ANALYSIS OF SOME NOVEL UTERINE EXTRACELLULAR MATRIX PROTEINS AND A GROWTH FACTOR

A Dissertation

by

SAEED Y. AL RAMADAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2007

Major Subject: Veterinary Anatomy

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ABSTRACT

Analysis of Some Novel Uterine Extracellular Matrix Proteins and a Growth Factor. (May 2007)

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This dissertation focused on two classes of molecules implicated in processes of implantation and placentation in sheep and pigs. Study one examined the temporal/spatial distribution of several Small Integrin-Binding Ligand, N-Linked Glycoprotein (SIBLING) family members in cyclic and pregnant ovine uterus. Studies two and three evaluated the relationships between progesterone (P4) and estrogen (E2) and their receptors (PGR and ESR1, respectively) on FGF7 mRNA expression within the endometrium and placenta of pigs.

Study one showed that dentin sialophosphoprotein (DSPP) was first detected in luminal epithelium (LE) of Day 15 cyclic and pregnant sheep. Stromal expression of DSPP was first detected on Day 20 of pregnancy in stratum compactum and remained prominent in stroma through Day 120. Stromal DSPP protein was positively influenced by the conceptus based upon analysis of a unilaterally pregnant ewe model system. Immunoreactive dentin matrix protein 1 (DMP1), matrix extracellular phospoglycoprotein (MEPE) were localized to

the stroma of cyclic and pregnant sheep, however, these proteins appeared to be constitutively expressed. BSP was not detected in ovine endometrium.

Study two determined the effects of E2, P4, P4+E2, P4+the PGR antagonist (ZK137, 316), and P4+E2+ZK on FGF7 mRNA expression in uterine LE of ovariectomized pigs. Results indicate that P4 is permissive to FGF7 mRNA expression by down-regulating PGR in LE; P4 stimulates PGR-positive uterine stromal cells to release an as yet unidentified progestamedin that induces FGF7 mRNA expression by LE; E2 and P4 can induce FGF7 mRNA in the absence of PGR rendered nonfunctional by ZK.

Study three showed the expression of ESR1, PGR and FGF7 in the uterine and placental tissue of pregnant pigs from Day 20 through 85. Results reveal a positive correlation between stromal cell expression of PGR and FGF7 mRNA which suggests that P4 is permissive to FGF7 mRNA expression by down-regulating PGR in LE. FGF7 mRNA in later pregnancy is maintained by the release of progestamedin from PGR-positive stromal cells. A novel finding was the presence of ESR1 in porcine placenta on Days 20 through Day 85 of pregnancy suggesting that E2 may play important roles in the placental biology of the pig.

DEDICATION

To my mother, who counts days and hours to see me home. Thank you mother for your patience, consistent prayers, encouragement, and support

To my wonderful wife, Fatemah and our children Sarah, Kawthar, Ahmed, Abdullah and Zainab for all their love, sacrifice and patience with me during this period of my life

To my brothers and sisters for all their long distance support

To all my friends for their continuous support and encouragement

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Last but not least, I would like to thank my sponsor, King Faisal University, for giving me the opportunity to pursue my Ph.D. degree in a pioneer university in the field of veterinary biomedicine.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

This dissertation is focused on the expression of several proteins which are thought to play a role in the uterus during early pregnancy. The following literature review first considers events occurring in the uterus of the two animal models, sheep and pig, used in these investigations including maternal recognition of pregnancy, implantation and placentation. This is followed by an overview of members of two protein families investigated in this dissertation including the SIBLING family of extracellular matrix proteins and fibroblast growth factor 7, a member of the fibroblast growth factor family.

Maternal Recognition of Pregnancy

In domestic mammals, ovulation is spontaneous and the reproductive cycles are dependent on the uterus, because it is the source of the luteolytic factors that lead to the structural and functional regression of the corpus luteum (CL), which is the main source of the pregnancy hormone, progesterone (P4). During early diestrus, P4 from the newly formed CL stimulates accumulation of phospholipids in the uterine LE that can liberate arachidonic acid for synthesis and secretion of the luteolytic factor, prostaglandin F2α (PGF) (McCracken *et al.* 1999). Oxytocin, secreted from the posterior pituitary and/or CL, then induces release of PGF pulses from the LE which results in luteolysis and declining P4 levels (Wathes & Lamming 1995). However, in case of pregnancy and before any intercellular contacts are

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This dissertation follows the style of *Reproduction.*

established, signals from the embryo act on the endometrium to prevent uterine release of luteolytic PGF to sustain the function of the CL during the early stages of pregnancy, a process referred to as 'maternal recognition of pregnancy' (Spencer & Bazer 2004).

Maternal recognition of pregnancy in sheep

During diestrus of sheep, P4 levels increase and act via progesterone receptor (PGR) to block the expression of E2 receptor α (ESR1) and oxytocin receptor (OTR) in the LE and superficial ductal glandular epithelium (sGE). Therefore, ESR1 and OTR expression are not detected in the LE between Days 5 and 11 of the cycle, i.e., during most of diestrus (McCracken *et al.* 1984). Moreover, continuous exposure of the uterus to P4 for 8 to 10 days down-regulates expression of PGR in the LE and sGE after Days 11 to 12, allowing for rapid increases in expression of ESR1 on Day 13 followed by OTR on Day 14 in LE and sGE (Spencer *et al*. 1995 *a,b*). Oxytocin, secreted at Day 9 of the estrous cycle and pregnancy from the posterior pituitary and/or CL, then induces release of luteolytic PGF pulses from the endometrium on Days 14 to 16 (Wathes & Lamming 1995, Gray *et al*. 2000). In sheep, however, the source of luteolytic PGF pulses is the LE and sGE (Gray *et al*. 2000), because they express the oxytocin receptors (OTR) (Wathes & Lamming 1995) and are the only uterine cell types that express cyclooxygenase 2 (COX-2), a rate limiting enzyme in the synthesis of prostaglandins (Kim *et al*. 2003). These luteolytic pulses of PGF make the CL undergo regression, allowing the ewe to return to estrus and complete the 17-day estrous cycle. In the sheep interferon tau (IFNτ), which is secreted by trophectoderm of the elongated conceptus between Days 10 to 25 with maximal production on Days 14 to 16, is the signal for the maternal recognition of pregnancy (Bazer 1992, Roberts *et al*. 1999). Further, IFNτ

appears to be the sole factor produced by the conceptus that prevents development of the endometrial luteolytic mechanism (Spencer & Bazer 2004). It is most likely that IFNτ acts in a paracrine fashion on endometrial LE and sGE to suppress transcription of ERα which presumably inhibits OTR genes to abolish the development of the endometrial luteolytic mechanism (Spencer *et al.* 1995*a*, Spencer & Bazer 1996, Fleming *et al*. 2001, Spencer *et al.* 2004*a*). However, the 5' promoter/enhancer region of the ovine OTR gene was cloned and the binding site for ESR1 was not found; rather proximal SP1 sites mediated ESR1 action (Fleming et al. 2006). Thus IFNτ may directly inhibit OTR gene transcription.

Maternal recognition of pregnancy in pig

In the pig, establishment of pregnancy begins about 11-12 days after the start of estrus. The ability of pig conceptuses to synthesize and release estrogens during this period, as well as the ability of exogenous estrogens to induce pseudopregnancy when administered from Day 11-15 of the estrous cycle, provides evidence for an involvement of estradiol-17β (E2) in the maternal recognition of pregnancy in the pig. It had also been reported (Geisert *et al*. 1982*a*, Bazer *et al.* 1986, Vallet & Christenson 1996) that E2 derived from the conceptus or from administration to cyclic gilts stimulates uterine secretion of calcium and total proteins on Day 11-12. The proposed role for estrogen in the maintenance of pregnancy in the pig is the prevention of luteolysis through reorientation of endometrial PGF release from an endocrine to an exocrine mechanism, i.e. redirection the PGF secretion from directly into the blood circulation to the uterine lumen (Perry *et al*. 1973, Bazer & Thatcher 1977, Geisert *et al*. 1982*b*). It was also reported that estrogen is secreted by the pig conceptuses in a biphasic pattern with the initial surge of estrogen secretion occurring on Day 12 and the

second phase of a high level of estrogen secretion occurring on Days 15-20. Geisert et al. (1990) suggested that the second surge of estrogen secretion is necessary for prolonged CL maintenance. Another proposed mechanism of estrogen action is that it possibly acts directly or indirectly on the CL by a mechanism not involving the uterus (Ziecik 2002). This theory was based on the findings from intact cyclic gilts treated with anti-luteolytic doses of estrogen on Day 12 after estrus resulted in a delay in luteal regression and was accompanied by a three-fold increase in luteinizing hormone receptor concentration at the CL. Similar effects could be obtained from gilts hysterectomized on Days 6-9 after estrus and treated with estradiol benzoate on Day 12 (Garverick *et al*. 1982). These findings lead Ziecik (2002) to conclude that estrogen may increase the LH receptor levels without involving the uterus but through effects on circulating concentrations of the pituitary hormones. According to this theory, it is possible that LH can stimulate prostaglandin secretion from endometrium of pigs through increasing expression of some angiogenic growth factors, like vascular endothelial growth factor (VEGF). However, no further details were given for the mechanism of how these growth factors work to maintain the CL and hence, the pregnancy. Finally, based on the assumptions that E2 could change PGE2:PGF2α secretion ratio (Zhang & Davis 1991) and that PGE2 has a luteotrophic/anti-luteolytic effect (Akinlosotu *et al*. 1986; 1988), E2 may protect the CL from the luteolytic effects of PGF through increasing the PGE2:PGF2α ratio.

Implantation

During maternal recognition of pregnancy, the uterine environment is changed from a non-receptive to a receptive state. These changes require structural and functional modulation of the endometrium in order to support intimate contact of the balstocyst with the uterine

wall in a process referred to as implantation, which will culminate with the formation of placenta. This reproductive strategy ensures efficient nutrition and protection of the embryo(s) and thus promotes their survival. However, this process is evolutionarily recent and therefore the mechanism is highly diverse among taxonomic groups. In contrast, the processes between fertilization and the initiation of implantation are highly conserved. These processes include shedding of the zona pellucida, followed by orientation, apposition, attachment and firm adhesion of the blastocyst to the endometrium (Lee & DeMayo 2004, Spencer *et al*. 2004*a*). In fact, these processes require both that the uterus has undergone a specified sequence of differentiation controlled by ovarian steroids and that the blastocyst has reached a precise stage of activation and both the blastocyst and the uterus must operate in synchrony. They must express, at the same time, the precise molecular repertoire required for adhesive interaction between TR and LE (Kimber 2000).

Regardless the types of the implantation and placentation, the adhesion process begins with a close interaction of embryonic trophoblast, the outermost extraembryonic cell layer, and endometrial LE cells which involves intimate interactions of apical surfaces of the LE of the uterus and trophoblast cells from the embryonic side. This event is mediated through the binding of adhesion molecules secreted from the glandular epithelium (GE) and/or LE to receptors on the apical surface of the conceptus trophectoderm and LE (Bowen & Burghardt 2000). For the embryo to implant, it must first come in contact with the apical surface of the LE (apposition), after which it undergoes firmer attachment. Attachment may continue throughout pregnancy in the epitheliochorial placentation of pig or be a transient event, as in the mouse and human (Kimber 2000, Kimber and Spanswick 2000).

In primates and some subprimate species such as rodents, implantation is marked by a significant degradation of part of the maternal endometrial tissues by the invasive trophoblast to bring the fetal membranes and blood vessels in close contact to the maternal blood circulation. However, for species with lesser invasive types of implantation, like the domestic animals, the alterations in the endometrial surface are minimal or involve molecular interactions at the apical surfaces of apposing uterine and conceptus epithelia. For example in the pig, the shift in the status from non-receptive to receptive is marked by a decrease in PGR and ESR1 in the LE, an increase in the expression of some extracellular matrix proteins and/or changes in the pattern of the integrin expression (Geisert *et al*. 1993, 1994). In contrast, stromal, myometrial and some vascular cells maintain these steroid receptors, which are likely mediate the action of these steroid hormones through the secretion of specific factors (estromedins and progestamedins) that act on the LE (Spencer & Bazer 2002, Spencer *et al*. 2004*a*). However, the paradigm of loss of PGR in uterine epithelia immediately before implantation is common across mammals (Carson *et al.* 2000, Spencer *et al*. 2004*a*) and can be directly correlated with reduced expression of certain genes, such as the antiadhesive protein MUC1 or induction of others like secreted phosphoprotein 1 (SPP1, also known as osteopontin) (Johnson *et al*. 2001, 2003*a*).

Implantation in sheep

Areas of contact between the trophectoderm and the uterine epithelium can be detected at Day 15 of pregnancy. On Day 16, uterine lavage to recover the conceptus causes superficial structural damage to the conceptus at this time. The interdigitation of the trophectoderm and endometrial LE occurs in both the caruncular and intercaruncular areas of

the endometrium. Adhesion of the trophectoderm to the endometrial LE progresses along the uterine horn and appears to be completed around day 22 (Bowen & Burghardt 2000, Spencer *et al*. 2004*b*). In the intercaruncular spaces, the trophoblast develops fingerlike villi or papillae, which penetrate into the mouths of the superficial ducts of the uterine glands at Days 15–18 then disappear at Day 20 (Guillomot *et al*. 1981, Wooding *et al*. 1982). These trophoblastic differentiations are hypothesized to anchor the peri-attachment conceptus and absorb the histotrophic secretions of the glands (Guillomot *et al*. 1981). Furthermore, the trophoblast papillae are hypothesized to facilitate the formation of more robust adhesive interactions between the trophoblast and endometrial LE (Wooding *et al*. 1982, Spencer *et al*. 2004*b*).

At this stage of implantation, two morphologically and functionally distinct cell types have been recognized in the trophectoderm of the placenta of ruminant animals. These are the mononucleate trophoblast cells and the giant binucleate trophoblast cells (Igwebuike 2006). The trophoblast giant binucleate cells (BNC) are thought to be differentiated from the mononuclear trophoblast by Day 16 (Hoffman & Wooding 1993).Giant BNCs typically exhibit the capacity for migration or invasion, although the degree of migratory activity varies among species while mononuclear trophoblast cells are able to adhere to the endometrial LE (Hoffman & Wooding 1993, Guillomot 1995). Interestingly, maximum expression of IFNτ could be observed at Day 14 and the level decreased by Day 16 of pregnancy. Moreover, the expression of IFNτ was arrested in the regions of trophoblast which have established cellular contacts with the uterine epithelium during the implantation process (Guillomot *et al*. 1990). At least two main functions for BNC have been reported: 1) to form a hybrid fetomaternal syncytium essential for successful implantation and subsequent

placentomal growth; 2) to synthesize and secrete protein and steroid hormones, such as placental lactogen and P4 that regulate maternal physiology (Wooding 1982, Hoffman & Wooding 1993, Spencer *et al*. 2004*b*).

Implantation in pig

Between Days 12 to 13 after fertilization the attachment process commences (Keys & King, 1990). However, implantation in this species remains superficial. However, the trophoblast and LE become closely apposed and the adhesion process appears to be facilitated by the modification of the glycocalices associated with epithelial and chorionic cells. The apical surfaces of these cells are characterized by loss of microvilli on embryonic cells and reduction in the thickness of anti-adhesion molecules like MUC1 enhancing access for blastocyst attachment (Bowen *et al*. 1996). Interestingly, although the pig demonstrates true epitheliochorial placental development, the barrier to invasiveness is a property resident in the LE because, like blastocysts of other species, the pig blastocyst is inherently invasive and will invade and undergo syncytium formation when transplanted to an ectopic site (reviewed in Burghardt *et al*. 2002). It is noteworthy that both the conceptus and uterine LE express a number of common integrin subunits including α1, α 3, α 4, α 5, α v, ß1, ß3, and ß5, and of these, the α 4, α 5, α v, ß1, ß3 andß5 subunits are detected at sites of initial attachment between uterine LE and TR on Days 12–15 of pregnancy (Bowen *et al*. 1996).

Once the union between the trophectoderm and LE cells is established, conceptus microvilli reform and begin to interdigitate with counterparts on uterine epithelial cells to consolidate association (Burghardt *et al*. 1997; King 1993). The blood vessels that develop from the blastocyst and those from the uterus do not traverse respective epithelia, so the

integrity of both the maternal and fetal tissues is not disrupted (Carson *et al*. 2000). It is noteworthy that estrogens produced by the conceptus stimulate increases in ultrastructural features associated with synthesis and secretory activity, accumulation of glycogen, variation in nuclear position, elaboration of a thick glycocalyx, and reduced cellular degeneration in LE (Keys & King 1992).

Placentation

Placentation is the growth and formation of the placenta (which consists of four layers, the chorion, amnion, yolk sac and allantoic sac) and the development of the uterine capacity to supply the nutrients and oxygen required by the fetus. Although the placentas of all eutherian species provide common functional purpose, there are species variations in the gross and microscopic levels. In ruminant ungulates, the placenta is classified as cotyledonary on the basis of its gross anatomical features and it exhibits discrete areas of attachment, the placentomes, which are formed by interaction of patches of the chorioallantois with the endometrium. The fetal portion of the placentome is the cotyledon, while the maternal contact sites are the caruncles. The richly vascularized chorioallantois is lined on its external surface by cells of the trophectodermal epithelium. These cells assume specialized functions and are referred to as trophoblast cells. In ruminants, chorionic binucleate cells migrate across the fetomaternal space and fuse with and become incorporated into the LE, forming hybrid of tri-and multinucleated syncytia (Wooding *et al*. 1993). On the other hand, pigs have diffuse, non-invasive placenta classified as being epitheliochorial where the uterine epithelium of the pig inhibits trophoblast cell migration so that fetal and maternal tissues remain separate and intact throughout gestation (King 1993).

In general, implantation and placentation are dependent on the establishment and maintenance of an adequate fetomaternal contact. Consequently, functional coordination of trophoblast, LE, stroma, immune cells, and vascular endothelial cells must occur during different stages of placental development. This type of coordination has been considered to be mediated by hormones, transcription factors, growth factors, cell adhesion molecules, and extracellular matrix proteins (Regnault *et al*. 2002, Wei *et al*. 2004). In the next section, focus will be directed toward SIBLING proteins, a group of related extracellular matrix proteins, and Fibroblast Growth Factor 7, a member of the large fibroblast growth factor family which were investigated in this dissertation research.

The Small Integrin-Binding Ligand *N*-Linked Glycoprotein Family

The small integrin-binding ligand *N*-linked glycoprotein (SIBLING) gene family includes five closely related members: bone sialoprotein (BSP), secreted phosphoprotein 1 (SPP1), dentin matrix protein 1 (DMP1), matrix extracellular phosphoglycoprotein (MEPE), and dentin sialophosphoprotein (DSPP). A sixth protein, enamelin (ENAM) has been identified as a more distant member of the family (Fisher & Fedarko 2003).

SIBLING proteins are found in the bone and dentin and are secreted into the extracellular matrices (ECM) during the formation and mineralization of these tissues (Fisher & Fedarko 2003; Qin *et al*. 2004). In the human genome SIBLING protein genes are clustered within a 375 kb region on human chromosome 4q21, in chromosome 14 of the rat, and chromosome 5 of the mouse (Huq *et al*. 2005). Moreover, it was reported that SIBLNG protein genes have similar exon structures. For example, exon 1 is always noncoding, exon 2 is the leader sequence and the first two amino acids of the mature protein, exons 3 and 5

usually contain the consensus sequence (SSEE) for casein kinase II (CKII) phosphorylation. Exon 4 is usually proline rich, and among the acidic proteins, is the only significantly positive-charged domain. However, the majority of each protein is encoded by the last one or two exons and contains the integrin-binding tripeptide Arg-Gly-Asp (RGD) (Fisher & Fedarko 2003; Huq *et al*. 2005).

The dominant view of protein structure-function is that an amino acid sequence specifies a three-dimensional (3-D) structure that is a prerequisite for protein function. In contrast, many proteins display functions requiring intrinsic disorder. Moreover, proteins from eukaryotes apparently have more intrinsic disorder than those from prokaryotes with typically 30% of eukaryotic proteins having disordered regions of length $\geq$ 50 consecutive residues (Dunker *et al*. 2002). Of the SIBLING proteins, BSP and SPP1 have been reported to be completely unstructured and flexible in solution based on NMR spectroscopy studies (Fisher *et al*. 2001). Recently, it was also reported that dentin phosphoprotein (DPP), a domain encoded by the *DSPP* gene, is a uniformly flexible molecule (Cross *et al*. 2005). The lack of structure may be a useful property in molecular recognition because disordered regions enable high specificity coupled with low affinity. For example, one protein can bind to differently shaped partners, different disordered sequences can fold to bind a common binding site, faster rates of association can be achieved by reducing dependence on orientation factors and by enlarging target sizes, and faster rates of dissociation may be achieved by unzippering mechanisms (Huq *et al*. 2005; Dunker *et al*. 2001, 2002). The properties displayed by disordered proteins explain the multifunctionality reported for the SIBLING proteins. For example, SPP1 and BSP are able to rapidly associate with a number of different binding partners, e.g. integrin and factor H, as well as the mineral phase of bones

and teeth and these features may also be reflected in broad tissue distribution of SPP1 (Huq *et al*. 2005).

Because of their primary association with cells that produce mineralized extracellular matrix, SIBLING proteins have been hypothesized to play roles in mineralization. However, there is now accumulating evidence showing that members of this protein family can be synthesized and secreted in nonmineralized tissues. DMP1 protein was localized in mouse brain, pancreas, and kidney by immunohistochemistry (Terasawa *et al*. 2004). Moreover, the expression of BSP, SPP1, DMP1, MEPE and DSPP were detected in the salivary gland of the mouse and the kidney of the monkey (Ogbureke & Fisher 2004, 2005). SPP1 expression could also be detected at the luminal surfaces of different organs and cell types including the gastrointestinal tract, gall bladder, pancreas, urinary and reproductive tracts, lung, breast, salivary glands, and sweat glands and by leukocytes, smooth muscle cells (Brown *et al.* 1992, Ashkar *et al*. 2000, Garlow *et al*. 2002). Additionally, there are extensive reports describing SIBLING protein expression in different cancer tissues (Hotte *et al*. 2002, Fisher *et al*. 2004, Rangaswami *et al*. 2006, Chaplet *et al*. 2003, 2006, Karadag *et al*. 2004).

SIBLINGs in reproduction

In the reproductive tract, SPP1 is hypothesized to be a critical component of pregnancy in different animal species and its role in reproduction of humans, rabbits, rodents, sheep, goats and pigs has been the focus of a number of studies during the past few years (Johnson *et al*. 1999a, 2000, 2001, 2003*a*, Garlow *et al*. 2002, Joyce *et al*. 2005, White *et al*. 2005, 2006). The precise physiological role of SPP1 in pregnancy has not been elucidated fully, but there is growing evidence that SPP1 influences the uterine environment as: 1) a

component of histotroph required for adhesion and signal transduction at the uterine-placental interface; 2) a gene product expressed by uterine stroma as it decidualizes in response to conceptus invasion; and 3) a product of resident placental and uterine immune cells that regulates their behavior and cytokine production (Johnson *et al*. 2003*a*). Early studies focused on the role of epithelial-derived SPP1 as a P4-induced secretory product of uterine glandular epithelium (GE) that binds receptors on the apical surface of uterine LE and conceptus trophectoderm to stabilize adhesion between uterus and conceptus for implantation (Garlow *et al*. 2002, Johnson *et al*. 2000, 2001). Later studies were focused on the expression of SPP1 in the stroma of sheep, which provided novel evidence that stromal SPP1 expression is linked to a decidualization-like differentiation response during pregnancy (Johnson *et al*. 2003*a*). In this respect, *SPP1* mRNA and protein have been detected in decidualized stroma of species that undergo invasive implantation, including mice, baboons, and humans and is considered to be a gene marker for decidualization (Burghardt *et al*. 2006, Carson *et al*. 2002, Briese *et al*. 2005).

