

BIKUNIN PROTEIN AND GENE EXPRESSION IN THE
PORCINE ENDOMETRIUM DURING THE ESTROUS
CYCLE AND EARLY PREGNANCY

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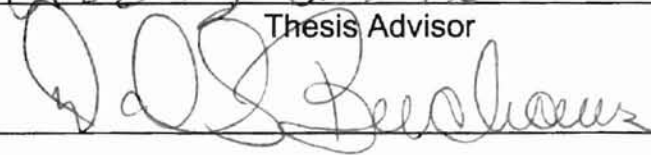
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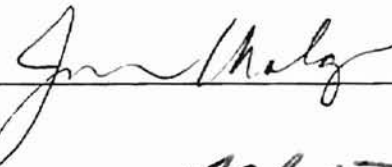
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Chapter I

INTRODUCTION

Mammalian embryos undergo a series of rapid changes in the first week of pregnancy including cleavage, compaction, blastocoele formation, hatching from the zona pellucida, and finally implantation. During the second week of gestation, the time of establishment of pregnancy in pigs, the conceptuses undergo dynamic morphological changes allowing for an increase in surface area available for placental attachment and nutrient uptake (Geisert and Yelich, 1997). This period of rapid transformation, attachment, and placentation coincides with the time of greatest embryonic loss in the pig. Since this time period is so critical for early conceptus development and survival, it is important that we understand the conceptus and uterine factors involved in these dynamic processes. Porcine embryos, which are non-invasive in the uterus, have been shown to be highly invasive outside of the uterine environment. This suggests the presence of factors in the uterine endometrium, which function to inhibit conceptus invasion. Recently, the inter- α -trypsin inhibitor family of protease inhibitors has been detected in the pig endometrium during this time of elongation and attachment, suggesting their role in conceptus development. This review of literature will cover development of the porcine conceptus including estrogen synthesis from

the conceptus, embryonic mortality in the pig, conceptus-uterine epithelial interactions, as well as uterine secretions involved in the maintenance of pregnancy. Finally, the inter- α -trypsin inhibitor family of proteins will be reviewed along with their possible roles in porcine conceptus development.

Chapter II

LITERATURE REVIEW

Development of the Porcine Conceptus

Prior to the period of implantation, porcine embryos undergo a rapid, morphological transformation during early pregnancy. Between Day 10 and 12 of gestation, spherical conceptuses develop from 3mm to 10mm in diameter with the outside layer of trophoctodermal cells being cuboidal in shape (Geisert et al., 1982b; Mattson et al., 1990). This increase in conceptus diameter is a result of cellular hyperplasia, which is evident from the increase in conceptus DNA content and amount of cellular mitosis (Geisert et al., 1982b, Pusateri et al., 1990). On approximately Day 12 of gestation, the conceptus rapidly expands, within 2 to 3 hours, from 10 mm spherical, to 20-40 mm tubular and finally to a 100 mm filamentous morphology (Geisert et al., 1982b). In 1990, Mattson et al. proposed that this dramatic and expansive elongation is due in part to a rearrangement of the trophoctodermal filamentous actin (f-actin) cytoskeleton, rather than an increase in cellular hyperplasia. The process of trophoblastic elongation functions to increase the surface area of the placenta for conceptus nutrient uptake from the uterus (Geisert and Yelich, 1997).

On Day 12, which is the time of maternal recognition of pregnancy in pigs, the conceptus begins to synthesize estrogens (Dhindsa and Dziuk, 1968; Gadsby et al., 1980; Fischer et al., 1985). In the pig, estrogen administration is luteotrophic (Gardner et al., 1963; Frank et al., 1977), prolonging the life of the corpus luteum by redirecting $\text{PGF}_{2\alpha}$, a luteolytic factor, from uterine venous blood into the lumen of the uterus (Bazer and Thatcher, 1977). Two enzymes involved in the synthesis of estrogen, are present in the trophoctoderm. These enzymes, cytochromes P450 17α -hydroxylase and aromatase peak at the stage of tubular development (Conley et al., 1992). Cytochrome P450 17α -hydroxylase is important for the metabolism of progesterone to 17α -hydroxyprogesterone, while aromatase is important for conversion of testosterone to estradiol- 17β . Gene expression for 17α -hydroxylase is first detected in 2- to 4-mm spherical conceptuses, with the greatest degree of expression in 7-mm conceptuses (Yelich et al., 1997b). Aromatase gene expression starts to increase in the 6-mm spherical conceptuses, which is consistent with the time of increased conceptus estradiol synthesis (Yelich et al., 1997b).

