and formation of accessory corpora lutea (ACL) by d 12 (see Chapter Three).

Gilts treated with RU486 had greater plasma E2 concentrations from d 2 to d 7 (T1) and from d 8 to d 11 of pregnancy (T1 and T2) when compared with control gilts. Plasma E2 concentrations in T1 gilts were nearly 5-fold greater than control gilts by d 10. Ovarian follicular growth in RU486 treated gilts did occur and would explain the increase in plasma E2 concentrations during early pregnancy. The removal of the P4 negative feedback on GnRH secretion in RU486 treated gilts would theoretically enable greater FSH and LH secretion by the anterior pituitary. The increase in LH and FSH would stimulate greater follicular development and E2 secretion by recruited follicles. The sharp decline in plasma E2 concentrations that occurred between d 10 and d 11 in T1 gilts may indicate that the gilts had a LH surge and ovulation of selected follicles. This response contributed to the increase in CL number and plasma P4 concentration in RU486 treated gilts.

Greater plasma E2 concentrations in response to RU486 as result of follicular growth have also been reported in the human and primate (Ledger et al., 1992; van Uem et al., 1989). Ovarian follicles in the pig rarely grow beyond 4 mm in diameter during pregnancy. High blood P4 concentrations result in decreased GnRH release by the hypothalamus. Treating gilts with RU486 (T1 and T2) may have removed the negative effect that P4 has on the hypothalamus and increased GnRH release and FSH and LH secretion, thereby, promoting follicular growth.

Progesterone may have an autocrine effect on the ovary as well, influencing follicular recruitment in the pig on a local level. In a study by Flowers et al. (1991) $PGF_{2\alpha}$ was administered to gilts on d 12 of the estrous cycle to initiate luteolysis. Following a sharp decline in blood P4 concentrations, a 52% increase in E2 was observed

in blood samples from the uteroovarian vein. The increase in E2 was not associated with a release of FSH and LH from the anterior pituitary. This study demonstrated that during the follicular phase of the pig estrous cycle, the increase in E2 production precedes the shift in FSH and LH secretion. Flowers et al. (1991) suggested that factors other than FSH and LH may be involved in the initial recruitment of follicles or that declining P4 levels may remove a negative effect that P4 has on the ovary. It is possible that P4 sensitizes the ovary to FSH and LH. Slomczynska et al. (2000) reported localization of PGRs within granulosa cells of the pig ovary indicating that P4 may act locally to regulate ovarian function in the pig. Chan and Tan (1986) reported that P4 antagonized the stimulatory effect of gonadotrophins on estrogen production in cultured pig granulosa cells. By injecting gilts with RU486 we may have removed the negative effect that P4 has on the ovary resulting in ovarian follicular growth and greater follicle E2 secretion.

RU486 reportedly has contraceptive effects by blocking ovulation at the ovarian level in the mouse (Loutradis et al., 1991) and at the hypothalamic level in the human (Ledger et al., 1992) and primate (van Uem et al., 1989). Injection of 400 mg of RU486 on d 3, 4 and 5 (T1) and d 6 and d 7 (T2) of pregnancy in the pig resulted in ovarian follicular growth and greater blood E2 concentrations (d 8 to d 11), when compared with control gilts. Gilts treated with RU486 also tended to have a greater number of CL on d 12 compared with d 8 of pregnancy. A sharp decline in plasma E2 concentrations was observed between d 10 to d 11 in T1 gilts, indicative of ovulation and luteinization of follicular cells. When injecting rats with RU486, Tebar et al. (1998) reported greater blood E2 concentrations, an E2-induced GnRH release by the hypothalamus and a LH surge resulting in a shortened estrous cycle. Increasing blood E2 concentrations in

RU486 treated gilts may have resulted in a surge release of LH from the anterior pituitary between d 8 and d 12 of pregnancy, resulting in ovulation of recruited follicles and formation of ACL. Multiple oocytes were flushed exclusively from the uterine horns of three of five T1 gilts on d 12 (see Chapter Three).

On day 8, uterine weight was less than controls in RU486 treated gilts. Uterine weight was greater on d 12 so that the weight was similar to control gilts. Concannon et al. (1988) reported reduced endometrial thickness, glandular development and epithelial cell size in response to RU486 in the dog. Morphological changes in uterine endometrium were not assessed in this study.

CONCLUSIONS

Treatment of gilts with RU486 on d 3, 4 and 5 (T1) and on d 6 and d 7 (T2) of pregnancy resulted in greater ovarian follicular diameters and greater plasma E2 concentrations during early pregnancy when compared with control gilts. The response was previously reported in the human (Ledger et al., 1992) and primate (van Uem et al., 1989). Gilts treated with RU486 tended to have a greater number of CL (d 12) and greater plasma P4 concentrations (d 8 to d 11) than control gilts. The greater follicular diameters, number of CL, plasma E2 and P4 concentrations observed in treated gilts may indicate that RU486 removed the negative effect that P4 has on the hypothalamus and/or ovary. The loss of negative feedback caused follicular growth and ovulation. Oocytes were flushed from uterine horns in three of five T1 gilts on d 12 of pregnancy. The greater ovarian weight in RU486 treated gilts is likely due to increases in ovarian activity

that occurred in response to RU486 (follicular growth and CL formation). In conclusion, RU486 can effectively remove the negative effect that P4 has on the ovary and/or hypothalamus, resulting in follicular growth and ovulation during early pregnancy in the pig.

Table 4.1 Least squares means for ovarian weight (g), follicle diameter (mm), number of corpora lutea (CL) and uterine weight (g) of gilts on d 8 and d 12 of pregnancy. Gilts were either not treated (control), treated with RU486 on d 3, 4 and 5 (T1) or treated with RU486 on d 6 and d 7 (T2) of pregnancy.

Day of Pregnancy	d 8			d 12			P <[1]				
Treatment	control	T1	T2	control	T1	T2	Trt	Day	Trt*Day	C1	C2
Ovarian weight (g)	16.9 ± 1.6	16.8 ± 1.5	17.1 ± 1.6	15.3 ± 1.3	19.1 ± 1.5	22.5 ± 1.5	NS	NS	NS	0.05	NS
Follicle diameter (mm)	4.0 ± 0.7	5.3 ± 0.7	4.5 ± 0.8	3.3 ± 0.7	6.0 ± 0.7	5.4 ± 0.7	0.05	NS	NS	0.05	NS
Number of CL	13.2 ± 1.3	14.0 ± 1.3	12.5 ± 1.5	14.2 ± 1.2	19.5 ± 1.5	17.4 ± 1.3	NS	0.01	NS	NS	NS
Uterine weight (g)	768.4 ± 65.0	500.7 ± 65.0	679.7 ± 72.7	802.1 ± 59.4	714.4 ± 65.3	882.1 ± 65.0	0.05	0.01	NS	NS	0.05

[1] Overall type 1 error rate (P value) for treatment (Trt), Day, Trt by Day (Trt*Day) and contrasts denoted as C1 [control vs RU486 treated (T1 and T2) gilts] and C2 (T1 vs T2 gilts).

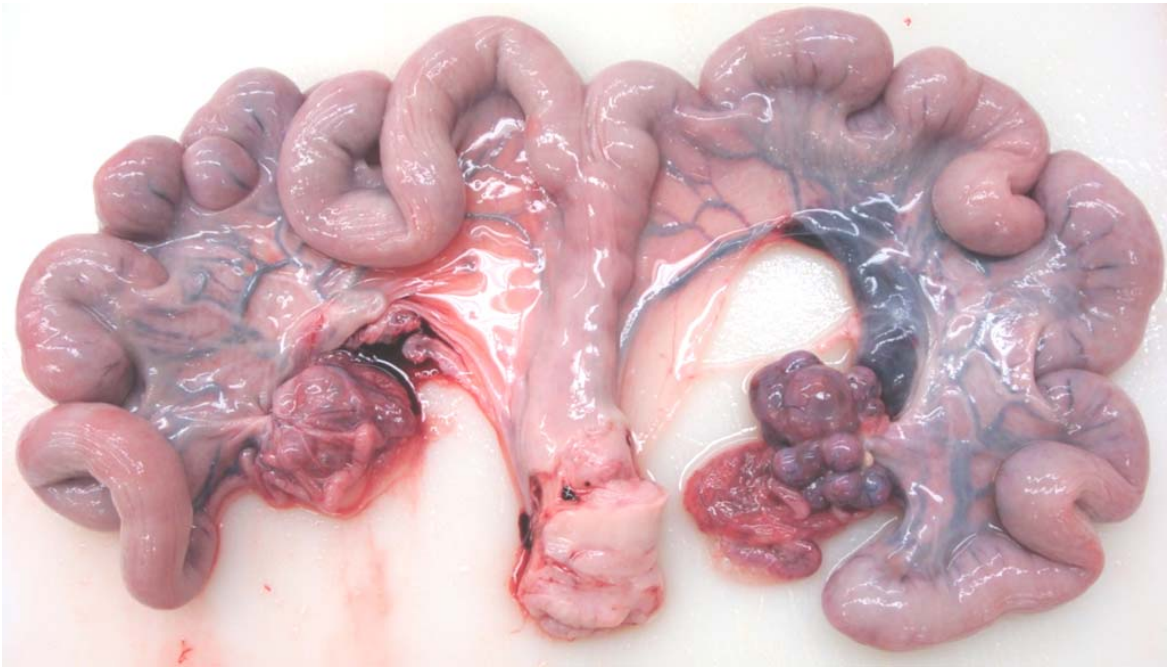


Figure 4.1 Uterus removed from a RU486 treated gilt before dissection. During dissection the ovaries were removed and the uterus was weighed. The ovaries were then weighed, the number of CL counted and ovarian follicles measured.

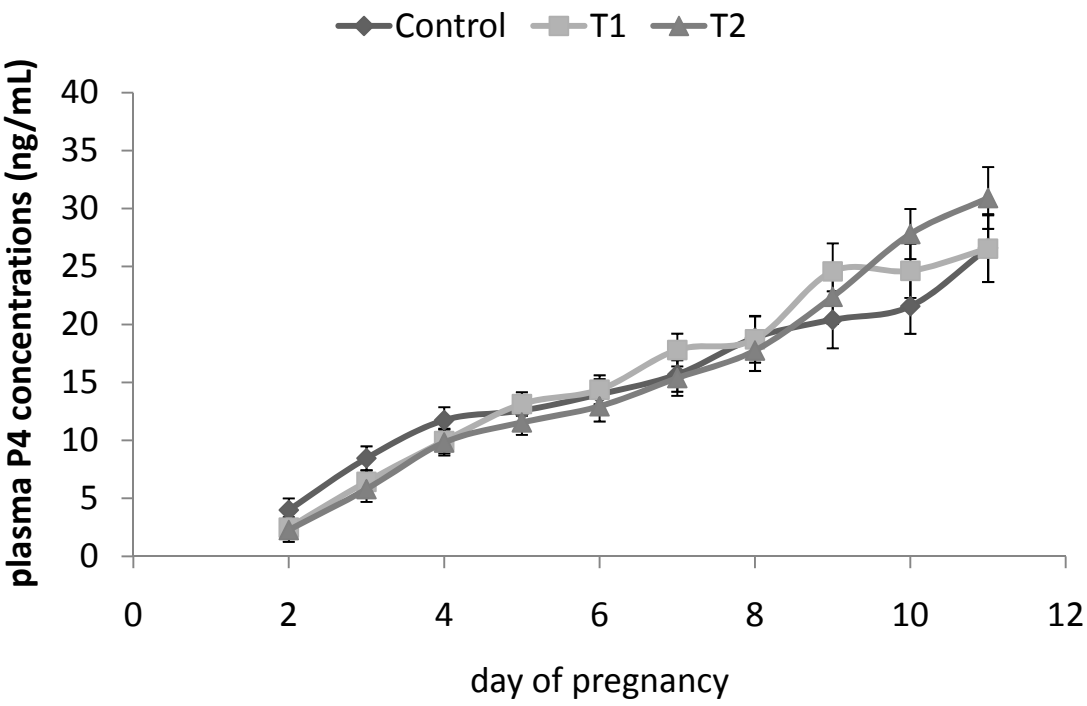


Figure 4.2 Plasma progesterone (P4) concentrations (ng/mL) from d 2 to d 11 of pregnancy. There was an effect of day ($P < 0.001$) and a tendency for a treatment by day interaction for plasma P4 concentrations. Gilts treated with RU486, T1 and T2, tended to have greater plasma P4 concentrations than control gilts from d 8 to d 11 of pregnancy ($P = 0.064$).

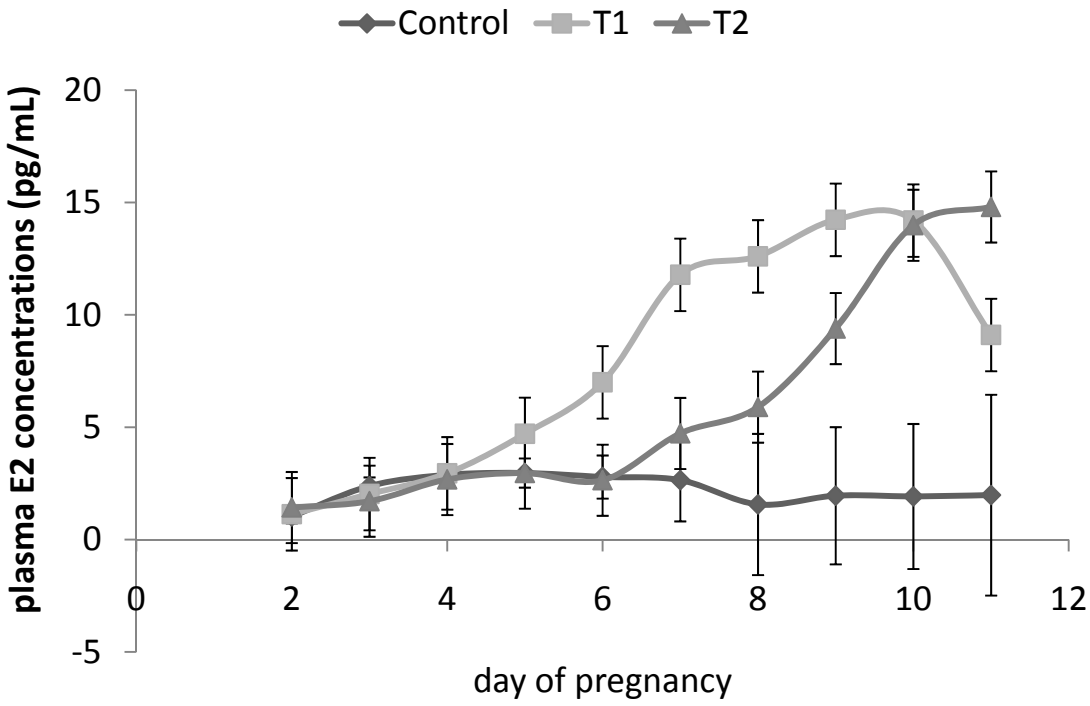


Figure 4.3 Plasma estradiol-17β (E2) concentrations (pg/mL) from d 2 to d 11 of pregnancy. There was a treatment by day interaction ($P < 0.001$). Gilts treated with RU486 on d 3, 4 and 5 (T1) had greater plasma E2 concentrations than control and T2 gilts from d 2 to d 7 of pregnancy. There was an effect of treatment ($P < 0.05$) as gilts treated with RU486, T1 and T2, had greater plasma E2 concentrations compared with control gilts from d 8 to d 11 of pregnancy.

