

Maternal recognition of pregnancy signal or endocrine disruptor: The two faces of oestrogen during establishment of pregnancy in the pig

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Timing of conceptus growth and attachment to the uterine luminal epithelium is regulated by progesterone secretion from the corpus luteum and by expression of progesterone receptor in the uterine epithelia and stroma. Conceptus growth and uterine attachment are temporally associated with the disappearance of progesterone receptors from uterine epithelia. While the loss of progesterone receptor from the endometrial epithelia on day 10 of the oestrous cycle and pregnancy has been well documented, the factors involved with cell specific down-regulation of progesterone receptor are yet to be established. We propose that several progesterone stimulated factors activate nuclear factor kappa B (NF- κ B) within the uterine epithelia, which leads to inhibition of progesterone receptor and concomitant stimulation of endometrial genes expressed during early conceptus development. Although oestrogens secreted by pig conceptuses function to establish pregnancy, timing of endometrial exposure to oestrogen is critical. Early oestrogen administration alters the pattern of gene expression through the NF- κ B system desynchronising the uterine environment for conceptus implantation resulting in later embryonic loss.

Introduction

The fundamental mechanisms for the regulation of the oestrous cycle and establishment of pregnancy in the pig are well established. For years we have known that following ovulation of the ovarian Graafian follicles during oestrus, synthesis and release of progesterone from developing corpora lutea (CL) is the principal driver of endometrial secretion and receptivity for early conceptus development and implantation (see review Geisert and Yelich, 1997). In the absence of conceptuses, progesterone stimulates synthesis and pulsatile release of endometrial derived prostaglandin $F_{2\alpha}$ (PGF_{2 α}) into the uterine vasculature after day 12 of the oestrous cycle to induce CL regression on days 15-16 and a return to oestrus between days 18-21 in the sow. During pregnancy, CL are maintained in the sow following rapid elongation of the developing conceptuses throughout the uterine horns on day 12 (Geisert *et al.* 1982). Conceptus

secretion of the "maternal recognition of pregnancy signal", oestrogen, redirects endometrial $\text{PGF}_{2\alpha}$ secretion into the uterine lumen where it is sequestered and metabolised to prevent luteolysis (see review Bazer *et al.*, 1982).

In the pig endometrium, a clear spatiotemporal association exists between the redirection of $\text{PGF}_{2\alpha}$ release, decline in progesterone receptor (PR) from the luminal (LE) and glandular (GE) epithelium, and receptivity for conceptus implantation. Down-regulation of PR in endometrial epithelia is a conserved event among mammals that is associated with opening of the implantation window in the mouse (Tan *et al.*, 1999), pig (Geisert *et al.*, 1994), sheep (Spencer and Bazer, 1995), and human (Lessey *et al.*, 1988). Although down-regulation of PR within LE and GE has been well documented, cellular and molecular mechanisms responsible for specific loss of PR from uterine epithelia are relatively unknown. Unlocking the pathway controlling uterine epithelial PR expression would provide a significant insight into the mechanisms regulating the oestrous cycle and implantation in the pig. Possible clues to the compartmental regulation of endometrial PR down-regulation have begun to emerge with recent reports of a mutual negative interaction between the transcription factor, nuclear factor kappa B (NF- κ B) and PR (Kalkhoven *et al.*, 1996; McKay and Cidlowski, 1998). Loss of PR in the endometrial epithelia is correlated with increased activation of components of the NF- κ B signalling pathway (Kalkhoven *et al.*, 1996; King *et al.*, 2001; Page *et al.*, 2002), which functions to activate transcription of many genes involved with inflammatory type reactions and release of cytokines associated with establishment of pregnancy in the pig.

Secretion of interleukin-1 β and oestrogen by the developing porcine conceptuses may function to regulate NF- κ B during the establishment of pregnancy in the pig. However, although oestrogen plays a major function in preventing luteolysis and inducing uterine secretions necessary for changes in conceptus morphology for implantation in pigs, the inappropriate timing of uterine exposure to oestrogen (*i.e.*, delivered prior to the normal time of conceptus secretion on day 11 to 12 of gestation) has a detrimental effect on conceptus survival (Pope *et al.*, 1986; Morgan *et al.*, 1987). Premature exposure of the endometrium to oestrogen disrupts the normal expression pattern of uterine and/or conceptus genes during trophoblast attachment, desynchronising the carefully orchestrated temporal and spatial interaction of the uterine environment and conceptuses resulting in early embryonic death. Oestrogen is a major player in controlling NF- κ B activation (Kalaitzidis and Gilmore, 2005) and therefore serves to modulate cytokine release to prevent outbreak of a disease state if NF- κ B activation is not held in check (Ali and Mann, 2004). It is reasonable to propose that oestrogen serves to modulate unchecked NF- κ B activation and therefore prevent inappropriate downstream cytokine release that would lead to an inflammatory "disease" state during implantation.

Role of progesterone in regulation of oestrous cycle and early pregnancy

Development of a uterine environment that is synchronous with the developing conceptuses is paramount to embryo survival and establishment of pregnancy. Spatiotemporal changes in the uterine luminal environment are controlled by the rapid and sustained increase in plasma progesterone following ovulation in pigs. The role of progesterone in regulating length of the oestrous cycle was demonstrated through its ability to shorten the length of the oestrous cycle following progesterone supplementation shortly after ovulation in cattle (Garrett *et al.*, 1988b) and sheep (Ottobre *et al.*, 1980). In contrast, administration of the antiprogestin, mifepristone, on days 3 to 5 of the sheep oestrous cycle lengthens the oestrous cycle (Morgan *et al.*, 1993). During pregnancy, administration of progesterone prior to the postovulatory period of CL development accelerates conceptus development in cattle (Garrett *et al.*, 1988a) and sheep (Lawson and Cahill, 1983).