It is noteworthy that the endometrial stromal cells in pregnant ewes were reported to change following conceptus attachment, and exhibited morphological changes about the time when the expression of *SPP1* mRNA and protein was observed in uterine stromal compartment. In contrast, neither morphological changes in uterine stroma nor induction of *SPP1* mRNA and protein were detected during porcine pregnancy (Johnson *et al*. 2003*b*). Although both sheep and pigs have noninvasive implantation, there are subtle differences in the in type of placentation. Pig conceptuses undergo true epitheliochorial placentation in which LE remains morphologically intact throughout pregnancy and the conceptus trophectoderm simply attaches to the apical LE surface without contacting uterine stromal

cells. In contrast, sheep have synepitheliochorial placentation where LE cell disintegration results in areas of epithelial erosion into which trophectoderm binucleate cells migrate and eventually fuse with remaining epithelial cells to form syncytia; however, invasion into the stroma does not occur. Therefore, Johnson and coworkers (2003*b*) have suggested that the uterine stroma of sheep undergoes a program of differentiation that has features that are similar to decidualization in invasive implanting species. Porcine stroma exhibits a type of differentiation that is more limited than that observed in sheep, rodents, or primates. Results suggest that uterine stromal decidualization and decidualization-like responses are common to species with different types of placentation, but the extent is variable and correlates with the depth of trophoblast invasion during implantation (Johnson *et al*. 2003*b*).

SIBLINGs and MMPs

Matrix metalloproteinases (MMPs) form a family of structurally and functionally related zinc-dependent proteolytic enzymes. They are synthesized as secreted or transmembrane proenzymes and processed to an active form by the removal of an amino-terminal propeptide. Collectively, these MMPs are capable *in vitro* and *in vivo* of degrading many extracellular matrix protein components such as interstitial and basement membrane collagens, proteoglycans, fibronectin and laminin (Massova *et al.* 1998, Bode *et al.* 1999). To date, at least 26 members of the MMP family have been reported and all contain a propeptide and a catalytic domain. Due to their potential for tissue damage if inappropriately activated, the MMPs are tightly regulated at different levels. These include: 1) the transcriptional level, depending on various cis-elements present in their gene promoter, and various factors (e.g. hormones, cytokines, and growth factors) able to induce gene expression; 2) at the secretion

level, depending on packaging in secretion granules; or 3) at protein level by either an activation of the zymogen form or by inhibition of the active form through tissue inhibitor of matrix metalloproteinase (TIMPs). Further, regulation might depend on the stabilization of the MMPs by posttranslational modification such as glycosylation (Van den Steen *et al*. 2001, Cohen *et al*. 2006). In addition, nitric oxide (NO) and peroxynitrite are involved in the cleavage of the MMPs proenzymes, leading to the formation of the enzymes with biological activity in different cell types (Zhang *et al*. 2004, Biondi *et al*. 2005). Regardless of the regulatory procedure, it is widely accepted that the inhibitory propeptide must be removed before the MMP can be enzymatically active. After the removal of the propeptide, the MMP typically remains active until a TIMP binds and inactivates the protease.

To date, several MMP members have been reported in association with implantation and placentation events including MMP1, MMP2, MMP3, MMP7 and MMP9, MMP14, MMP15, MMP26. However, MMP2 and MMP9 are the most studied in trophoblastic cell invasion (Isaka *et al*. 2003, Cohen *et al*. 2006). Interestingly, there is a report showing that three members of the SIBLING family can specifically bind and activate three different MMPs. Activation includes both making the proMMPs enzymatically active and the reactivation of the TIMP inhibited MMPs (Fedarko *et al*. 2004). Bone sialoprotein was shown to specifically bind proMMP-2 and active MMP-2, while SPP1 binds proMMP-3 and active MMP-3, and dentin matrix protein-1 binds proMMP-9 and active MMP-9. Subsequent studies confirmed these findings by colocalizing different MMPs to their partner SIBLING family members in different tissues (Ogbureke & Fisher 2004, 2005; Karadag *et al*. 2005). Collectively, these studies indicate that, even in the presence of TIMPs, MMPs may be enzymatically active in regions of locally high concentrations of specific SIBLINGs. It is

noteworthy that these SIBLING proteins have been correlated with cancer progression and metastasis (Fisher *et al.* 2004); it is therefore interesting to consider that these proteins may be locally activating their corresponding proteases in implantation and trophoblastic cell invasion.

SIBLINGs and factor H

Factor H is a multidomain, multifunctional plasma protein that was first isolated in 1965, and is present in human plasma at a concentration of about 500 µg/mL. In the immune system, Factor H is a central discriminator of self and non-self structures by acting as a regulator of the early activation cascade of the complement system (Zipfel, 2001). The complement system found in the blood of mammals is composed of about 26 proteins that combine with antibodies or cell surfaces as part of host humoral surveillance and plays a role in inflammation, immune adherence and cell lysis. The complement system can be activated via two distinct pathways: the classical (antibody initiated) pathway and the alternate pathway. The classical and alternate pathways consist of a series of humoral protease activation cascades that result in cell lysis (Maarten *et al*. 1985). One of the complement components, C3, plays a central role in the complement system, and is common to both pathways. C3 along with other plasma glycoproteins engage in an activation cascade that results in lysis of foreign cells or in their opsonization as a prelude to phagocytosis. Cleavage of C3 by C3 convertases gives rise to two activated fragments, the anaphylatoxin C3a-a vasoactive peptide and C3b. Surface-bound C3b acts as a cofactor in the formation of C5 convertase and thus can complete activation of the complement cascade (Maarten 1985, Liszewski *et al*. 1991). Basically, all cells are subject to low levels of this attack all the time,

but only cells that cannot inactivate the early steps of the cascade, C3b convertase formation, are killed. Cells that are destined to become transformed and escape the complement system may up-regulate genes that help to control this aspect of immune surveillance. The ability to bind and use the natural alternate complement pathway (ACP) inhibitory actions of Factor H would be one method of escape. Factor H inhibits the production of C3b by inhibiting the binding of Factor B to membrane-bound C3b, thereby preventing cleavage of B to Bb and production of the C3 convertase, C3b2b. Factor H also accelerates Factor I-mediated cleavage of C3b and sterically inhibits C5 binding to C3b (Bozas *et al*. 1993).

Findings from a number of studies have shown that some of the SIBLING proteins are expressed by malignant tissue and trophectoderm and along the fetal-maternal interface (Bianco *et al*. 1991, Rowe *et al*. 2000, Hotte, 2002, Burghardt *et al*. 2002, Johnson *et al*. 2003*c*, Fisher *et al*. 2004). In fact, trophoblasts and metastasizing cancer cells are exposed to the highest levels of complement because they are in direct contact with host blood. To survive, trophoblasts and neoplasms need to directly control the complement activity on their surfaces and thus aid their escape not only from direct lysis but also from being opsonized by the alternative complement pathway. Additionally, macrophages, the effector cells in immune surveillance, are activated by the alternate complement pathway. Thus, agents that inhibit or down-regulate complement decrease both the direct lysis pathway of complement as well as macrophage activation, and potentially promote trophectoderm and tumor cell survival (Mevorach *et al*. 1998). The expression of SIBLING proteins in tumor and /or trophectoderm cells could provide such a selective advantage for survival. In this regard, some SIBLING members have been reported to bind strongly to Factor H and block the lytic activity through either the $\alpha v\beta 3$ integrin or CD44. Binding of Factor H to SPP1, BSP or

DMP1 was localized to the cell surface and all three proteins were shown to act in conjunction with Factor I, a serum protease that, when complexed to appropriate cofactors will lead to digestion of the bound C3b in a series of proteolytic steps and that will result in sequestration of Factor H to the cell surface and inhibition of complement-mediated cell lysis (Fedarko *et al.* 2000, Jain *et al*. 2002).

SIBLINGs and integrins

Integrins are a large family of heterodimeric transmembrane glycoproteins that attach cells to extracellular matrix proteins (Hynes 1992). The central role of integrins in the implantation adhesion cascade is thought to stem from their ability to bind ECM ligand(s) to mediate adhesion, cause cytoskeletal reorganization to stabilize adhesion, and transduce cellular signals through numerous signaling intermediates (Burghardt *et al*. 1997, Johnson *et al*. 2001). Integrins consist of non-covalently linked α and β subunits, each of which has a relatively large extracellular and a short cytoplasmic domains connected by a transmembrane segment. The N-terminal domains of the α and β subunits associate to form the integrin headpiece which contains the ligand binding site. The C-terminal segment traverses the plasma membrane and this portion of the β subunit mediates interaction with the cytoskeleton as well as with the signaling proteins (Giancotti 2003). Integrins can mediate "outside-in" and "inside-out" signaling in two processes referred to as signaling and activation, respectively (Giancotti and Ruoslahti, 1999). Ligand binding through an integrin receptor results in specific effects on cytoskeletal organization and activation of signaling pathways which lead to regulation of gene expression. In addition, integrin cytoplasmic domains transduce cell type-specific signals that modulate ligand binding affinity (Burghardt *et al*.

2002). Many members of the integrin family, including α5β1, α8β1, αIIbβ3, αvβ3, αvβ5, αvβ6 and αvβ8, recognize an Arg-Gly-Asp (RGD) motif within their ligands. These ligands include Fn (fibronectin), fibrinogen, vitronectin, SIBLING proteins, and many other large glycoproteins. In fact, peptides containing this motif can efficiently block these integrin-ligand interactions (Fisher & Fedarko 2003, Takagi 2004). The RGD sequence is a very important functional motif found in all members of the SIBLING family (Fisher *et al*. 2001). SPP1, and presumably other SIBLINGs have additional non-RGD integrin binding sequences which suggests additional integrin-SIBLING interactions.

In the reproductive tract, some members of the integrin family have been reported to have spatial and temporal relationships with blastocyst development and implantation (Burghardt *et al*. 1997). Many unexplained cases of infertility in human are accompanied by abnormal expression of integrins (Lessey & Castelbaum 2002). The integrin family represents a clear example of a set of genes with potentially overlapping functions. Most integrin ligands can be bound by multiple integrins and most integrins can bind more than one ligand (Hynes 1992). Furthermore, since integrins are α and β heterodimers, ablation of the gene for a single subunit usually has effects on other subunits, therefore the null mutations of either subunit for some integrins leads to peri-implantation lethality (Hynes 1996).

During the periimplantation period of pregnancy in ewes, integrin subunits α (v, 4, 5) and β (1, 3 and 5) were found to be constitutively expressed on the apical surface of the LE and were not influenced by pregnancy or presence of the conceptus. Moreover, these subunits were also detected on the apical surface of conceptus trophoblast (Johnson *et al*. 2001). Therefore, in the sheep, receptivity to implantation does not appear to involve changes

in either temporal or spatial patterns of integrin expression, but may depend on expression of other glycoproteins and ECM proteins, such as galectin-15, SPP1 and fibronectin, which are ligands for heterodimers of these integrins (Johnson *et al*. 2003a, Gray *et al*. 2004). In addition, down regulation of the anti-adhesive MUC1 and/or other mucins may remove a barrier that sterically hinders the ability of integrins and other glycoproteins to interact with counter receptors or extracellular matrix proteins at the maternal-conceptus interface (Aplin *et al.* 2001, Brayman *et al.* 2004).

In the pig, both the conceptus and uterine LE express a number of common integrin subunits including α1, α3, α4, α5, αv, β1, β3, and β5, and of these, the α4, α5, αv, β1, β3 and β5 subunits are detected at sites of initial attachment between uterine LE and trophectoderm on Days 12-15 of pregnancy. Additionally, α4, α5, and β1 are modulated during the estrous cycle and exhibit a similar expression pattern in ovariectomized gilts receiving P4 or P4 plus E2 replacement therapy (Bowen *et al*. 1996, Burghardt *et al*. 1997).

Fibroblast Growth Factors

Fibroblast growth factors (FGFs) are small polypeptide growth factors, all of which share common structural characteristics, and most of which can bind to heparin and to heparan sulfate moieties of cell surface and extracellular matrix heparan sulfate proteoglycans (HSPGs). However, many FGFs contain signal peptides for secretion and are therefore secreted into the extracellular environment (Powers *et al.* 2000). In addition, FGFs bind specific receptor tyrosine kinases, i.e., fibroblast growth factor receptors (FGFRs), that induce receptor dimerization and kinase activation, ultimately resulting in the activation of various signal transduction cascades which are implicated in diverse functions including

morphogenesis, cellular differentiation, angiogenesis, tissue remodeling, inflammation, and oncogenesis (Powers *et al.* 2000, Kim 2001). To date, twenty-two distinct FGFs have been discovered, numbered consecutively from 1 to 22 that range in molecular mass from 17 to 34 kDa, and share 13–71% amino acid sequence identity. FGFs can be classified into several subfamilies, according to sequence homology within a conserved 120-amino acid core (Kim 2001).

One of the FGFs, FGF7 also known as keratinocyte growth factor, has been detected in the uterine wall of primate and subprimate species (Pekonen *et al*. 1993, Matsui *et al*. 1997, Slayden *et al*. 2000, Ka *et al*. 2000). Due to the high level of identity of FGFs in their structural and biological properties, FGF7 has been placed in an FGF subfamily that includes FGF10 and FGF22 (Emoto *et al*. 1997, Igarashi *et al*. 1998, Umemori 2004). FGF7 was first isolated as an epithelial cell mitogen from the conditioned medium of the human embryonic lung fibroblast cell line, M426 (Rubin *et al*. 1989). Later, stromal cells from a variety of sources were found to express FGF7 in culture. These included fibroblasts from human lung, skin, mammary gland, stomach, bladder, prostate (Rubin *et al*. 1995), and smooth muscle cells (Winkles *et al*. 1997).

Initially, FGF7 was shown to stimulate DNA synthesis in BALB/MK mouse keratinocytes and subsequently exhibited mitogenic activity for a wide variety of epithelial cells (Rubin *et al*. 1989, 1995). It had long been postulated that epithelial cell proliferation during development as well as in adult organs was mediated by diffusible substances released from the underlying mesenchymal tissue (Cunha *et al*. 1983, Schor & Schor 1987). The combination of FGF7 expression by stromal cells in most of the tissue types and cultures and its activity specifically on epithelial cells supported the hypothesis that FGF7 functioned as a

paracrine mediator of mesenchymal-epithelial communication (Finch & Rubin 2004). Neonatal mice, injected with FGF7 showed a 5- to 6-fold increase in uterine epithelial growth (Hom *et al*.1998).

In sheep uterus, FGF7 is expressed in the developing neonatal and in adult ovine uterus (Chen 2000, Taylor *et al*. 2001). In adult ewes, the spatial pattern of FGF7 expression showed that this protein is located to the deeper layers of the uterine stroma in cyclic and pregnant animals which led the authors to hypothesis that FGF7 is a factor that functions in regulating development of deeper uterine glands (Chen *et al*. 2000). Moreover, *FGFR2IIIb* mRNA was also localized by *in situ* hybridization at the LE during the estrous cycle and pregnancy in adult ewes; and semiquantitative RT-PCR analyses indicate that expression of *FGFR2IIIb* and *FGF-7* mRNA is constitutive during the estrous cycle and pregnancy. Based on these results, it is not clear if any of the female sex steroids have any effect on the expression of these two genes (Chen *et al*. 2000). However, in the porcine uterus *FGF7* mRNA expression was localized in endometrial epithelia and *FGF7* receptor, *FGFR2IIIb*, expression was also localized in endometrial epithelia and conceptus trophectoderm, suggesting that FGF7 mediates an epithelial-epithelial interaction in an autocrine and/or paracrine manner in the pig uterus (Ka *et al*. 2000).

Fibroblast growth factor receptors

FGF activity on responsive cells is mediated by a family of high-affinity tyrosine kinase receptors, fibroblast growth factor receptors (FGFRs) that are encoded by four structurally related genes (*FGFR1–4*) (Ornitz *et al*. 1996, Beer *et al*. 2000). FGFRs contain an extracellular ligand binding domain, a single transmembrane domain, and an intracellular

tyrosine kinase domain. The extracellular domain determines ligand binding specificity and mediates ligand-induced receptor dimerization and contains three immunoglobulin-like (Ig-like) domains (Lee *et al*. 1989). Dimerization, in turn, results in one or more trans-phosphorylation events and the subsequent activation of the receptor (Ornitz *et al*. 1996). Indeed, the specificity of FGF-FGFR binding is determined in part by alternative exons corresponding to the carboxy terminal half of the third Ig domain and an adjacent ~20 residues of downstream sequence in FGFRs. These alternative exons, designated IIIa, IIIb, and IIIc, generate receptor variants with different ligand-binding properties (Powers *et al*. 2000).

Expression cloning of the FGF7 receptor revealed that it was encoded by IIIb variants of the *BEK/FGFR2* gene (FGFR2IIIb) (Miki *et al*. 1991). Interestingly, binding studies demonstrated that FGF7 did not interact with any other FGFR variant (Ornitz *et al*. 1996). However, FGF10 bound preferentially to the FGFR2b receptor variant (Igarashi *et al*. 1998), although it also associated with the IIIb splice variant of FGFR1 (Beer *et al*. 2000). Although the target cell and receptor-binding specificity of FGF22 has not been reported, there is speculation that the primary receptor for FGF22 would be the FGFR2b variant as well (Umemori *et al*. 2004). FGFR2b isoforms are found primarily in epithelial cells, and the restricted pattern of FGFR2b distribution and remarkable specificity of FGF7 for FGFR2b isoforms account for the predominant epithelial activity of FGF7 (Finch & Rubin 2004).

FGF7 and heparin binding

While FGF family members can be quite divergent in their amino acid sequences and expression, binding to heparin and HSPGs is a defining feature of the family. Heparin/HSPG

binding has long been recognized to have a major impact on FGF activity. Recent crystallographic work showed that heparin molecules stabilize the formation of FGF-FGFR complexes by binding both to ligand and receptor molecules (Pellegrini *et al*. 2000). It has been also reported that heparin enhanced the binding of individual FGF and FGFR molecules to each other, and facilitated the association of higher order FGF-FGFR complexes (Schlessinger *et al*. 2000). Interestingly, FGF7 binding to FGF2IIIb is enhanced by heparin when the level of FGF7 is at physiological concentrations, but binding could be blocked when the level of FGF7 is above physiological levels (Berman *et al*. 1999). Nevertheless, HSPGs and heparin are potent modulators of FGF activity. They can protect FGFs from thermal denaturation and proteolytic degradation, and binding of FGF to extracellular matrix HSPGs provides a reservoir from which FGFs can be rapidly released in response to specific triggering events (Saksela 1988, Pineda-Lucena 1994, Berman 1999).

Rationale, Hypotheses and Objectives

SIBLING proteins are found in bone and dentin; and are secreted into the extracellular matrix (ECM) during the formation and mineralization of these tissues (Fisher & Fedarko 2003). Previous studies from sheep, pig, goats and mice (Garlow *et al.* 2002, Johnson *et al*. 2003*c*, Joyce *et al*. 2005, White *et al*. 2005, 2006) that indicate one SIBLING family member (i.e., SPP1) is expressed and functions in the uterus of these animals. Therefore, we hypothesized that other SIBLING proteins DSPP, DMP1, MEPE and BSP may also be found and function as a matrix protein in the female reproductive tract. Sheep were used as a model for our study. *Objective 1 of this research was to analyze the*

expression of extracellular matrix proteins, DSPP, DMP1, MEPE and BSP in the endometrium of the cyclic and pregnant sheep.

FGF7 has been well characterized as a mesenchymal-released factor that binds to a receptor in the epithelium to mediate several functions such as proliferation, differentiation and migration (Szebenyi & Fallon 1999). In pigs, it has been shown that FGF7 is synthesized by uterine LE and secreted into the uterine lumen where it is hypothesized to bind its receptor, FGFR2IIIb, which is found in the LE and TR and act in an autocrine and/or paracrine manner to mediate interactions between the uterus and conceptus (Ka *et al*. 2000). *In vitro* studies using pig endometrial explant cultures, showed that estrogen, but not progesterone could increase FGF7 expression (Ka *et al*. 2001). However, the organ structure and multiple cell types of the uterus and the overlapping events of steroid hormones secreted from the conceptus and the ovary complicate *in vivo* gene expression studies, compared to *in vitro* studies. In order to study changes in FGF7 expression in relation to specific or combined steroidal treatments, gene expression in the uterus was analyzed by *in situ* hybridization in the context of ER and PR expression. In addition, there is evidence in the uterus suggesting that P4 binds PR in stromal cells and induces expression of paracrine factors, progestamedins that will then induce gene expression in neighboring epithelial cells (Roberts & Bazer 1988). We hypothesized that FGF7 expression in endometrial LE is up-regulated by estrogen via ER present in endometrial epithelia and progesterone is also required as a permissive hormone to allow estrogen to stimulate FGF7 expression or to stimulate PR positive stromal cells to release, a yet to be defined, progestamedin which in turn will upregulate FGF7 expression in LE. Therefore, *Objective 2 of this research was to investigate the effects of exogenous steroids (P4 and E2) administration on the expression of*

FGF7mRNA in ovariectomized pigs and to determine the role of progesterone on the expression of FGF7 using the synthetic progesterone antagonist, ZK137, 316.

Previous studies showed the critical role of the estrogen in regulating FGF7 in the pig uterus in early pregnancy (Ka *et al*. 2000). In another study the same group showed that E2, could increase FGF7 expression *in vitro* in a dose-dependent manner (Ka *et al*. 2001). The effects of E2 and P4 could be elicited via ER and PR respectively (Cooke *et al*. 1998*a*, Spencer & Bazer 2002). In the pig, there are several reports regarding ER and PR expression in the uterus (Geisert *et al*. 1993, 1994, Sierralta & Thole, 1996, Sukjumlong *et al*. 2004, Sukjumlong 2005). Although informative, those reports have provided no information concerning the endometrial ER and PR expression beyond day 20 of the pregnancy. The proposed hypothesis is that both ER and PR expression are similar to early pregnancy and that estrogen and progesterone continue to regulate FGF7 expression through out the pregnancy. *Objective 3 of this research was to study the expression and the cellular localization of PR, ER and FGF7 mRNA within the endometrium and placenta of pigs.*

CHAPTER II

EXPRESSION OF SOME MEMBERS OF THE SIBLING PROTEIN FAMILY IN THE UTERUS OF SHEEP

Overview

The small integrin-binding ligand, N-linked glycoprotein (SIBLING) family of genetically related extracellular matrix proteins including secreted phosphoprotein 1 (SPP1), dentin matrix protein 1 (DMP1), bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), and matrix extracellular phosphoglycoprotein (MEPE). These proteins are somewhat similar in character, being secreted, phosphorylated, and sulfated acidic sialoproteins and capable of binding integrins and hydroxyapatite. Members of the family have been described as matrix constituents of bone and/or teeth. One family member, SPP1, is more widely expressed and has been observed in a number of epithelial cell types and in immune cells. Moreover, some of the SIBLING proteins have been detected in nonmineralized tissues including brain, pancreas, kidney and salivary glands. Studies of SPP1 in domestic species indicate that stromal SPP1 expression during pregnancy correlates with the extent of uterine LE cell displacement and depth of trophoblast invasion during implantation. Based upon findings with SPP1, we initiated an analysis of the expression of other SIBLING family members in sheep uterus and report here the spatial and temporal detection of DSPP, DMP1, MEPE and BSP proteins in the endometrium of cyclic and pregnant sheep. Additionally, sheep were made unilaterally pregnant via ligation of one horn and removal of the ipsilateral ovary to generate non-gravid and gravid uterine horns thereby allowing investigation of the influence of the conceptus on endometrial expression of

SIBLING proteins. RNA extracted from endometrium of a Day 40 pregnant sheep was reverse transcribed into cDNA using random *DSPP* primers and the enzyme SuperScript III RT. The cDNA was amplified and the PCR product was subcloned and sequenced, and the blasting results showed 94%, 86% and 84% identities to cow, pig and dog DSPP mRNA respectively. Frozen sections of ovine uterine cross sections obtained from cyclic pregnant and unilaterally pregnant sheep were processed for immunofluorescence staining using rabbit antisera to the SIBLINGs. DSPP staining was first detected on LE of Day 15 cyclic and pregnant animals. In pregnant animals, starting from Day 20 DSPP expression was restricted to the stratum compactum stroma, increased by Day 30 and remained prominent in caruncular and intercaruncular stroma through Day 120 of pregnancy. Stromal staining was not detected in cyclic sheep. DSPP expression in the gravid horn of unilaterally pregnant sheep was dramatically upregulated as compared to the non-gravid horn. DMP1 and MEPE proteins were constitutively localized to the stroma of cyclic and pregnant ewes while no BSP was detected. These studies indicate that DSPP protein is upregulated in conjunction with pregnancy-related stromal changes and matrix remodeling in sheep and upregulated under the influence of the conceptus. Moreover we also reported the presence of DMP1 and MEPE proteins in the stratum compactum stroma of the ovine uterus. We hypothesize that the SIBLING proteins DSPP and SPP1 increase in the stroma of pregnant sheep as part of a uterine response to implantation and placentation. The role of DMP1 and MEPE are unknown but might be important to limit the invasive migration of trophoblast cells.