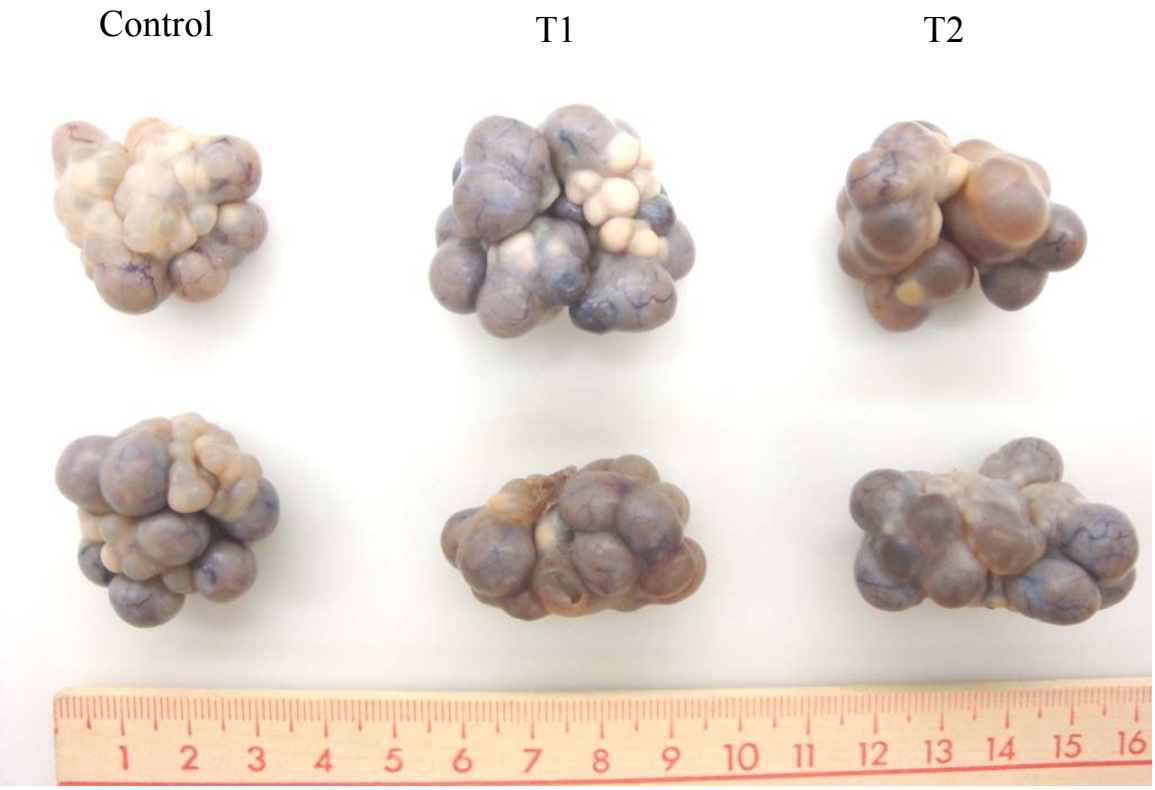


Figure 4.4 Ovaries removed from control and RU486 treated (T1 and T2) gilts on d 12 of pregnancy. Centimeters (cm) are represented by the numbers along the bottom.

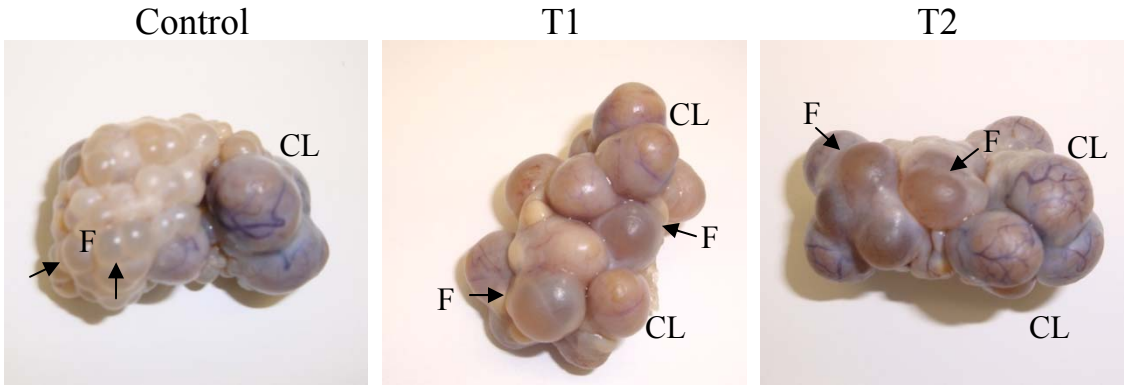


Figure 4.5 Ovaries removed from a control and RU486 treated (T1 and T2) gilts on d 12 of pregnancy. Although ovaries of control gilts contain many small follicles (F; 4 mm), large pre-ovulatory follicles (F) can be seen amongst corpora lutea (CL) on ovaries of T1 and T2 gilts.

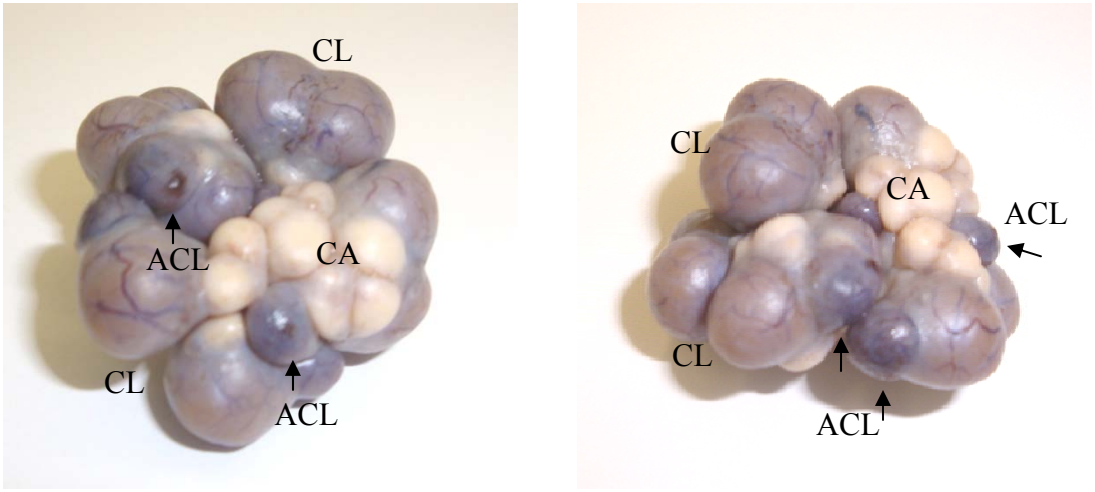


Figure 4.6 Ovaries removed from a gilt on d 12 of pregnancy after receiving 400 mg of RU486 on d 3, 4 and 5 (T1). Small accessory corpora lutea (ACL) can be seen amongst mature corpora lutea (CL) and corpora albicantia (CA) indicating that gilts may have ovulated in response to RU486. Accessory corpora lutea appear dark purple in color and contain a small brown structure or stigma, indicating a possible site of ovulation.

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTION FOR RESEARCH

CONCLUSIONS

Progesterone (P4) down-regulates expression of the progesterone receptor (PGR) receptor exclusively within the epithelium of the pig uterus before d 7 of both the estrous cycle and pregnancy. The loss of PGR protein from luminal (LE) and surface glandular epithelium (GE) begins near d 8 and is completely lost by d 12 (Geisert et al., 1994). Progesterone does not down-regulate expression of the PGR in the myometrium or stroma (Geisert et al., 1994; Ka et al., 2007; Persson et al., 1997; Sukjumlong et al., 2005). Down-regulation of the PGR is considered necessary for both pregnant and cyclic pigs. In the absence of the conceptus, the loss of PGR from the epithelium is associated with uterine $PGF_{2\alpha}$ production and luteolysis, allowing the female to recycle and ovulate for another attempt at conception (Spencer and Bazer, 2002). The loss of PGR is also associated with a decrease in mucin-1 (MUC-1) expression, a large glycoprotein that inhibits conceptus attachment to the uterus (Bowen et al., 1996; Surveyor et al., 1995). As the PGR is lost form the uterine epithelium, MUC-1 is no longer expressed, allowing the conceptus to attach to the uterus and establish pregnancy. Although down-regulation

of the PGR is considered necessary for establishment of pregnancy in the pig, the mechanism by which P4 down-regulates the PGR exclusively within the uterine epithelium but not within the myometrium or stroma is not understood. It has been suggested that P4 may increase expression of receptor activator for nuclear factor-kappa B ligand (RANKL) in the pig uterine epithelium. RANKL is a proinflammatory cytokine that can activate nuclear factor-kappa B (NF-κB), a transcription factor that is able to bind DNA and regulate gene expression. Upon activation, NF-κB is thought to enter the epithelial cell nucleus, bind the PGR gene and down-regulate expression of the PGR in the uterine epithelium.

In the study described in Chapter Three we injected gilts with RU486, a progesterone antagonist, to investigate how P4 regulates expression of the PGR within the uterine epithelium and how disruption of that mechanism might effect conceptus development. In the first treatment (T1), we injected gilts with 400 mg of RU486 (i.m.) on d 3, 4 and 5 of pregnancy. In the second treatment (T2), we injected gilts with 400 mg of RU486 on d 6 and d 7 of pregnancy. Blood was collected daily and the uterus and ovaries were then removed on both d 8 and d 12 of pregnancy. Following removal of the uterus, conceptuses were flushed from the uterine horns.

Progesterone may regulate follicular growth by acting on both the hypothalamus and/or ovary in the pig (Guthrie, 1995; Guthrie and Copper, 1996). Pig ovarian follicles do not grow to pre-ovulatory size during the luteal phase of the estrous cycle or pregnancy; a time with blood P4 concentrations are high. After removal of the ovaries, gross anatomical observations revealed increased ovarian activity in response to RU486 (Chapter Four). Gilts treated with RU486 (T1 and T2) had greater ovarian weights,

follicular diameters and number of CL when compared with controls. Gilts treated with RU486 also had greater plasma estradiol (E2) concentrations during early pregnancy. Although pregnancy was lost by d 12, numerous oocytes were flushed from three of five gilts that were treated with RU486 on d 3, 4 and 5 of pregnancy. In conclusion, RU486 effectively removed the negative effect that P4 has on the ovary and/or hypothalamus, resulting in follicular growth and ovulation during early pregnancy in the pig.

Although injecting gilts with RU486 on d 3, 4 and 5 resulted in complete loss of pregnancy, 60% of gilts treated with RU468 on d 6 and d 7 maintained pregnancy and had elongated conceptuses on d 12. This would suggest the P4 action during the first 6 days of pregnancy may be enough to support conceptus development until d 12.

Compared with non-treated control gilts, endometrial PGR mRNA expression was greater in both RU486 treatments on d 8 and d 12 indicating that P4 does down-regulate expression of the PGR during early pregnancy. When localizing PGR-B protein within the uterus, a large decrease in PGR protein occurred in the LE and surface GE of control gilts from d 8 to d 12 in parallel with reduced endometrial PGR mRNA expression. The loss of PGR protein occurred in a cell by cell manner as adjacent epithelial cells may be stained either positive or negative for PGR protein. Although PGR-B protein also decreased from d 8 to d 12 in the deep GE, the loss of protein was less prominent, indicating that PGR down-regulation decreases in strength from the LE to the deep GE (Figure 5.1, p. 132). For the most part, the myometrium and stroma maintained PGR protein during early pregnancy. Compared with control gilts, gilts treated with RU486 (T1 and T2) had reduced PGR protein on d 8, indicating that RU486 enhanced the degradation of PGR protein from the epithelium. By d 12, PGR protein in T1 gilts

increased well above controls in parallel with increase PGR mRNA expression. In gilts that were treated later in pregnancy with RU486 (d 6 and d 7; T2), PGR protein continued to decrease from d 8 to d 12 despite of increased PGR mRNA expression, indicating that RU486 continued to enhance PGR protein degradation.

Endometrial expression of RANKL was greatest in RU486 treated (T1 and T2) gilts when compared with control gilts on both d 8 and d 12 of pregnancy. Given the fact that RU486 gilts had blocked P4 action, this result suggests that P4 does not induce expression of RANKL in the pig uterine epithelium to indirectly down-regulate the PGR. Activation of NF-κB did not differ between treatments on d 8, a time when PGR mRNA expression was largely different between treatments. Activation of NF-κB, therefore, did not appear to be involved in PGR down-regulation. Nuclear factor-kappa B was highly activated on d 12 in treatments that allowed for normal conceptus development (control and T2). Release of interleukin-1β (IL-1β) by the elongating conceptus activates NF-κB in the uterine epithelium near d 11 of pregnancy in the pig (Ross et al., 2003). Although RANKL and NF-κB do not appear to mediate P4-induced PGR down-regulation, activation of NF-κB does occur in the pig uterus and may be necessary for conceptuses to establish pregnancy.

Uterine epithelial expression of prostaglandin-endoperoxidase synthase 2 (PTGS2) and fibroblast growth factor 7 (FGF-7) is necessary for establishment of pregnancy in the pig (Ashworth et al., 2006; Ka et al., 2007). Near d 12 of pregnancy, PTGS2 and FGF-7 mRNA expression is enhanced in the uterine epithelium in response to the conceptus (Ashworth et al., 2006; Ka et al., 2007). In this study, PTGS2 and FGF-7 were highly expressed in the endometrium of gilts that had elongated conceptuses.

Figures 5.2 (p. 133) and 5.3 (p. 134) represent a mechanistic model for the underlying factors that control establishment of pregnancy in the pig. Blood P4 concentrations increase during the first week after ovulation and cause down-regulation of PGR mRNA expression in the uterine epithelium before d 7. Progesterone receptor protein begins to disappear from the LE near d 8. Progesterone receptor down-regulation decreases in strength from the surface epithelium to the deep GE. By d 12, PGR protein is completely lost from the LE and the surface GE and to a less extent, in the deep GE. During the estrous cycle and pregnancy, PTGS2 and FGF-7 mRNA expression increases within the endometrium near d 5 and d 9, respectively (Ashworth et al., 2006; Ka et al., 2007). Near d 12, as the conceptus releases E2 as the maternal recognition of pregnancy signal, FGF-7 mRNA expression increases within the LE, resulting in elevated levels within the endometrium (Ka et al., 2007). Interleukin-1 beta is also released by the conceptus near d 12, activating NF-κB within the uterine epithelium before attachment (Ross et al., 2003). Activation of NF-κB is thought to enhance expression of PTGS2 mRNA within the LE, resulting in elevated levels within the endometrium on d 12 (Ashworth et al., 2006). Endometrial expression of RANKL increases near d 10 of the estrous cycle and pregnancy (Geisert and Ross, unpublished results) and could be a result of PGR down-regulation and loss of P4 action within the epithelium. If the conceptus is not present to release the maternal recognition of pregnancy signal (E2), $PGF_{2\alpha}$ is released into the uterine vasculature resulting in luteolysis (Bazer and Thatcher, 1977). As blood P4 concentrations decrease, epithelial expression of PGR presumably increases (removal of negative effect of P4 on PGR) resulting in greater epithelial PGR protein

after d 18 of the estrous cycle. Blood E2 concentrations increase near d 20 as follicles are recruited and later selected for ovulation.

After injecting gilts with RU486 on d 3, 4 and 5 of pregnancy (T1; Figure 5.2, p. 133 and Figure 5.4, p. 135), endometrial PGR-B expression was maintained on both d 8 and d 12. RU486 effectively blocked P4-induced PGR down-regulation within the uterine epithelium. Progesterone receptor protein was slightly reduced within the epithelium on d 8, perhaps because of enhanced degradation of PGR by RU486. Progesterone receptor protein later increased within the epithelium and was greater than controls on d 12. The increase in PGR protein may be a result of blocking PGR down-regulation and maintaining greater PGR mRNA expression. Endometrial expression of RANKL was also greater on d 8 and d 12 and may have resulted from blocking P4 action with RU486. Treating gilts with RU486 on d 3, 4 and 5 caused the pigs to lose pregnancy by d 12. The loss of conceptuses on d 12 resulted in reduced endometrial FGF-7 mRNA expression and NF-κB activation within the epithelium. The loss of NF-κB activation may have resulted in a decrease in endometrial PTGS2 expression on d 12. RU486 blocked P4 action on the hypothalamus and/or ovary. Numerous follicles were recruited and blood E2 concentrations began to increase after d 3 of pregnancy resulting in ovulation between d 8 and d 12.