Treatment of gilts with progesterone on days 2 and 3 of pregnancy can advance both uterine secretions and conceptus development (Vallet *et al.*, 1998; Vallet and Christenson, 2004), while treatment with mifepristone slows conceptus development (Vallet and Christenson, 2004). Alteration in length of the oestrous cycle and acceleration of conceptus development during pregnancy following progesterone administration is not as dynamic in the gilt as in the ewe and cow. The more pronounced change in cattle and sheep occurs because concentrations of progesterone in plasma do not increase significantly until days 4 to 5 of the oestrous cycle compared with the rapid increase within 24–36 h after ovulation in pigs.

Cell-specific expression of progesterone receptors in the uterus

Progesterone's role in timing both luteolysis during the oestrous cycle and conceptus expansion and implantation during early pregnancy is associated with cell-specific changes in expression of endometrial PR. Sustained stimulation of the uterus by progesterone over 7 to 8 days causes a loss of PR from LE and GE. The ability of progesterone to down-regulate its own receptor within the uterine epithelia is the foundation of the classical McCracken model for luteolysis in sheep (McCracken *et al.*, 1984). There is a clear relationship between loss of PR in LE and GE with luteal regression in cyclic gilts and the onset of rapid conceptus development in pregnant animals. Expression of PR in endometrial LE and GE is down-regulated by day 10 of the oestrous cycle and pregnancy, whereas expression of PR is maintained in stromal cells and myometrium throughout pregnancy (Geisert *et al.*, 1994). Removal of PR from endometrial LE and GE is associated with loss of the large mucin, MUC-1, from the LE apical surface exposing integrins for trophoblast attachment (Bowen *et al.*, 1996). This is also the period when the endometrium releases many cytokines and growth factors to support early development and trophoblast expansion of pig conceptuses on day 12 of pregnancy (see Geisert and Yelich, 1997). Although loss of PR from the uterine epithelium is well established, the mechanism responsible for inhibiting epithelial PR expression during the mid luteal phase (day 10) of the oestrous cycle and early pregnancy is not known. It is clear that down-regulation of epithelial PR precedes the dramatic changes in endometrial secretion and rapid trophoblast expansion and oestrogen secretion by the porcine conceptuses on days 11 to 12 of pregnancy (Geisert and Yelich, 1997).

The processes of implantation in mammals mirror an acute phase inflammatory response as components of the kallikrein-kininogen-kinin system are expressed during implantation in the mouse (Figuroa *et al.*, 2001). There is also pregnancy-specific activation of the kallikrein-kininogen-kinin system in the pig uterus (Vonnahme *et al.*, 1999), including expression of the bradykinin β_2 receptor gene in the pig endometrial LE and GE during the period of conceptus elongation and implantation (Allen *et al.*, 2002). Since kinins induce increased microvascular permeability and vascular growth (Fabre *et al.*, 1999), the kallikrein-kininogen-kinin system likely plays a major role in uterine and placental angiogenesis essential for conceptus survival. A proteoglycan family of acute phase proteins, inter- α -trypsin inhibitors (I α 1), form the LE extracellular matrix (ECM) in the porcine endometrium (Geisert *et al.*, 2003). We propose that the biological role of I α 1 heavy chains is to stabilise the ECM during trophoblast attachment to endometrial LE and release of the serine protease inhibitor, bikunin, which serves as an additional protease inhibitor to prevent conceptus invasion (see Geisert *et al.*, 2004). It is evident that many factors involved with inflammatory type reactions are present during establishment of pregnancy in the pig, which may be triggered through activation of the NF- κ B system.

Uterine NF- κ B activation

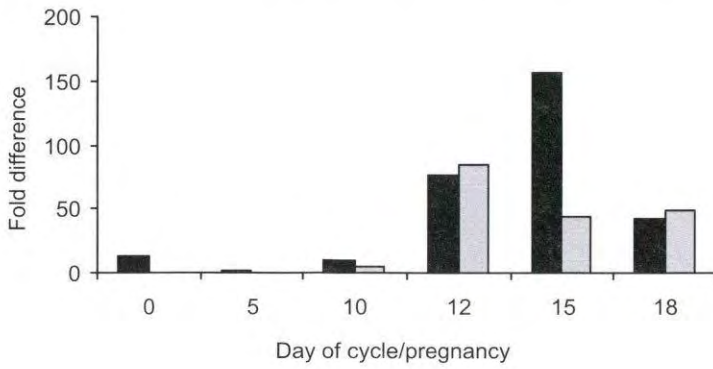
The NF- κ B family of transcription factors regulates tissue immune function, as well as inflammatory and acute phase responses (McKay and Cidlowski, 1998). NF- κ B activation targets a