Introduction

The small integrin-binding ligand *N*-linked glycoprotein (SIBLING) gene family includes DSPP, DMP1, BSP, MEPE, and SPP1 (also known as osteopontin). A sixth protein, enamelin (ENAM) has recently been identified as a more distant member of the family. (Fisher & Fedarko 2003). SIBLING proteins are found in the bone and dentin and are secreted into the extracellular matrices (ECM) during the formation and mineralization of these tissues (Butler *et al.* 2002; Fisher & Fedarko 2003). SPP1 DMP1, BSP, DSPP, and MEPE are clustered within a 375 kb region of human chromosome 4q21, on chromosome 14 of the rat, and chromosome 5 of the mouse. Moreover, SIBLNG genes have similar exon structures, e.g., exon 1 is always noncoding, exon 2 is the leader sequence and the first two amino acids of the mature protein, exon 3 and 5 usually contains the consensus sequence (SSEE) for casein kinase II (CKII) phosphorylation, exon 4 is usually proline-rich, and among the acidic proteins, is the only significantly positive-charged domain while the majority of each protein is encoded by the last one or two exons and contains the integrin-binding tripeptide Arg-Gly-Asp (RGD) (Huq *et al.* 2005).

DSPP is a compound gene encoding for two proteins, namely dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) (also known as phosphophoryn, PP) (MacDougall *et al.* 1997). The *DSPP* gene undergoes several post-transcriptional modifications such as alternative splicing as several transcripts of various length have been identified (Yamakoshi *et al.* 2003). There is also speculation that after post-translational modification, DSPP is synthesized as a "pro" form and may be proteolytically cleaved to DSP and DPP moieties (Ritchie & Wang 2000). DMP1 was first discovered by cDNA cloning with the use of a rat odontoblast mRNA library (George *et al.* 1999). Because DMP1 contains large number of

acidic domains, DMP1 was initially thought to participate only in matrix mineralization. However, more recent studies have indicated that DMP1 may act as a transcription factor for activation of osteoblast-specific genes like osteocalcin and as a regulator of DSPP (Narayanan et al. 2003, 2006). BSP was first purified and characterized from fetal calf bone (Fisher *et al.* 1983) and has been shown to be involved in cell attachment, cell signaling, hydroxyapatite binding, hydroxyapatite nucleation and collagen binding (Ganss *et al.* 1999). MEPE was first described in bone marrow and tumors causing osteomalacia as tumor-secreted phosphaturic factor (Rowe *et al.* 2000, 2005). The best known SIBLING family member is SPP1 which was first described as a bone matrix protein (Prince *et al.* 1987) but has since been described in a wide variety of tissues.

While SIBLING protein expression was initially associated with cells that produce mineralized extracellular matrix and were hypothesized to play a role in mineralization, it was soon recognized that members of this protein family are also produced in nonmineralized tissues. For example, while SPP1 was identified as a bone matrix protein, it was independently identified as 2ar, a product inducible by tumor promoters and growth factors in a variety of cultured mouse cell lines (Smith and Denhart, 1987) and as early T lymphocyte activation 1 (ETA-1) a protein that is abundantly expressed after activation of T cells (Patarca 1989). Later, SPP1 was found to be deposited as a prominent layer at the luminal surfaces of specific populations of epithelial cells of the gastrointestinal tract, gall bladder, pancreas, urinary and reproductive tracts, lung, breast, salivary glands, and sweat glands (Brown *et al*. 1992). It has recently received considerable attention as an extracellular matrix protein present in multiple tissue-level compartments of the uterus where it may participate as a critical role in implantation, stromal differentiation, and placentation

(Johnson *et al.* 2003*c*). The multiple functional roles of SPP1 in the uterus are thought to result from both the spatial temporal patterns of expression and the posttranslational modifications of the protein (Johnson *et al.* 1999*a*, 1999*b*, 2003*a*, 2003*b*, 2003*c*, White *et al.*2006).

Similarly, other SIBLING family members, including DMP1, BSP, DSPP, and MEPE have been identified in a wide variety of non-mineralizing tissue (Rowe *et al.* 2000, Terasawa *et al.* 2004; Ogbureke & Fisher, 2004, 2005). Bianco *et al.* 1991 also reported the expression of BSP in the TR of human placenta. Therefore in light of the growing evidence for the expression of SIBLING proteins in diverse tissue types and the expression of SPP1 expression throughout pregnancy in the uterus, the expression of other SIBLING proteins was analyzed in cyclic and pregnant ovine uterus. We report here, initial findings relative to expression of DSPP, DMP1, BSP and MEPE in the ovine uterus.

Materials and Methods

Animals and tissue collection

All experimental and surgical procedures complied with the Guide for Care and Use of Laboratory Animals and were approved by the Agricultural Animal Care and Use Committee of Texas A&M University. Mature western range ewes (n=64) were observed daily for estrous behavior using vasectomized rams. After experiencing at least two estrous cycles of normal duration (16–18 days), animals were assigned randomly on Day 0 (estrus/mating) to cyclic or pregnant status. For pregnancy, ewes were bred to intact rams at estrus (day 0) and 12 h and 24 h after the onset of the estrus. Ewes were randomly assigned to be hysterectomized (n = 4 ewes/day) on Days 10, 13, or 15 of the estrous cycle and Days

13, 15, 20, 25, 30, 35, 40, 45, 50, 60, 80, 100 and 120 of pregnancy. Days of hysterectomy were chosen to represent previously defined morphological and developmental changes in intercaruncular and caruncular regions of the uterus during pregnancy in sheep. Pregnancy was confirmed by the presence of an apparently normal conceptus in uterine flushes (Days 11 and 15), or the presence of conceptus tissue on Days 20 through 120.

Another group of ewes was made unilaterally pregnant in order to further evaluate the local effect of the conceptus on SIBLING proteins that might be expressed exclusively in pregnant animals based upon previous findings of SPP1 (Johnson *et al.* 2003*c*). In this model, one uterine horn was manipulated to produce a non-gravid horn while the other uterine horn remained unmodified to supply the pregnancy with normal placentation (Bazer *et al.* 1979). Sexually mature ewes (n = 3) were checked daily for estrous behavior as described above. Following a second estrous cycle of normal duration, the ovary ipsilateral to the right uterine horn was removed. A double ligature was placed on the base of the right uterine horn at the uterine bifurcation. At the following estrus (Day 0), ewes were mated to intact rams. Ewes were then hysterectomized on either Day 40, 80 or 120 of gestation.

At hysterectomy, several sections (~0.5 cm^3) from the middle of each uterine horn were fixed in 4% paraformaldehyde in PBS (pH 7.2) and were embedded in Paraplast-Plus (Oxford Laboratory, St. Louis, MO). In addition, several other sections were embedded in Tissue-Tek Optimal Cutting Temperature (OCT) compound (Miles, Oneonta, NY), snap-frozen in liquid nitrogen, and stored at -80^oC until analysis.

Immunocytochemical analyses

Localization of DSPP, DMP1, BSP and MEPE proteins by immunofluorescence microscopy were performed as previously described (Johnson *et al.* 1999a). Rabbit polyclonal antisera to DSPP (LF-151), DMP1 (LF-148), BSP (LF-83) or MEPE (LF-155) were a gift of Dr. Larry Fisher, Matrix Biochemistry Laboratory, National Institute of Dental and Craniofacial Research, National Institutes of Health. Details regarding these rabbit polyclonal antisera derived against human antigen sequences can be found at Appendix A. Frozen sections (~10 μm) of endometrium were cut with a cryostat (Hacker-BrightOTF, Hacker Instruments, Inc., Winnsboro, SC, USA) and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). Sections were fixed in – 20 ºC methanol for 10 min, permeabilized at room temperature with 0.3% Tween 20 in 0.02M PBS (rinse solution), and blocked in antibody dilution buffer (2 parts 0.02M PBS, 1.0% BSA, 0.3% Tween 20 [pH 8.0] and one part glycerol) containing 10% normal goat serum for 1 h at room temperature. Sections were then dipped in rinse solution at room temperature and incubated overnight at 4 ºC with 2 μg/ml of rabbit anti DSPP (LF-151), DMP1 (LF-148), BSP (LF-83) or MEPE (LF-155) antibodies. Antibody-protein complex were detected with fluorescein-conjugated goat anti-rabbit secondary antibody (Chemicon International, Temecula, CA). Slides were then overlaid with a coverglass and Prolong antifade mounting reagent (Molecular Probes).

To support results obtained by immunofluorescence, the expression of DSPP protein was evaluated by immunohistochemistry in paraformaldehyde-fixed, paraffin-embedded, uterine tissue cross-sections (4 μm) using procedures described previously (Spencer *et al* 1995a). Briefly, boiling citrate buffer was used for antigen retrieval of DSPP and protein was

detected with rabbit anti-human DSPP IgG (2 μg/ml; LF-151, a gift from Dr. Larry Fisher, NIH) and visualized with a Super ABC Rabbit IgG Kit (Biomeda, Foster City, CA). For negative controls for primary antibody staining, primary antibodies were substituted with rabbit IgG at the same concentration as the primary antibodies.

DSPP and BSP cDNA cloning

An ovine *DSPP* cDNA of 850 base pairs (bp) was amplified as described previously (Ka *et al*. 2000). Briefly, polymerase chain reaction (PCR) was performed with primers to a porcine *DSPP* cDNA sequence (GenBank accession no. AY161862; forward, 5´-TTCCAGTTCCTCAAATCAAGC; reverse, 5´ TCCTCTTCATCTGCTCCATTC) and a reverse-transcribed template from Day 40 pregnant ovine endometrial total RNA and cloned into the pCR II vector (Invitrogen, Carlsbad, CA). A *BSP* cDNA of 180 bp was amplified using PCR with primers to a bovine cDNA sequence (GenBank accession no NM_174084; forward, 5´-TGGAGAGGAAGACGGTGAAG; reverse, 5´ TCATTGGTGCCTGTTTGTTC) and cloned into the pCR II vector.

Photomicrography

Digital photomicrographs of representative brightfield illumination (immunohistochemistry) images were evaluated with a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY) fitted with an Axiocam HR digital camera. Digital images of immunohistochemistry were recorded using Axiovision 4.3 software. All immunohistochemistry figures were assembled using Adobe Photoshop 8.0 (Adobe Systems Inc., San Jose, CA).

Results

Three of four SIBLING proteins (DMP1, MEPE and DSPP, but not BSP) were detected in ovine uterus by immunofluorescence analyses. Immunoreactive signals were absent in uterine sections in which rabbit IgG replaced primary antibody.

DMP1 and MEPE

High levels of immunoreactive DMP1 were localized to the stratum compactum stroma of cyclic and pregnant ewes (Fig. 1). The fluorescence intensity of DMP1 staining in LE and GE was not detectably different from background levels of staining when primary antibody was replaced with irrelevant rabbit IgG (Fig.1).

DMP1 was also detected along the endothelial cells lining the blood vessels and the allantoic membrane (Fig.2). Similar to DMP1, immunofluorescence staining for MEPE revealed that the protein was constitutively expressed in the stromal layer of both cyclic and pregnant ewes while low to undetectable staining was observed in the LE (Fig.3).

DSPP

Immunoreactive DSPP was detected in the LE and GE of Day 15 cyclic and pregnant ewes by immunofluorescence (Fig. 4). An identical pattern of staining was observed in samples processed for immunohistochemistry using the Super ABC peroxidase kit, data shown in Appendix B. DSPP signal in LE and GE was significantly reduced after Day 20 of pregnancy, however, staining was detected in cells of the stratum compactum stroma increased until Day 40 and remained elevated in the stratum compactum stroma through Day 120 (Fig. 4). Since DSPP was the only SIBLING protein analyzed in which stromal

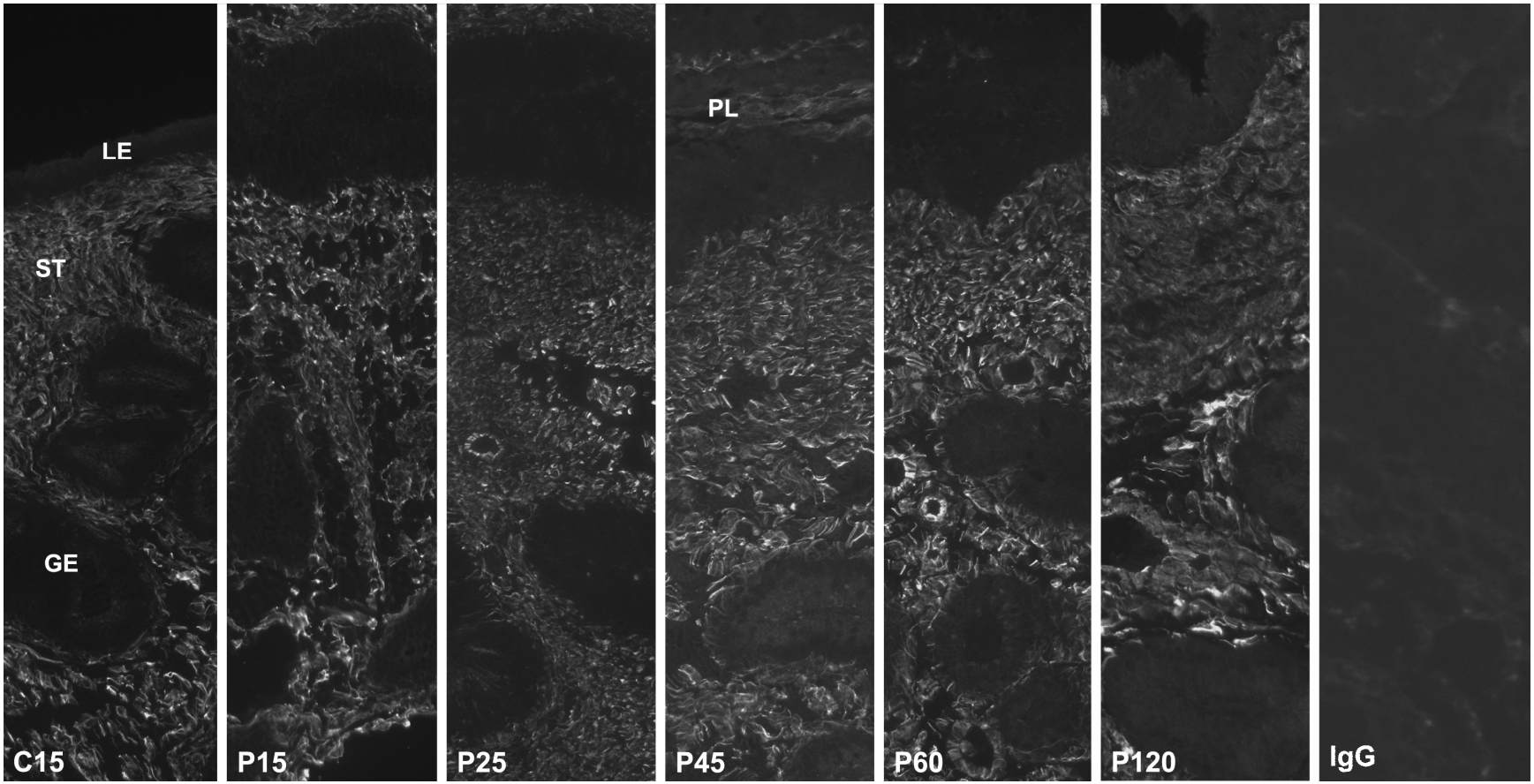


Figure 1. DMP1 immunofluorescence staining of endometrium from cyclic and pregnant ewes. Note the strong constitutive DMP1 expression in the stroma (ST). Normal rabbit IgG serves as a negative control when used in Day 35 pregnant tissue. LE, Luminal epithelium; GE, glandular epithelium; PL, placenta. Width of each field, 330 μm.

expression was upregulated exclusively during pregnancy, the effect of the conceptus on DSPP protein was analyzed. Immunofluorescence analysis of the unilaterally pregnant sheep uterus indicated that DSPP protein in the gravid horn was significantly upregulated as compared to the non-gravid horn on Days 40 through 120 (Fig.5). A cDNA for DSPP was amplified and the PCR product of 830 bp was subcloned and sequenced and analysis of sequence homology indicated 94%, 86% and 84% identities to cow, pig and dog DSPP mRNA respectively. However, the probe failed to provide a detectable signal for *in situ* hybridization analysis.

BSP

Neither BSP protein nor mRNA was detected in either cyclic or pregnant ovine uterus.

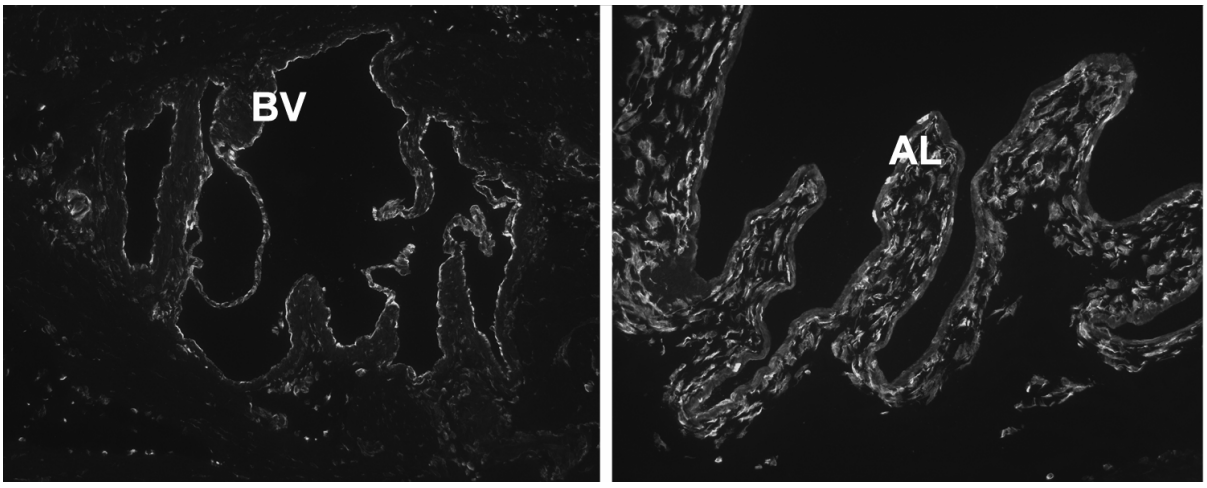


Figure 2. High levels of DMP1 immunoreactivity were detected by immunofluorescence in the endothelial cells of the blood vessels (left panel) and in the allantoic membrane (right panel). Width of each field, 690 μm.

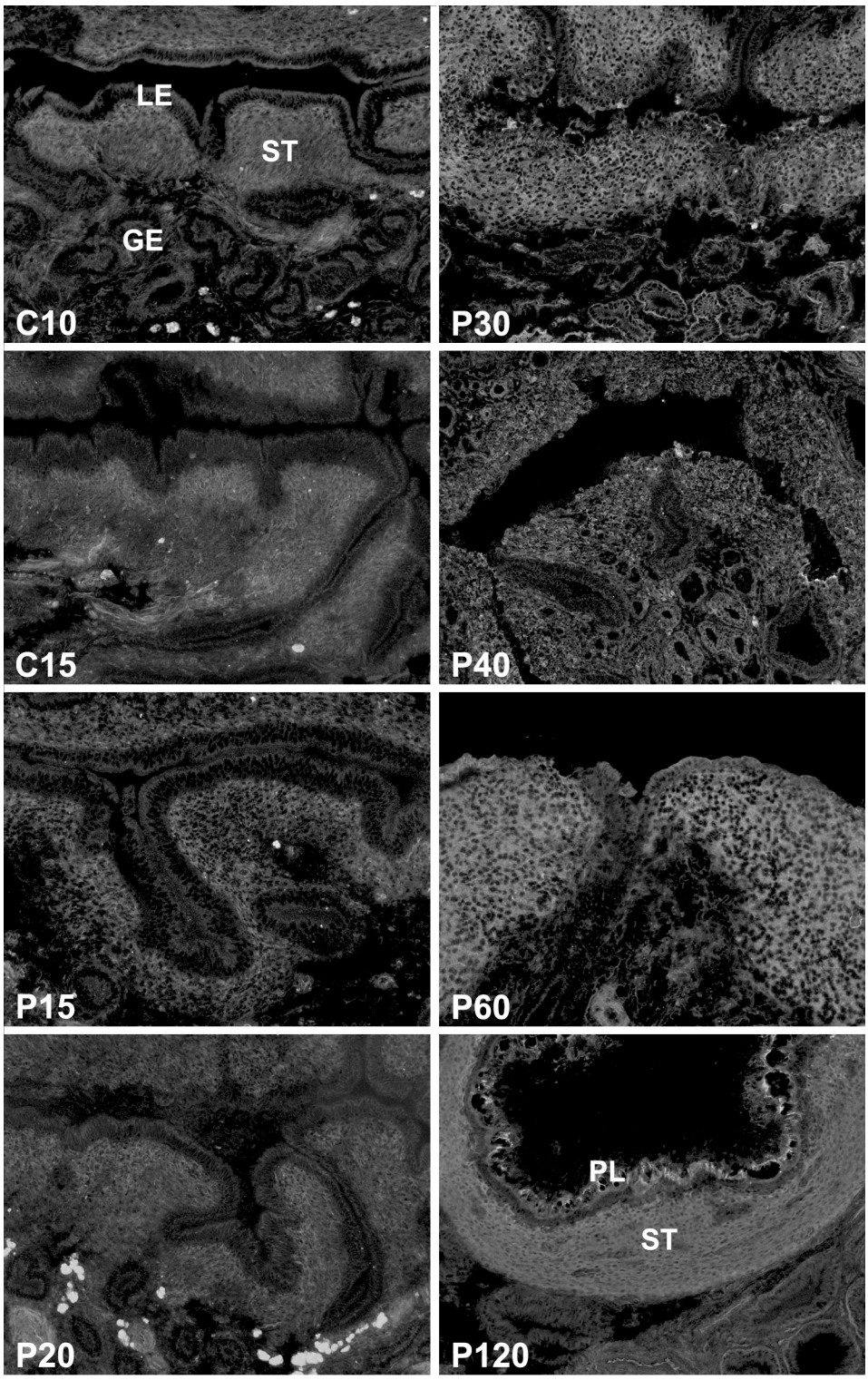


Figure 3. MEPE immunofluorescence staining of endometrium from cyclic and pregnant ewes. Note the strong MEPE expression in the stroma (ST) of both cyclic and pregnant sheep. LE, Luminal epithelium; GE, glandular epithelium; PL, placenta. Width of each field, 690 μm.