Injecting gilts with RU486 on d 6 and d 7 of pregnancy (T2) resulted in characteristics that were similar to both control gilts and gilts treated with RU486 on d 3, 4 and 5 of pregnancy (T1) (Figure 5.2, p. 133 and Figure 5.5, p. 136). Interruption of the PGR down-regulation mechanism (RU486 on d 6 and d 7) resulted in greater PGR expression on d 8 and d 12 when compared with control gilts, however, expression was

slightly less than T1 gilts. Again, PGR protein slightly decreased within the epithelium on d 8, possibly through enhanced degradation of the PGR by RU486. Although endometrial PGR expression was greater than control gilts, PGR protein continued to decrease reaching levels similar to control gilts by d 12. This may indicate that RU486 continued to enhance PGR protein degradation within T2 gilts. Blocking P4 action resulted in greater endometrial RANKL expression on both d 8 and d 12 of pregnancy when compared with controls. The majority of T2 gilts, (60%) maintained pregnancy until day 12. Endometrial FGF-7 mRNA expression and activation of NF-κB within the uterine epithelium was similar to control gilts; increasing from d 8 to d 12. The presence of elongating conceptuses on d 12 of pregnancy may have enhanced endometrial FGF-7 mRNA expression and activation of NF-κB within the uterine epithelium. Endometrial PTGS2 mRNA expression was greater on d 12, possibly enhanced by activation of NF-κB within the epithelium. RU486 blocked P4s action on the hypothalamus and/or ovary. Numerous follicles were recruited and blood E2 concentrations began to increase after d 6 of pregnancy.

The P4 antagonist, RU486, effectively maintained PGR expression within the pig uterus, indicating that P4 does control expression of its own receptor. How P4 down-regulates the PGR exclusively within the uterine epithelium during the estrous cycle and early pregnancy is not understood. Down-regulation of the PGR occurs in a cell by cell manner within the epithelium and tends to decrease in strength from the surface to the deep glands, observations made in this study and others. These common trends of PGR down-regulation may be clues to how this mechanism occurs. An alternative hypothesis, is that PGR positive stroma cells may release a progestamedin in response to P4 that may

act on LE and GE, either down-regulating PGR and (or) allowing the epithelia to respond to P4 following PGR down-regulation (Ka et al., 2007). Indeed, P4 does cause down-regulation of its own receptor within the epithelium and the underlying stroma cells maintain PGR in the pig uterus (Geisert et al., 1994; Ka et al., 2007), phenomena also observed in this study. Although NF-κB was not activated during PGR down-regulation, NF-κB was activated within the uterine epithelium on d 12 of pregnancy and in the presence of the conceptus. Clearly, uncovering the mechanism controlling PGR down-regulation in the uterine epithelium or why NF-κB is activated by the conceptus before uterine attachment would allow a greater understanding of uterine mechanisms responsible for establishment of pregnancy and increase reproductive efficiency in the pig.

FUTURE DIRECTION FOR RESEARCH

Establishment of pregnancy in the pig depends on the development of the conceptus and the uterine environment, as asynchrony between the conceptus and uterus is detrimental to conceptus survival. Some conceptuses may be developmentally behind other conceptuses (timing of ovulation and fertilization), resulting in asynchrony. Investigators, therefore, have focused on understanding factors that control uterine receptivity, such as progesterone (P4)-induced progesterone receptor (PGR) down-regulation. Down-regulation of the PGR in the uterine epithelium is associated with the loss of a large glycoprotein, mucin 1 (MUC-1), from the luminal epithelial (LE) surface, a mechanism considered necessary for conceptuses to attach to the uterus and establishment of pregnancy in the pig. How P4 down-regulates the PGR in the uterine epithelium is not understood. Theoretically, if investigators are able to alter this process

and delay uterine receptivity, conceptuses will have more time to develop before attaching to the uterus, resulting in a greater number of conceptuses that establish pregnancy and ultimately, larger litter sizes. Before investigators can control the mechanisms that allow for establishment of pregnancy in the pig, such as PGR down-regulation, the factors that regulate these processes must be uncovered and better understood.

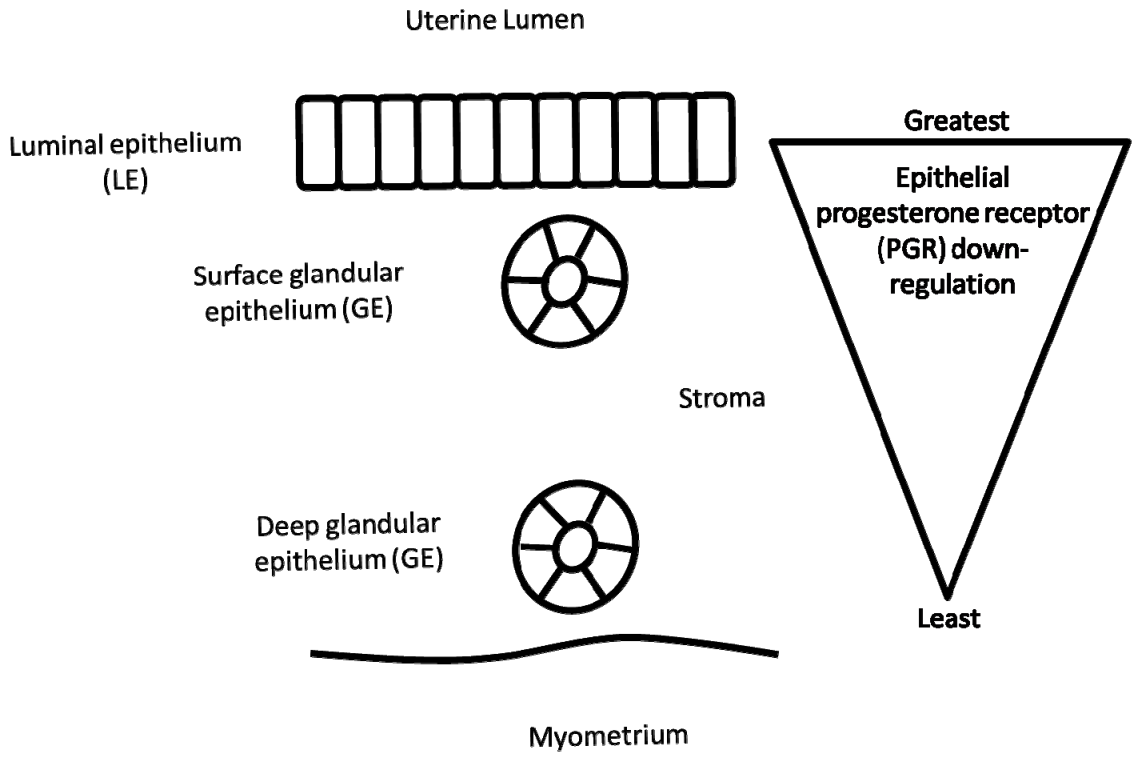


Figure 5.1 Model for progesterone receptor (PGR) down-regulation in the uterine epithelium. Progesterone receptor down-regulation decreases in strength from the luminal epithelium (LE) to the deep glandular epithelium (GE). Progesterone receptor protein begins to disappear from the LE near d 8 of the estrous cycle and pregnancy. By d 12, PGR protein is completely lost from the LE and the surface GE and to a less extent, in the deep GE. The myometrium and stroma maintain PGR during the estrous cycle and pregnancy.

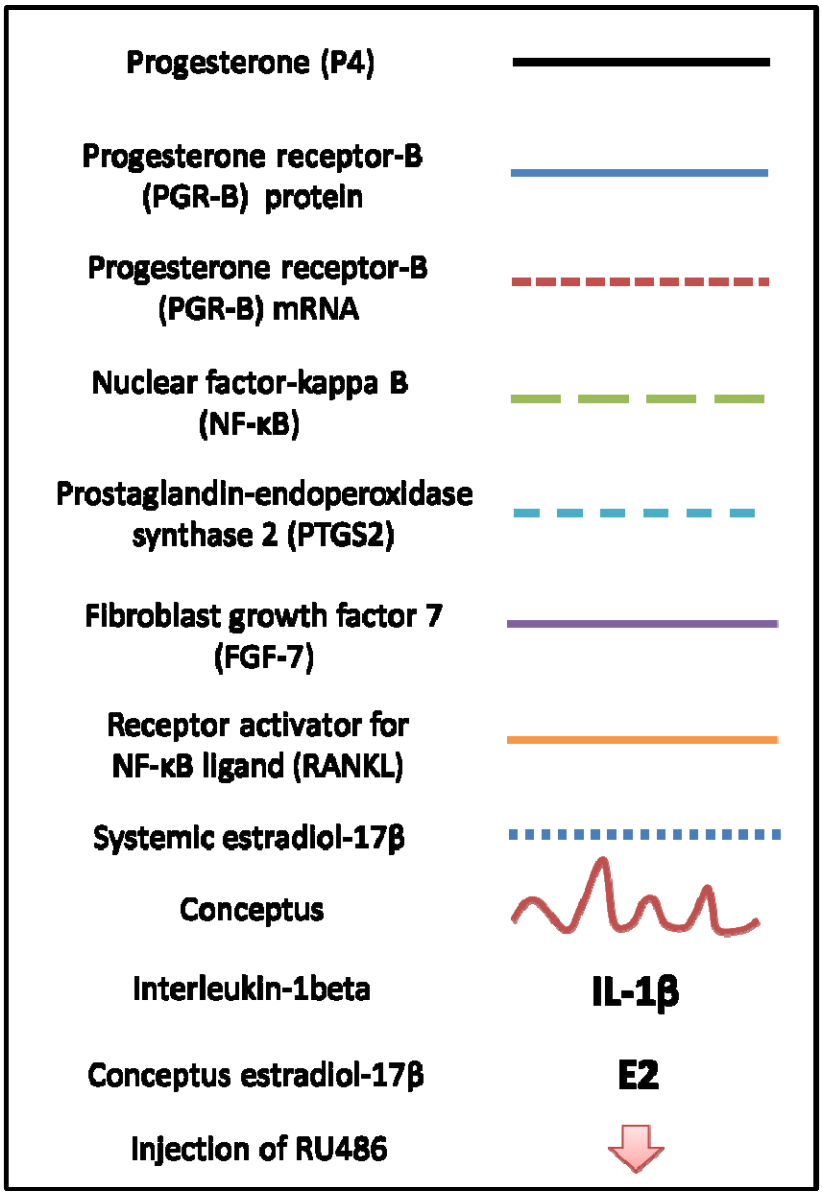


Figure 5.2 Key for mechanistic models (Figures 5.3, 5.4 and 5.5) for the underlying factors that control establishment of pregnancy in the pig with and without injections of RU486.

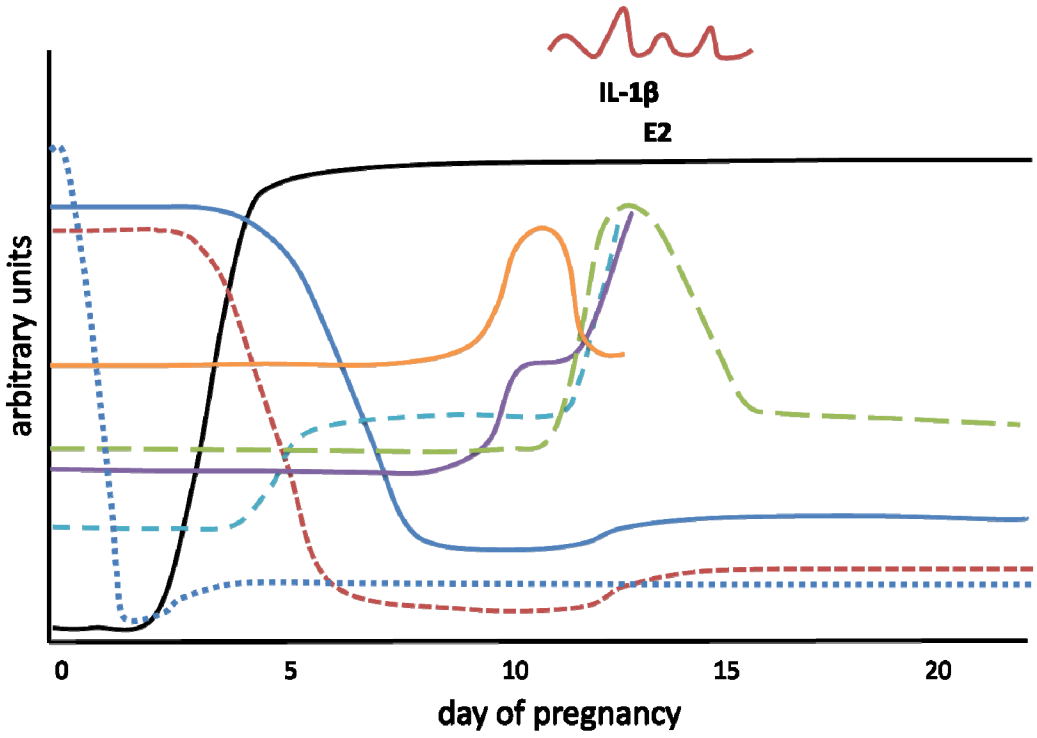


Figure 5.3 A mechanistic model for the underlying factors that control establishment of pregnancy in the pig. Blood progesterone (P4) concentrations increase during the first week after ovulation and cause down-regulation of progesterone receptor (PGR) mRNA expression in the uterine epithelium before d 7. Progesterone receptor protein begins to disappear from the luminal epithelium (LE) near d 8. By d 12, PGR protein is completely lost from the LE and the surface glandular epithelium (GE) and to a less extent, in the deep GE. During both the estrous cycle and pregnancy, prostaglandin-endoperoxidase synthase 2 (PTGS2) and fibroblast growth factor 7 (FGF-7) mRNA expression increases within the endometrium near d 5 and d 9, respectively (Ashworth et al., 2006; Ka et al., 2007). Near d 12, as the conceptus releases estradiol (E2) as the maternal recognition of pregnancy signal, FGF-7 mRNA expression increases within the LE, resulting in elevated levels within the endometrium (Ka et al., 2007). Interleukin-1 beta (IL-1β) is also released by the conceptus near d 12, activating nuclear factor-kappa B (NF-κB) within the uterine epithelium before attachment (Ross et al., 2003). Activation of NF-κB presumably enhances PTGS2 mRNA expression within the LE, resulting in elevated levels within the endometrium on d 12 (Ashworth et al., 2006). Endometrial expression of receptor activator for NF-κB ligand (RANKL) increases near d 10 of the estrous cycle and pregnancy (Geisert and Ross, unpublished results) and could be a result of PGR down-regulation and loss of P4 action within the epithelium.