Trophoblastic elongation in the porcine conceptus is occurring coincident with not only conceptus estrogen synthesis, but with the expression of various growth factors and ligand receptors. In 6-mm conceptuses, which is the stage of development just prior to mesodermal outgrowth, gene expression for brachyury, a specific marker for mesodermal cells, is detected (Yelich et al., 1997b). Increased gene expression in the growth factor brachyury, may be necessary for

the activation of 17 α -hydroxylase and aromatase, and therefore, the conceptus synthesis of estrogen (Yelich et al., 1997b).

Another growth factor, which may be important for conceptus development in vitro is insulin-like growth factor-I (IGF-I). IGF-I is a cell mitogen that promotes cell division and differentiation (Letcher et al., 1989). Porcine uterine tissues synthesize and secrete IGF-I (Tavakkol et al., 1988) with highest concentrations detected between days 10 and 12 of pregnancy, coincident with the time of conceptus estrogen synthesis and elongation (Letcher et al., 1989). Ko et al. (1994) proposed that IGFs might influence conceptus estrogen synthesis through regulation of aromatase P450 gene expression. Preimplantation porcine blastocysts possess IGF-II/mannose-6-phosphate (IGF-II/M6P) receptors (Chastant et al., 1994) which may stimulate cellular growth and differentiation of the conceptus. IGF-I receptors are also located in the uterine endometrial and myometrial tissues during early pregnancy, suggesting a physiological role of IGF-I on uterine secretory activity and, subsequently, conceptus development (Hofig et al., 1991).

Retinol binding protein (RBP), a major component of the uterine histotroph secreted in response to progesterone during the period of conceptus elongation, transports vitamin A, in the form of retinol, to the developing conceptus (Adams et al., 1981; Trout et al., 1992). Continued secretion of progesterone by the corpora lutea may ensure that RBP will be present throughout the majority of gestation (Adams et al., 1981). Retinol, under the control of various enzymes,

can be oxidized to retinoic acid (Blomhoff et al., 1990), a cellular morphogen shown to function in trophoblastic remodeling and conceptus elongation. Retinoic acid acts through its receptor, retinoic acid receptor (RAR), which binds to enhancer elements of the DNA to activate gene transcription (De Luca, 1991), thereby influencing the production of cell surface adhesion molecules (Agura et al., 1992) and extracellular matrix components (De Luca, 1991). RAR α , RAR β and RAR γ , three isoforms of retinoic acid receptor are present in the day 10-12 porcine conceptus (Yelich et al., 1997a). RAR α gene expression is increased from the 4mm-8mm spherical stage and shows a marked increase during elongation (Yelich et al., 1997a).

RBP is produced by the elongating conceptus with gene expression (Trout et al., 1991) and secretion (Harney et al., 1990) localized to the trophectoderm and the inner cell mass. An increase in RBP gene expression is detected during the growth of spherical conceptuses from 2-8 mm. The RBP expression then declines during late spherical to early tubular stages of development followed by a dramatic increase during elongation (Yelich et al., 1997b). The rise in RBP gene expression may help protect the developing embryo from teratogenic or embryocidal effects of excessive retinol (Trout et al., 1992).

Yelich et al. (1997b) demonstrated increasing expression of transforming growth factor- β 3 (TGF β) during spherical conceptus development from 2mm-8mm. Expression remained high through the filamentous stage of development. TGF β , a major regulator of cell growth and differentiation, may be involved in

mesodermal migration (Gupta et al., 1996) and has been shown to modify the extracellular matrix (Roberts et al., 1990).

Geisert and Yelich (1997) proposed a model for the role of retinol and other growth factors in trophoblastic elongation. Estrogen synthesized by the early conceptus stimulates endometrial estrogen receptor, which initiates the release of uterine RBP (uRBP). The uRBP functions to transport retinol directly to the conceptus or transfer it to the embryonic RBP (eRBP). Free retinol, which has not bound to RBP, is oxidized to RA. The RA turns on its receptor, thereby activating TGF β and leukemia inhibiting factor (LIF), a haematopoietic cytokine, both of which play a role in remodeling of the extracellular matrix.

Anegon and coworkers (1994) suggested leukemia inhibiting factor (LIF) activity, detected at peak levels in porcine uterine luminal fluids on days 7 and 13 of the estrous cycle and day 12 of pregnancy, may play a role in conceptus development and implantation. Another cytokine thought to be involved in placentation and fetal growth and development, colony-stimulating factor 1 (CSF-1), has been localized to the uterine surface and glandular epithelium, with the highest levels of expression occurring after day 30 of gestation (Tuo et al., 1995).

Embryonic Mortality in the Pig

Embryonic mortality, before day 20 of gestation, is a major cause limiting litter size in pigs. In 1923, Corner et al. determined that counting the number of

corpora lutea on each ovary is a good indicator of ovulation rate, and can be used to compare to the number of fetuses present, therefore determining the rate of embryonic mortality. It is estimated that 30-40% of the oocytes ovulated do not develop into piglets (see review, Pope and First, 1985).