variety of genes, including those for cell adhesion molecules, cytokines, growth factors and immunoreceptors. NF- κ B normally exists as a cytoplasmic heterodimer composed of various Rel family proteins (Ghosh *et al.*, 1998). The heterodimer of p50 and p65 is the most abundant form of NF- κ B in eukaryotes. NF- κ B is sequestered in an inactive form within the cytoplasm through binding by inhibitors of NF- κ B, I κ Bs (Ali and Mann, 2004). NF- κ B is regulated through receptor activation by variety of stimuli such as bacterial endotoxin lipopolysaccharide, oxidative stress, radiation exposure, and specific cytokines, particularly, interleukin-1 β (IL-1 β) and tumour necrosis factor- α (TNF α). IL-1 β and TNF α receptor activation stimulates the classical pathway for phosphorylation of two serines in I κ B by I κ B kinase composed of two catalytic units, IKK α and IKK β and the regulatory subunit IKK γ . Phosphorylation of I κ B results in its release from the NF- κ B complex and degradation by polyubiquitination in the 26S proteasome (Ghosh *et al.*, 1998). Release of I κ B allows nuclear translocation of NF- κ B (p65:p50) for binding to specific κ B-sites in the promoter region of target genes to activate transcription. Activation of nuclear κ B sites also stimulates I κ B to bind NF- κ B and translocate as an inactive complex back to the cytoplasm (negative feedback loop). Endometrial gene expression for p65 and p50 increases between days 5 to 12 in the pig (J.W. Ross and R.D. Geisert, unpublished). Genes containing κ B sites that are transcriptionally regulated by NF- κ B include many cytokines (TNF α , IL-1, IL-2, IL-6, IL-12, LIF and GM-CSF), chemokines (IL-8 and RANTES), and enzymes such as cyclooxygenase-2 (COX-2) (Ali and Mann, 2004). Steroid hormone regulation of uterine receptivity for implantation is modulated through activation of a cascade of events involving many of the NF- κ B regulated adhesion factors, growth factors and cytokines (see Carson *et al.*, 2000; Spencer *et al.*, 2004).

A role for NF- κ B activation in regulating uterine function during the oestrous cycle and early pregnancy is emerging in a number of species. Endometrial NF- κ B activation is proposed to be involved with implantation in the human (King *et al.* 2001; Page *et al.*, 2002) and mouse (Nakamura *et al.*, 2004). Endometrial expression of NF- κ B proteins is compartmentalised to GE of women (Laird *et al.*, 2000; King *et al.*, 2001) and mice (Nakamura *et al.*, 2004). The porcine endometrium secretes a number of NF- κ B regulated cytokines (IL-6 and LIF) and growth factors such as keratinocyte growth factor (KGF) during the period of rapid conceptus development between days 12 and 15 of pregnancy (see Geisert and Yelich, 1997). There is an 80-fold increase in endometrial COX-2 gene expression from day 5 to day 12 of the oestrous cycle and pregnancy (M.D. Ashworth and R.D. Geisert, unpublished). The increase in endometrial COX-2 gene expression between days 10 and 15 of the oestrous cycle and pregnancy is restricted to uterine LE and GE (Fig. 1) directly after loss of epithelial PR expression (Geisert *et al.*, 1994). The presence of NF- κ B in uterine epithelia and the increase in COX-2, the inducible form of cyclooxygenase, in LE and GE indicates a role for NF- κ B transcription factors in regulation of uterine functions during the oestrous cycle and pregnancy.

The importance of endometrial NF- κ B activation in the establishment of pregnancy is further indicated by conceptus synthesis of a cytokine activator of NF- κ B, IL-1 β (Tuo *et al.*, 1996; Ross *et al.*, 2003). IL-1 β could serve an important function in regulating cross-talk between porcine conceptuses and the endometrium. Our laboratory has established that IL-1 β is temporally associated with both rapid conceptus elongation and an increase in endometrial IL-1 receptor type I and IL-1 receptor-accessory protein gene expression (Ross *et al.*, 2003). Interestingly, IL-1 receptor type I and II are both molecular markers for uterine receptivity in mice (Reese *et al.*, 2001). Expression of IL-1 β increases prior to initiation of blastocyst implantation in the mouse (Takacs and Kauma, 1996), and it may be an initiator of conceptus-uterine interaction during pregnancy in women (Lindhard *et al.*, 2002). The transient expression of conceptus IL-1 β also suggests an autocrine function to induce the rapid morphological transformation of pig conceptuses from spherical to filamentous forms on day 12 of pregnancy (Geisert *et al.*, 1982). Indeed, porcine conceptus IL-1 β gene expression and release of IL-1 β protein into the uterine lumen is temporally and spatially