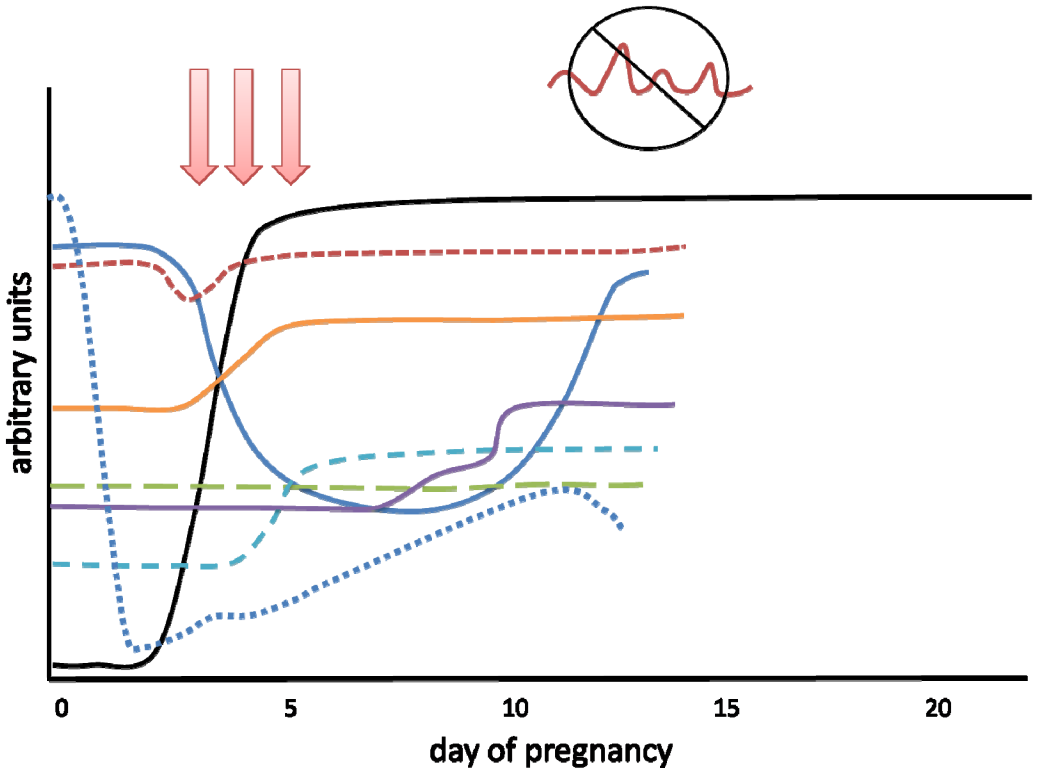


Figure 5.4 A mechanistic model for the underlying factors that control establishment of pregnancy in the pig after treatment with RU486 on d 3, 4 and 5 of pregnancy (T1). After injecting gilts with RU486 on d 3, 4 and 5 of pregnancy, endometrial PGR-B mRNA expression was maintained on both d 8 and d 12. RU486 effectively blocked P4-induced PGR down-regulation within the uterine epithelium. Progesterone receptor protein was slightly reduced within the epithelium on d 8, perhaps because of enhanced degradation of PGR by RU486. Progesterone receptor protein later increased within the epithelium and was greater than controls on d 12. The increase in PGR protein may be a result of blocking PGR down-regulation and maintaining greater PGR mRNA expression. Endometrial expression of RANKL was also greater on d 8 and d 12 and may have resulted from blocking P4 action with RU486. Treating gilts with RU486 on d 3, 4 and 5 caused the pigs to lose pregnancy by d 12. The loss of conceptuses may have resulted in reduced endometrial FGF-7 mRNA expression and NF-κB activation within the epithelium. The loss of NF-κB activation may have resulted in a decrease in endometrial PTGS2 expression on d 12. RU486 blocked P4 action on the hypothalamus and/or ovary. Numerous follicles were recruited and blood E2 concentrations began to increase after d 3 of pregnancy resulting in ovulation between d 8 and d 12.

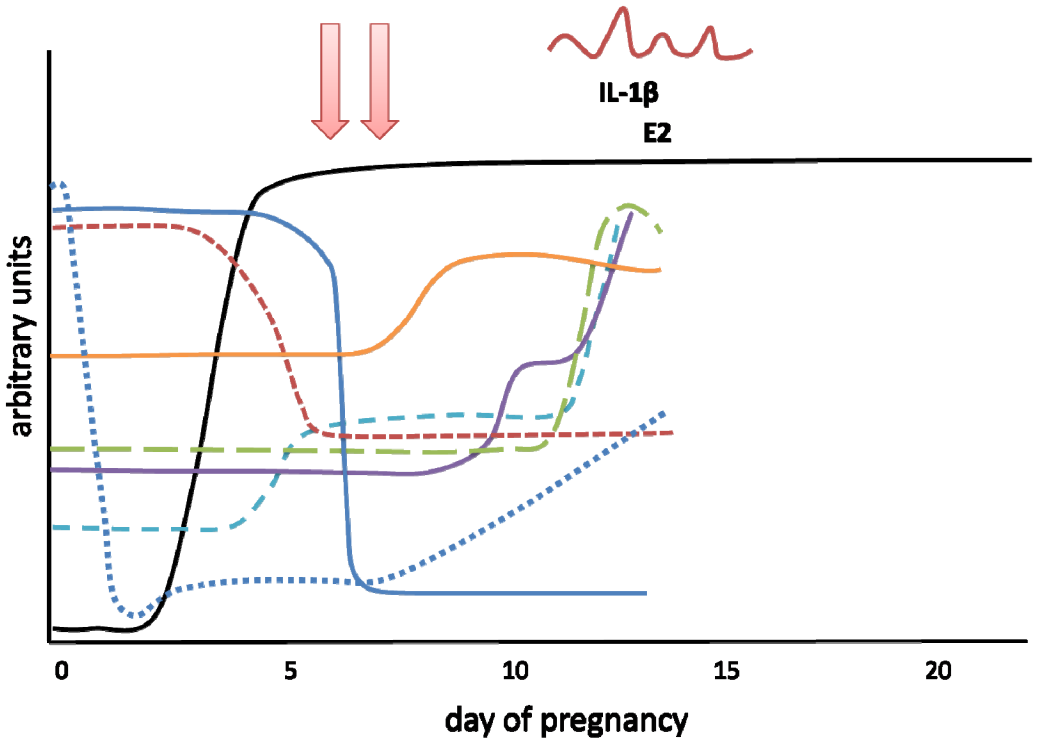


Figure 5.5 A mechanistic model for the underlying factors that control establishment of pregnancy in the pig after treatment with RU486 on d 6 and d 7 of pregnancy (T2). Injecting gilts with RU486 on d 6 and d 7 of pregnancy (T2) resulted in characteristics that were similar to both control gilts and gilts treated with RU486 on d 3, 4 and 5 of pregnancy (T1). Interruption of the PGR down-regulation mechanism (RU486 on d 6 and d 7) resulted in greater PGR expression on d 8 and d 12 when compared with control gilts, however, expression was slightly less than T1 gilts. Again, PGR protein slightly decreased within the epithelium on d 8, possibly through enhanced degradation of the PGR by RU486. Although endometrial PGR expression was greater than control gilts, PGR protein continued to decrease reaching levels similar to control gilts by d 12. This may indicate that RU486 continued to enhance PGR protein degradation within T2 gilts. Blocking P4 action resulted in greater endometrial RANKL expression on both d 8 and d 12 of pregnancy when compared with controls. The majority of T2 gilts, (60%) maintained pregnancy until day 12. Endometrial FGF-7 mRNA expression and activation of NF-κB within the uterine epithelium was similar to control gilts; increasing from d 8 to d 12. The presence of elongating conceptuses on d 12 of pregnancy may have enhanced endometrial FGF-7 mRNA expression and activation of NF-κB within the uterine epithelium. Endometrial PTGS2 mRNA expression was greater on d 12, possibly enhanced by activation of NF-κB within the epithelium. RU486 blocked P4s action on the hypothalamus and/or ovary. Numerous follicles were recruited and blood E2 concentrations began to increase after d 6 of pregnancy.

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APPENDIX A

EFFECT OF RU486, A PROGESTERONE ANTAGONIST, ON UTERINE PROGESTERONE RECEPTOR A AND B (PGR-AB) MRNA EXPRESSION AND PROTEIN DURING EARLY PREGNANCY IN PIGS

Progesterone controls expression of progesterone receptors (PGRs) in the uterus and down-regulates PGRs exclusively within endometrial epithelium but not within the myometrium or stroma before conceptus implantation. Progesterone receptor down-regulation is thought to allow conceptus attachment to the uterine surface and establishment of pregnancy. Progesterone induced PGR down-regulation in the uterine epithelium occurs before implantation in a number of mammalian species including humans (Okulicz and Scarrell, 1998), cattle (Kimmins and MacLaren, 2001), sheep (Spencer and Bazer, 1995), western spotted skunk (Mead and Eroschenko, 1995), baboons (Hild-Petito et al., 1992), rhesus monkeys (Okulicz and Scarrell, 1998), mice (Tan et al., 1999), and pigs (Geisert et al., 1994). In the pig, P4 causes down-regulation of PGR-A and PGR-B in the luminal epithelium (LE) and glandular epithelium (GE) near d 8 of the estrous cycle and pregnancy (Geisert et al., 1994). Full down-regulation of PGRs occurs by d 12 (Geisert et al., 1994; Persson et al., 1997; Sukjumlong et al., 2005).

Decreased staining of PGR protein was detected as early as d 7 in the uterine epithelium during the estrous cycle, indicating that PGR down-regulation, at the transcriptional level, occurs before d 7 (Geisert et al., 1994).

Mifepristone (RU486) was used to determine the effect of a P4 antagonist on PGR mRNA abundance and protein during early pregnancy in the gilt uterus. Theoretically, administering RU486 gilts during early pregnancy should block P4 action and maintain expression of PGR in the uterus. The RU486 was administered to gilts on d 3, 4 and 5 to determine the effect of blocking PGR down-regulation before it begins (T1) and on d 6 and d 7 to determine the effect of block PGR down-regulation following initiation of the mechanism (T2). Gilts were then sacrificed on d 8 and d 12 of pregnancy to assay for PGR mRNA and protein.

In Chapter Three, we used antibodies and RT-PCR assays that only detected PGR-B. Here, we used a second set of antibodies and RT-PCR probes to both PGR-A and PGR-B. Given the molecular structure of PGR-A, it cannot be easily distinguished from PGR-B (although the reverse is not true). Thus this experiment examines both PGR-A and PGR-B collectively to differentiate the results from PGR-B.

A primary monoclonal mouse anti-progesterone receptor antibody (PR-2C5, Invitrogen, Camarillo, CA), was diluted in 1 X PBS (1:200) and used in combination with a Vectastain elite ABC kit (6102, Vector Laboratories) for IHC (see Chapter Three).

Two sections per gilt were blindly scored for PGR-AB protein within nuclei of LE, surface GE, deep GE, myometrium and stromal cells by two independent investigators using a Leica light microscope at 400 X magnification. Because cell types differed by percentage of nuclei stained rather than intensity of staining, each cell type

was scored on a 0 to 4 scale were 0 indicated 0% of nuclei and 4 indicated 100% of nuclei stained for PGR protein.

A primer set for PGR-AB was designed based on the porcine nucleotide sequence (Table A.1, p. 162). Within the RT-PCR plate, a high, medium and low concentration (sequential 1:4 dilutions) of pooled cDNA was ran in triplicate and used as standards. A no template control (NTC) was also ran in triplicate with the plate. The equation: efficiency = $10^{(-1/slope)}$ was used to calculate the amplification efficiency of the RT-PCR assay. Using the efficiency and mean C_T value of the medium control for each RT-PCR assay, fold change differences in PCR product between the sample were calculated using the equation: fold change = efficiency $^{(\text{mean medium control } C_T - \text{mean sample } C_T)}$.

The experiment was conducted as a completely randomized design with two factors (treatment and day of pregnancy). Fold change over medium control of endometrial gene expression and IHC score were analyzed using the general linear models (GLM) procedure of the Statistical Analysis System (SAS institute Inc. Cary, NC, USA). The dependent variables in the model statement included treatment, day and their interaction.

Treatment affected expression of PGR-AB ($P < 0.001$) during early pregnancy. Endometrial expression of PGR-AB was greater in T1 (10.7 ± 1.1) and T2 (7.0 ± 1.0) gilts when compared with controls (3.5 ± 1.0) gilts (lsmeans ± SEM indicate fold change over medium control; Figure A.1, p. 163).

There was a tendency for a treatment by day interaction ($P = 0.095$) for PGR-AB protein in LE. Protein decreased from d 8 (1.9 ± 0.4) to d 12 (1.3 ± 0.5) in control gilts when compared with RU486 treated (T1 and T2) gilts. Although on d 8 T1 (0.9 ± 0.4)

and T2 (0.9 ± 0.5) gilts contained reduced PGR-AB protein compared with controls, protein levels increased for T1 (2.1 ± 0.5) and T2 (1.9 ± 0.4) gilts and surpassed that of control gilts by d 12 (0 indicates 0% of nuclei and 4 indicates 100% of nuclei containing PGR protein; Figure A.2, p. 164).

There was a tendency for treatment by day interaction ($P = 0.07$) for PGR-AB protein in the surface GE. Progesterone receptor protein decreased from d 8 (2.6 ± 0.3) to d 12 (1.8 ± 0.3) of pregnancy in control gilts while increased from d 8 (2.3 ± 0.3) to d 12 (2.9 ± 0.3) in T1 gilts. Progesterone receptor protein did not change from d 8 (2.3 ± 0.3) to d 12 (2.3 ± 0.3) in T2 gilts (Figure A.3, p. 165).

There was a treatment by day interaction for PGR-AB protein in the deep GE ($P < 0.01$). Progesterone receptor protein decreased from d 8 (3.1 ± 0.3) to d 12 (2.3 ± 0.3) in the deep GE of control gilts. Compared to controls, gilts treated with RU486, T1 (2.2 ± 0.3) and T2 (2.0 ± 0.3), had reduced PGR-AB protein on d 8, however, protein increased in T1 (3.0 ± 0.3) and T2 (2.9 ± 0.3) gilts, surpassing that of control gilts by d 12 (Figure A.4, p. 166). There was no effect of day or treatment on PGR-AB protein in the myometrium or stroma (Figure A.5, p. 167 and Figure A.6, p. 168).

Geisert et al. (1994) reported intense staining of PGR-AB protein in the LE and GE during estrus (d 0) and on d 5 of the estrous cycle. Although the concentration of PGR protein did not change in the myometrium or stroma, decreased staining was detected as early as d 7 in the epithelium. After d 7, PGR-AB protein continued to decrease and remained low in the pig LE and surface GE from d 12 to d 18 of the estrous cycle and pregnancy (Geisert et al., 1994).

The pattern of expression for PGR-AB was similar to PGR-B (see Chapter Three). Treatment of gilts with RU486 (T1 and T2) increased endometrial PGR mRNA expression on d 8 and d 12 of pregnancy when compared with control gilts. This supports the general concept that P4 causes down-regulation of PGRs in the pig uterine epithelium near d 8 and fully down-regulates PGRs by d 12 (Geisert et al., 1994). Progesterone receptor expression increased in both T1 and T2 gilts indicating that RU486 can effectively block PGR down-regulation before and during the mechanism, respectively.

Similar to PGR-B, endometrial PGR-AB mRNA expression on d 8 was greatest in T1 gilts when compared with control gilts while T2 gilts were intermediate. Earlier treatment with RU486 (T1; d 3, 4 and 5 of pregnancy), a time when PGR down-regulation begins, in combination with a greater number of RU486 injections, may account for greater PGR expression in endometrium of T1 gilts when compared with T2 gilts on d 8. Although endometrial PGR-AB expression did not change, there was a slight decrease in endometrial PGR-B expression from d 8 to d 12 of pregnancy in T1 gilts (see Chapter Three).

The intermediate expression of PGRs on d 8 in T2 gilts may indicate the return of PGR expression, in response to RU486, following an initial P4-induced down-regulation. The expression of PGRs within T2 gilts did not reach that of T1 gilts by d 12 of pregnancy. This may indicate that timing (interruption of PGR down-regulation) and/or number of RU486 injections during early pregnancy were not sufficient to increase expression of PGRs within T2 gilts to levels similar to T1 gilts.

A significant decrease in endometrial PGR-B or PGR-AB expression from d 8 to d 12 of pregnancy in control gilts did not exist; this would indicate that PGR down-regulation at the transcriptional level, had already occurred in control animals before d 8. Geisert et al. (1994) reported decreased staining of PGR-AB protein in the epithelium by d 7 of the estrous cycle. The loss of PGR expression in the epithelium before d 7 may account for decreased staining of PGR-AB protein observed by Geisert et al. 1994. Although there is a slight increase in endometrial PGR-B and PGR-AB expression from d 8 to d 12 in control gilts, this increase was not significant and levels remained low compared with T1 and T2 gilts.

Similar to PGR-B, when localizing PGR-AB protein using IHC we observed a decrease in PGR protein in the LE of control gilts from d 8 to d 12 of pregnancy (Figure A.2, p. 164). The loss of PGR protein from the LE of control gilts coincides with reduced endometrial PGR expression when compared with RU486 treated (T1 and T2) gilts. These results further support observations made by Geisert et al. (1994) as they reported decreased staining of PGR protein in the epithelium near d 8 and full down-regulation of the protein d 12 of pregnancy.

Similar to PGR-B, treatment of gilts with RU486 (T1 and T2) reduced PGR-AB protein within LE on d 8 of pregnancy when compared with control gilts (Figure A.2, p. 164). Zaytseva et al. (1993) reported a similar effect on PGR protein within the human decidual when using RU486 to terminate pregnancy. After administering 600 mg of the antagonist, Zaytseva et al. (1993) observed decreased decidual PGR protein within 12 h. Binding of RU486 to PGRs may have enhanced the degradation of the protein, possibly

by the 26S proteasome pathway (Lange et al., 1999), resulting in reduced PGR protein in the LE of treated gilts on d 8.