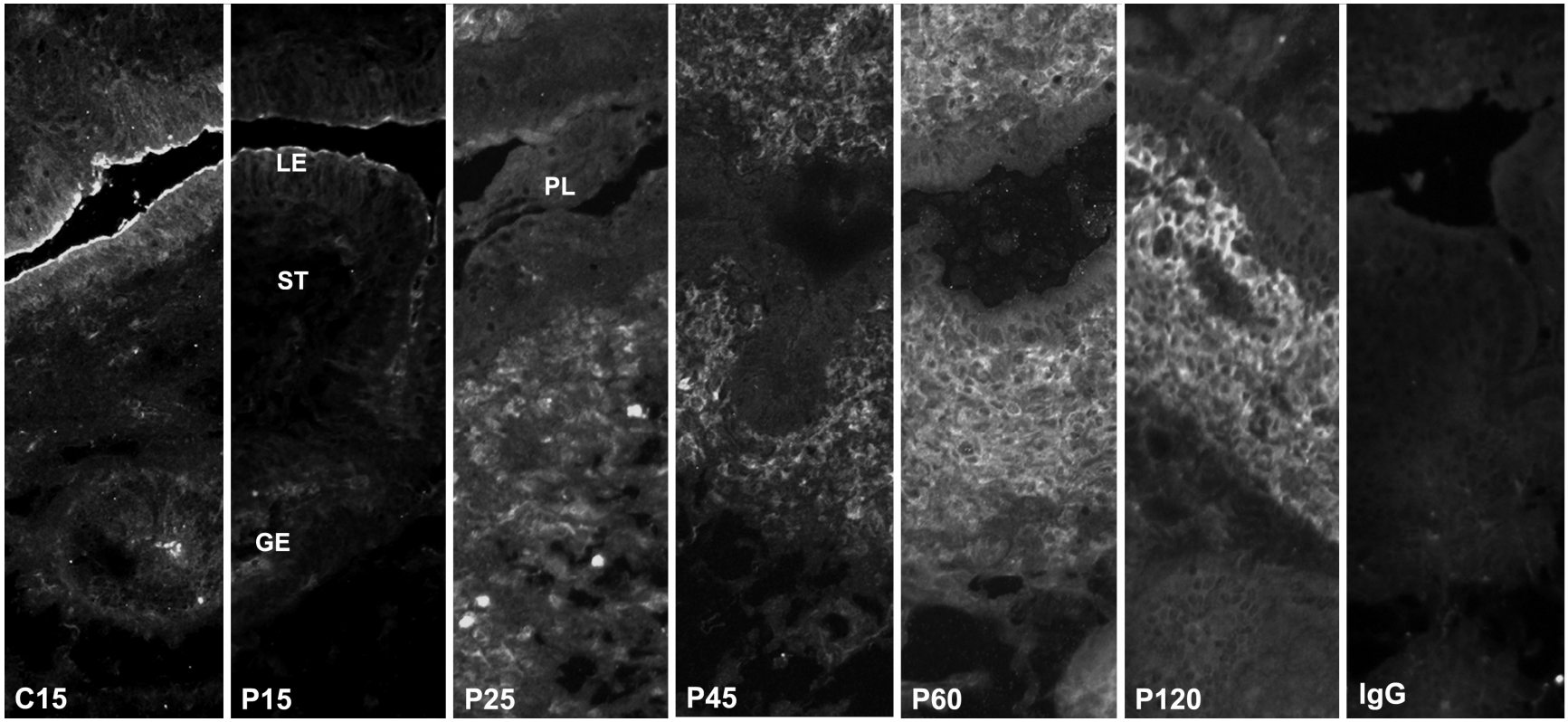


Figure 4. DSPP protein expression in ovine endometrium of cyclic (C) and pregnant (P) ewes. DSPP was detected using immunofluorescence staining of frozen sections. Note that there was some immunoreactivity at the luminal epithelium, LE of cyclic and early pregnant uterus. Stratum compactum stroma, ST, showed progressive increasing immunoreactivity starting from Day 20 through Day 40 and remained high through Day 120 pregnancy. Normal rabbit IgG serves as a negative control when used in Day 35 pregnant tissue. PL, placenta. Width of each field, 330 μm.

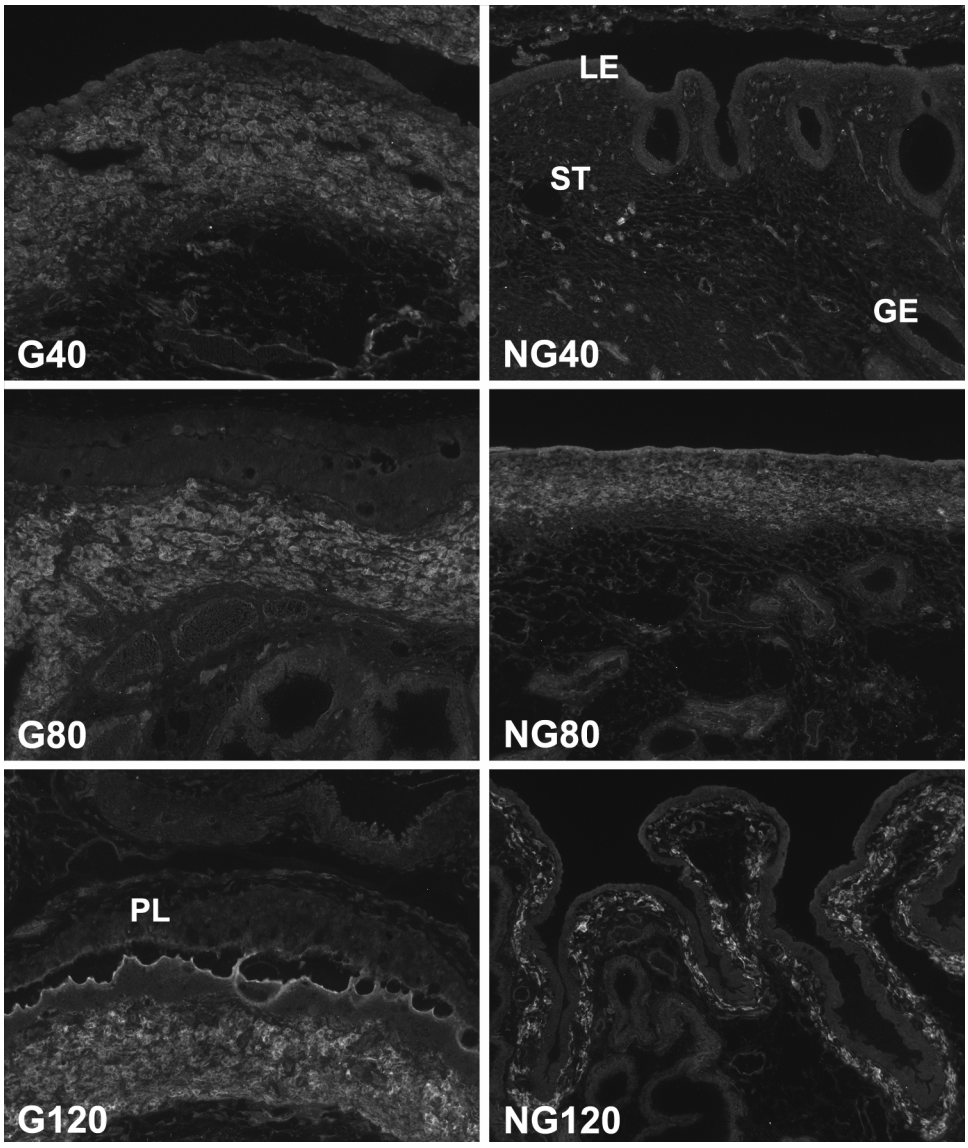


Figure 5. DSPP immunofluorescence staining of endometrium from gravid and non gravid horns of Day 40, 80 and 120 pregnant ewes. DSPP expression in the stroma (ST) of gravid horns showed increased upregulation in comparison to non-gravid horns suggesting potential effects of the conceptus on stromal expression of DSPP. LE, Luminal epithelium. Width of each field, 690 μm.

Discussion

This study presents the first evidence that members of SIBLING proteins other than SPP1 are expressed in the ovine uterus suggesting additional roles for these proteins in non-mineralized tissues. While BSP protein or mRNA were not detected in ovine uterus, DMP1 and MEPE were present in uterine stroma in cyclic and pregnant sheep and the pattern of expression did not change during the cycle and pregnancy. In contrast, DSPP protein exhibited a pattern in uterine stroma that is remarkably similar to that previously reported for SPP1 (Johnson *et al*. 2003*a,b*).

All of the SIBLINGs expressed in ovine endometrium are characterized as being secreted, phosphorylated, and sulfated proteins, and with the exception of MEPE, are acidic in character (Fisher *et al*. 2001). Each of the SIBLINGs contains the integrin-binding RGD tripeptide, however, the function of most SIBLINGs is poorly understood and thus the significance of their expression in ovine uterus remains to be determined.

To date the best known and well studied SIBLING member in the female reproductive tract of different primate and subprimate species is SPP1. Comprehensive analyses of the temporal and spatial patterns of SPP1 mRNA and protein expression within the uteri of multiple species has led to the suggestion that SPP1 has diverse biological functions in the uterus throughout the estrous cycle and pregnancy beginning at the peri-implantation period and extending to the end of gestation (Johnson *et al.* 2003*a*). In ovine uterus, at least two different tissue-level compartments express SPP1. A 70 kDa full length protein is secreted by uterine glands into the uterine lumen during pregnancy under the control of progesterone and is proteolytically cleaved to a 40 kDa fragment that persists along the entire maternal/conceptus interface during pregnancy in

sheep (Johnson *et al*. 2003*c*). A full length 70 kDa SPP1 is also induced in uterine stroma once the conceptus begins to form stable contacts with the uterine LE and expression also continues to be present in endometrial stromal throughout pregnancy (Johnson *et al*. 2003*a,b,c*).

Similar to SPP1, it appears that immunoreactive DSPP is present at the surface of the LE during the peri-implantation period. It is possible therefore that DSPP in the uterine lumen may function in a manner similar to that hypothesized for the 40 kDa SPP1 protein. The SPP1 fragment is thought to function as a bridging ligand with mechanosensory properties linking apical surfaces of both LE and conceptus trophectoderm (Johnson *et al*. 2003*a*).

The similarity of stromal DSPP immunoreactivity to that of stromal SPP1 is striking. Similarly, results obtained with the unilaterally pregnant sheep model system showing that increased DSPP immunoreactivity in the gravid uterine horn compared to the non-gravid horn suggests the influence of the conceptus on this response as has been found for SPP1 (Johnson *et al*. 2006). Stromal DSPP appears to be another marker of stromal changes that appear to be associated with a decidualization-like response of ovine endometrial stroma (Johnson *et al*. 2003*b*) which is also associated with the upregulation of the RGD-binding αvβ3 integrin and several proteins regarded as markers of decidualization in species with invasive implantation. The upregulation of both SPP1 and DSPP may reflect matrix remodeling and changes in the mechanical properties of the stroma needed to accommodate tension and shear forces that increase during pregnancy. Studies are needed to determine how these SIBLINGs influence mechanical properties of the uterine stroma during pregnancy. In addition, several SIBLING members including

SPP1 have been shown to bind and activate matrix metalloproteinases (MMP) (Fedarko *et al.* 2004). Several MMPs including MMP-1, -2, -3 and -9 (Salamonson *et al.* 1991, 1993, Vagoni *et al.* 1998) have previously been reported to be secreted by ovine endometrial cells and placenta. To date, however, no specific MMP has been associated with DSPP (Fedarko *et al.* 2004) nor has any SIBLING protein been directly associated with any MMP in ovine uterus. Further studies are needed to evaluate possible relationships between ovine uterine SIBLING proteins and MMPs.

The detection of DMP1 and MEPE in cyclic and pregnant ovine endometrial stroma is novel. As noted previously, several SIBLINGs expressed in non-mineralizing tissue including BSP, DMP1 and SPP1 binds with high affinity and specifically activates proMMP-2, proMMP-9 and proMMP-3 respectively. Additionally, these same proteins bind to complement Factor H with even higher affinity (Fedarko *et al*. 2004). Interactions among DMP1, pro-MMP9 and Factor H are hypothesized to influence activation of MMPs to effect local turnover of ECM. A role for DMP1 in the local activation of MMPs in ovine uterine stroma remains to be evaluated.

MEPE is the most recently identified member of the SIBLING family initially identified as a dentin/bone protein regulating mineralization (Fisher & Fedarko 2003) but more recently found in salivary gland and kidney (Ogbureke & Fisher 2004) and in tumors that exhibit altered phosphate homeostasis (Berndt & Kumar 2006). While no specific MMP partner for MEPE has been identified to date, it is speculated that this SIBLING may also play a role in MMP regulation.

In summary, this is the first identification of three new SIBLING family members in ovine uterus, however, the functional role of these ECM proteins remains to be

determined. Future challenges are to determine: a) whether any of the SIBINGs expressed in ovine uterus are associated with MMP partners, b) specific integrin partners that are capable of binding each of the SIBLINGS, and c) the potential mechanosensory/mechanotransduction roles of these SIBLINGs, particularly DSPP, in endometrial function.

CHAPTER III

REGULATION OF FIBROBLAST GROWTH FACTOR 7 IN THE PIG UTERUS BY PROGESTERONE AND ESTRADIOL

Overview

Fibroblast growth factor 7 (FGF7) stimulates cell proliferation, differentiation, migration and angiogenesis. The consensus has been that FGF7 is expressed by mesenchymal cells and acts via FGF receptor 2IIIb (FGFR2) on epithelia to mediate epithelial-mesenchymal interactions. The pig uterus is unique in that FGF7 is expressed by LE and FGFR2 is expressed on LE, glandular epithelium (GE) and trophectoderm to effect proliferation and differentiated cell functions during conceptus development and implantation. *FGF7* expression by uterine LE of pigs increases between Days 9 and 12 of the estrous cycle and also pregnancy as concentrations of progesterone increase, progesterone receptors (PGR) in uterine epithelia decrease and conceptuses secrete estradiol for pregnancy recognition. Further, estradiol increases expression of FGF7 in epithelial cells of pig uterine explants. This study investigated involvement of progesterone and estradiol and their receptors on expression of FGF7 in the pig uterus *in vivo*. Pigs were ovariectomized on Day 4 of the estrous cycle and injected i.m. daily from Days 4-12 with either corn oil (CO), progesterone (P4), P4 and ZK317,316, a PGR antagonist, (PZK), estradiol-17β (E2), P4 and E2 (PE), or P4 and ZK and E2 (PZKE). All gilts (n = 5/trt) were hysterectomized on Day 12. In the absence of P4 and E2, the LE of CO-treated pigs expressed PGR which precluded expression of *FGF7* mRNA. In P4-

treated pigs, PGR were down-regulated in LE, but maintained in stromal cells, allowing production of a putative progestamedin from stromal cells to induce *FGF7* in LE. For pigs treated with PZK, uterine PGR are not functional in stromal cells which precluded P4-induction of a progestamedin to stimulate expression of *FGF7* in LE. Injection of E2 alone did not induce *FGF7* in LE because LE was PGR-positive. However, treatment with PE allowed both down-regulation of PGR and induction of *FGF7* in LE via either direct effects of E2 via estrogen receptor alpha (ESR1), effects of a progestamedin, or a combination of those events. Finally, LE of pigs treated with PZKE expressed *FGF7* indicating that E2 can directly induce *FGF7* expression in the absence of functional PGR. These results suggest that: 1) P4 is permissive to FGF7 expression by down-regulating PGR in LE; 2) P4 stimulates PGR positive uterine stromal cells to release an unidentified progestamedin that induces FGF7 expression by LE; 3) E2 and P4 can induce FGF7 when PGR are rendered nonfunctional by ZK and 4) E2 from conceptuses interacts via ESR1 in LE to induce maximal expression of FGF7 in LE on Day 12 of pregnancy in pigs.

Introduction

Fibroblast growth factor 7 (FGF7), also called keratinocyte growth factor, is a member of the FGF superfamily which has been reported to stimulate cell proliferation, differentiation, migration and vascular angiogenesis (Szebenyi & Fallon 1999). The preponderance of studies show that FGF7 is expressed in cells of mesenchymal (stromal) origin. However, FGF7 receptors (FGF receptor 2IIIb, FGFR2) are present only on epithelial cells (Rubin *et al.* 1995). Therefore, the prevailing opinion is that

mesenchymal-derived FGF7 binds receptors on epithelia to mediate epithelial-mesenchymal interactions in various organs, including the reproductive tract (Rubin *et al.* 1995, Cooke *et al.* 1998*b*). In sharp contrast to this consensus opinion, results of these studies demonstrated that *FGF7* mRNA is expressed in endometrial epithelia of pigs, that *FGFR2* mRNA is present in both the endometrial epithelia and conceptus trophectoderm, and that FGF7 stimulates trophectoderm, but not endometrial epithelial cells, to undergo proliferation and differentiation, suggesting that FGF7 is a paracrine mediator of interactions between the uterus and conceptus (Ka *et al*. 2000, 2001, Ka & Bazer 2005).

Several factors including cytokines, growth factors and hormones, are known to affect FGF7 expression in various tissues. Interleukin-1, interleukin-6, platelet-derived growth factor-β and transforming growth factor-α increase *FGF7* mRNA expression [7, 8] (Chedid *et al.* 1994; Li & Tseng 1997). Parrott and co-workers (2000) showed that FGF7 and hepatocyte growth factor (HGF) stimulate FGF7 expression in ovarian surface epithelium via a positive autocrine feedback mechanism. Steroid hormones also regulate FGF7 expression in reproductive organs. In the uterine endometrium of rhesus monkeys, progesterone (P4) increases stromal expression of FGF7 where it may mediate P4-induced increases in epithelial cell proliferation and spiral artery development (Koji *et al*. 1994). In mice, estrogen increases mammary gland expression of FGF7 where it may play a role in gland development (Pedchenko & Imagawa 2000). Androgens also stimulate FGF7 expression in stromal cells of the prostate (Yan *et al*. 1992). It is significant that the promoter region of the *FGF7* gene contains various regulatory factor binding sites, including steroid response elements for estrogen and glucocorticoids in

humans (Finch *et al*. 1995, Zhou & Finch 1999) and for androgens in rats (Fasciana *et al*. 1996).

Northern blot and *in situ* hybridization analyses of *FGF7* in pig endometrium indicated expression during the estrous cycle and peri-implantation period. *FGF7* expression was first detected in LE between Days 9 and 12 of both the estrous cycle and pregnancy, peaked on Day 12 of pregnancy, and remained high through Day 20 (Ka *et al*. 2000). Because the increase in FGF7 expression on Day 12 of pregnancy was significantly amplified over Day 12 of the estrous cycle, subsequent studies using an endometrial explant culture system were performed to determine hormonal and/or cytokine regulation of FGF7. In these studies, estradiol (E2), but not P4, increased *FGF7* mRNA (Ka *et al*. 2001). However, the peri-implantation period of pregnancy is highly complex, and cannot be duplicated in *in vitro* culture systems. In pigs the uterine environment is influenced by the overlapping events of E2 release by conceptuses for maternal recognition of pregnancy, and by extended exposure to P4, the hormone of pregnancy, to mediate developmental changes in the uterus conducive to successful implantation and placentation (Bazer & Thatcher 1977, Bazer *et al*. 1998). It is known that estrogen receptor α (ESR1) is present in uterine endometrial epithelial cells between Days 12 and 15 of the estrous cycle and pregnancy in the pig, whereas P4 receptors (PGR) are undetectable in epithelial cells (Geisert *et al* 1993, 1994). Given that E2 is secreted by pig conceptuses into the uterine lumen (Bazer & Thatcher 1977, Bazer *et al*. 1998) it is likely that FGF7 expression in LE is up-regulated by E2 via ESR1 present in endometrial epithelia. However, since P4 is the dominant hormone during diestrus of the estrous cycle and during pregnancy and FGF7 is expressed by LE between Days 12 and

15 of both the estrous cycle and pregnancy, it is possible that P4 is required as a permissive hormone to allow E2 to stimulate FGF7 expression by endometrial LE. Therefore, we hypothesized that P4 is required as a permissive hormone for E2-induced FGF7 expression in LE of pig endometrium and conducted a study to investigate the relationship between P4 and E2 and their receptors on FGF7 expression *in vivo*.

Materials and Methods

Animals and tissue collection

All experimental and surgical procedures complied with the Guide for Care and Use of Laboratory Animals and were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University. A total of 26 sexually mature gilts of similar age, weight and genetic background were ovariectomized on Day 4 after the onset of estrus (Day 0) and assigned randomly to be treated daily at 0700 h from Day 4 through Day 12 with either: 1) progesterone (P4, 200 mg in corn oil vehicle (CO); Sigma, St. Louis, MO) alone (5 gilts); 2) P4 plus ZK137,316 (75 mg; ZK, PGR antagonist, generously provided by Dr. Kristoff Chwalisz, Schering, AG, Berlin, Germany) (5 gilts); 3) P4 plus estradiol-17β (E2, 100 μg; Sigma) (5gilts); 4) P4 plus ZK plus E2 (5 gilts); 5) estradiol benzoate (E2, 5 mg in 5 ml of corn oil/day) (3 gilts) or 6) CO alone (3 gilts). All gilts were hysterectomized on Day 12, and uterine flushings obtained by introducing and recovering 20 ml sterile saline per uterine horn at hysterectomy. The flushings were clarified by centrifugation (3000 x g for 10 min at 4^{o}C), aliquoted, and frozen at -80^{o}C until analyzed. Several tissue sections (~0.5 cm) from the middle of each uterine horn were fixed in 4% paraformaldehyde in PBS (pH 7.2) and embedded in Paraplast-Plus

(Oxford Laboratory, St. Louis, MO). The remaining endometrium was physically dissected from the myometrium, frozen in liquid nitrogen, and stored at -80 C for RNA extraction.

Northern blot hybridization analyses

Total cellular RNA was isolated from endometrial tissues using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. Total endometrial RNA (20 ug) was loaded onto a 1.2 % agarose gel, electrophoresed and transferred to a 0.2 u nylon membrane as described previously (Spencer & Bazer 1995). The blot was then hybridized with [^{32}P]-radiolabeled antisense cRNA probes generated from a linearized porcine *FGF7* partial cDNA (Ka et. al 2000). Autoradiographs of northern blots were prepared using Kodak X-OMAT x-ray film (Eastman Kodak Co., Rochester, NY).

In situ *hybridization analyses*

The location of *FGF7* mRNA in uterine tissue sections was determined by *in situ* hybridization analysis as described previously (Johnson *et al*. 1999a). Briefly, deparaffinized, rehydrated, and deproteinated uterine tissue sections (~ 5 μm) were hybridized with radiolabeled antisense or sense porcine *FGF7* cRNA probes that were generated from linearized plasmid templates using *in vitro* transcription with [^{35}S]-UTP (Perkin Elmer Life Sciences, Inc). Plasmid templates were partial cDNAs for porcine *FGF7* (Ka *et al*. 2000). After hybridization, washes and digestion with RNase A, autoradiography was performed using Kodak NTB-2 liquid photographic emulsion

(Eastman Kodak, Rochester, NY). Slides were exposed at 4°C for 2 wk, developed in Kodak D-19 developer, counterstained with Harris modified hematoxylin (Fisher Scientific, Fairlawn, NJ), dehydrated through a graded series of alcohol to Citrisolv (Decon Laboratories, Inc, King of Prussia, PA) and protected with cover slips.

Western blot analysis

FGF7 was purified from 1 mg total protein from each uterine flushing by incubating with 200 µl heparin-beaded agarose (Sigma) at 4°C overnight and washing three times with Hank's balanced salt solution (Sigma; 10,000 x g for 5 min). Proteins bound to heparin-beaded agarose were denatured in Laemmli buffer and separated by SDS-PAGE, transferred to nitrocellulose, and blocked with 5% nonfat milk-TBST (Tris-buffered saline, 0.1% Tween-20) as described previously (Ka *et al*. 2000). Blots were incubated with a goat polyclonal antibody against human FGF7 synthetic peptide (2 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) or normal goat IgG (Sigma) as primary antibody in 2% milk-TBST overnight at 4 C, rinsed for 30 min at room temperature with TBST, incubated with peroxidase-conjugated rabbit anti-goat IgG (1:10,000; Zymed Laboratories Inc., San Francisco, CA) as a secondary antibody for 1 h at room temperature, and then rinsed again for 30 min at room temperature with TBST. Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham/Pharmacia, Arlington Heights, NY) according to the manufacturer's recommendations.