A



B

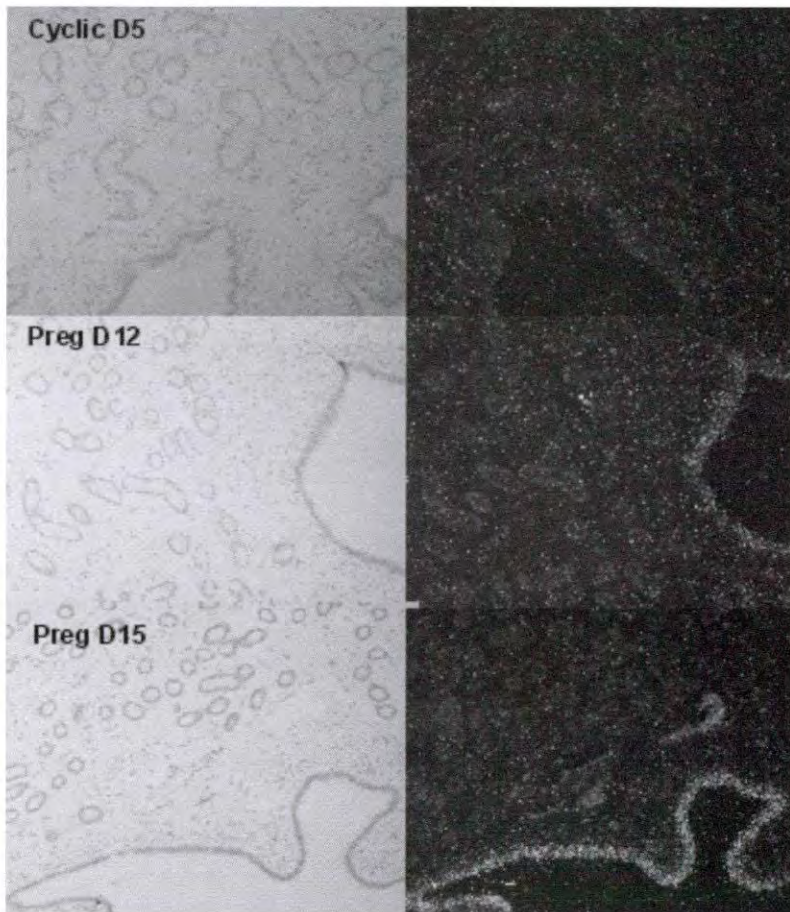


Fig.1 A) Fold change in endometrial COX-2 gene expression during oestrous cycle (black bars); and pregnancy (grey bars). B) *In situ* hybridisation of endometrial COX-2 gene expression in the luminal and glandular epithelium during the oestrous cycle (day 5) and early pregnancy (days 12 and 15) of the pig.

associated with rapid trophoblast elongation (Ross *et al.*, 2003). IL-1 β , an inducer of phospholipase A2 (Kol *et al.*, 2002), regulates the release of arachidonic acid from the phospholipid bilayer of cells resulting in an increase in membrane fluidity necessary for tissue remodelling during elongation of the conceptus and its conversion to prostaglandins which affect placental attachment during the establishment of pregnancy (Kraeling *et al.*, 1985). Inhibition of NF- κ B activation prevents abundant expression of COX-2 gene expression in human trophoblast cells (Kniss *et al.*, 2001). Filamentous (day 12) porcine conceptuses express high levels of COX-2 mRNA (Wilson *et al.*, 2002) that is temporally associated with IL-1 β expression. Indomethacin blocks NF- κ B activation of COX-2 expression through inhibition of I κ B α kinase activity and suppression of I κ B α degradation (Takada *et al.*, 2004). While inhibition of conceptus prostaglandin production with indomethacin does not effect trophoblast elongation (Geisert *et al.*, 1986), prostaglandin synthesis or NF- κ B activation is essential for placental attachment and survival following elongation (Kraeling *et al.*, 1985). Implantation in mice and rats require PG synthesis in the oestrogen primed uterus. The importance of endometrial COX-2 gene expression during implantation and decidualisation was established using COX-2 null mice that have an altered inflammatory response resulting in reproductive defects in ovulation, fertilisation and implantation (Lim *et al.*, 1997). These effects are COX-2 specific because COX-1 deficient mice are fertile and only exhibit a defect in parturition (Gross *et al.*, 1998). We hypothesise that IL-1 β signalling is critical to trophoblast elongation (Ross *et al.*, 2003) and induction of endometrial events responsible for cell adhesion and cytokine production associated with establishment and maintenance of pregnancy.

Model for progesterone regulation of uterine PR

Endometrial synthesis and release of PGF_{2 α} into the uterine vasculature stimulates luteolysis in pigs. Prostaglandin synthesis is regulated through constituent expression of COX-1 and/or the inducible COX-2 (Smith *et al.*, 2000). A slight increase in endometrial COX-1 gene and protein expression occurs after day 12 of both the oestrous cycle and pregnancy (M.D. Ashworth and R.D. Geisert, unpublished); however, during the same period there is a dramatic 80-fold increase in endometrial COX-2 gene expression and a 10-fold increase in COX-2 protein. While increased expression of COX-2 on day 12 of pregnancy is consistent with conceptus secretion of IL-1 β and activation of endometrial NF- κ B on day 12 of pregnancy, the increase in COX-2 expression in cyclic females was unexpected. What could induce NF- κ B activity and COX-2 expression within the uterine epithelium on day 10 in the absence of conceptuses? Recent information has revealed an interaction between NF- κ B and PR that may explain how the timing of endometrial COX-2 expression on day 10 is under uterine control. Progesterone exerts an inhibitory effect on the synthesis of the proinflammatory chemokine, COX-2, by blocking the NF- κ B pathway (McKay and Cidlowski, 1998) and loss of endometrial progesterone stimulation enhances *in vivo* chemokine release (Jones *et al.*, 1997). Mutual repression between PR and NF- κ B activation has been reported and this mechanism could control cytokine release for establishment and maintenance of pregnancy as previously discussed (Kalkhoven *et al.*, 1996; McKay and Cidlowski, 1998). Progesterone has been proposed to inhibit NF- κ B activation through its receptor by either directly interfering with NF- κ B binding to its consensus DNA response element, inhibiting transcription of ligands and receptors that activate NF- κ B or increasing expression of binding proteins that inhibit NF- κ B activity (Davies *et al.*, 2004). Therefore, the presence of PR in cells could inhibit NF- κ B activation until either: 1) withdrawal of progesterone as would be the case during parturition (Wickelgren, 2004); or 2) PR is down-regulated in progesterone sensitive epithelial cells.