Unlike PGR-B, PGR-AB protein synthesis increased within the LE of both T1 and T2 gilts from d 8 to d 12 of pregnancy, coinciding with greater endometrial PGR-AB expression when compared with controls. Although PGR-B protein increased within T1 gilts from d 8 to d 12, PGR-B protein decreased in the LE of T2 gilts reaching levels similar to controls by d 12 (see Chapter Three). Not fully understood, differences in degradation of PGR-B and PGR-A protein when bound to RU486 may account for observed variation of PGR-B and PGR-AB protein within LE of T2 pigs on d 12 of pregnancy.

The loss of PGR (PGR-B and PGR-AB) protein in the surface GE and deep GE of control gilts decreased in strength when compared to the LE. Similar to the LE, PGR-AB protein decreased from d 8 to d 12 in the surface and deep GE of controls gilts, parallel with reduced endometrial PGR-AB expression. Although PGR-B protein decreased in the surface GE of control gilts from d 8 to d 12, this trend was not observed within the deep GE as levels were similar between days. These results are consistent with the initial observation made by Geisert et al. (1994) as loss of PGR protein also occurred within the surface GE and to a less extent, within the deep GE by d 12 of pregnancy.

Similar to PGR-B (see Chapter Three), treating gilts with RU486 (T1 and T2) resulted in a slight decrease in PGR-AB protein in the surface GE and deep GE on d 8 when compared with control gilts, indicating RU486 enhanced the degradation of PGR protein within surface and deep GE.

In RU486 treated (T1 and T2) gilts, PGR-B protein levels in the surface GE resembled PGR-B protein levels in the LE but to a less extent. The PGR-B protein in the surface GE remained high for T1 gilts, however, continued to decrease from d 8 to d 12 in T2 gilts reaching levels similar to controls by d 12 (see Chapter Three). The PGR-AB protein levels in the surface GE also resembled PGR-AB protein levels in the LE, but again, the response was not as strong. The PGR-AB protein in the surface GE of T1 gilts slightly increased between d 8 to d 12, however, was similar between days in T2 gilts.

In the deep GE, the strength of PGR (PGR-B and PGR-AB) protein loss or protein synthesis was less than both the LE and surface GE. Although PGR-B protein in the deep GE of RU486 treated gilts was similar between days (d 8 and d 12) and resembled that of controls on d 12, PGR-AB protein increased from d 8 to d 12 in the deep GE of both T1 and T2 gilts and surpassed that of controls by d 12.

Not fully understood, the differences observed between PGR-B and PGR-AB protein within the uterine epithelium in response to progesterone or RU486 may include but are not limited to protein degradation, protein synthesis and location within the uterus.

Occurring exclusively in the LE and GE near d 8 of the estrous cycle and pregnancy, PGR down-regulation does not occur within the stoma or myometrium of the pig uterus (Geisert et al., 1994; Sukjumlong et al., 2005). Although PGR-AB protein reportedly attenuates within the stroma, decreasing in staining intensity from proestrus (d 17 to d 20) to late diestrous (d 5 to d 16), PGR is maintained throughout the estrous cycle and pregnancy (Geisert et al., 1994; Sukjumlong et al., 2005). In this study we observed a similar effect, as PGR-AB protein slightly decreased in the stroma of control gilts from

d 8 to d 12, however, this decrease was not significant nor as extreme as the epithelium and consistent with previous reports (Geisert et al., 1994; Sukjumlong et al., 2005). When localizing PGR-B in the uterus of control gilts, we did not observe any changes in stroma PGR-B protein levels between d 8 and d 12.

When localizing PGR-AB in the pig uterus, Sukjumlong et al. (2005) reported decreased staining intensity for PGR protein as well as number of PGR positive myometrial cells from estrus to d 11 of pregnancy. In this study, the number of PGR-AB positive myometrial cells did not change between days or treatments (Figure A.6, p. 168), however, a slight decrease in myometrial PGR-B protein was observed in control gilts from d 8 to d 12 of pregnancy (see Chapter Three). This may indicate that fluctuations in PGRs do occur in the myometrium and may be a common feature in the pig uterus.

Treatment of gilts with RU486 on d 3, 4 and 5 (T1) and on d 6 and d 7 (T2) increased endometrial PGR-AB and PGR-B (see Chapter Three) mRNA expression on both d 8 and d 12 of pregnancy when compared with control gilts. This supports the general concept that P4 down regulates PGRs in the uterine epithelium near d 8 of pregnancy and fully down-regulates PGRs by d 12 (Geisert et al., 1994; Ka et al., 2007; Persson et al., 1997; Sukjumlong et al., 2005). Earlier treatment or greater number of injections of RU486 in T1 gilts may have increased PGR mRNA expression over T2 gilts.

The uterine PGR-AB and PGR-B protein levels were similar in the fact that they tended to decrease from the LE to the deep GE of control gilts between d 8 and d 12 of pregnancy, parallel with reduced endometrial PGR expression when compared with RU486 treated (T1 and T2) gilts. Treating gilts with RU486, T1 and T2, resulted in

reduced PGR-AB and PGR-B protein levels in the uterine epithelium on d 8 when compared with control gilts, indicating that RU486 enhance PGR protein degradation possibly the 26S proteosome pathway. The PGR-B and the PGR-AB protein tended to be greater in the epithelium of T1 gilts on d 12 when compared with control gilts, parallel with increased PGR expression (protein synthesis). The PGR-B and PGR-AB protein levels were dissimilar in the uterine epithelium of T2 gilts on d 12. The PGR-B protein tended to decrease in the epithelium from d 8 to d 12 in T2 gilts, reaching levels similar to control gilts by d 12. The PGR-AB protein tended to stay the same (surface GE) or increase (LE and deep GE) from d 8 to d 12 in T2 gilts.

Not fully understood, the differences observed between PGR-B and PGR-AB protein within the uterine epithelium in response to progesterone or RU486 may include but are not limited to protein degradation, protein synthesis and location within the uterus.

Although PGR down-regulation is exclusive to the uterine epithelium, slight fluctuations in PGR-AB protein in the stroma and PGR-B protein in the myometrium were observed in this study and may indicated differential regulation of PGR isoforms in these cell types. A better understanding of PGR isoforms and there modulation during PGR down-regulation as well as other uterine artifacts that control establishment of pregnancy may help to reduce reproductive inefficiency in the pig as well as other species.

Table A.1 Accession number, primer sequences (forward and reverse primers; 5' to 3') and location of primers within the GenBank sequence for PCR amplification of PGR-AB.

Accession Number	Gene	Primer	Primer sequence	Primer location
GQ903679	PGR-AB	Forward	gattcagaagccagccagag	2673 to 2693
		Reverse	gatgcttcatccccacagat	2736 to 2755

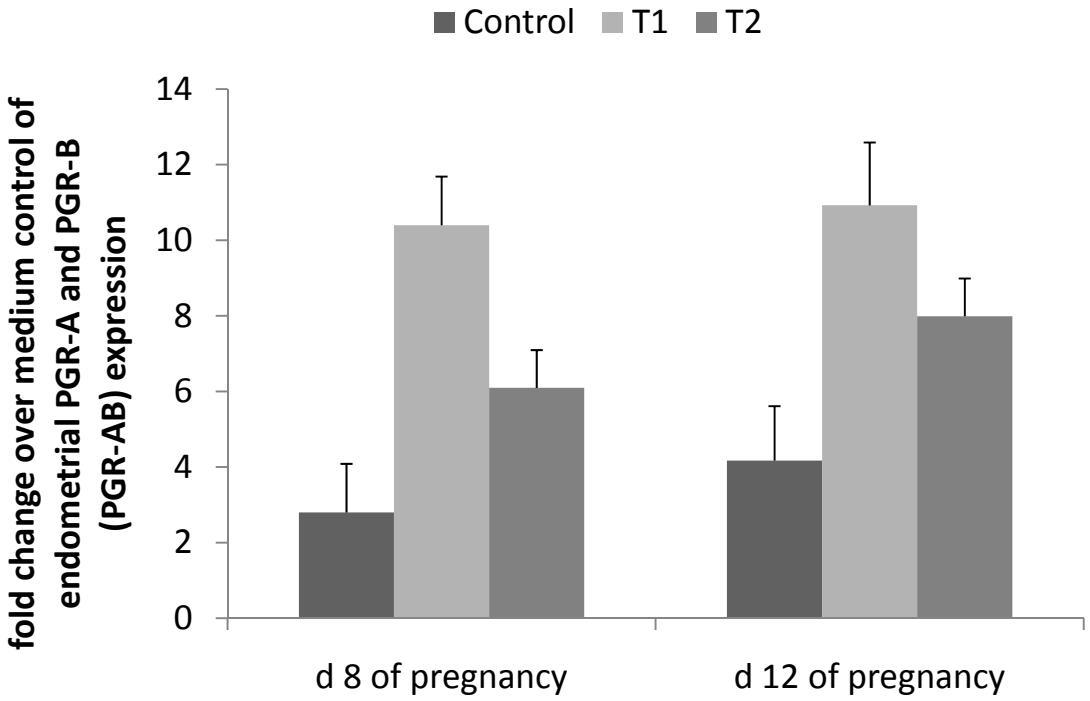

Figure A.1 Least squares means for fold change over medium control of progesterone receptor A and progesterone receptor B (PGR-AB) expression in endometrium of control gilts (control; n = 9), gilts treated with RU486 on d 3, 4 and 5 (T1; n = 9) and gilts treated with RU486 on d 6 and d 7 (T2; n = 9) of pregnancy. Endometrium was removed on d 8 and d 12 of pregnancy (n = 4 to 5). Treatment affected endometrial PGR-AB expression ($P < 0.001$). Similar to PGR-B (see Chapter Three), expression was greater in RU486 treated (T1 and T2) gilts compared with control gilts. Although PGR-B expression slightly decreased from d 8 to d 12, PGR-AB expression remained high between days in T1 gilts.

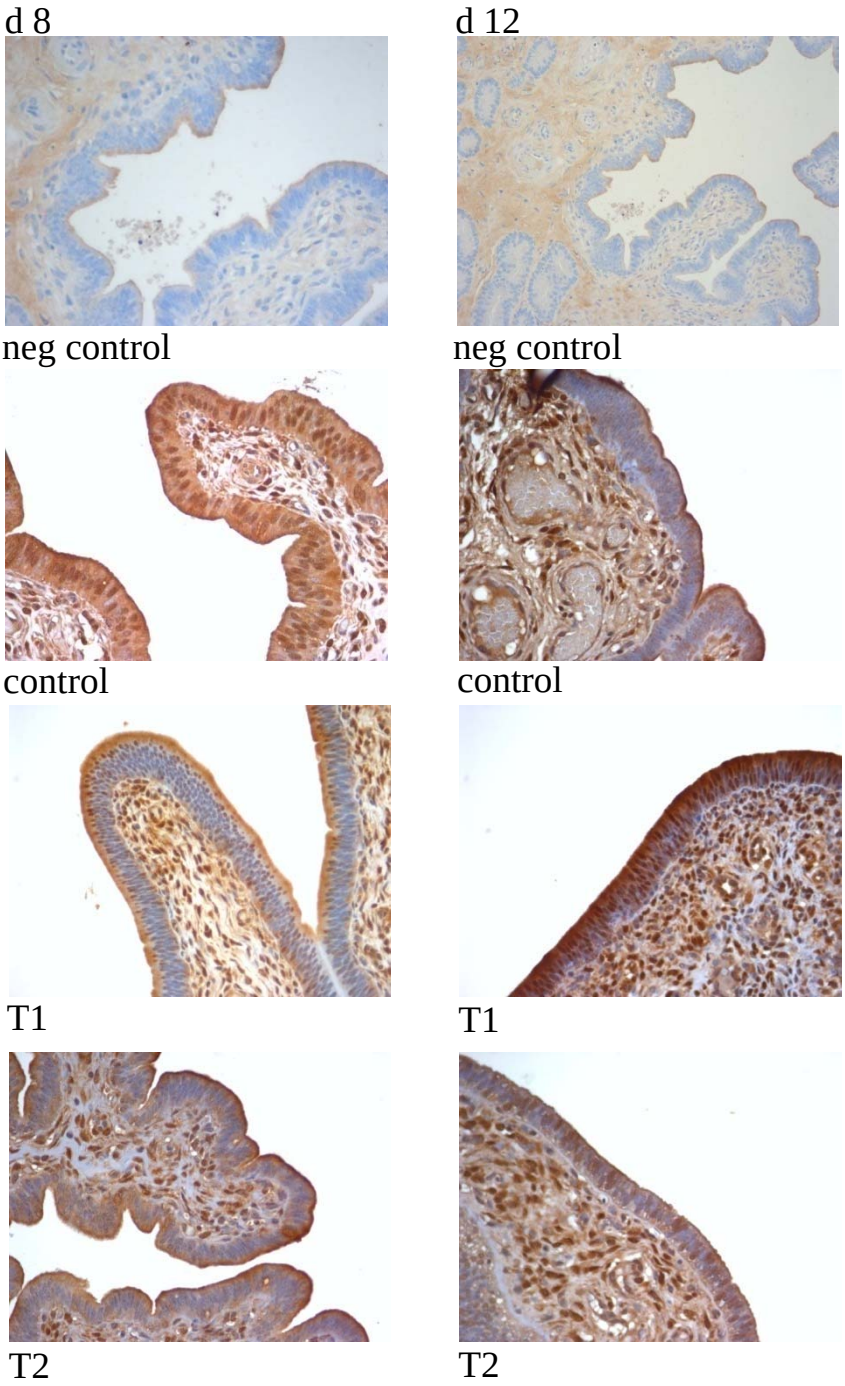

Figure A.2 Progesterone receptor A and progesterone receptor B (PGR-AB) protein in the luminal epithelial (LE) of control gilts, gilts treated with RU486 on d 3, 4 and 5 (T1) and gilts treated with RU486 on d 6 and d 7 (T2) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy. Images were taken with a Leica light microscope at 400 X magnification.

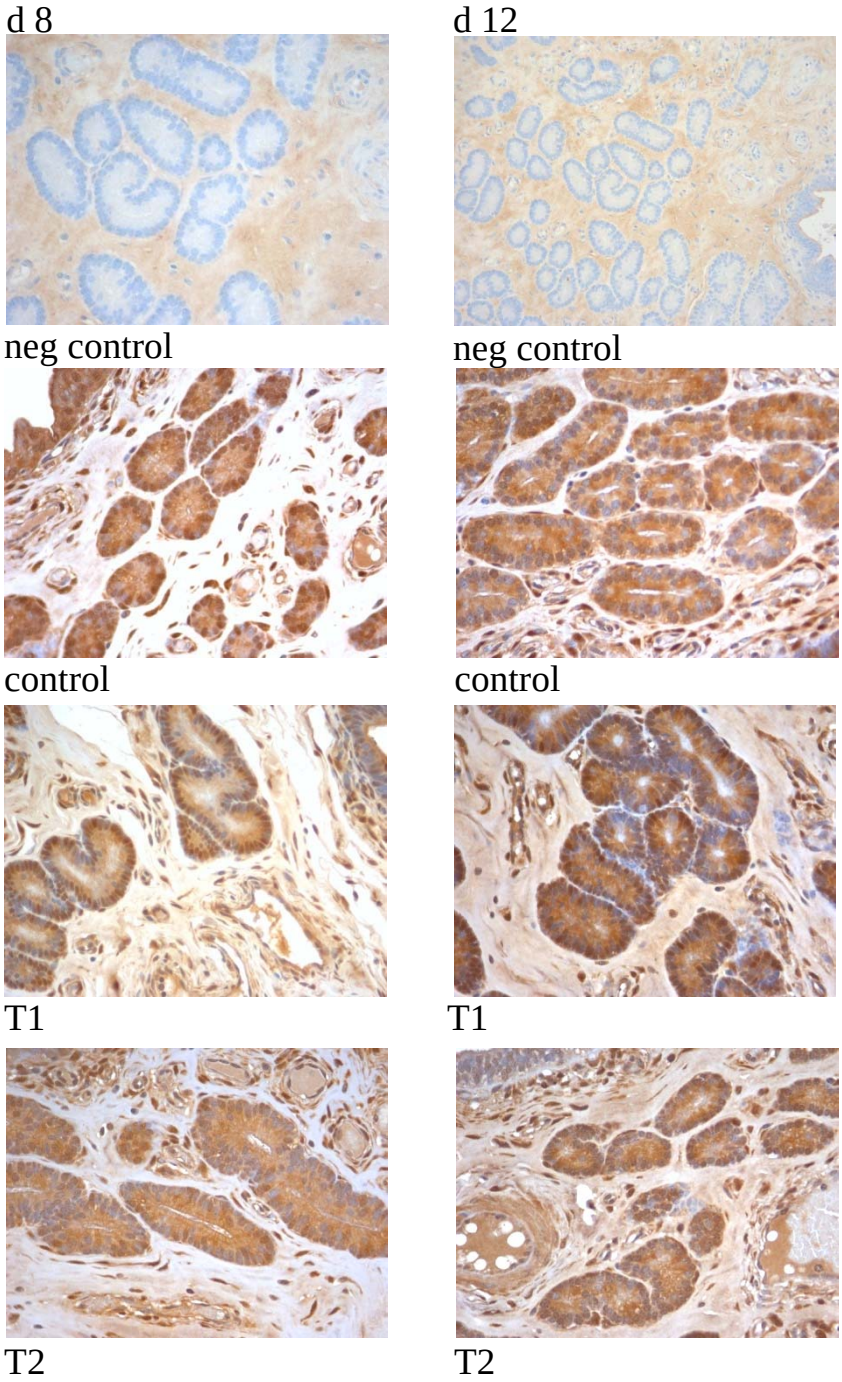

Figure A.3 Progesterone receptor A and progesterone receptor B (PGR-AB) protein in the surface glandular epithelium (GE) of control gilts, gilts treated with RU486 on d 3, 4 and 5 (T1) and gilts treated with RU486 on d 6 and d 7 (T2) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy. Images were taken with a Leica light microscope at 400 X magnification.