Immunohistochemistry

The expression of PGR and ESR1 proteins was evaluated in paraformaldehyde-fixed, paraffin-embedded, uterine tissue cross-sections (4 μm) using procedures described previously (Spencer *et al*. 1999). Briefly, boiling citrate buffer was used for antigen retrieval of PGR, and Pronase E (0.5 mg/ml in PBS) was used to retrieve ESR1 antigen. Proteins were detected with mouse anti-human PGR IgG (2 μg/ml; 2C5 PGR, Zymed Labs, San Francisco, CA) and rat anti human-ESR1 IgG (2 μg/ml; H222, Dr. Chris Nolan, Abbott Laboratories, Chicago, IL) and visualized with a Super ABC Mouse/Rat IgG Kit (Biomeda, Foster City, CA). For negative controls, primary antibodies were substituted with mouse (for PGR) or rat (for ESR1) IgG at the same concentration as the primary antibodies.

Photomicrography

Digital photomicrographs of representative brightfield (immunohistochemistry) or brightfield and darkfield illumination (*in situ*) images were evaluated with a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY) fitted with an Axiocam HR digital camera. Digital images of immunohistochemistry and *in situ* hybridization were recorded using Axiovision 4.3 software. All immunohistochemistry and *in situ* hybridization figures were assembled using Adobe Photoshop 8.0 (Adobe Systems Inc., San Jose, CA).

Results

Steady-State Levels of FGF7 mRNA in Pig Endometrium and FGF7 Protein in the Uterine Lumen

The *FGF7* cRNA detected a single transcript of ~ 2.4 kb in northern blot analysis of pig endometrial total RNA (Fig. 6). *FGF7* mRNA was detectable in endometria of ovariectomized pigs treated with P4 alone, but was not detectable in P4-treated pigs that were also treated with ZK, the PGR antagonist. Results of western blotting to detect FGF7 protein purified from uterine flushings using heparin-coated agarose beads are shown in Figure 7. An immunoreactive FGF7 protein of 17-kDa was detected in flushings from pigs treated with P4 and E2 and two molecular weight variants of FGF7 proteins, 17 kDa and 25 kDa were detected in flushings from pigs treated with P4, E2 and ZK indicating secretion of FGF7 into the uterine lumen of pigs (Fig. 7).

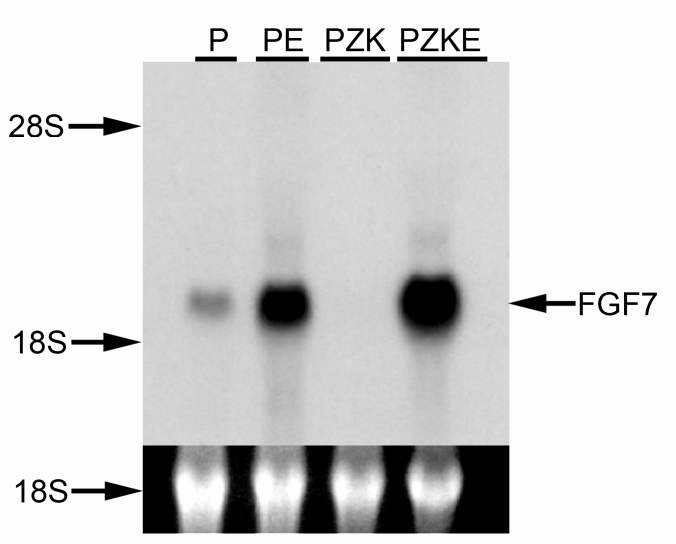


Figure 6. Northern blot analysis of FGF7 mRNA (20 μg) in endometria from ovariectomized pigs treated with progesterone (P4), P4 + estradiol-17□ (PE), P4 + ZK137,316 (PZK), and PZKE. Positions of the 28S (4.7 kb) and 18S (1.8 kb) rRNAs are indicated. A single transcript of *FGF7* (~ 2.4 kb) was detected.

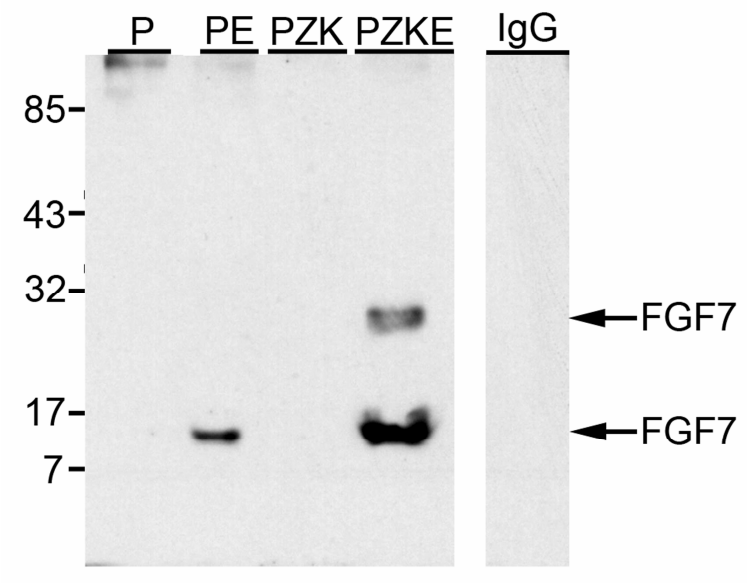
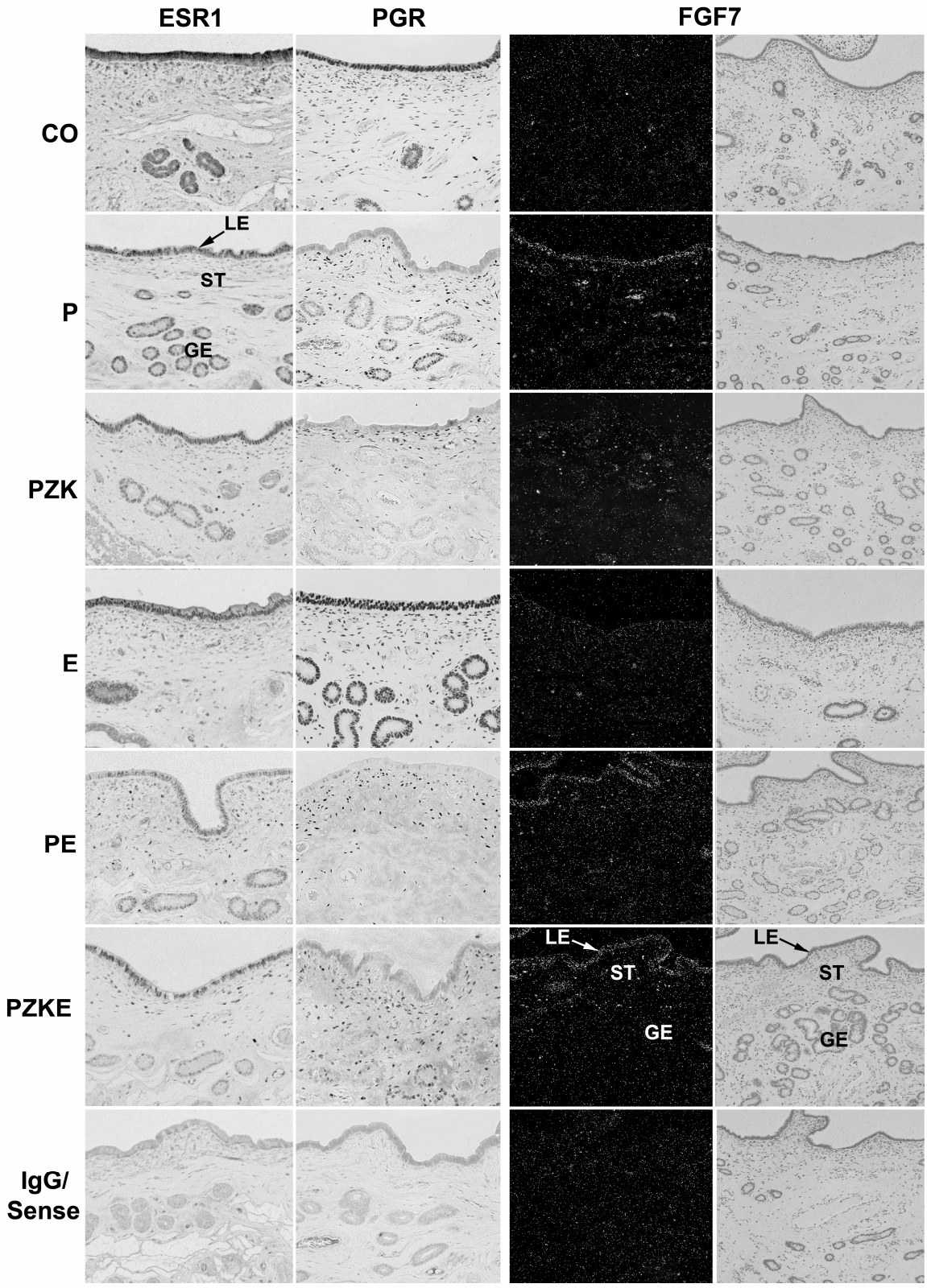


Figure 7. Western blot analysis of FGF7 in porcine uterine luminal flushings. Positions of prestained molecular weight standards are indicated. Immunoreactive FGF7 was detected (arrows) in uterine flushings from ovariectomized pigs treated with progesterone + estradiol-17β (PE) and P4 + ZK137,316 + E2 (PZKE).

Interrelationships between FGF7 mRNA, PGR and ESR1 in pig endometrium (Fig. 8)

In situ hybridization analysis of pig endometrium localized *FGF7* mRNA to LE of ovariectomized pigs treated with P4, P4 and E2, or P4, E2 and ZK. However, *FGF7* mRNA was not detected in endometrial LE of pigs treated with CO vehicle, P4 and ZK, or E2 alone. Immunohistochemistry for PGR revealed expression in endometrial LE of pigs treated with CO vehicle and also E2, but expression of PGR was absent in LE of pigs treated with P4 alone or P4 in combination with either ZK and/or E2. In contrast, PGR was expressed in endometrial stromal cells of pigs in all treatment groups. Immunohistochemistry for ESR1 revealed expression in LE, stromal cells and GE of all pigs in all treatment groups. In the following section, the relationships between

Figure 8. Interrelationships between FGF7 mRNA, progesterone receptor protein and E2 receptor protein in pig endometrium. First Column: Nuclear immunostaining for ESR1 protein was detected in luminal (LE), glandular (GE) epithelia (GE) and stromal cells (ST) of endometria from all treatment groups. A section from an estradiol valerate-treated pig stained with nonimmune rat IgG served as a negative control. Width of each field, 540μm. Second Column: Nuclear immunostaining for PGR protein was detected in luminal epithelium (LE) of corn oil (CO) and estradiol-17β treated (E2) pigs, whereas PGR were present in stroma cells (ST) of endometria from all treatment groups. A section from an E2-treated pig stained with nonimmune mouse IgG (IgG) served as a negative control. Width of each field, 540μm. Third and Fourth Columns: *In situ* hybridization analysis of FGF7 mRNA expression in pig endometrium. The left and right panels represent corresponding brightfield and darkfield images, respectively, of endometria from each treatment group. A representative section from a progesterone-treated pig hybridized with a radiolabeled sense cRNA probe (Sense) served as a negative control. Note that FGF7 mRNA is detectable only in LE and the hybridization signal is evident in endometria from pigs treated with progesterone (P4), P4 + estradiol-17 β (PE) and P4 + ZK 137,316 + estradiol-17β (PZKE) treatments. Width of each field, 690 μm.


ESR1
PGR
FGF7
CO
P
PZK
E
PE
PZKE
IgG/
Sense
LE
ST
GE
LE
ST
GE
LE
ST
GE

treatment, PGR and ESR1 status, and *FGF7* mRNA expression are summarized by treatment group.

Ovariectomized pigs injected with corn oil (Fig. 8)

In the absence of ovarian steroid hormones following ovariectomy, the absence of P4 and E2 resulted in default expression of both PGR and ESR1 in endometrial LE, stromal cells and GE. The presence of PGR in LE is considered to preclude expression of *FGF7* mRNA in CO-treated pigs.

Ovariectomized pigs injected with progesterone (Fig. 8)

Continuous 9 day exposure of pigs to P4 resulted in down-regulation of PGR in endometrial LE; but not stromal cells, and did not affect expression of ESR1 in LE, GE or stromal cells. The combined effects of P4-induced down-regulation of PGR in LE with maintenance of PGR in stromal cells is considered to enable P4 to interact with PGR in stromal cells to induce synthesis and secretion of a putative progestamedin that induced *FGF7* in LE.

Ovariectomized pigs treated with progesterone and ZK137,316 (Fig. 8)

It appears that PGR in LE and stromal cells are differentially sensitive to effects of ZK. Although ZK did not inhibit P4-induced down-regulation of PGR in endometrial LE, ZK bound PGR in endometrial stromal cells to render stromal cell unresponsive to P4. Therefore, it is considered that a progestamedin was not synthesized and released by

stromal cells in response to P4 to mediate induction of *FGF7* mRNA in LE of pigs receiving both P4 and ZK.

Ovariectomized pigs treated with estradiol 17β (Fig. 8)

The absence of P4 to down-regulate PGR and the effect of E2 to induce PGR, resulted in expression of PGR in LE which is considered to prevent induction of *FGF7* mRNA in LE by E2.

Ovariectomized pigs treated with progesterone and estradiol 17β (Fig. 8)

Treatment of pigs with both P4 to down-regulated PGR in LE allows E2 to induce FGF7 mRNA expression in endometrial LE. Further, the interaction of P4 with PGR in stromal cells is considered to allow induction of a progestamedin that acts on LE to induce expression of *FGF7* mRNA, or expression of *FGF7* mRNA may be induced in this treatment group by a combination of these two mechanisms.

Ovariectomized pigs treated with progesterone, ZK and estradiol 17β (Fig. 8)

The action of ZK on PGR in stromal cells inhibits production of the putative progestamedin necessary to induce expression of *FGF7* in LE. Therefore, in the absence of functional PGR, E2 acting via ESR1 is considered sufficient to induce expression of *FGF7* mRNA in pig endometrial LE.

Discussion

The results of the present study in pig endometrium indicate that: 1) P4 is permissive to FGF7 expression through its action of down-regulating PGR in LE; 2) P4 stimulates PGR positive uterine stromal cells to release an as yet unidentified progestamedin that induces FGF7 expression by LE; 3) combined effects of E2 and P4 can induce FGF7 due to the absence of PGR down-regulation by P4 or the presence of ZK to block PGR function; and 4) E2 from conceptuses interacts via ESR1 in LE to induce maximal expression of FGF7 in LE on Day 12 of pregnancy in pigs. The treatment group receiving both P4 and E2 is most physiological with P4 from the CL being permissive to actions of E2 from pig conceptuses during the peri-implantation period. A model summarizing the present working hypothesis for the hormonal regulation and role of uterine FGF7 during early pregnancy in pigs is presented in Figure 9. The mechanism(s) responsible for lack of expression of FGF7 by endometrial GE is not known.

Progesterone, the hormone of pregnancy in all mammals, is critical to control temporal and spatial (cell-specific) changes in gene expression within the uterus that ensure synchrony between uterine and conceptus (embryo/fetus and associated membranes) development (Spencer *et al*. 2004*a*). Indeed, treatment with exogenous P4 significantly alters the expression of a number of genes in rodent, primate and sheep uteri as measured by microarray (Ace & Okulicz 2004, Jeong *et al* 2005, Gray *et al*. 2006). Although similar studies have not been performed in pigs, P4 increases expression of calbindin-D9k (Yun *et al*. 2004), vascular endothelial growth factor (Welter *et al*. 2003), FGF2 and two of its receptors, FGFR1 and FGFR2 (Welter *et al*. 2004), and α4, α5 and

β1 integrin receptor subunits (Bowen *et al* 1996), as well as suppress expression of MUC1 (Bowen *et al* 1996). Importantly, P4 increases expression of various uterine secretory proteins, components of histotroph, hypothesized to support conceptus development in pigs (Roberts & Bazer 1988, Simmen *et al*. 1990, Harney *et al*. 1993). Previous studies with endometrial explant cultures and *in vivo* steroid replacement experiments, failed to demonstrate FGF7 regulation by P4 in the pig uterus because investigators assumed that increases in FGF7 expression during the estrous cycle and pregnancy were most likely to be influenced by P4 (Ka *et al*. 2001, Wollenhaupt *et al*. 2005). Results of the present study clearly indicate that FGF7 is induced in the uterine LE of ovariectomized pigs treated with P4 and that expression of FGF7 is blocked by ZK, a PGR antagonist.

The permissive effects of P4 on FGF7 expression are mediated by PGR in pig endometrium (Kastner *et al*. 1990, Cheon *et al*. 2002). In most mammalian uteri, PGR are expressed in endometrial epithelia and stroma cells during the early to mid-luteal phase, allowing direct regulation of genes by P4. However, in sheep, the presence of PGR in LE precludes expression of most P4-regulated genes in LE until continuous exposure of the endometrium to P4 down-regulates PGR expression exclusively in LE and GE (Spencer *et al*. 2004*b*). This paradigm of loss of PGR in uterine epithelia prior to implantation appears to be common across mammals (Carson *et al*. 2000) and predicates that endocrine effects of ovarian P4 on endometrial epithelia during the peri-implantation period are mediated indirectly by either P4-indiced paracrine-acting factors (progestamedins) produced by the PGR-positive stromal cells, or by induction of factors in LE that simultaneously down-regulate PGR and either allow or stimulate expression of

endometrial genes (Johnson *et al*. 2000, Geisert *et al* 2005). In pigs, expression of PGR in endometrial LE and GE is down-regulated by Day 10 of the estrous cycle and pregnancy, whereas expression of PGR is maintained in stromal cells and myometrium (Geisert *et al*. 1994). Removal of PGR from LE correlates with loss of MUC1 and expression of secreted phosphatase 1 (SPP1; also known as osteopontin) on the apical surface of LE, exposing integrins to extracellular matrix proteins for trophoblast attachment to the uterus (Bowen *et al*. 1996, White *et al*. 2005). This is also the period when the endometrium releases many cytokines and growth factors into the uterine lumen of pigs to support conceptus development and trophoblast elongation (Geisert & Yelich 1997). Although loss of PGR in endometrial epithelia of pigs is well established (Geisert *et al*. 1994), the present results are the first to effectively dissect P4 regulation of gene expression in LE during the estrous cycle and early pregnancy.

Three conclusions can be inferred from results of the present study. First, similar to that reported in sheep (Spencer & Bazer 1995), P4 negatively autoregulates PGR in LE, but not PGR in endometrial stromal cells of pigs. Expression of PGR in LE and stromal cells was detected in pigs receiving CO, but was down-regulated in LE of all pigs treated with P4. Second, similar to results for P4-regulated genes in endometrial epithelia of sheep (Spencer & Bazer 1995, Johnson *et al*. 2000), the presence of PGR in pig LE precludes induction of *FGF7*. All pigs with PGR in LE, i.e., those treated with CO alone or E2 alone, failed to express *FGF7* in LE. Third, the combined effects of P4-induced down-regulation of PGR in LE and P4 interaction with PGR in stromal cells, resulted expression of a putative progestamedin(s) from stromal cells that acted on LE to induce *FGF7*. Pigs treated with P4 exhibited down-regulation PGR in LE concomitant with

induction of FGF7. Further, FGF7 expression required functional stromal PGR because ZK-treatment ablated P4 -induced FGF7 expression. However, in the absence of functional PGR (treatment with P4, E2 and ZK), E2 alone, interacting with ESR1, induced FGF7 expression.

The mechanisms by which P4 both down-regulates PGR and up-regulates expression of other genes within the uterine LE of pigs are not understood. Induction of stromal-derived progestamedins is one explanation for these phenomena; however, an attractive alternative hypothesis has been described by Geisert and colleagues (Geisert *et al*. 2005). They proposed that P4 interacts with PGR in LE to stimulate factors that activate nuclear factor kappa B (NF-κB) which then functions to both inhibit PGR expression and activate transcription of genes believed to be involved with implantation (Geisert *et al*. 2005). The results of the present study are consistent with either of these theories of endometrial gene regulation by P4.

Estrogens, secreted by pig conceptuses on Day 12 of gestation, are the maternal recognition signal that switches secretion of endometrial prostaglandin F2α from an endocrine to exocrine direction to prevent CL regression (Bazer & Thatcher 1977). In addition, conceptus estrogens modulate uterine gene expression to support uterine secretions and the controlled inflammatory-like events that characterize changes in conceptus morphology and uterine remodeling for implantation in pigs (Geisert *et al*. 1982*b*). Indeed, secreted SPP1 is induced by estrogen in LE, and initially localizes to LE in close proximity to the Day 12 implanting conceptus (White *et al*. 2005), whereas conceptus secretion of estrogens correlates with conceptus secretion of interleukin-1 beta (IL1B) and may modulate uterine responses to this cytokine (Ross *et al*. 2003). The

importance of estrogen to early survival of pig conceptuses is underscored by pregnancy loss in response to premature exposure of the pregnant uterus to estrogen. Administration of estrogen on Days 9 and 10 of pig pregnancy is associated with altered expression of SPP1 and cyclooxygenase 1 in LE (Geisert *et al*. 2005, Ashworth *et al*. 2006), and degeneration of conceptuses by Day 15 (Morgan *et al*. 1987). In pigs, ESR1 are readily detectable in LE from Days 5 through 12 of the estrous cycle and pregnancy, then decrease, but remains detectable until Day 15 (Geisert *et al*. 1993). The presence of ESR1 on LE provides a mechanism by which secretion of estrogen by the elongating pig conceptus can stimulate changes in uterine function necessary for maintenance of pregnancy (Geisert *et al*. 1993). Previous reports, using endometrial explant culture and *in vivo* steroid replacement experiments, strongly suggested that induction of FGF7 in uterine LE during early pregnancy in pigs is stimulated primarily by conceptus estrogens (Ka *et al*. 2001, Wollenhaupt *et al*. 2005). Results of the present study support previous reports and further indicate that FGF7 is induced in the uterine LE of ovariectomized pigs injected E2, but only when progesterone has down-regulated PGR in LE. The uterine LE of pigs treated with E2 alone does not express *FGF7* mRNA, likely due to the lack of progesterone to down-regulate PGR in LE, whereas pigs treated with a combination of E2 and P4 exhibit *FGF7* expression. Further, functional stromal PGR, and therefore progestamedins, are not required for E2 induction of *FGF7* because the addition of ZK did not alter effects of the combination of P4 and E2.

There is a discrepancy between results of the present study and previous reports that estrogen in the absence of progesterone can increase endometrial expression of FGF7 (Ka *et al*. 2001, Wollenhaupt *et al*. 2005). The study by Ka et al (2001) used endometrial

explant cultures from Day 9 of the estrous cycle, a day when PGR are significantly reduced compared to Days 0 to 5 (Geisert *et al*. 1994, Sukjumlong *et al*. 2005). It is likely that PGR were already decreased to levels inadequate to prevent induction of *FGF7*. Although the study by Wollenhaupt *et al.* (2005) employed ovariectomized steroid-replaced pigs and detected a significant increase in endometrial *FGF7* mRNA in response to estradiol benzoate over progesterone treatment, levels of expression of *FGF7* mRNA overlapped between gilts treated with vehicle and estradiol benzoate. Further, effects of estradiol benzoate alone did not differ from the combined effects of progesterone and estradiol benzoate which did not differ from treatment with either vehicle or progesterone alone on FGF7 expression. Interestingly, increases in FGF7 protein were detected primarily in the vascular smooth muscle cells and endothelium, whereas weak expression in LE was not affected due to the different treatments (Wollenhaupt *et al*. 2005). In contrast, the present study utilized ZK, a PGR antagonist, to dissect the hormonal regulation of FGF7 and *in situ* hybridization analyses to understand cell-specific changes in *FGF7* expression. The results clearly indicate the complex and overlapping regulation of FGF7 in endometrial LE by P4 and E2.