Down-regulation of PR in endometrial epithelia explains loss of endometrial NF- κ B inhibition, but how is this compartmentalised to LE and GE of the uterus while maintaining biologically essential PR expression in the stroma and myometrium throughout pregnancy? One hypothesis for the specificity of PR loss in epithelia is that activation of NF- κ B occurs through

progesterone-stimulated endometrial expression of receptor activator of nuclear factor κ B ligand (RANKL). RANKL and its cognate receptor (RANK) have a critical role in bone remodelling and mammary gland development (Holstead-Jones *et al.*, 2002; Cao and Karin, 2003). Deletion of RANKL gene expression causes severe bone osteoporosis and underdeveloped lobuloalveolar buds within the mammary gland (Holstead-Jones *et al.*, 2002). In the mammary epithelium, RANKL acts via RANK, which is constitutively expressed to stimulate NF- κ B up-regulation of cyclin D1 (see Cao and Karin, 2003). Activation of RANKL expression in mammary epithelium is induced by prolactin and progesterone, but progesterone stimulation may require oestrogen priming (Srivastava *et al.*, 2003). The presence of the prolactin receptor in the porcine endometrium during early pregnancy (Young *et al.*, 1990) indicates that basal concentrations of prolactin could regulate RANKL as proposed in the mammary gland (Srivastava *et al.*, 2003). Mulac-Jericevic *et al.* (2003) demonstrated that initial activation of RANKL is PR dependent. The pig endometrium expresses RANK during the oestrous cycle and early pregnancy (J.W. Ross and R.D. Geisert, unpublished). Although further studies are needed to establish the cellular localisation and time specific changes in endometrial RANKL and RANK expression, these results support our hypothesis that down-regulation of PR in endometrial LE and GE may be regulated through progesterone and prolactin driven secretion of RANKL (Fig. 2). Constitutive expression of RANK in uterine epithelia would allow RANKL activation of NF- κ B leading to epithelial cell specific loss of PR followed by activation of NF- κ B stimulated genes such as keratinocyte growth factor (KGF) and COX-2, while PR is maintained in stromal cells and myometrium.

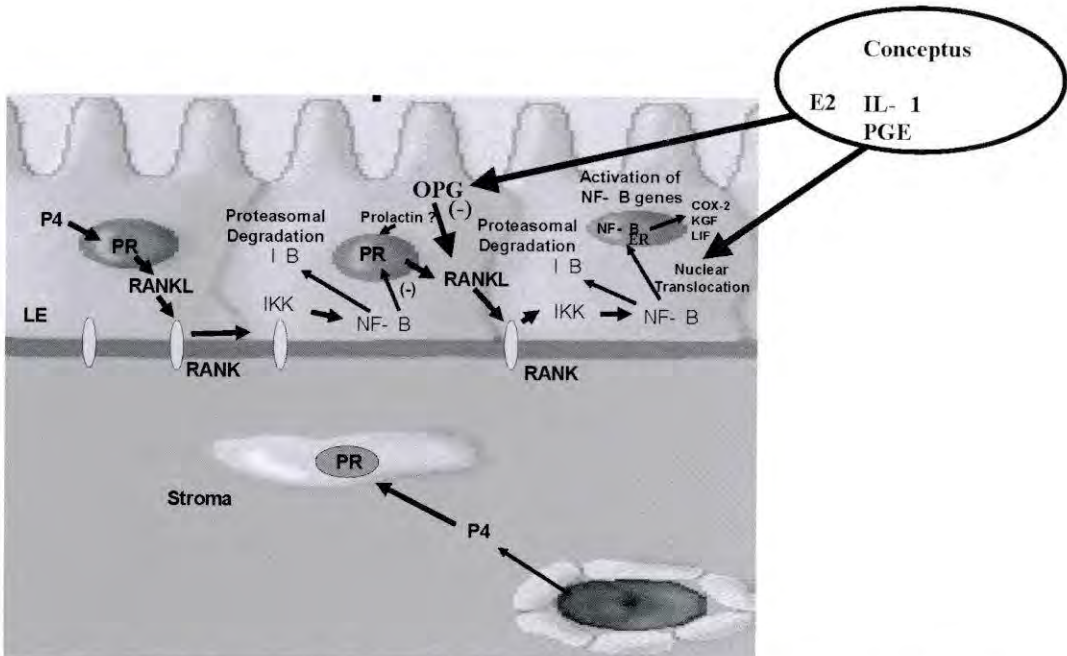


Fig. 2 Model depicting progesterone (P4) stimulation of RANKL expression to induce NF- κ B activation of PR loss in GE and LE followed by increased expression COX-2, KGF, LIF and OPN at the time of rapid conceptus expansion. Conceptus expression of IL-1 β and PGE continues to stimulate NF- κ B activation during early pregnancy. Conceptus oestrogen (E2) secretion modulates NF- κ B gene activation either directly through binding ER within LE and GE or stimulates expression of the decoy receptor osteoprotegerin (OPG) which competes with RANKL for binding to RANK.