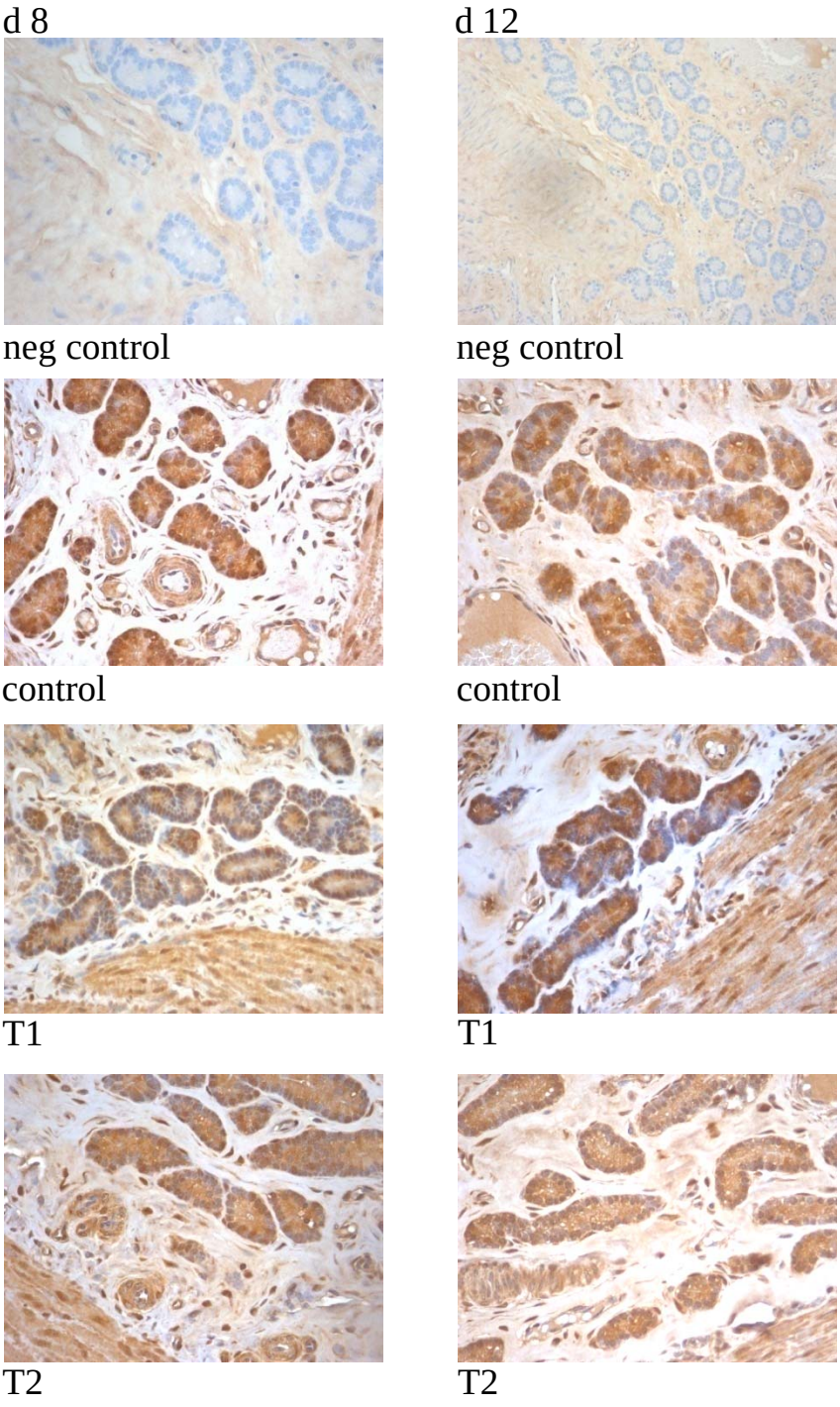

Figure A.4 Progesterone receptor A and progesterone receptor B (PGR-AB) protein in the deep glandular epithelium (GE) of control gilts, gilts treated with RU486 on d 3, 4 and 5 (T1) and gilts treated with RU486 on d 6 and d 7 (T2) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy. Images were taken with a Leica light microscope at 400 X magnification.

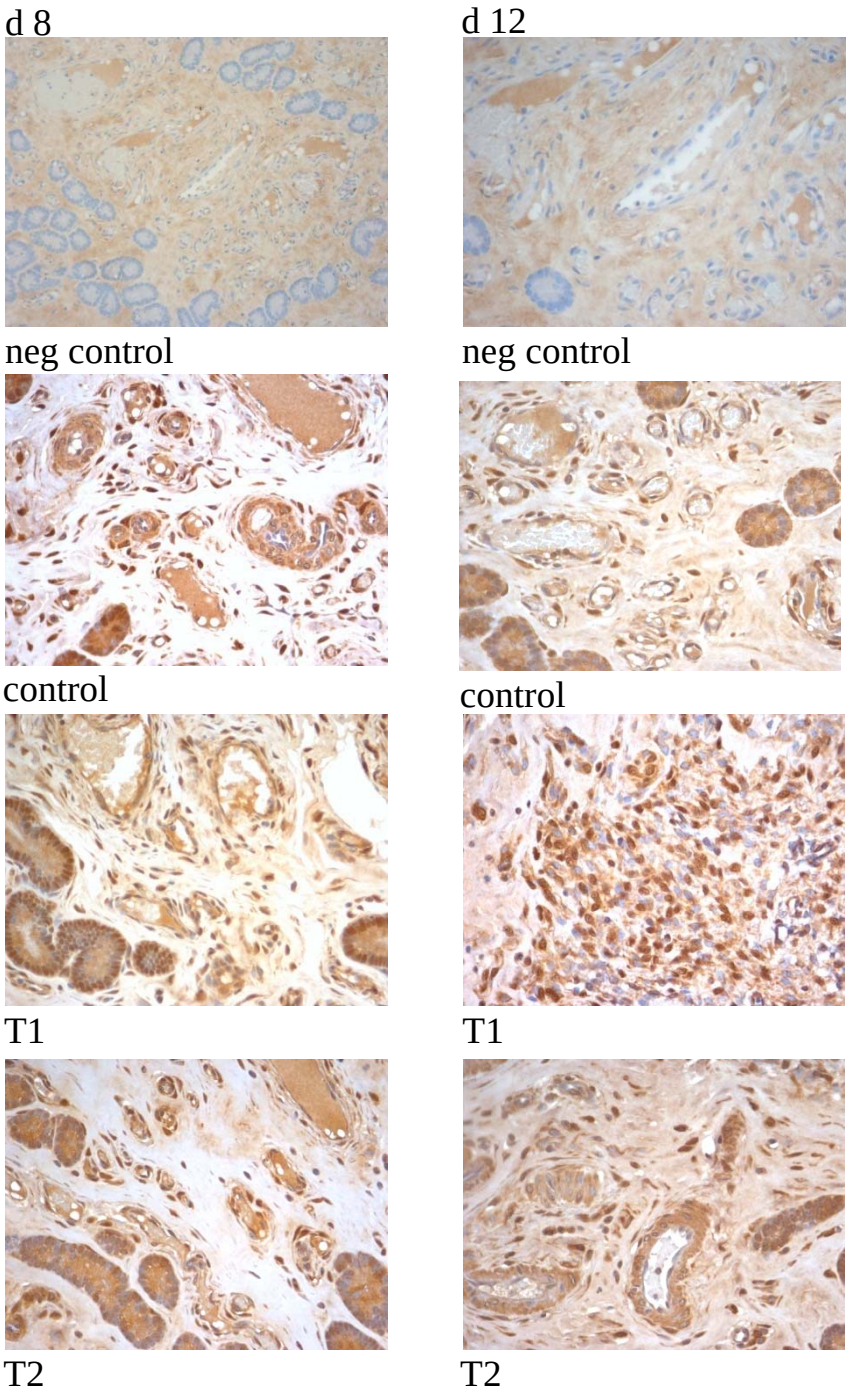

Figure A.5 Progesterone receptor A and progesterone receptor B (PGR-AB) protein in the stroma of control gilts, gilts treated with RU486 on d 3, 4 and 5 (T1) and gilts treated with RU486 on d 6 and d 7 (T2) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy. Images were taken with a Leica light microscope at 400 X magnification.

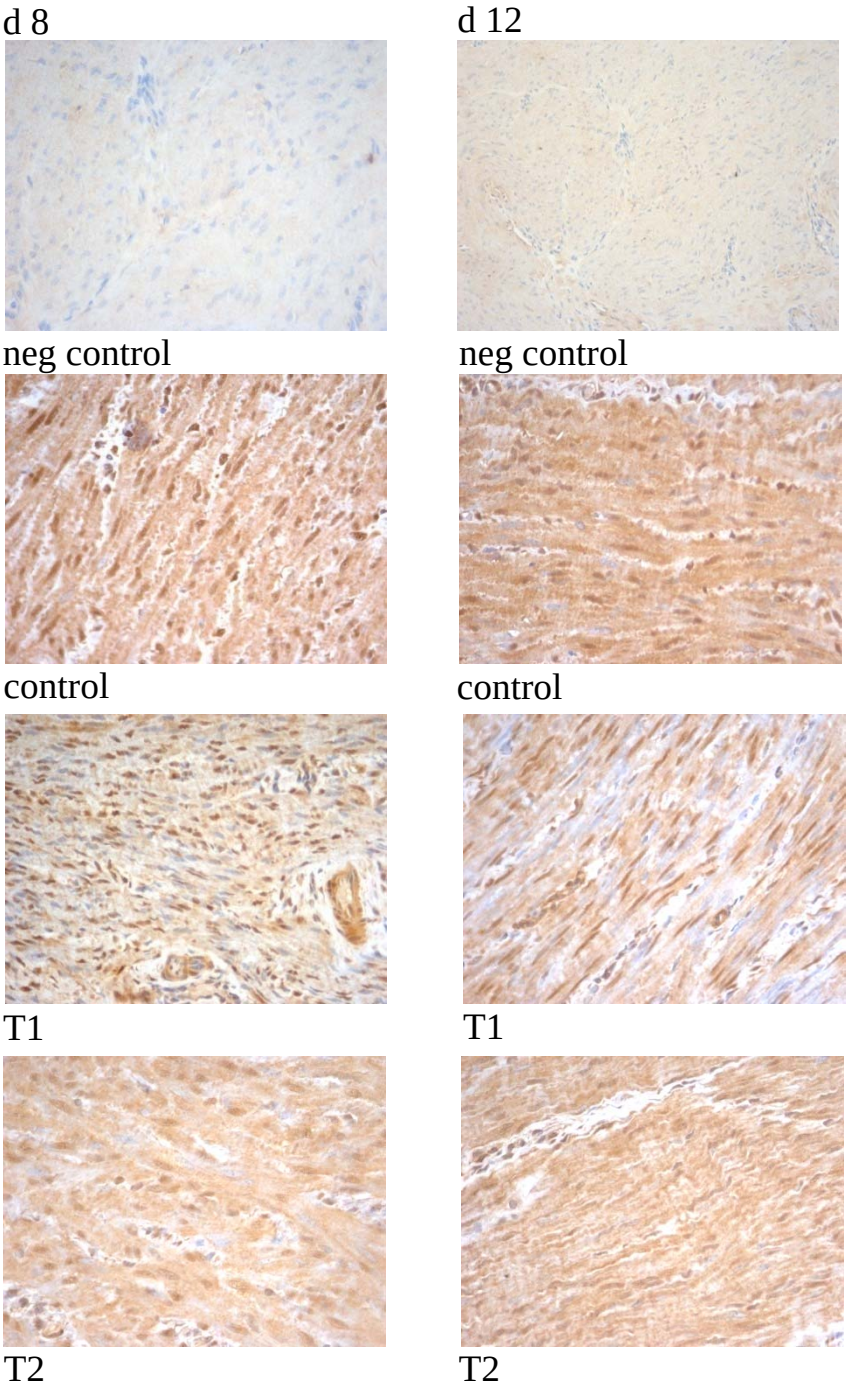

Figure A.6 Progesterone receptor A and progesterone receptor B (PGR-AB) protein in the myometrium of control gilts, gilts treated with RU486 on d 3, 4 and 5 (T1) and gilts treated with RU486 on d 6 and d 7 (T2) of pregnancy. The uterus was removed on d 8 and d 12 of pregnancy. Images were taken with a Leica light microscope at 400 X magnification.

APPENDIX B

EFFECT OF ESTRADIOL CYPIONATE AND INTERLEUKIN-1 BETA (IL-1β) ON UTERINE ACTIVATION OF NUCLEAR FACTOR-KAPPA B IN PIGS

Bazer and Thatcher (1977) first proposed that pig conceptuses secrete estradiol (E2) as the maternal recognition of pregnancy signal. Porcine conceptuses will elongate and begin releasing E2 between d 11 and d 12 of pregnancy. The E2 reroutes $PGF_{2\alpha}$ into the uterine lumen. Redirection of $PGF_{2\alpha}$ into the uterine lumen rather than the vasculature protects the corpora lutea (CL) from luteolysis, and maintains progesterone (P4) synthesis and secretion for pregnancy. Conceptus E2 also regulates secretion of uterine factors such as fibroblast growth factor 7 (FGF-7) and SPP1 (osteopontin) that are proposed to aid in establishment of pregnancy in the pig.

The cytokine interleukin-1 beta (IL-1β) is secreted in addition to E2 by porcine conceptuses during trophoblastic elongation (Tou et al., 1996; Ross et al., 2003). Porcine conceptus expression of IL-1β was first identified by Tou et al. (1996). They found that expression was greatest on d 11, d 12 and d 13 of pregnancy. Compared with day 11, gene expression of IL-1β sharply declines by d 15 and is reduced 2000-fold (Ross et al.,

2003). Conceptus release of IL-1β is not exclusive to the pig and is also secreted by the mouse and human conceptus during establishment of pregnancy (Lindhard et al., 2002; Takacs and Kauma, 1996).

Interleukin-1β is an important mediator of the inflammatory response and can activate nuclear factor –kappa B (NF-κB). Nuclear factor kappa B is a transcription factor that controls expression of many different genes involved the immune response such as prostaglandin-endoperoxide synthase 2 (PTGS2). Activation of NF-κB by IL-1β from the conceptus is thought to be necessary for establishment of pregnancy through its effects on endometrial PTGS2 expression (Ashworth et al., 2006).