Pope et al. (1990) reported the effects of asynchronous development of blastocysts, within litters, on the rate of embryonic mortality. Embryos at day 6 of gestation transplanted into pregnant gilts at day 7 of gestation showed a higher degree of variability among blastocysts, as well as a lower degree of development, when compared to day 7 embryos transferred into day 6 recipients. These experiments suggest that the asynchronous development is due to the more developed embryos synthesizing estrogens sooner, thereby changing the protein secretions in the uterus.

Evidence suggests that the advancement of the endometrial cell surface and uterine glandular secretions, by the administration of exogenous estradiol on days 9 and 10 of pregnancy, is associated with embryonic mortality (Morgan et al., 1987a, 1987b; Blair et al., 1991; Geisert et al., 1991). These biochemical alterations in the uterine lumen result in embryonic mortality by day 30 (Pope et al., 1986a). In the gilt, the uterine glycocalyx thickens between days 12 and 18 of pregnancy, and placental attachment occurs between days 13 and 18 of gestation (Dantzer et al., 1985; Stroband and Van der Lende, 1990). In areas of placental attachment, the uterine glycocalyx is reduced (Dantzer et al., 1985; Blair et al, 1991), proving to be an important part of implantation. When gilts are

administered estradiol valerate on days 9 and 10 of gestation, the uterine glycocalyx is shed on approximately day 14 of gestation (Blair et al., 1991), which correlates with the time of embryonic mortality.

Superovulation and unilateral hysterectomy-ovariectomy (UHO) are methods that have been used to evaluate uterine capacity. Superovulation results in a greater number of embryos at day 30 of gestation, however, the number of embryos at term does not differ significantly when compared to controls (Christenson et al., 1987). This indicates that uterine capacity plays a role in embryo development later in gestation.

Gilts undergoing UHO have approximately half the uterine space available for embryonic growth. However, the single ovary remaining in UHO gilts compensates for the loss of the ovary and ovulation rates are similar to control intact animals (Dziuk, 1968). Between days 25 of gestation and term, when the uterine capacity becomes restricted due to increased embryonic growth, approximately 30% of the embryos are lost (Dziuk, 1968; Christenson et al., 1987).

Biensen and coworkers (1998) examined the impact of either a Meishan or Yorkshire uterus on Meishan or Yorkshire fetal and placental development. Meishan placenta showed a two-fold increase in vascular density between days 90 and 110, with no increase in placental surface area. While the Yorkshire placenta showed constant vasculature from days 90-110, the surface area of the placenta doubled in size. This suggests that Meishan fetal growth is dependent

on the increased vasculature of the placenta, while Yorkshire fetal growth relies on an increase in placental surface area.

Wilson and coworkers (1998) reported that Meishan and Yorkshire fetuses recovered from Meishan uteri were similar in weight; however, Meishan fetuses were lighter than Yorkshire fetuses when recovered from Yorkshire uteri. The weight of Day 90 and term Meishan placentas were similar, while Yorkshire placentas were approximately 70% larger at term than at Day 90. Given that uterine capacity sets the upper limit on litter size, it has been suggested that Meishan pigs have a greater potential for increased litter size due to the decreased surface area of endometrium required per conceptus.

Maternal Recognition of Pregnancy

The term "maternal recognition of pregnancy", first coined by Short in 1969, can be defined as the signal produced by the conceptus which prolongs the normal lifespan of the CL (Geisert et al., 1990). The corpora lutea of pigs are required to maintain pregnancy throughout gestation. In pigs, $\text{PGF}_{2\alpha}$ is the luteolytic agent (Moeljono et al., 1976), while estrogen of trophoblastic origin is the luteostatic agent (Perry et al., 1976). Since only 18% of $\text{PGF}_{2\alpha}$ is metabolized by the lungs to the inactive metabolite, 15 keto-13,14 dihydro-prostaglandin $\text{F}_{2\alpha}$ in this species (Davis et al., 1979), the pig has both a local and

systemic luteolytic pathway. Therefore, the presence of at least two conceptuses is required in each uterine horn for pregnancy to be maintained (Dziuk, 1968).