Conceptus regulation of uterine NF- κ B expression

The importance of regulating NF- κ B activation during pregnancy is evident from the large increase in IL-1 β secretion during rapid trophoblast elongation throughout the uterus on day 12 of pregnancy (Ross *et al.*, 2003). Factors, conceptus or uterine, that activate conceptus IL-1 β synthesis are not currently known. Activation of uterine NF- κ B through endometrial RANKL expression could be involved with triggering the acute expression of conceptus IL-1 β . Loss of PR from the endometrial LE and GE, and the increase in endometrial IL-1 receptor type I and IL-1 receptor-accessory protein gene expression represent a pathway for cross-talk between conceptus and uterus (Ross *et al.*, 2003). One would predict that the extremely high uterine luminal IL-1 β content during early pregnancy would induce a strong inflammatory response in the uterus. However, only a select set of genes involved with a proinflammatory response are increased, while expression of other NF- κ B regulated genes is not detected. A classical inflammatory reaction does not occur and endometrial expression of inflammatory type cytokines, TNF α and IL-1 β , are not increased during the period of implantation (Ross *et al.*, 2003; M.D. Ashworth and R.D. Geisert, unpublished). It is clear that the expression of conceptus IL-1 β decreases acutely following rapid elongation of the conceptuses to prevent development of a chronic endometrial response that would result in rejection of the developing conceptuses. Therefore, conceptuses are likely secrete many factors to control the extent of NF- κ B activation during establishment of pregnancy.

Oestrogens secreted by pig conceptuses have been established as the maternal recognition signal that switches secretion of endometrial PGF_{2 α} from an endocrine to an exocrine direction to prevent CL regression (Bazer *et al.*, 1982). In addition to a role in CL maintenance, conceptus oestrogens may modulate uterine responses to IL-1 β activation of NF- κ B. Conceptus secretion of oestrogens mirrors the increase and decrease of uterine luminal IL-1 β between days 12 and 15 of pregnancy (Ross *et al.*, 2003). Because of the clinical pathology that can emerge if aberrant NF- κ B activity occurs in tissues, the anti-inflammatory activity of oestrogens has received increased attention in studies of inflammatory diseases (Kalaitzidis and Gilmore, 2005). Oestrogens regulate tissue gene expression via both oestrogen receptor- α and - β . Recently, ER α was proposed to suppress NF- κ B inflammatory activity through directly inhibiting intracellular transport of p65 to the nucleus (Ghisletti *et al.*, 2005), but this effect could occur at several points in the NF- κ B activation pathway. Expression of ER may inhibit activity of the kinase that phosphorylates I κ B, inhibit degradation of I κ B, block DNA binding to the NF- κ B dimer or compete for coactivators of NF- κ B (see review, Kalaitzidis and Gilmore, 2005). Activation of NF- κ B in osteoclasts by RANKL can also be inhibited by oestrogen-induction of expression of osteoprotegerin (OPG), which is a soluble TNF receptor family member that competes with RANKL for binding to RANK (Holstead-Jones *et al.*, 2002). The presence of ER α in porcine uterine epithelia (Geisert *et al.*, 1993; Sukjumlong *et al.*, 2004) and ER β in trophoblast (Kowalski *et al.*, 2002) suggests that oestrogen can potentially differentially regulate NF- κ B genes through the different ERs expressed in the conceptus and endometrium during the period of conceptus IL-1 β secretion. Increased transcription of NF- κ B activated genes may depend upon both the type of ER present and the specific pathway by which NF- κ B is activated (Cao and Karin, 2003). Activation of NF- κ B is consistent with the increase of KGF expression in porcine uterine LE on day 10 of the oestrous cycle (Ka *et al.*, 2000). Li and Rinehart (1998) demonstrated that IL-1 β stimulates KGF expression in human endometrial fibroblasts through activation of NF- κ B while chronic exposure to oestrogen induces a similar response. During pregnancy, expression of KGF in the uterine LE is enhanced at the time of conceptus IL-1 β secretion; however, oestrogen alone can also increase KGF expression in the pig (Ka *et al.*, 2001). Endometrial activation of NF- κ B provides a unique model to explain epithelial PR down-regulation and expression of

many cytokines involved with early conceptus development and implantation. Conceptus expression of IL-1 β would be consistent with continued NF- κ B activation with oestrogen providing a modulating role to prevent a full inflammatory reaction that would be detrimental to conceptus survival (Fig. 2).

Secretion of IL-1 β and oestrogen by pig conceptuses may also establish immunological interactions between the endometrium and conceptus during placental attachment. Because pro-IL-1 β lacks a signal sequence, its activation and secretion requires cleavage by an intracellular cysteine protease (Fantuzzi and Dinarello, 1999). IL-1 β -converting enzyme (ICE), also known as caspase-1, transforms IL-1 β to its biologically active form. Conceptus expression of ICE occurs coincidentally with IL-1 β secretion on days 12 and 13 of pregnancy (J.W. Ross, M.D. Ashworth and R.D. Geisert, unpublished). Although IL-1 β secretion is minimal on day 15 of pregnancy, there is a second sustained phase of oestrogen secretion by conceptuses that is necessary to maintain CL function beyond 28 days of gestation (Geisert *et al.*, 1987). The second increase of conceptus oestrogen secretion is temporally associated with a pregnancy specific 8-fold increase in endometrial ICE gene expression (M.D. Ashworth, J.W. Ross and R.D. Geisert, unpublished). The increase in endometrial ICE expression is not associated with endometrial release of IL-1 β for which the uterine lumen content is greatly decreased on day 15 and 18 of pregnancy suggesting involvement of an alternate substrate (Ross *et al.*, 2003). Another substrate for ICE, prointerleukin 18 (Pro-IL-18), has structural similarities to Pro-IL-1 β and is involved with modulation of the immune system through induction of interferon- γ (Fantuzzi and Dinarello, 1999). Although similar to IL-1 β , IL-18 binds to a unique IL-18 receptor and therefore does not function through the NF- κ B pathway (Lee *et al.*, 2004). IL-18 is expressed by endometrial epithelia (Yoshino *et al.*, 2001) and is suggested to regulate maternal-embryo interplay during establishment of pregnancy. Porcine endometrial IL-18 gene expression increases 4-fold from days 10 to 15 of the oestrous cycle and pregnancy with expression increasing by 10-fold on day 18 of pregnancy (M.D. Ashworth and R.D. Geisert, unpublished). Although speculative, increased expression of interferon- γ following trophoblast elongation and during attachment to the uterine surface from day 15 to 18 of gestation (see review, Cencic and La Bonnardiére, 2002) may be the result of increased endometrial expression of ICE, and possibly the release of IL-18 that induces secretion of interferon- γ by conceptuses to modulate the maternal immune system at the interface between trophoctoderm and uterine LE (Cencic and La Bonnardiére, 2002).