The release of E2 by the conceptus may suppress the immune response initiated by conceptus secretion of IL-1β and hamper NF-κB activation. Simultaneous release of IL-1β and E2 in the uterus is thought to provide an appropriate cytokine and leukocyte environment permissive to establishment of pregnancy. Theoretically, treating gilts with E2 and later infusing IL-1β into the uterus should reduce NF-κB activation compared with IL-1β alone.

To test this hypothesis, cyclic gilts were treated with either corn oil (CO; n = 4) or received 5 mg of estradiol cypionate in corn oil (EC; n = 4) by intramuscular (i.m.) injection on d 11 of the estrous cycle. On day 12, gilts were subjected to mid-ventral laparotomy where a ligature was placed at the bottom of each uterine horn near the uterine bifurcation. One uterine horn was infused with 2 mL of saline and the second uterine horn was infused with 15 mg of recombinant porcine IL-1β (Cell Sciences Inc, Canton, MA). Uterine horns were removed at 4 and 36 h post-infusion and dissected. Uterine tissue was fixed in 10% buffered formalin phosphate (Fisher Scientific; Fair

Lawn, NJ). After 48 hours, sections were imbedded in paraffin in preparation of immunohistochemistry (IHC).

During IHC, a rabbit polyclonal antibody, able to bind the p65 subunit of NF-κB, was used as a primary antibody (sc-372, Santa Cruz Biotechnology, INC; Santa Cruz, CA) and a donkey anti-rabbit monoclonal antibody (711-225-152, Jackson ImmunoResearch Laboratories, INC, West Grove, PA) containing a Cy2 conjugate was used as a secondary antibody (see Chapter Three).

Nuclear factor-kappa B activation in uterine epithelial cells was blindly scored by three independent investigators using a Leica light microscope with a green fluorescent protein filter (GFP) at 400 X magnification. Two tissue sections per pig were scored for NF-κB activation (nuclear localization) in the luminal epithelial (LE), glandular epithelial near the lumen (surface GE) and glandular epithelial distal to the lumen (deep GE) on a 0 to 5 scale (0 indicating no nuclear localization and 5 indicating complete nuclear localization; Figure B.1, p. 174).

Immunoflourescents score was analyzed using the general linear models (GLM) procedure of the Statistical Analysis System (SAS institute Inc. Cary, NC, USA). Least squares means (lsmean) and standard errors were generated using the LSMeans statement of SAS. All reported means are the adjusted least squares means ± standard error of the lsmean (SEM) and the significance was declared when $P < 0.05$.

Treatment of gilts with EC had no effect on NF-κB activation within the LE, surface GE or deep GE cells of the pig uterus. Activation increased slightly within LE cells of IL-1β-infused horns of gilts pretreated with EC (3.4 ± 0.4) compared with corn oil (CO) treated (2.2 ± 0.4) gilts (lsmean ± SEM; 0 indicating no nuclear localization and

5 indicating complete nuclear localization). Although there was no effect of IL-1β infusion on NF-κB activation in deep GE cells there was an effect in the surface GE ($P < 0.05$) as activation increased with and without injection of EC. There was an effect of treatment ($P < 0.01$) as LE cells of uterine horns infused with IL-1β (2.8 ± 0.3) had greater NF-κB activation compared with saline infused (1.6 ± 0.3) horns (CO and EC treated gilts combined; Figure B.2, p. 175). There was a treatment by hour interaction ($P < 0.005$) as NF-κB activation was greatest in the LE within 4 h (3.5 ± 0.4) compared with 36 h (2.0 ± 0.4) of IL-lβ infusion (CO and EC treated gilts combined; Figure B.2, p. 175). Figure B.3 (p. 176) displays images of NF-κB activation within the LE of uterine horns pretreated with CO or EC and later infused with IL-1β or saline. Within LE cells of IL-1β infused horn, nuclei become indistinguishable from the cytoplasm indicating that NF-κB has translocated into the nucleus following activation.

In response to IL-1β, NF-κB was localized to both the cytoplasm and nucleus of uterine epithelial cells, indicating that IL-1β can initiate an inflammatory response in the LE (Figure B.3, p. 176) and surface GE of the pig uterus. Activation (nuclear localization) of NF-κB, temporally associated with conceptus expression of IL-1β, has also been reported in the mouse and human endometrium during implantation (Lindhard et al., 2002; Nakamura et al., 2004; Page et al., 2002; Takacs and Kauma, 1996). Injecting gilts with EC prior to infusion of IL-1β or saline did not have an effect on NF-κB activation in the pig uterus. These results would indicate that EC did not have an effect on the inflammatory response (NF-κB activation) initiated by IL-1β. Activation (nuclear localization) of NF-κB increased significantly within 4 h of IL-1β infusion but decreased to levels similar to saline infusion by 36 h. This response may represent

transient NF-κB activation after the IL-1β infusion. Nuclear factor-kappa B is transiently activated to rapidly regulate gene expression (Arsura et al., 2003; Thompson et al., 1995; Uehara et al., 1999).

The pro-inflammatory cytokine, IL-1β, can effectively activate NF-κB within the LE and surface GE of the pig uterus. Tou et al. (1996) reported that the pig conceptus will release IL-1β near d 12 of pregnancy. The release of IL-1β is temporally associated with conceptus secretion of E2 as the maternal recognition of pregnancy signal (Bazer and Thatcher, 1977). Conceptus release of E2 does not appear to suppress IL-1β stimulated activation of NF-κB. The combined effects of E2, IL-1β, and transient activation of NF-κB near d 12 of gestation may be necessary for successful establishment of pregnancy in the pig.

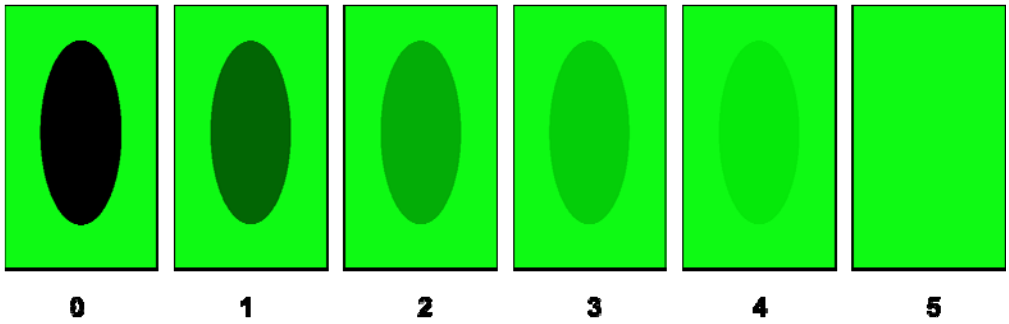

Figure B.1 Scale used by investigators to score the activation of NF-κB in gilt uterine epithelia. Activation (nuclear localization) was scored on a 0 to 5 scale where 0 indicated no nuclear localization and 5 indicated complete nuclear localization.

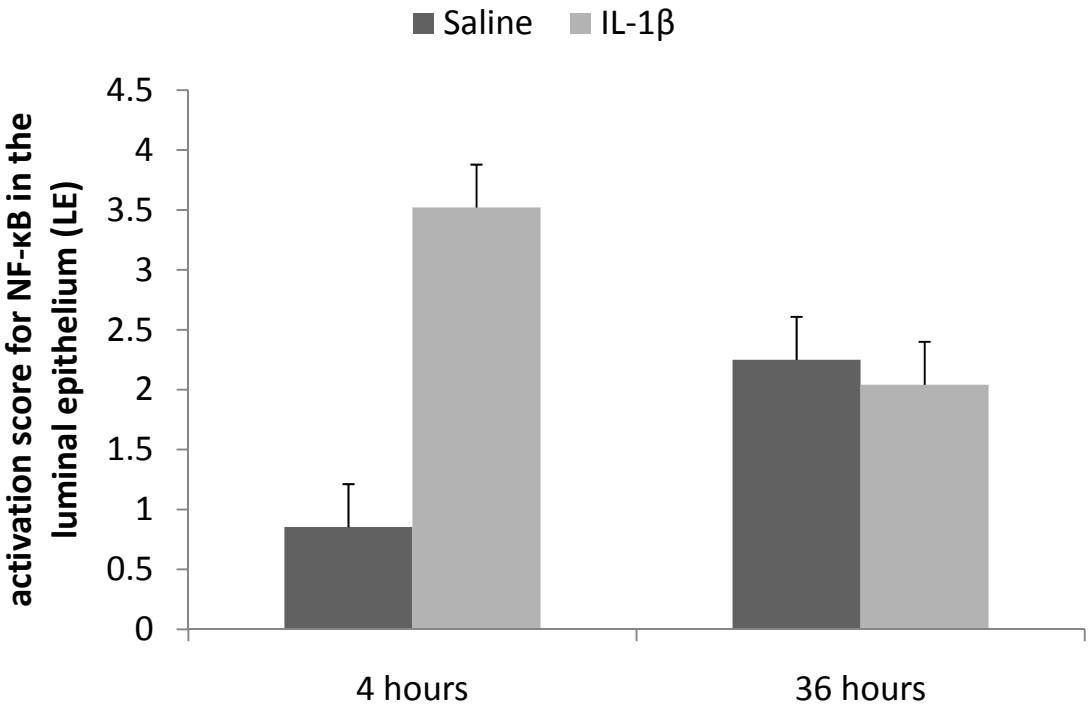

Figure B.2 Least squares means for nuclear localization (activation) score for nuclear factor-kappa B (NF-κB) in the luminal epithelium (LE). Gilts were injected with either estradiol cypionate in corn oil (EC; n = 4) or corn oil (CO; n = 4) and one uterine horn was infused with saline while the other infused with interleukin-1 beta (IL-1β). Hysterectomies were performed at 4 and 36 h following infusion. Activation (nuclear localization) was scored on a 0 to 5 scale where 0 indicated no nuclear localization and 5 indicated complete nuclear localization. There was an effect of treatment ($P < 0.01$) on NF-κB activation in the LE. Uterine horns infused with IL-1β had increased NF-κB activation compared with saline infused horns (CO and EC treated gilts combined). There was a treatment by hour interaction ($P < 0.005$) as NF-κB activation was greater within 4 h compared with 36 h of IL-lβ infusion (CO and EC treated gilts combined).

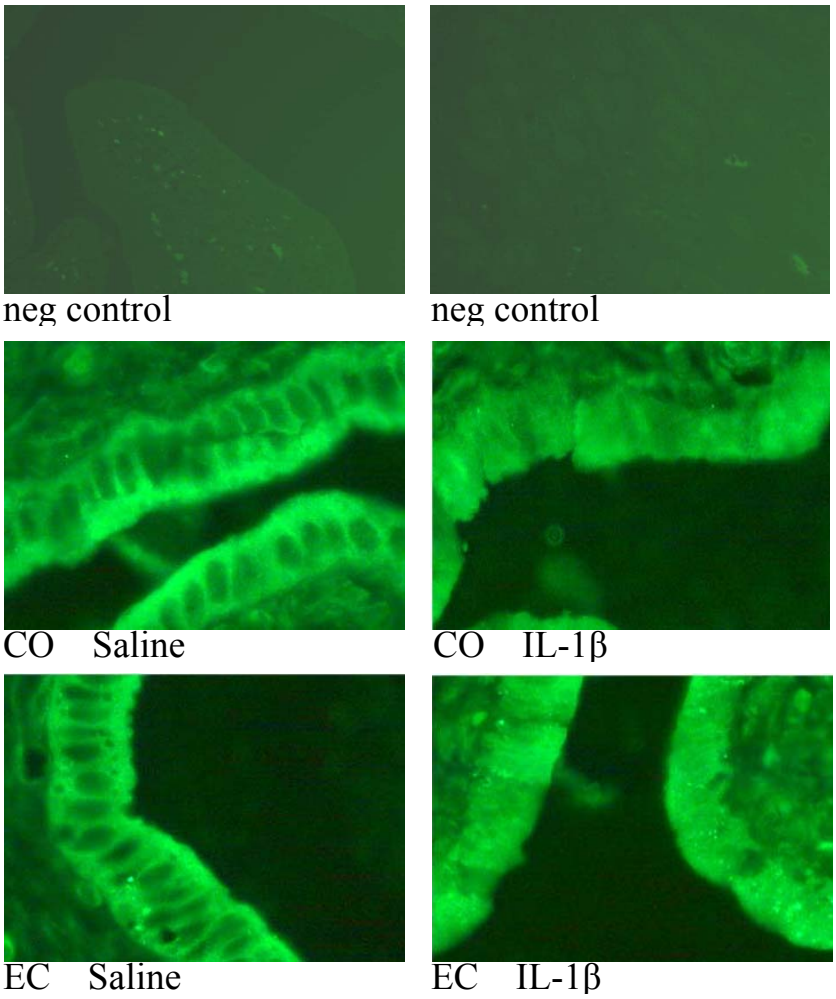

Figure B.3 Activation (nuclear localization) of NF-κB in the luminal epithelium (LE) of uterine horns 4 h after infusion with saline or interleukin-1 beta (IL-1β). Gilts were injected with either corn oil (CO) or 5 mg of estradiol cypionate in corn oil (EC) 24 h before infusion. There was no effect of EC injection on NF-κB activation (nuclear localization); however, infusion of IL-1β resulted in complete activation of NF-κB within LE cells of the pig uterus 4 h after infusion. Images were taken with a Leica light microscope using a green fluorescent protein (GFP) filter at 400 X magnification.

APPENDIX C

UTERINE ACTIVATION OF NUCLEAR FACTOR-KAPPA B DURING THE ESTROUS CYCLE AND EARLY PREGNANCY IN PIGS

During establishment of pregnancy in the pig, numerous conceptuses will undergo rapid trophoblast elongation near d 12 of gestation and attach to the uterine luminal epithelial (LE) surface. Previous studies in the human and mouse indicate that as the conceptus nears the uterus, it releases interleukin-1 beta (IL-1β), a pro-imflammatory cytokine that activates nuclear factor-kappa B (NF-κB) (Lindhard et al., 2002; Takacs and Kauma, 1996). Nuclear factor-kappa B is a transcription factor thought to modify the uterine environment by regulating gene expression in preparation for conceptus attachment (Ashworth et al., 2006). Near d 11, the pig conceptus will also secrete IL-1β (Ross et al., 2003; Tou et al., 1996). Although IL-1β is initially expressed on d 11, expression is reduced nearly 2000-fold by d 15 of pregnancy (Ross et al., 2003). The IL-1β protein is greatest in the uterine lumen on d 12 of gestation, coincident with conceptus trophoblast elongation prior to attachment (Ross et al., 2003).

Binding of IL-1β to its epithelial cell surface receptor activates NF-κB by increasing phosphorylation of IκB (inhibitor of NF-κB), a protein that sequesters NF-κB in the cytoplasm (McKay and Cidlowski, 1999). Once phosphorylated, IκB will release

NF-κB allowing the transcription factor to translocate into the epithelial cell nucleus. In the nucleus, NF-κB increases expression of many genes involved in inflammatory-type reactions, such as prostaglandin-endoperoxide synthase 2 (PTGS2), that are considered necessary for establishment of pregnancy (Ashworth et al., 2006; Kraeling et al., 1985). The purpose of this experiment was to determine if development of the pig conceptus during establishment of pregnancy is coincident with the activation of NF-κB (nuclear localization) in uterine luminal epithelial (LE) cells.

The uterus from both cyclic (d 0, 5, 7.5, 10, 12, 13, 15, and 17) and pregnant (d 10, 12, 13, 15, and 17) pigs was removed by midventral laparotomy. Uterine tissue from 2 to 8 pigs per treatment was fixed in 10% buffered formalin phosphate (Fisher Scientific). After 48 hours, sections were imbedded in paraffin in preparation of immunohistochemistry (IHC).

During IHC, a rabbit polyclonal antibody, able to bind the p65 subunit of NF-κB, was used as a primary antibody (sc-372, Santa Cruz Biotechnology, INC; Santa Cruz, CA) and a donkey anti-rabbit monoclonal antibody (711-225-152, Jackson ImmunoResearch Laboratories, INC, West Grove, PA) containing a Cy2 conjugate was used as a secondary antibody (see Materials and Methods; Chapter Three).