The FGF7 synthesized and secreted by uterine epithelial cells into the uterine lumen can stimulate proliferation and differentiation of conceptus trophectoderm (Ka *et al*. 2000, 2001) by influencing DNA synthesis, as well as motility, differentiation, cytoprotection and antiapoptotic effects on cells (Finch *et al*. 1989). Western blotting detected an immunoreactive FGF7 protein of about 25-kDa only in uterine flushings of pigs treated with E2. Interestingly, a 17-kDa FGF7 protein was also detected in uterine flushings which may be a cleavage fragment of the native 25 kDa FGF7 protein. These

results indicate that E2 regulates secretion of FGF7 from LE cells. It is known that estrogens of conceptus origin or administered exogenously induce pseudopregnancy, modulate redirection of PGF2α secretion from primarily endocrine into the underlying vasculature to exocrine into the uterine lumen (Bazer & Thatcher 1977), and increase secretory activity of endometrial epithelia directly or by increasing uterine expression of prolactin receptors on uterine epithelia (Young *et al*. 1989). Therefore, we hypothesize that FGF7 secretion is regulated by conceptus estrogens during pregnancy, and ovarian estrogens during the estrous cycle. The detailed cellular mechanism by which estrogen affects epithelial FGF7 secretion remains to be determined.

In summary, the uterine environment of early pregnancy in pigs is complex and influenced by overlapping actions of conceptus estrogens for pregnancy recognition in concert with permissive effects of P4 acting on uterine epithelia to down-regulate PGR and/or via PGR expressed by uterine stromal cells to stimulate expression of a progestamedin(s) to mediate developmental changes in uterine functions necessary for establishment and maintenance of pregnancy. The dynamic regulation of FGF7 by estrogen and progesterone reflects this complexity, and strongly suggests important roles for FGF7 in stimulating proliferation and differentiation of the pig conceptus during elongation and implantation. Results of the present study provide new insights into the intricate interplay between the actions of estrogen and progesterone to modify gene expression in the peri-implantation pig uterus. A similar interplay between pregnancy recognition signals and progesterone have been reported for sheep (Johnson *et al*.1999b, Song *et al*. 2005,2006); However, the magnitude and extent of the influence that conceptus estrogens impart on the early pregnant uterus are remarkable and unique to the

pig. Clearly, further studies are warranted to further dissect the endocrine and paracrine regulation of gene expression in the uterus of pregnant pigs.

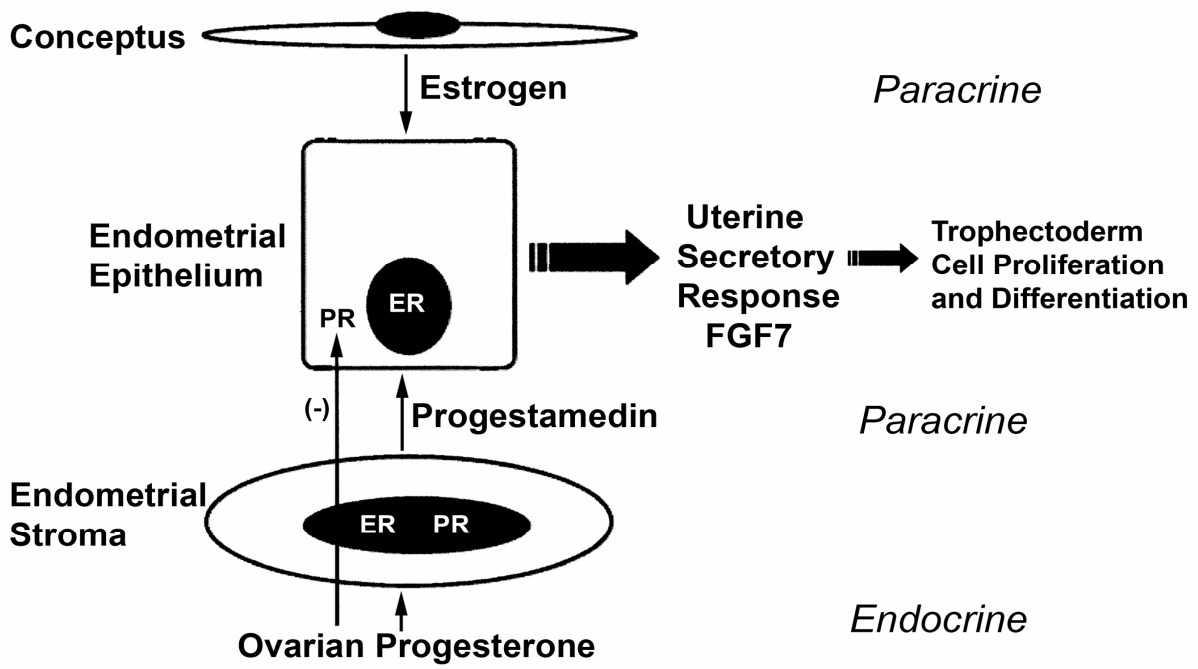


Figure 9. Schematic illustrating the proposed hormonal regulation of FGF7 expression in pregnant uterine endometrium of pigs. During early pregnancy, continuous exposure to progesterone down-regulates progesterone receptor (PGR) in endometrial epithelia to remove PGR-dependent inhibition of expression of most progesterone-regulated genes, e.g., LE. Therefore, the endocrine effects of progesterone to induce expression of FGF7 in endometrial LE and its secretion into the uterine lumen are mediated by paracrine-acting factors (progestamedins) produced by the PGR-positive stromal cells. Further, in PGR negative epithelial cells, E2, produced by pig conceptuses, binds E2 receptors in LE to induce FGF7 expression. The combined endocrine/paracrine effects of ovarian progesterone and conceptus E2 are likely responsible for the high levels of FGF7 expression in endometria and uterine lumen on Day 12 of pregnancy. During the estrous cycle, FGF7 expression increases in LE during the progesterone-dominated luteal phase, whereas maximal levels of FGF7 are attained on Day 12 of pregnancy after PGR are down-regulated and when LE is stimulated by both high levels of E2 released by pig conceptuses for pregnancy recognition, and progesterone can stimulate secretion of a progestamedin(s) by uterine stromal cells. It is hypothesized that secreted FGF7 acts on the conceptus to stimulate proliferation and differentiation of trophectoderm.

CHAPTER IV

ENDOMETRIAL AND PLACENTAL CHANGES IN THE PORCINE ESTROGEN AND P4 RECEPTORS DURING PREGNANCY AND THEIR ROLE ON FGF7 mRNA EXPRESSION

Overview

The objective of this study was to investigate the temporal and spatial expression of the estrogen receptor (ESR1) and PGR and correlates these to FGF7 expression in the uterus from Day 20 through Day 85 of pig pregnancy. As assessed by immunohistochemistry, ESR1 was readily detectable in the LE and GE in all days examined while PGR was undetectable. However, PGR were more abundant in the stromal cells at Day 20 than during later days of pregnancy. The current data showed also reduction of FGF7 in LE later in pregnancy. This data suggests that these changes may be related to soluble progestamedin levels released by PR positive stromal cells which are in turn affected by P4. An interesting finding in this study is the presence of ESR1 in placenta of the pig suggesting that estrogen may play important roles in the biology of pregnancy through out the gestation in the pig.

Introduction

Fibroblast growth factor-7 (FGF7) also known as keratinocyte growth factor, was originally identified in the conditioned medium of a human embryonic fibroblast cell line and identified as a mitogen that is particularly active on keratinocytes and specific for

epithelial cells (Rubin et al. 1989). Later studies showed that FGF7 is a paracrine mediator of epithelial-mesenchymal interactions in various organs, including those of the reproductive tract (Rubin et al. 1995). In the pig, however, it has been shown that FGF7 is produced and secreted by uterine epithelial cells into the uterine lumen and may functions to stimulate the proliferation and differentiation of conceptus trophectoderm (Ka *et al.* 2000). FGF7 expression was first observed in LE on Day 9 of the estrous cycle or pregnancy, peaked on Day 12 of pregnancy during a period when the concentrations of P4 (P4) increases and conceptuses releases estradiol-17β (E2) for pregnancy recognition (Ka *et al.* 2000). Subsequent studies using an endometrial explant culture system were performed to determine hormonal and/or cytokine regulation of FGF7. In these studies, E2, but not P4, increased *FGF7* mRNA (Ka *et al*. 2001).

The objective of the current study, was to investigate the extent to which E2 and P4 and their receptors contribute to *FGF7* mRNA expression in the uterus of the pigs during later pregnancy, i.e., from Days 20 through 85. We also assessed these same endpoints using short-term (Day 15) and long-term (Day 90) pseudopregnancy models. These experiments further support the role for steroid hormones and P4 in particular, as important regulators of *FGF7* mRNA expression from Day 20 though 85 of pregnancy in the pig.

Materials and Methods

Animals and tissue collection

All experimental and surgical procedures complied with the Guide for Care and Use of Laboratory Animals and were approved by the Agricultural Animal Care and Use

Committee of Texas A&M University. Sexually mature crossbred gilts were observed daily for estrous behavior. Gilts exhibiting at least two estrous cycles of normal duration (18–21 days) were assigned randomly to cyclic, pregnant or pseudopregnant status. Gilts for pregnancy were mated with crossbred boars. Gilts were hysterectomized on either Days 5 or 12 of the estrous cycle (Day 0 = estrus) or Days 9, 12, 13, 14, 15, 20, 25, 30, 35, 40, 60, or 85 of pregnancy (n = 3 gilts per day). Pregnancy was confirmed by the presence of conceptuses of normal morphology in the uterine flushings on Days 9, 12, 13, 14, and 15 or by visual observation of conceptus tissues undergoing attachment and placentation from Days 20 through 85.

Two different pseudopregnancy models were used to evaluate uterine estrogen receptor alpha (ESR1), and PGR protein expression and their role on *FGF7* mRNA expression. In both models, cycling gilts were randomly assigned on Day 0 (estrus/mating) to pseudopregnant status (Bazer, 1992). For the model referred to as "short-term pseudopregnancy," gilts received i.m. injections of estradiol benzoate (5 mg in 5 ml of corn oil/day) or corn oil alone (n =4/treatment) on Days 11, 12, 13, and 14 of the estrous cycle. Gilts were then ovariohysterectomized on Day 15. For the second pseudopregnancy model referred to as "long-term pseudopregnancy," gilts received i.m. injections of estradiol benzoate (5 mg in 5 ml of corn oil/day) on Days 11, 12, 13, 14 and 15 of the estrous cycle and were subsequently ovariohysterectomized on Day 90 of pseudopregnancy. At hysterectomy, several sections (0.5 cm) from the middle portion of each uterine horn of both pregnant and pseudopregnant gilts were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2) for 24 h and then embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO).

In situ *hybridization analysis*

The location of *FGF7* mRNA in uterine tissue sections was determined by *in situ* hybridization analysis as described previously (Ka *et al.* 2000). Briefly, deparaffinized, rehydrated, and deproteinated uterine tissue sections (~ 5 μm) were hybridized with radiolabeled antisense or sense porcine *FGF7* cRNA probes that were generated from linearized plasmid templates using *in vitro* transcription with [^{35}S]-UTP (Perkin Elmer Life Sciences, Inc). After hybridization, washes and digestion with RNase A, autoradiography was performed using Kodak NTB-2 liquid photographic emulsion (Eastman Kodak, Rochester, NY). Slides were exposed at 4̣C for 2 wks, developed in Kodak D-19 developer, counterstained with Harris modified hematoxylin (Fisher Scientific, Fairlawn, NJ), dehydrated through a graded series of alcohol to Citrisolv (Decon Laboratories, Inc , King of Prussia, PA) and protected with cover slips.

Immunohistochemistry

The expression of PGR and ESR1 antigens were evaluated in paraformaldehyde-fixed, paraffin-embedded, uterine tissue cross-sections (4 μm) using the procedures described previously (Spencer *et al.* 1995). Briefly a boiling citrate buffer antigen retrieval protocol was used to retrieve the PGR and Pronase E (0.5 mg/ml in PBS) was used to retrieve ESR1 antigen. The proteins were detected by using mouse anti-human PGR (2 μg/ml; 2C5 PR, Zymed Labs, San Francisco, CA) and rat anti human- ESR1 (2 μg/ml; H222, Dr. Chris Nolan, Abbott Laboratories, Chicago, IL) and a Super ABC Mouse/Rat IgG Kit (Biomeda, Foster City, CA). For negative controls, primary

antibodies were substituted with mouse (for PR) or rat (for ER) IgG at the same concentration as the primary antibodies.

Photomicrography

Digital photomicrographs of representative brightfield illumination (immunohistochemistry) or brightfield and darkfield illumination (*in situ*) images were evaluated with a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY) fitted with an Axiocam HR digital camera. Digital images of immunohistochemistry and *in situ* hybridization were recorded using Axiovision 4.3 software. All immunohistochemistry and *in situ* hybridization figures were assembled using Adobe Photoshop 8.0 (Adobe Systems Inc., San Jose, CA).

Results

ER expression in pig endometrium during pregnancy

Endometrial ESR1 expression was evaluated by immunohistochemistry during the interval from Day 20 through Day 85 of pregnancy in the pig. In order to validate ESR1 immunohistochemical results of ESR1 staining during later pregnancy, ESR1 staining was also performed on tissues| from Days 5 and 15 in cyclic pigs as well as Day 15 of pregnancy. Consistent with earlier findings (Geisert *et al*. 1993), ER expression was undetectable in the LE and stroma of Day 5 cyclic endometrium whereas ER was present in LE and stroma of Day 12 cyclic and Day 15 pregnancy (Figure 10). From Day 20 to Day 85 of pig pregnancy, ESR1 was readily detectable in the LE and GE while stromal nuclear ESR1 staining remained high until Day 40 when it began to decline through Day

85 when relatively few immunostained stromal cells were detected. It is noteworthy that ER-positive cells were detected in the placenta in all days examined, Day 20 through 85. The nuclei of both trophoblast and allantois showed high levels of ESR1 (Figure 11).

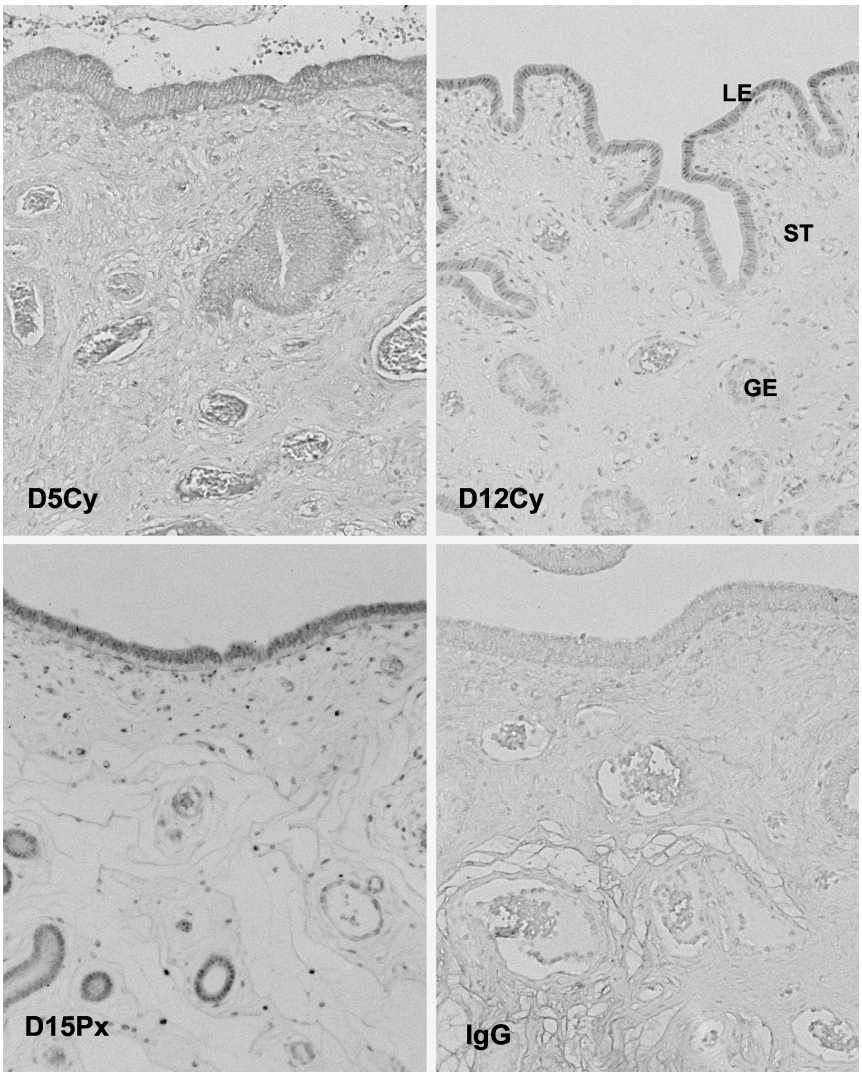


Figure 10. Immunohistochemical localization of nuclear ESR1 protein in paraffin-embedded pig endometrium. Photomicrographs show the distribution of ESR1 in cyclic and early pregnant pigs. Nuclear immunostaining was detected in the luminal epithelium (LE), glandular epithelium (GE) and stroma (ST) of Day 12 cyclic and Day 15 pregnant pigs, whereas ESR1 protein was absent form endometrial compartments of Day 5 cyclic animals. Sections from Day15 pregnant pigs incubated with nonimmune rat IgG (IgG) serve as negative controls. Width of each field 540 μm.

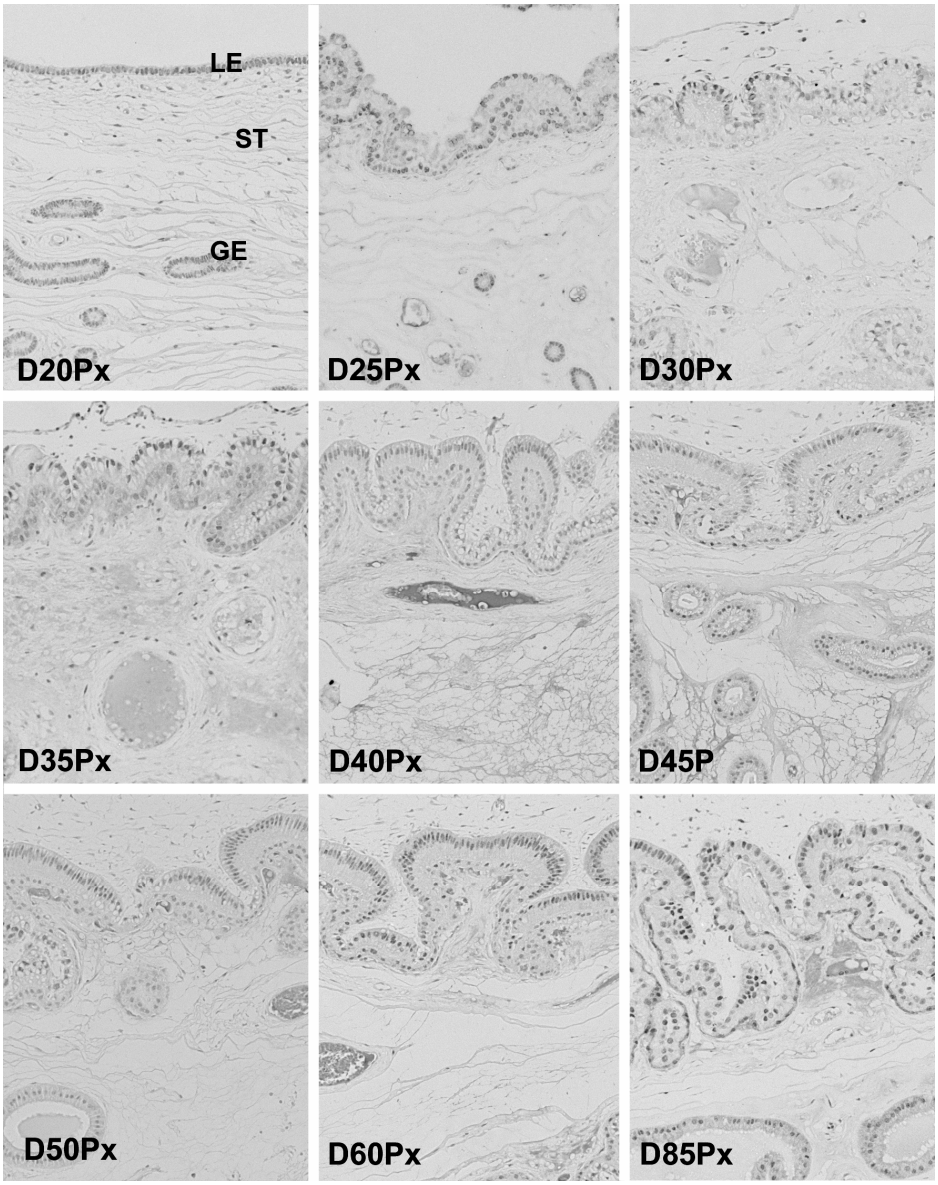


Figure 11. Immunohistochemical localization of nuclear ESR1 protein in paraffin-embedded pig endometrium. Photomicrographs show the effects of advancement of pregnancy on the presence and distribution of nuclear ESR1 protein in porcine endometrium and extraembryonic membranes. Nuclear ESR1 immunostaining was detected with variable intensity in the three endometrial cellular compartments, the luminal epithelium (LE), glandular epithelium (GE) and stroma (ST) while it is consistently present in the trophectoderm (TR) and other extraembryonic membranes during all days examined. Width of each field is 540 μm.

PGR expression in pig endometrium during pregnancy

Immunohistochemical analysis of PGR was also examined in endometrium from Day 20 through Day 85 of pregnancy. Validation of immunoreactive PGR was confirmed by PGR staining of endometrial tissue from Days 5 and 9 of the estrous cycle and Day 15 of pregnancy. As reported previously by Geisert *et al*. 1994, PGR-positive nuclei in LE and GE were detected at highest levels on Day 5 and decreased to undetectable levels after Day 9 of the cycle and were also absent on Day 15 of pregnancy. Further, PGR nuclear staining in stroma was present throughout the estrous cycle and early pregnancy (Figure 12). The absence of PGR staining in LE and GE continued from Day 20 through Day 85 of pregnancy. Stromal PGR was high on Day 20 through Day 40 of pregnancy, however, as was seen with stromal ER, nuclear PGR staining declined after Day 40 and by Day 85, relatively few PR-positive stromal cells were detected (Figure 13).