The role of oestrogen as an endocrine disruptor during early pregnancy

Although conceptus oestrogens play a major role in preventing luteolysis and inducing uterine changes in secretion and morphology necessary for implantation, the pig endometrium is very sensitive to the timing of oestrogen stimulation. Exposure of the endometrium to oestrogen before the normal period of conceptus secretion on days 11 and 12 of gestation is detrimental to conceptus survival. Consumption of feed containing the oestrogenic mycotoxin, zearalenone, causes total embryonic loss in swine (Long and Diekman, 1986). Pope *et al.* (1986) first demonstrated that administration of oestrogen to gilts on days 9 and 10 of gestation resulted in complete loss of conceptuses before day 30 of pregnancy; however, no adverse effects occurred when oestrogen was administered on days 12 to 13 (coinciding with endogenous conceptus oestrogen secretion). Our laboratory demonstrated that premature exposure of the pregnant uterus to oestrogen does not effect elongation of conceptuses on day 12, but results in degeneration of conceptuses on day 15 of pregnancy (Morgan *et al.*, 1987). We established that administration of oestrogen on days 9 and 10 of pregnancy is associated with breakdown of

the apical glycocalyx of endometrial LE which is essential for the heterologous cell-cell interactions necessary for conceptus attachment in the pig (Blair *et al.*, 1991).

The presence of ER α in endometrial LE and GE and the possible role of oestrogen in modulating NF- κ B stimulated genes suggests that premature exposure of the uterus to oestrogen may disrupt pregnancy through aberrant gene expression between days 12 and 15 of pregnancy as occurs in mice (Ma *et al.*, 2003). To evaluate this possibility, we utilised a 15K unigene porcine cDNA microarray constructed from porcine uterine, ovarian and conceptus tissues (Whitworth *et al.*, 2005), to determine the alterations in endometrial gene expression profiles following treatment of pregnant gilts with oestrogen on days 9 and 10 of pregnancy. Although a number of genes were either up or down regulated on days 10 and 15 of pregnancy, the vast majority of altered endometrial gene expression in response to early oestrogen treatment occurred on day 13 of pregnancy. Many of the differentially expressed genes modified by oestrogen are those known to be involved with cell attachment, immunology, transcriptional regulation and metabolism (Table 1). For example, expression of the osteopontin (OPN) gene was advanced by early oestrogen and this was confirmed in LE by *in situ* hybridisation (Fig. 3). Osteopontin, a proposed mediator of adhesion of trophoblast/chorion to endometrial LE through integrin receptors present on the LE apical surface (Johnson *et al.*, 2003), is stimulated by IL-1 β , TNF- α , interferon- γ and oestrogen. Therefore, alteration in OPN expression may interfere with glycocalyx formation required for conceptus attachment and implantation.

Table 1. Microarray analysis of endometrial genes altered by exogenous oestrogen administration on days 9 and 10 of gestation

	Putative identity	Fold change
Day 10	Retinol-binding protein 4	8.2
	Spermidine/spermine N1-acetyltransferase	6.8
	Integrin, beta 5	4.6
Day 13	Unknown	9.7
	Unknown	7.2
	Unknown	6.0
	F-actin capping protein alpha-1 subunit	4.9
	CD24a antigen	4.5
	Ubiquitin B precursor	4.4
	Osteopontin	3.4
	Aldo-keto reductase family 1	-13.9
	Aldose reductase	-9.6
	Stathmin 1	-4.5
	Plastin 1 (I isoform)	-4.4
	Programmed cell death 1 ligand 1	-4.1
	Neuromedin B	-3.7
Unknown	-3.5	
Day 15	Unknown	4.3
	Unknown	3.0

Negative value in fold change indicates gene down-regulated by oestrogen treatment

Early oestrogen administration also altered the pattern of COX-2 expression in endometrial LE (Ashworth *et al.*, 2004) suggesting effects on timing of NF- κ B activation. The role of COX-2 expression in LE during establishment of pregnancy has not been investigated; however, it is known that COX-2 null mice have an altered inflammatory response resulting in defects causing implantation failure (Lim *et al.*, 1997). Pharmacological inhibition of PG synthesis through