Nuclear factor-kappa B activation in luminal epithelial (LE) cells was blindly scored by three independent investigators using a Leica light microscope with a green fluorescent protein filter (GFP) at 400 X magnification. Two tissue sections per pig were scored for NF-κB activation (nuclear localization) on a 0 to 5 scale (0 indicating no nuclear localization and 5 indicating complete nuclear localization; see Appendix B).

Immunoflourescents score was analyzed using the general linear models (GLM) procedure of the Statistical Analysis System (SAS institute Inc. Cary, NC, USA). Least squares means (lsmean) and standard errors were generated using the LSMeans statement of SAS. All reported means are the adjusted least squares means ± standard error of the lsmean (SEM) and the significance was declared when $P < 0.05$.

There was an effect of status ($P < 0.01$) as NF-κB was activated more in the LE cells of pregnant pigs when compared with cyclic pigs. By d 12 and 13, there was an increase in NF-κB nuclear localization within LE cells of pregnant pigs when compared with cyclic pigs, however, nuclear localization of NF-κB increased in cyclic pigs after d 13, reaching levels similar to pregnant pigs by d 17 (Figure C.1, p. 182). Figure C.2 (p. 183) displays images of immunofluorescence for NF-κB in the uterine LE of both cyclic and pregnant pigs on d 10, d 12, d 13, d 15 and d 17 of the estrous cycle and pregnancy. On d 12 and d 13, NF-κB is localized more to the nucleus of LE cells in pregnant pigs when compared with cyclic pigs. On d 15 and d 17, nuclear localization of NF-κB increases in LE cells of cyclic pig reaching levels similar to pregnant pigs by d 17. Figure C.3 (p. 184) displays images of immunofluorescence for NF-κB in the uterine LE from both cyclic and pregnant pigs on d 10 and d 13 of the estrous cycle and pregnancy taken at 400 X magnification plus a zoom. Differences in nuclear localization of NF-κB in the LE can not be seen between d 10 of the estrous cycle and pregnancy, however, nuclear localization of NF-κB is greater in pregnant pigs on d 13 and when compared with cyclic pigs.

Interleukin 1 beta (IL-1β) is a pro-inflammatory cytokine that can activate NF-κB. During activation, NF-κB translocates from the cell cytoplasm to the nucleus and

increases expression of genes associated with the inflammatory response. In the human and mouse, conceptus secretion of IL-1β activates NF-κB in the uterus before implantation (Lindhard et al., 2002; Takacs and Kauma, 1996). Interleukin-1β is also thought to activate NF-κB in the pig uterus during early pregnancy (Ross et al., 2003; Tou et al., 1996). In this study, activation of NF-κB increased in uterine LE cells on d 12 and 13 of pregnancy in the pig. Activation of NF-κB on d 12 and 13 of pregnancy is temporally associated with the presence to the conceptus and secretion of IL-1β. Although activation of NF-κB was reduced on d 12 and 13 of the pig estrous cycle, in the absence of the conceptus, activation increased in cyclic pigs to levels similar to pregnant pigs by d 17. Reduced activation of NF-κB in the LE of cyclic pigs on d 12 and 13 when compared with pregnant pigs are likely due to the absence of conceptus and secretion of IL-1β. The increase in NF-κB activation observed in the LE of cyclic pigs after d 13 is not understood, however, is temporally associated with uterine epithelial production of $PGF_2\alpha$ and luteolysis.

It has been suggested that progesterone (P4) will regulate expression of it own receptor in the uterine epithelium by indirectly activating NF-κB during the estrous cycle and pregnancy. Upon activation, NF-κB is thought to bind up stream of the PGR gene and down-regulate transcription of the PGR. In the study described in Chapter Three, we injected gilts with RU486 to investigate how P4 regulates expression of the PGR within the uterine epithelium and how disruption of that mechanism might effect conceptus development. Although no differences in uterine NF-κB activation were observed between treatments during PGR down-regulation, indicating that NF-κB is not involved in this process, NF-κB was activated in uterine epithelial cells on d 12 of pregnancy in

treatments that were conducive to normal conceptus development. It was later confirmed that activation of NF-κB was induced by the conceptus. Figure 3.30 (p. 101) displays an image of a conceptus locally activating NF-κB in the uterine epithelium on d 12 of pregnancy. We later infused IL-1β into uterine horns of gilts after injecting them with estradiol cypionate to determine if IL-1β can activate NF-κB in the uterus in the presence of conceptus release of estradiol (Appendix B). Injection of estradiol cypionate had no effect on NF-κB activation as IL-1β drastically increased activation within 4 h of infusion (Appendix B; Figure B.2, p. 175 and Figure B.3, p. 176). In this study, activation of NF-κB increased in the LE cells of pregnant pigs (d 12 and 13) when compared with cyclic pigs (d 12 and 13) and was temporally associated with the presence of the conceptus and secretion of IL-1β. In the human and mouse, conceptus secretion of IL-1β is thought to initiate cross talk between the uterus and conceptus prior to attachment (Lindhard et al., 2002; Takacs and Kauma, 1996). The activation of NF-κB enhances prostaglandin-endoperoxidase synthase 2 (PTGS2) expression in the uterine epithelium. This is necessary for establishment of pregnancy. The dramatic increase in IL-1β expression by the conceptus and initiation of an inflammatory response in the uterus (NF-κB activation) is not fully understood. Further investigation of factors that may control establishment of pregnancy, such as the IL-1β/NF-κB system, may be necessary to improve reproductive performance in the pig as well as other species.

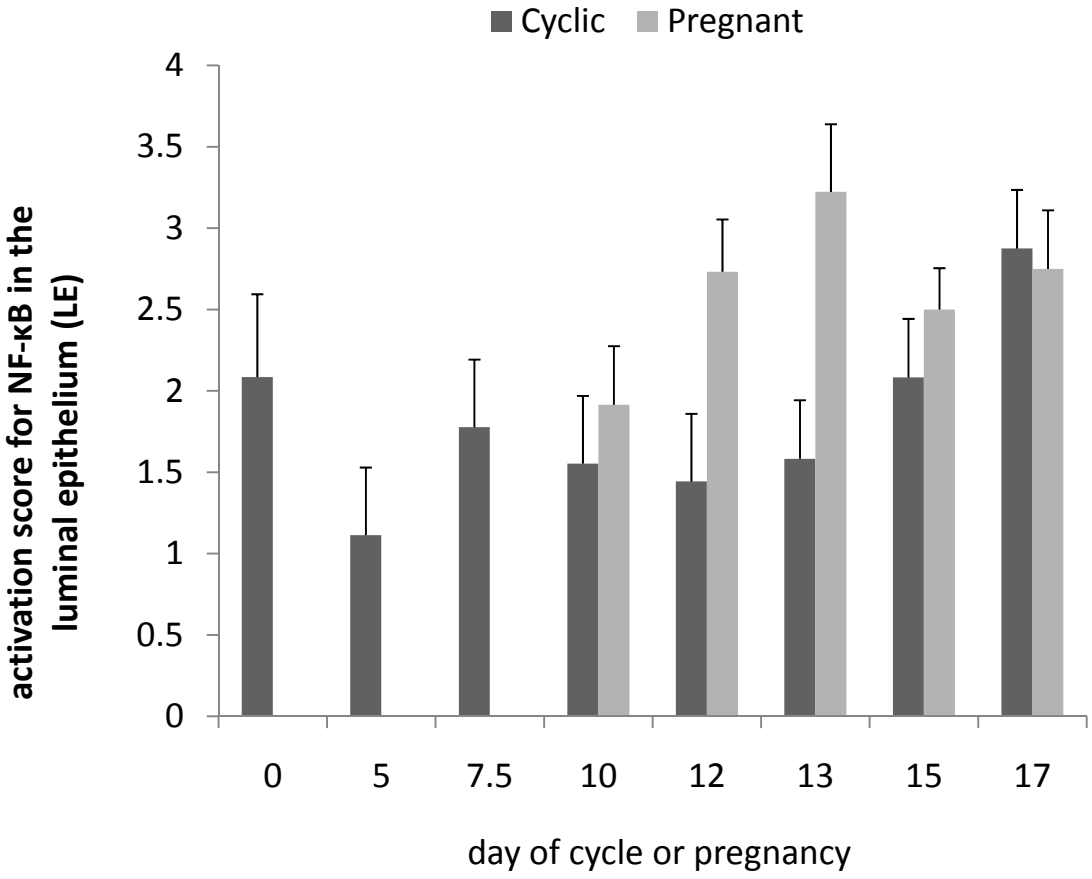

Figure C.1 Least squares means for nuclear localization (activation) score for nuclear factor-kappa B (NF-κB) in the uterine luminal epithelial (LE) of the pregnant and cyclic pigs. Activation was scored on a 0 to 5 scale where 0 indicated no nuclear localization and 10 indicated complete nuclear localization. There was an effect of status ($P < 0.01$) as NF-κB was activated more in the LE of pregnant pigs when compared to cyclic pigs. Activation was greater in the LE of pregnant pigs on d 12 and 13; however activation increased after d 13 in cyclic pigs, reaching levels similar to pregnant pigs by d 17.

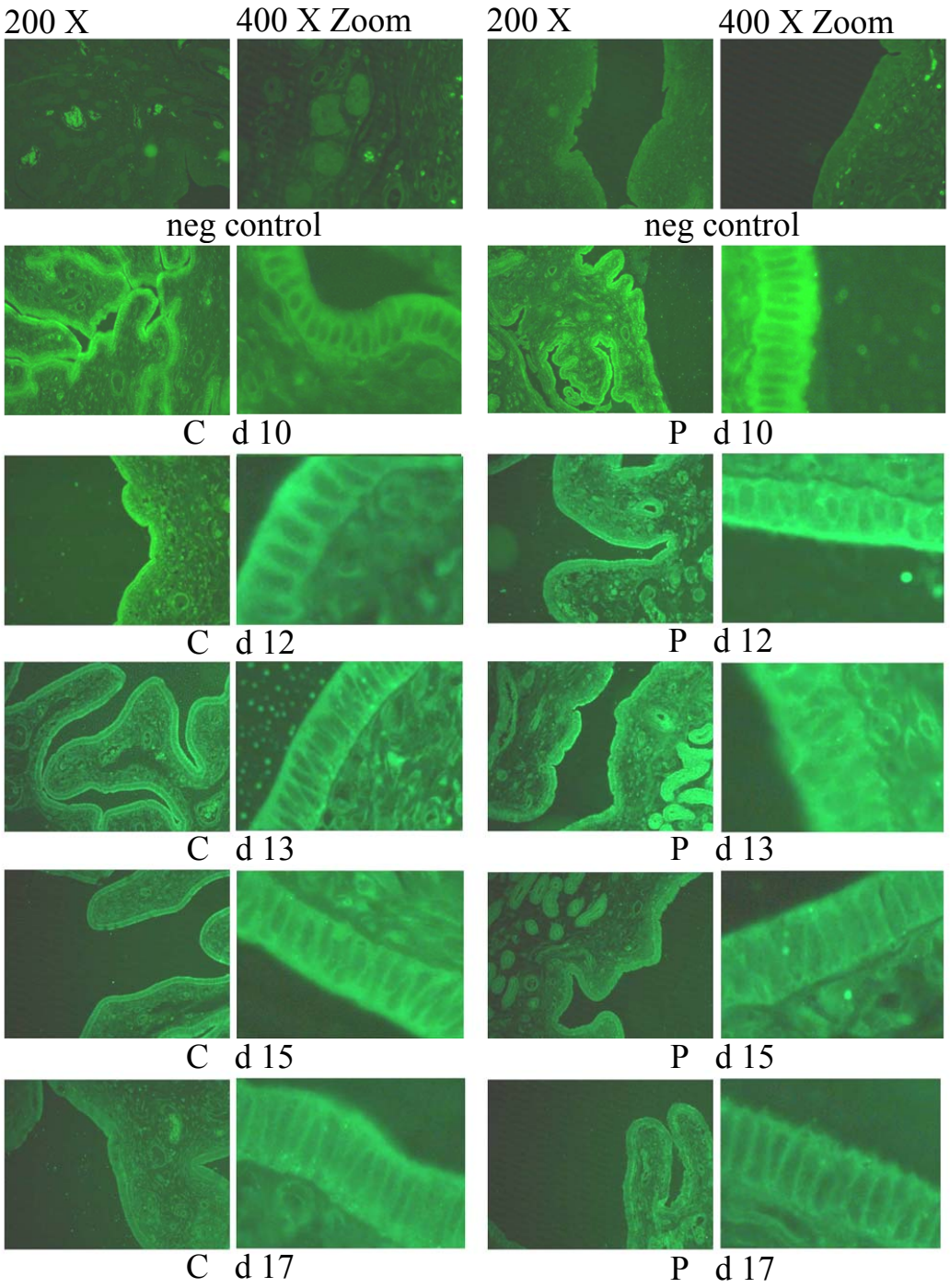

Figure C.2 Immunofluorescence for nuclear factor-kappa B (NF-κB) in the uterine luminal epithelium (LE) on d 10, 12, 13, 15 and 17 of the estrous cycle (C) and pregnancy (P). Activation (nuclear localization) of NF-κB increased in the LE on d 12 and d 13 of pregnancy when compared to the estrous cycle; however, activation increased after d 13 in the LE of the cyclic uterus, reaching levels similar to pregnant pigs by d 17. Images were taken with a Leica light microscope using a green fluorescent protein (GFP) filter.

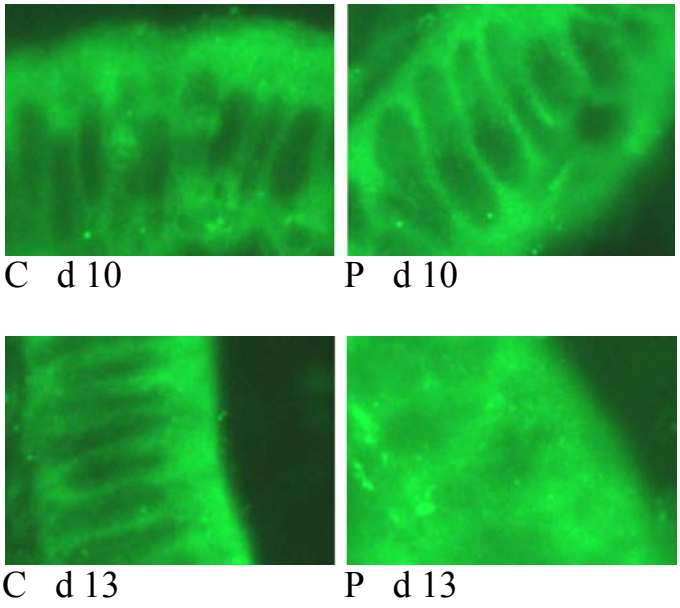

Figure C.3 Immunofluorescence for nuclear factor-kappa B (NF-κB) in the uterine luminal epithelium (LE) on d 10 and d 13 of the estrous cycle (C) and pregnancy (P). Activation was scored on a 0 to 5 scale where 0 indicated no nuclear localization and 10 indicated complete nuclear localization. Images were taken with a Leica light microscope using a green fluorescent protein (GFP) filter at 400 X magnification plus a zoom.

VITA

Daniel Joseph Mathew was born June 20, 1983 in Lafayette, Indiana. He grew up on the family swine farm in Wolcott, Indiana where his interest in animal agriculture sparked at an early age. Daniel later moved to Philadelphia, Tennessee and lived on a beef farm just west of the Smoky Mountains. Daniel attended the University of Tennessee-Knoxville, receiving his Bachelors in Animal Science with a minor in biology in 2006. While under the guidance of Drs. Neal Schrick and Lannett Edwards at the University of Tennessee Animal Science Department, Daniel became interested in early embryonic development and conceptus uterine interactions. Daniel will complete a Masters in Animal Science/Reproductive Physiology with an emphasis in swine from the University of Missouri in December, 2009, under the guidance of Drs. Matthew Lucy and Rodney Geisert. Daniel will pursue a PhD in Reproductive Physiology from the University of Missouri in the spring of 2010.