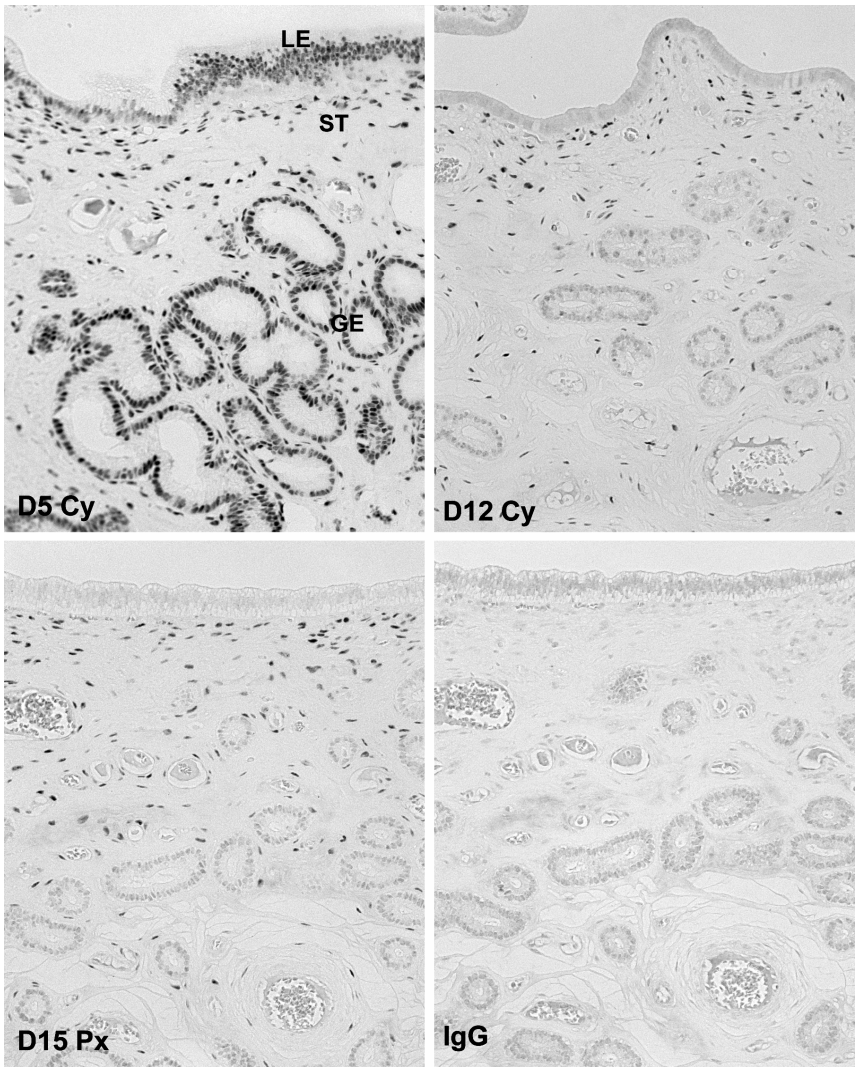


Figure 12. Immunohistochemical localization of nuclear PGR protein in paraffin-embedded pig endometrium. Photomicrographs show the distribution of PGR in the cyclic and early pregnant pigs. Nuclear immunostaining intensity was strong in the luminal epithelium (LE) and glandular epithelium (GE) of Day 5 cyclic pigs, whereas PGR protein was down-regulated in the LE of Day 12 cyclic and Day 15 pregnant pigs. Sections from Day 15 pregnant pigs incubated with nonimmune mouse IgG (IgG) serve as negative controls. Width of each field 540μm.

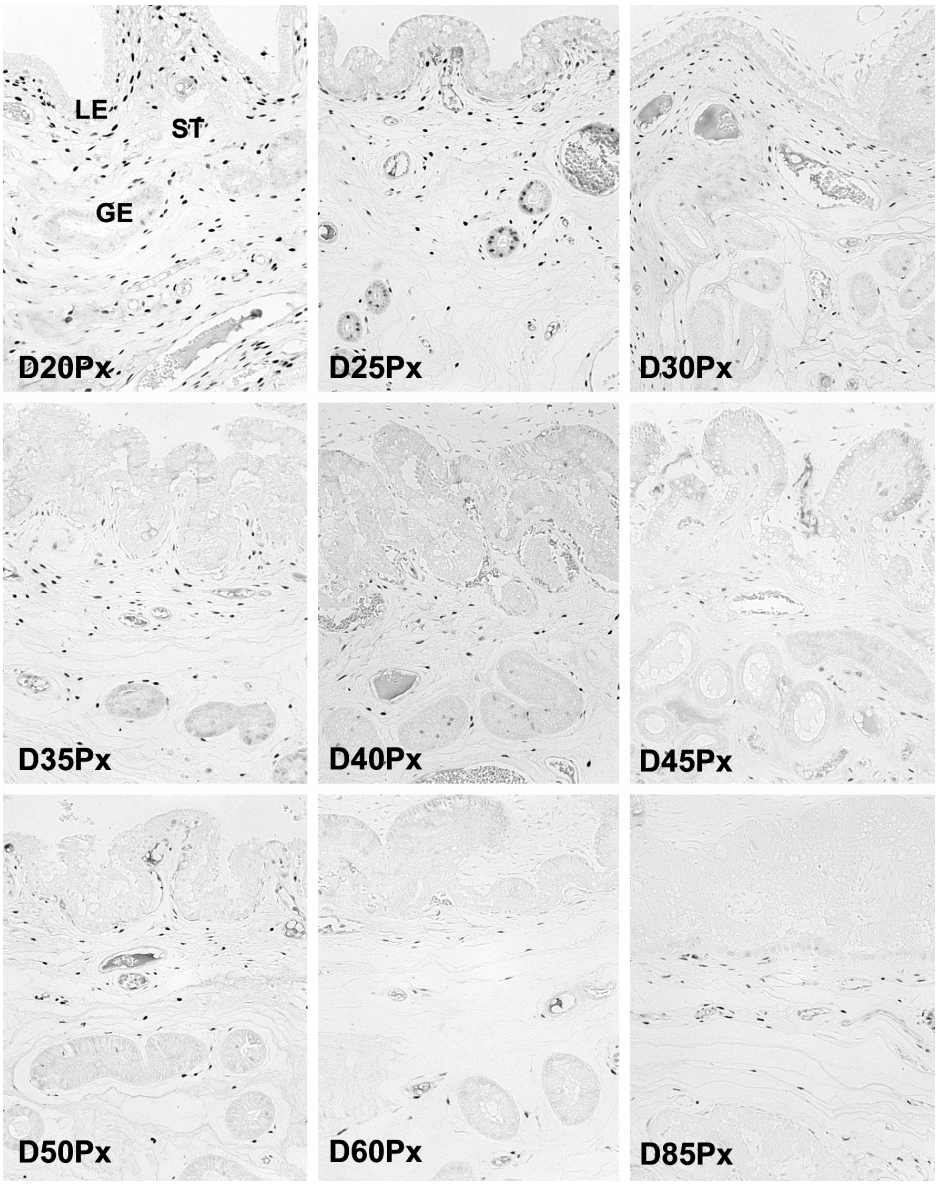
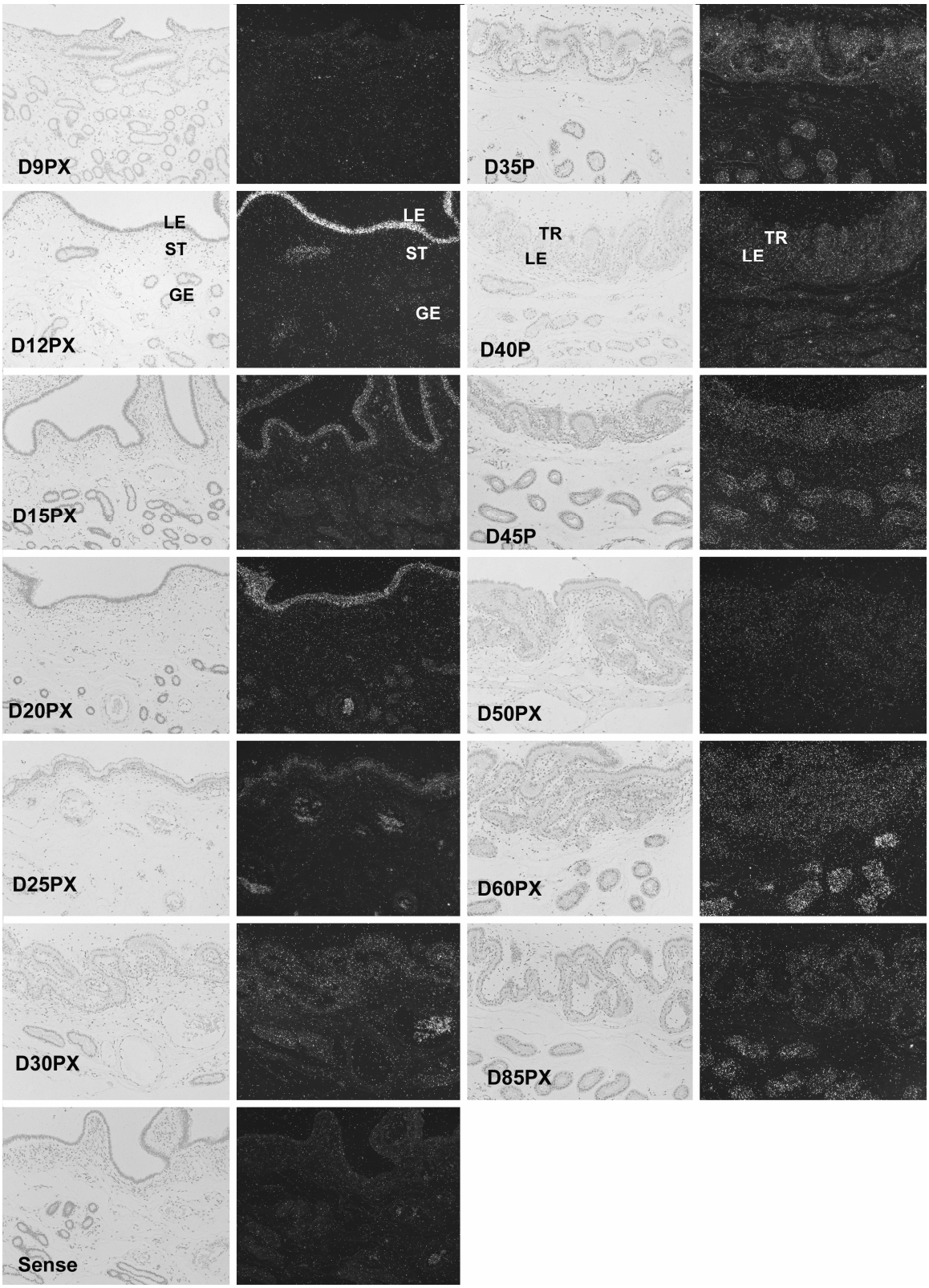


Figure 13. Immunohistochemical localization of nuclear PGR protein in paraffin-embedded pig endometrium. Photomicrographs show the effects of advancement of pregnancy on the presence and distribution of nuclear PGR protein in porcine endometrium and extraembryonic membranes. There was no nuclear immunostaining in the luminal (LE) or glandular epithelia (GE) while the stroma (ST) showed strong immunoreactivity in the Days 20-35 pregnancy which decline later but did not disappear. The trophectoderm and other extraembryonic membranes did not show any PGR immunostaining. Width of each field is 540 μm.

Figure 14. *In situ* hybridization analysis of FGF7 mRNA expression in the porcine endometrium. The left and right panels represent corresponding brightfield and darkfield images, respectively, of endometrium for each treatment. A representative section from Day15 pregnant pigs hybridized with a radiolabeled sense cRNA probe (Sense) serves as a negative control. FGF7 mRNA is detectable in LE of Days 12-85 of pregnancy. Note the the expression of FGF7 is gradually decrease to very low levels at the late stage of pregnancy. LE, lumenal epithelium; GE: glandular epithelium; ST: stroma; TR: trophectoderm. Width of each field, 870 μm.


D9PX
D35P
LE
ST
GE
D12PX
LE
ST
GE
TR
LE
D40P
TR
LE
D15PX
D45P
D20PX
D50PX
D25PX
D60PX
D30PX
D85PX
Sense

FGF7 mRNA expression in pig endometrium during pregnancy

FGF7 mRNA expression was analyzed from Days 9 through Day 85 of pregnancy. These data overlap and confirm previous data reported by Ka *et al.* (2000) in which *FGF7* mRNA was undetectable on Day 9 of pregnancy but highly expressed in LE on Days 12 and 15. In later pregnancy, *FGF7* mRNA expression levels were high on Days 20 and 25, however, expression levels declined during later pregnancy and were very low by Day 85 (Figure 14).

Analysis of ER, PGR and FGF7 mRNA during pseudopregnancy

Endometrial tissues from two different pseudopregnancy model systems were analyzed for ESR1 and PGR immunoreactivity and *FGF7 mRNA* expression and compared to a corn oil-treated control. Strong immunoreactive ESR1 was detected in the LE of both the corn oil control and the long-term pseudopregnant animals (Day 90) whereas ESR1 staining in the LE of the short-term pseudopregnant animals (Day 15) was considerably lower (Figure 15). PGR immunoreactivity was absent from the LE was and strong in the stroma in corn oil control, and both short-term and long-term pseudopregnant pigs (Fig 15). *FGF7 mRNA* expression was relatively high in the LE of corn oil-treated and short-term pseudopregnant animals whereas *FGF7 mRNA*, was absent in the long-term pseudopregnant animals.

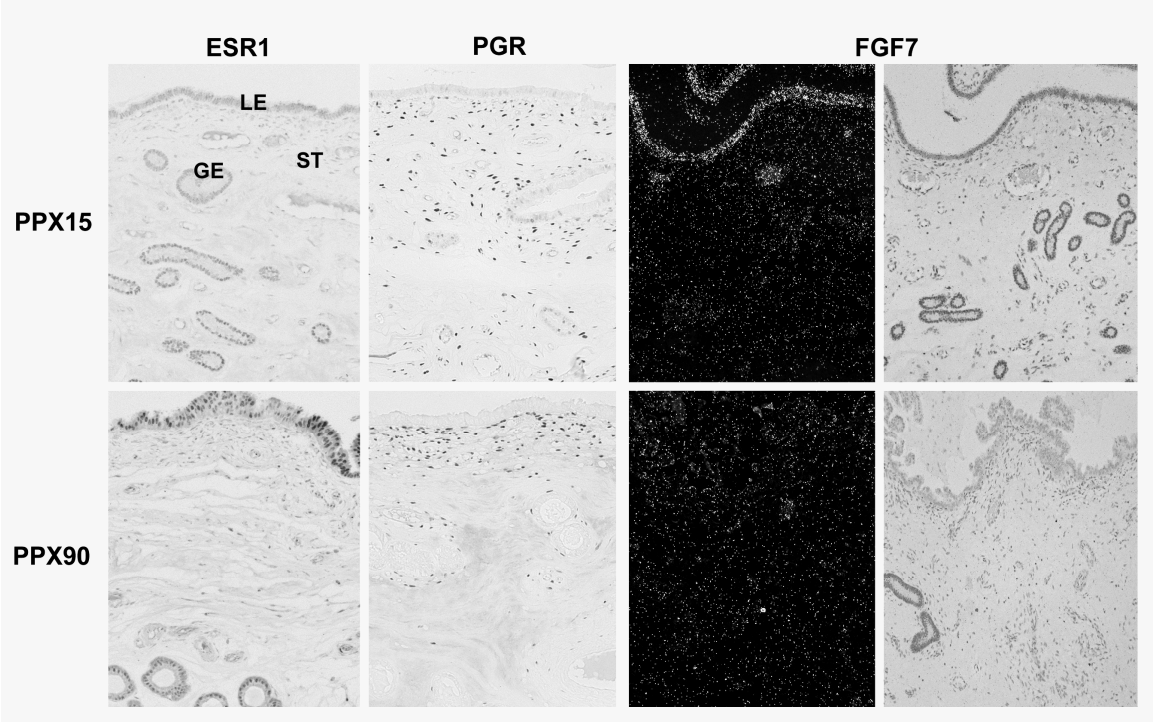


Figure 15. Interrelationships between FGF7 mRNA, progesterone receptor protein and ESR1 protein in pseudopregnant pig endometrium. First Column: Nuclear immunostaining for ESR1 protein was detected in luminal (LE), glandular (GE) epithelia (GE) and stromal cells (ST) of endometria from all treatment groups. Width of each field, 540μm. Second Column: Nuclear immunostaining for PGR protein was detected in stroma cells (ST) of endometria from both treatment groups while PGR was undetectable in the LE. Width of each field, 540 μm. Third and Fourth Columns: *In situ* hybridization analysis of *FGF7* mRNA expression in pig endometrium. The left and right panels represent corresponding brightfield and darkfield images, respectively, of endometria from each treatment group. Note that *FGF7* mRNA is detectable only in LE and the hybridization signal is evident in endometrial of short-term pseudopregnant pig only. Width of each field, 690 μm.

Discussion

The present study is an extension of results presented in the previous chapter in which the interplay between the actions of estrogen and P4 regulate FGF7 in the peri-implantation uterus. Reported here is an analysis of *FGF7* mRNA expression in the uterus of pregnant pigs later in pregnancy (Days 20 through Day 85) in the context of ESR1 and PGR in endometrial LE and stroma. The presence of *FGF7* mRNA in the LE supports the hypothesis that FGF7 plays an important paracrine role in the developing conceptus as hypothesized in earlier studies by Ka *et al*. (2000, 2001).

The observation that *FGF7* mRNA and protein were detected in the LE and uterine secretions respectively, and the presence of the FGF7 receptor, *FGFR2IIIb* mRNA in conceptus trophectoderm (Ka *et al*. 2000) is counter to a paradigm that described FGF7 as a mesenchymal factor released to act on the adjacent epithelium (Finch *et al.* 1989). Indeed, the hypothesis is that FGF7 in pig endometrial LE acts in an autocrine and/or paracrine manner to modulate the cellular activity of the trophectoderm and LE since *FGFR2IIIb* mRNA was also detected on the LE as well (Ka *et al*. 2000). Members of FGF growth factor family have been shown to affect cell growth, migration and protease synthesis directly and hence might be involved in tissue remodeling during pregnancy (Chai *et al.* 1998, Powers *et al.* 2000). In the present study, the decline in *FGF7* mRNA with the advancement of pregnancy is consistent with the reduced proliferative activity of trophectoderm cells as reported by Blankenship & King (1994), and this expression pattern supports a role of FGF7 in trophectoderm proliferation *in vivo* during the earlier stage of pregnancy.

To study the effect of E2 and P4 on the expression of *FGF7* mRNA, we examined ESR1 and PGR status in the pig endometrium since it is well established that both of these steroid hormones elicit their effects through ESR1 and PGR (Cooke *et al.* 1998*a*). As shown in Figs. 1 and 2, the expression of ESR1 varies with the physiological status as previously reported (Geisert *et al*. 1993). ESR1 expression was undetectable in the LE and stroma of Day 5 cyclic animals while it was detected on Day 12 of the cycle and on Day 15 of pregnancy. The current study further examined the expression of ESR1 and PGR from Day 20 to Day 85 of pregnancy. ESR1 was readily detectable in the LE and GE on all days examined while the population of stromal cells that were immunoreactive for ESR1 began to decline around Day 40 of pregnancy. On the other hand, PGR expression was not detected in the LE from Day 20 through Day 85 and the stroma showed decreasing levels of PR-positive cells with the progression of the pregnancy. Previous studies showed increased *FGF7* mRNA expression in the LE of the pig between Day 12 and 15 of the estrous cycle and pregnancy which peaked around Day 12 of pregnancy, the day which temporally coincides with the release of E2 from the conceptus for maternal recognition of pregnancy (Ka *et al.* 2000). In a later *in vitro* study, Ka *et al*. 2001 reported that E2 increased *FGF7* mRNA in a dose-dependent manner.

Results presented in the previous chapter indicate that, in ovariectomized pigs treated with steroid hormones, E2 increased *FGF7* mRNA expression if, and only if, PGR is downregulated in the LE. This is consistent with a similar report in sheep in which long-term exposure to P4 downregulated PGR in LE and while maintaining stromal PGR (Spencer *et al.* 2004*b*). Therefore, we hypothesized that the increase in

FGF7 mRNA in animals treated with P4 alone was due to a putative progestamedin released from PR-positive stromal cells.

The current data from pregnant pigs examined from Days 20 through 85 showed a reduction in the expression of *FGF7* mRNA in LE which also coincides with reduction in PGR staining within stromal cells. This reduction in *FGF7* mRNA in LE might be related to reduction of soluble progestamedin levels released by PR-positive stromal cells which are in turn affected by P4 levels, the main regulator of secretory activity during this period (Roberts & Bazer 1988). Soluble factors appear to act as mediators of mesenchymal-epithelial interaction under other physiological conditions such as inflammation and tissue repair (Sporn & Roberts 1986). Fibroblast cells produce a number of soluble factors which function as paracrine regulators of neighboring epithelial cells (Stoker & Perryman 1985). The biological activity of these soluble factors and the nature of the ECM surrounding these cells are mutually interdependent parameters in the sense that many soluble factors such as TGFβ appear to exert a primary effect on the matrix biosynthesis by their responsive target cells (Ignotz & Massague 1986) and the response of the cells to soluble growth factors may be as well modulated by the nature of the ECM (Schor & Schor 1987).

However, the results from the two pseudopregnant pig models showed expression of *FGF7* mRNA in LE at Day 15 (short-term pseudopregnant model) while *FGF7* mRNA was undetectable at Day 90 of the pseudopregnancy (long-term pseudo pregnant model). In these animals the ESR1 was detected on the LE of both days 15 and 90 while PGR was only detectable at the stroma. The results from the short-term pseudopregnant study support our current studies that indicate exogenous or CL P4 could regulate *FGF7*

mRNA expression through the induction of progestamedins. Although, the CL of pseudopregnant pigs appears to remain active as late as Day 110 of pseudopregnancy (Fuller W. Bazer, personal communication), the *FGF7* mRNA was not detected at Day 90 in the long term pseudopregnant model. The reason for this discrepancy remains to be determined. It is possible, that conceptus factors may be required to maintain *FGF7* mRNA levels. Endometrium from pregnant gilts secreted more protein, nondialyzable macromolecules, acid phosphatase activity, and retinol-binding protein than did endometrium from pseudopregnant pigs (Vallet *et al.* 1994, Vallet & Christenson 1996). Likewise, SPP1 is expressed by pig LE and high levels of *SPP1* mRNA are elevated in LE in proximity to conceptus tissue whereas lower *SPP1* mRNA expression was recorded in both short and long term pseudopregnant pigs (White *et al.* 2005). There is a wide array of growth factors and cytokines, including interleukin-1β, prostaglandin E, Type I and II interferons, and TFGβ-1, -2, and -3, all have been reported to be release by pig conceptus (Jaeger *et al.* 2001) which could influence *FGF7* mRNA levels in LE.

An interesting finding in this study is the presence of ESR1 in placenta of the pig. Previous studies reported estrogen receptor expression in the placenta of human (Bukovsky *et al.* 2003a, b), rat (Al Bader 2006) and cattle (Schuler 2005). In the pig estrogen is synthesized and released by the conceptus in a three phases. Between Days 11 and 12, pig conceptuses produce estrogen which believed to be a critical component for the signaling mechanism for maternal recognition of pregnancy, followed by a second phase between Days 15 and 25 of pregnancy (Bazer *et al.* 1998). Later in pregnancy, there is another sustained high level of estrogen starting from Day 70 up to parturition (Knight 1994). In domestic animals, maternal and conceptus steroid hormones have

important interrelated roles in mediating different cellular activity during various stages of pregnancy (Jaeger *et al.* 2001). The current results of ESR1 expression in the trophectoderm are the first to suggest that conceptus derived estrogen could exert effects on the fetal and maternal tissues by interacting with these receptors in an autocrine and paracrine manner.

In conclusion, investigations on pig FGF7 regulation in the context of ESR1 and PGR expression during pregnancy and in pseudopregnant pigs clearly point to a role of steroid hormones and P4, in particular, as important regulators of FGF7 expression in throughout pregnancy in the pig. Expression of ESR1 in the placenta throughout pregnancy suggests that E2 may play important roles in the biology of pregnancy through out the gestation in the pig.

CHAPTER V

SUMMARY AND CONCLUSIONS

SIBLING Proteins in the Uterus of Sheep

Immunocytochemical analysis of SIBLING proteins in the ovine uterus have uncovered evidence for the presence of DSPP, DMP1 and MEPE members of this protein family (but not BSP) in the uterus of sheep. Reagents were not available to search for a recently identified, more distantly related potential member of the SIBLING family, enamelin (ENAM) (Fisher & Fedarko 2003). The most striking finding of this study was the similarity in the expression pattern between DSPP and the previously reported expression of SPP1 in the sheep uterus (Johnson *et al*. 1999a, 2000, 2001. 2003*b*, 2006). While the multiple physiological roles of SPP1 within several tissue-level compartments of the uterus during pregnancy are in the early stages of being defined, there is growing evidence that SPP1 might be required for adhesion and signal transduction at the uterine-placental interface and/or as a gene product expressed by uterine stroma as it decidualizes in response to conceptus invasion. DSPP is a chimeric glycoprotein with dentin sialoprotein (DSP) on its N-terminus and dentin phosphoprotein (DPP) on its C-terminus (Ritchie and Wang 1996); however, the LF-151 antibody used to localize DSPP was developed against a bacterial recombinant peptide recognizing the N-terminal "part of the DSP portion of human DSPP". It has not yet been determined whether staining recognized a "pro" form of DSPP or if the DSPP was proteolytically cleaved to DSP and DPP moieties (Ritchie & Wang 2000). Therefore, it is important to determine whether the expression of DSPP detected by immunocytochemistry in the stroma is secreted into the

matrix or is in an intracellular proDSPP form, or both. Co-localization studies of the DSPP and a stromal-specific marker such as vimentin would help to address this issue. Alternatively, ultrastructural immunocytochemistry would be very useful if antigen preservation is adequate in tissues processed for electron microscopy.