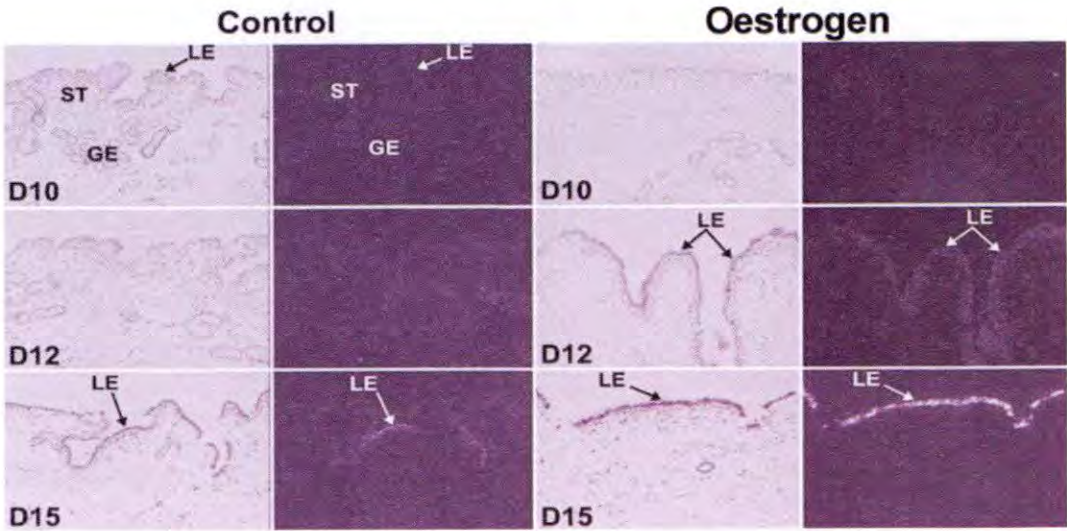


Fig. 3 *In situ* hybridisation of endometrial osteopontin gene expression in gilts treated with either vehicle (control) or oestrogen on days 9 and 10 of pregnancy. Stroma (ST), Luminal Epithelium (LE) and Glandular Epithelium (GE).

inhibition of COX does not affect trophoblast elongation in pigs (Geisert *et al.*, 1986), but it does increase embryonic mortality during the peri-implantation period (Kraeling *et al.*, 1985). Induction of COX-2 expression promotes PGE synthesis (Murakami and Kudo, 2004), and it is known that endometrial secretion of PGE increases during peri-implantation in pigs (see Bazer *et al.*, 1982), perhaps to stabilise the extracellular matrix, inflammation and immune functions.

Treatment of gilts with oestrogen may advance the uterine environment beyond the 24 h window of synchrony necessary for embryonic survival (Polge, 1982). Indeed early exposure of pregnant gilts to oestrogen disrupts the normal synchrony between the presence of insulin-like growth factors (IGF) in uterine lumen and rapid conceptus elongation on day 12. Concentrations of IGF-I are elevated within the uterine lumen prior to and during rapid trophoblast expansion on days 10 to 12 of pregnancy in pigs (Simmen *et al.*, 1995; Geisert *et al.*, 2001). Content of IGF-I in uterine flushings decreases dramatically following conceptus elongation and initiation of implantation on day 13 of gestation. The high lumen content of IGF-I is closely associated with the presence of IGF binding proteins (IGFBPs) (Lee *et al.*, 1998; Geisert *et al.*, 2001). Disappearance of uterine luminal IGFBPs on day 11 or 12 of either the oestrous cycle or pregnancy precedes the decline in IGF-I (Geisert *et al.*, 2001). Disappearance of IGFBP's from uterine luminal flushings is caused by an increase in IGFBP proteolysis rather than down-regulation of IGFBP mRNA (Lee *et al.*, 1998). The proteolysis of IGFBPs in the porcine uterine lumen may occur due to activation of serine proteases, tissue kallikrein and/or metalloproteinases (Lee *et al.*, 1998; Geisert *et al.*, 2001). IGF's are regulated and stabilised through tertiary binding to IGFBPs, thus degradation of IGFBP's within the uterine lumen may be responsible for the decreased content of IGF-I in uterine flushings collected after day 13. It is possible that IGFBPs sequester IGF-I in the uterine lumen for release during the period of conceptus differentiation and trophoblast elongation. The precise timing of the loss of uterine luminal IGFs following conceptus elongation suggests that release of IGFs on days 12 and 13 of pregnancy is critical for subsequent development and survival of pig conceptuses. Administra-

tion of oestrogen to gilts on days 9 and 10 of pregnancy causes premature proteolysis of uterine luminal IGFBPs on day 10 and an earlier (48 h) decline of IGF-I on days 10 and 11 (M.D. Ashworth and R.D. Geisert, unpublished). Although a causal effect of premature loss of IGFs with later conceptus deaths has not been established, we demonstrated that early oestrogen administration clearly results in a dramatic decline in IGFs before the critical period of conceptus elongation and differentiation. Thus, alterations in the normal synchrony between IGF release into the uterine lumen and conceptus development during early pregnancy may cause aberrant endometrial and/or conceptus gene expression leading to conceptus loss during the peri-implantation period.

Conclusion

Evidence indicating involvement of the NF- κ B system in regulating endometrial function during the oestrous cycle and early pregnancy has led to the development of a working hypothesis to explain how progesterone regulates the oestrous cycle and early conceptus development in the pig. This working hypothesis is supported by results summarised in this paper and provides the basis for novel approaches for mechanistic studies of uterine PR regulation and oestrogen effects on conceptus survival. Developing an understanding of NF- κ B regulation during early pregnancy and how this transcription factor modulates expression of genes essential for conceptus development and implantation will greatly enhance our ability to improve reproductive efficiencies in swine. Involvement of oestrogen in regulating NF- κ B activation provides a clue as to how disruption of NF- κ B activation by early oestrogen action on the uterus prevents normal interactions between the conceptus and uterine environment that leads to conceptus death.

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