In addition, further analysis the size of DSPP protein detected in uterine endometrium by Western blot analysis is warranted. This would be useful to determine whether the immunoreactive protein represents full length DSPP or a proteolytically processed protein. It is noteworthy that SPP1 is proteolytically cleaved by MMPs into fragments with different biological activity (Agnihotri et al. 2001, Gao et al. 2004). Johnson et al., (1999b, 2003c) have identified full length SPP1 and SPP1 fragments using SPP1 antibodies (i.e., LF-124 and LF-123) which recognizes an N-terminal RGD containing increased biological activity for cell attachment and motility and a carboxy-terminal fragment that identifies the full length protein and fragments with cell binding activity without promoting adhesion. Further analysis of the *in situ* processing of *DSPP* in the uterus is needed to determine if there is any type of functional redundancy between DSPP and SPP1. At the time of this investigation, however, antibodies were not available to analyze amino- and carboxy-terminal fragments of DSPP. It is obvious that a more comprehensive analysis of spatial and temporal patterns of *DSPP, DMP1 and MEPE* mRNA and proteins throughout the estrous cycle and pregnancy is needed in order to begin to address the functional relevance of this protein family in uterine function.

All the SIBLING proteins display the RGD motif that mediates cell attachment/signaling via its reaction with cell surface integrins (Qin et al. 2004). All five αv integrins, two β1 integrins (α5 and α8) and αIIbβ3 share the ability to recognize

ligands containing an RGD tripeptide active site (Humphries et al. 2006). Studies from sheep indicate the presence of αv, α4, α5, β1, β3, and β5 integrin subunit mRNA and protein expression by endometrium of both cyclic and pregnant ewes (Johnson *et a.l* 2001). Therefore, it is crucial to identify the specific integrin receptors capable of binding individual integrins. An *in vitro* focal adhesion assay that has been previously used by Johnson et al. (2001) involving matrix coated polystyrene beads would be useful to identify integrin binding partners of individual siblings on cultured endometrial stroma and or luminal epithelial cells.

This investigation revealed that DMP1 and MEPE proteins were detected in the stroma of cyclic and pregnant animals. Earlier reports linked DMP1 expression to the high affinity binding and activation of MMP-9 (Fedarko *et al.* 2004) (also known as gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase). The possible DMP1/MMP-9 relationship was not investigated in this study but is a logical next step to evaluate the potential role of DMP1 in the ovine uterus. While no specific MMP partner(s) for MEPE has been reported as yet, it is possible that a similar MMP relationship exists. The co-distribution of these two SIBLINGS suggests similarity in their biological roles in the uterus. An important future direction is an analysis of MMP distribution in the ovine uterus, and where indicated, colocalization analysis with appropriate SIBLING partners. As with DSPP, additional proteomic studies are needed to better characterize DMP1 and MEPE for further structural and functional studies. Moreover, using of the unilateral pregnancy model will be useful to evaluate the potential role of the conceptus on the expression of DMP1 and MEPE.

Placentomal and interplacentomal endometrium are evident after day 40 of pregnancy in sheep (Boshier 1969). However, there are few reports regarding differences in gene expression within these compartment-specific regions of the uterus such as that reported for glycosylation dependent cell adhesion molecule 1-like protein (Muniz et al. 2006). In the cow, which has cotyledonary type of placenta, the SPP1 expression showed compartmental differences (Pfarrer et al. 2002). Therefore, the compartmental variation in the SIBLING expression in the sheep should also be considered in any future studies.

FGF7 Expression in Steroid Treated Pigs

The objective of this project was to test the hypothesis that *FGF7* mRNA expression in endometrial LE of pigs is up-regulated by estrogen via ER present in endometrial epithelia and progesterone is also required as a permissive hormone to allow estrogen to stimulate *FGF7* mRNA expression or to stimulate PGR positive stromal cells to release, a yet to be defined, progestamedin which in turn will upregulate *FGF7* mRNA in LE. We found that:

1. In the absence of P4 and estrogen, the LE of CO-treated pigs expressed PGR which precluded expression of *FGF7* mRNA (Fig. 16).

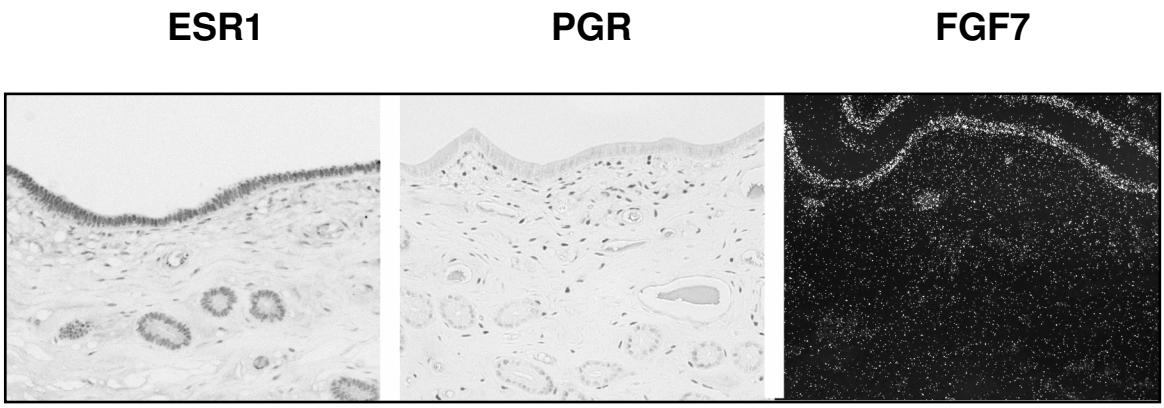


Figure 16. Porcine endometrium treated with corn oil.

2. In P4-treated pigs, PGR were down-regulated in LE, but maintained in stromal cells, allowing production of a putative progestamedin-induction of *FGF7* mRNA in LE (Fig. 17).

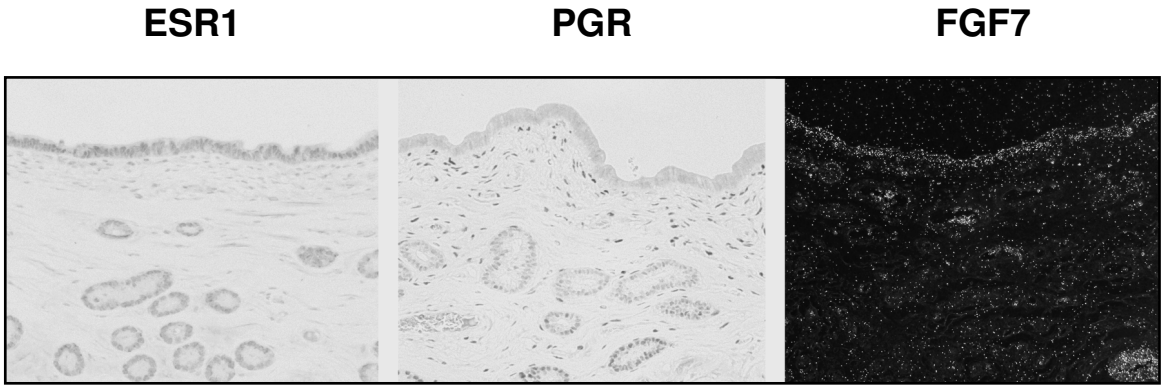


Figure 17. Porcine endometrium treated with P4 only.

3. For pigs treated with P4 and ZK, uterine PGR are not functional in stromal cells which precludes progestamedin-induction of *FGF7* mRNA in LE (Fig. 18).

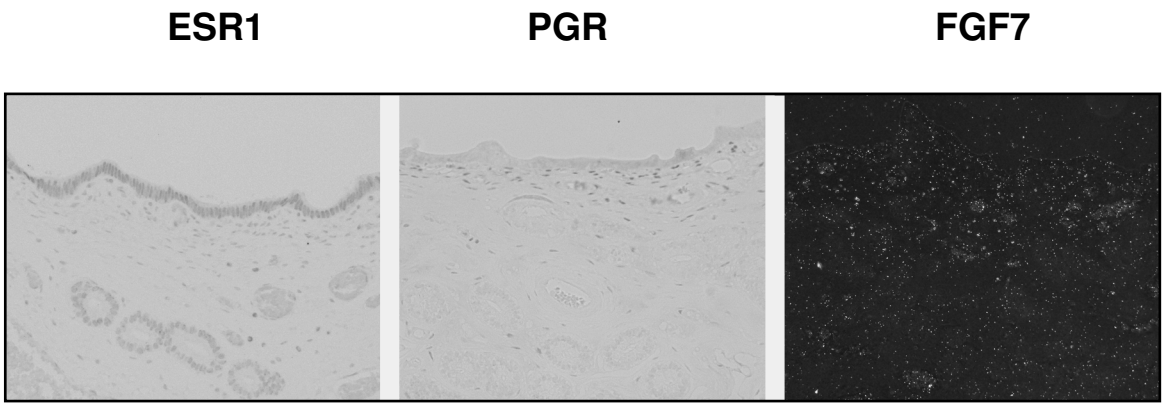


Figure 18. Porcine endometrium treated with P4+ZK.

4. Injection of estrogen alone did not induce *FGF7* mRNA in LE because LE was PGR-positive (Fig. 19).

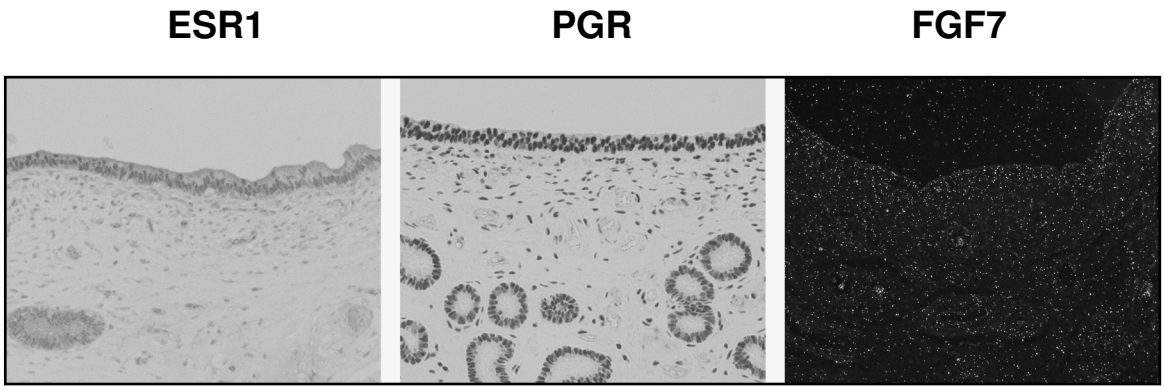


Figure 19. Porcine endometrium treated with estrogen alone.

5. Treatment with P4 and estrogen allowed both down-regulation of PGR and induction of *FGF7* mRNA in LE via either direct effects of estrogen on ESR1, effects of a progestamedin, or their combination (Fig. 20).

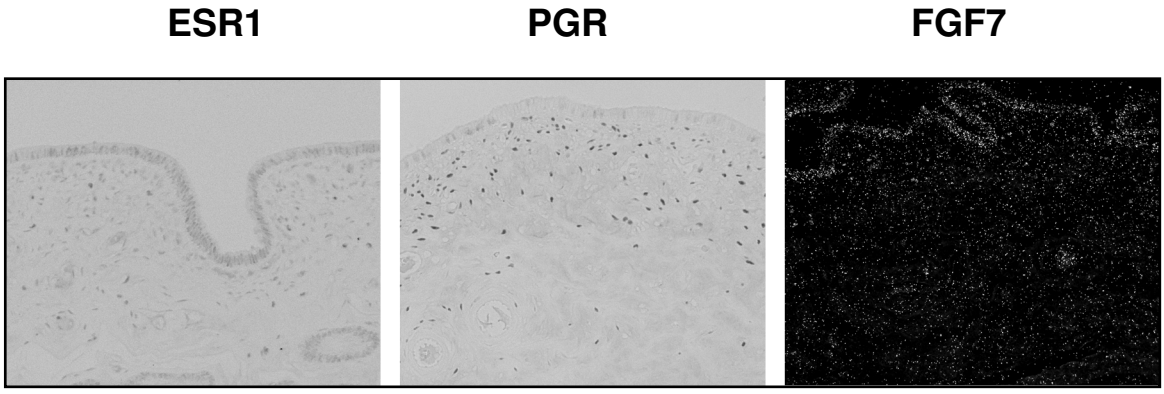


Figure 20. Endometrium of pig treated with estrogen and P4.

6. LE of pigs treated with PZKE expressed *FGF7* mRNA indicating estrogen can directly induce FGF7 in the absence of functional PGR (Fig. 21).

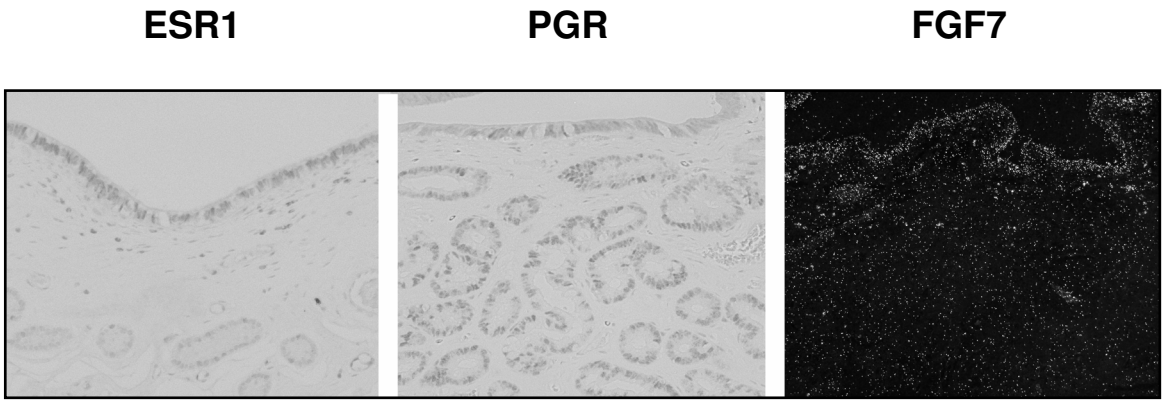


Figure 21. Endometrium of pig treated with estrogen, P4 and ZK.

Accordingly, we concluded that (Fig. 10) P4 is permissive to FGF7 expression by down-regulating PGR in LE. Moreover, P4 might stimulate PGR positive uterine stromal cells to release progestamedin that induces FGF7 expression by LE. Estrogen and P4 can induce FGF7 in the absence of PGR rendered nonfunctional by ZK. Finally, estrogen

from conceptuses interacts via ESR1 in LE to induce maximal expression of FGF7 on Day 12 of pregnancy in pigs.

An alternative hypothesis to the suggestion that a progestamedin is responsible for induction of *FGF7* mRNA by LE has been offered by Geisert et al. (2005) in which these authors hypothesize that P4 interacts with PGR in LE to stimulate factors that activate nuclear factor kappa B (NF-κB) which then functions to both inhibit PGR expression and activate transcription of genes believed to be involved with implantation (Geisert *et al*. 2005). Further investigations are required to explore these competing hypotheses. An LE – stromal coculture approach might be useful to search for a potential stromal factor that might function as a progestamedin. It might also be possible to utilize chemical (curcumin, PDTC, or salicylic acid) or biological inhibitors (NIK-KM or IKK-p DN) to NF-κB DNA binding activity in porcine LE.

ESR1, PGR and FGF7 Expression in Pregnant Pigs

The pattern of the expression of *FGF7* as it has been recorded in the previous researches (KA et al. 2000, 2001, 2005, Chapter III) indicating a tight control of the estrogen and P4 on this gene either directly or indirectly. The current study is further suggesting the roles of these steroid hormones. It is also showed that the critical importance of FGF7 in the early stages of pregnancy in the pig is gradually decreases as the pregnancy progresses and in fact, this study advances our knowledge of how estrogen and P4 and their receptor work under normal physiological conditions. At this point, two key issues in P4 role need to be addressed; they relate to how P4 induces PGR positive stromal cells to release any hypothesized progestamedin and how these progestamedin

induces the production of FGF7. Another, area of investigation will be the role of estrogen in FGF7 regulation. Unfortunately, the long-term pseudopregnancy model was not consistent with the pregnancy data. Therefore, further investigation on the possibility of the conceptus factors is required. Moreover, further work will be required to fully understand the regulation of FGF7 expression in the pig during different phases of pregnancy and both pseudopregnancy models by evaluating the differences in the magnitude of the changes in circulating P4 and estrogen compared to the level of FGF7 expression in the uterus.

In this study we also reported the expression of ESR1 in the trophectoderm, mesenchymal cells and allantois of the pig in all pregnancy days examined. These results suggest a potential function of estrogen in fetal tissues. In domestic animals, maternal and conceptus steroid hormones have important interrelated roles in mediating different cellular activity during various stages of pregnancy (Jaeger *et al.* 2001). Therefore, further studies to determine the role of ESR1 in placental tissues will allow us to better understand the role of estrogen, which is synthesized and released by the conceptus at later stages of pregnancy (Knight 1994).

In these studies, we also verified the presence of FGF7 protein by Western blot analysis. Two immunoreactive bands were detected; one band was of the reported size of 25 kDa while another immunoreactive band of 17 kDa was also detected. Preabsorption of the primary antibody with FGF7 before western analysis and neutralization of anti-FGF7 with excess blocking peptide will be useful to establish the identity of this band.

In conclusion, experiments in this dissertation have contributed toward the knowledge of how ESR1 and PGR expression in the LE and stroma could regulate gene

expression in the LE of the pig. Future experiments must be directed toward determining the cellular and molecular mechanisms by which the FGF7 might affect the LE and trophectoderm These experiments include: (1) infusing FGF7 in the uterus of pig before the implantation and monitor the growth of the conceptus as well as for any differentiation in the conceptus tissues; (2) inactivating FGF7 in the LE and monitor the consequences on the development of the conceptus and pregnancy and (3) determining the genes affected by FGF7 treatment in endometrial explant cultures.

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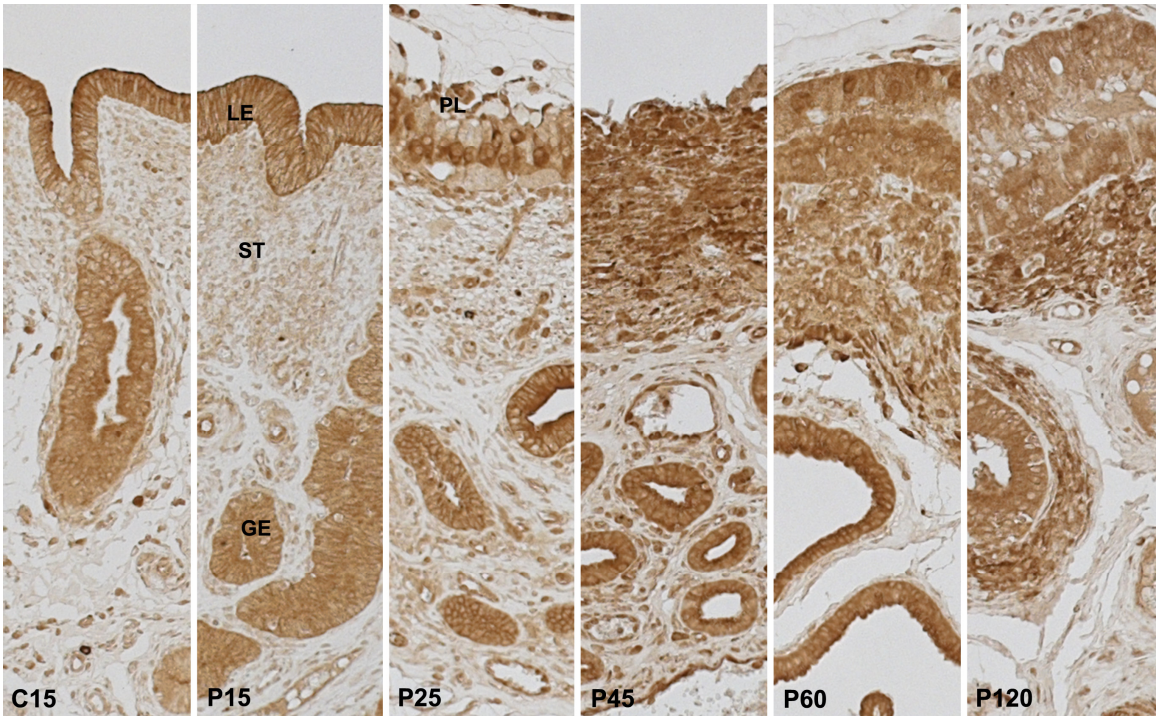
APPENDIX A

List of Antibodies Used to Detect SIBLING Proteins in Ovine Uterus:

Gene Product	Antiserum Name	Antigen Source	Known Species	Comment	References
Human dentin sialophosphoprotein (DSSP)	LF-151	part of DSP portion of human DSPP, bacterial recombinant	human and bovine, not mouse		
Human dentin matrix protein1 (DMP1)	LF-148	CEHPSRKIFRKSRISE and CLKNIEIESRKLTVDAYH conjugated to KLH	human and bovine, others not tested		
Human bone sialoprotein	LF-83	YESENGEPRGDNYRAYE D-[LPH]	hum, man, dog	epitope appears to be RAYED	Bianco et al. 1993 Mintz et al. 1993
Human MEPE	LF-155	last exon of human MEPE made in bacterial	human, others not test		

* Details regarding these and other rabbit polyclonal antisera derived against human antigen sequences can be found at the Matrix Biochemistry Laboratory website: http://csdb.nidcr.nih.gov/csdb/frame_mbu.htm

APPENDIX B



Immunohistochemical localization of DSPP protein in ovine endometrium. Photomicrographs show the effects of reproductive status on the presence and intensity of DSPP protein in ovine endometrium. Stromal immunostaining intensity was strong on Days 45 through 120 of pregnancy while it was absent from the stroma of cyclic and early pregnant animals. The LE and GE immunostaining suggests that the protein may be secreted into the uterine lumen. LE, luminal epithelia; ST: stroma; GE: glandular epithelia; PL: placenta.

VITA

Saeed Y. Al Ramadan was born in Al-Ahsa, east of Saudi Arabia. He received his DVM from College of Veterinary Medicine and Animal Resources, King Faisal University, Saudi Arabia in 1992. He worked in the Department of Veterinary Anatomy, King Faisal University from 1993 through 1999, teaching gross and microscopic anatomy to DVM students. Meanwhile, he got his Masters of Science degree in Veterinary Anatomy from King Faisal University in 1998. In the year of 1999 he was chosen by King Faisal University to receive a full scholarship to pursue a doctoral degree in veterinary anatomy. While studying in English Language Institute at Texas A&M University, Saeed showed an interest in biology of reproduction. He entered the graduate program of Dr. Robert C. Burghardt in January 2000 in the Department of Veterinary Anatomy and Public Health (currently, Department of Integrative Biosciences), Texas A&M University. His professional activities include currently being a trainee member of the Society for the Study of Reproduction (SSR) from 2001 to 2006 and a trainee member of the American Association of Anatomist from 2005-2006.

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