Between days 11 and 13 of gestation, during the time of trophoblastic elongation, pig conceptuses produce both estradiol-17 β (Perry et al., 1976; Fischer et al., 1985) and PGE₂ (Geisert et al., 1982a; Guthrie and Lewis, 1986), a vasodilator thought to be involved with immunosuppression of T cells (Croy et al., 1987; King, 1988). Estradiol-17 β is synthesized and released for a second time during days 15 and 18 of pregnancy, helping to maintain pregnancy until term. Ford and Christenson (1991) demonstrated that estradiol-17 β alone was not enough to protect the CL from an exogenous luteolytic dose of PGF_{2 α} . PGE₂, when administered at a ratio to PGF_{2 α} of 4:1, increased CL weight and P₄ content, thereby preventing luteolysis. Therefore, it is hypothesized that the roles of E₂ and PGE₂ in protecting CL from the luteolytic effects of PGF_{2 α} are independent of each other.

In 1977, Bazer and Thatcher first proposed the theory of maternal recognition of pregnancy in the pig. During the estrous cycle, the uterine endometrial tissue becomes an endocrine tissue releasing PGF_{2 α} into the uterine venous system where it then reaches the ovarian artery and finally the CL causing luteolysis. The release of estrogen from the elongating conceptus prevents CL regression by redirecting the transport of PGF_{2 α} into an exocrine direction, sequestering the luteolysin to the uterine lumen, rather than preventing its synthesis and secretion.

During days 12 to 13 of pregnancy, along with the increased synthesis of E_2 and PGE, there is a 3 - 4 fold increase in uterine blood flow (Ford and Christenson, 1979). Stice et al. (1987a) proposed that uterine blood flow fluctuations are a result of changes in uterine arterial tone and/or contractibility due to alterations in Ca^{2+} uptake. Estrogens, metabolized to catechol estrogens, have been shown to block uptake of Ca^{2+} by uterine arterial smooth muscle cells (Stice et al., 1987b), thus increasing uterine blood flow necessary for conceptus survival.

Conceptus-Uterine Epithelial Interactions

In the pig, a species that has a non-invasive, epitheliochorial type of placentation (King et al., 1982; Keys et al., 1990), placental attachment to the uterine epithelium occurs between days 13 and 18 of gestation (Perry et al., 1981). The large (200-1000 kDa), heavily glycosylated transmembrane protein, Muc-1, is an integral component of the uterine epithelium (Bowen et al., 1996, 1997). Much like the results seen by Braga and Gendler (1993) in uterine attachment and implantation in rodents, Muc-1 is down-regulated during the transition of the porcine uterus from the prereceptive to the receptive state, allowing for trophoblast attachment (Bowen et al., 1996). Muc-1 levels in the uterine epithelium are maximal at Day 0 (estrus) and Day 4; however, levels are undetectable by Day 10 of the estrous cycle or pregnancy (Bowen et al., 1996). Reduced staining of Muc-1 on the uterine luminal and glandular epithelium

correlates with increasing progesterone concentrations in both cyclic and pregnant gilts (Bowen et al., 1996, 1997), as well as the time of progesterone receptor down-regulation in the uterine epithelium (Geisert et al., 1994). These results are consistent with the theory of Surveyor et al., (1995) in which Muc-1 functions as an anti-adhesive molecule in the mouse uterus. Uterine receptivity may be due to the loss of Muc-1, which allows for various ligands to bind to their uterine epithelial cell surface receptors (Surveyor et al., 1995), resulting in implantation.

In order for there to be contact between the uterine epithelium and the conceptus, the epithelial cells of the uterus must possess receptors for the conceptus extracellular matrix (ECM) proteins. The major receptors for conceptus ECM proteins appear to be integrins, which are cell surface glycoproteins (Hyne, 1992). All integrins are composed of one of the 14 known α subunits and one of the 9 known β subunits (Bowen et al., 1996). The various heterodimers that form determine the specific function of the integrin (Hynes, 1992). Bowen et al. (1996, 1997) looked at the in vivo and in vitro expression of α_1 , α_3 , α_4 , α_5 , α_v , β_1 and β_3 integrin subunits in the endometrium and conceptus during the estrous cycle and early pregnancy. The integrin subunits expressed in the epithelium and trophoctoderm are α_4 , α_5 , α_v , β_1 and β_3 , members of the fibronectin and vitronectin families of receptors. Peak expression of α_4 , α_5 , and β_1 occurred between days 11-15, also known as the window of implantation, while α_v and β_3 expression remained consistently high independent of the day.

Steroid treatments had an effect on the expression of the α_1 , α_4 , α_5 , and β_1 integrin subunits (Bowen et al., 1996). Expression of α_4 , and α_5 was down-regulated by estrogen, while α_1 and β_1 expression was up-regulated by progesterone (Bowen et al., 1996).

Bowen and coworkers (1996) also reported the presence of fibronectin on the trophoctoderm, vitronectin on the trophoctoderm and uterine epithelium, and laminin and type IV collagen on the basal lamina of the uterine epithelium and trophoctoderm. Tuo and Bazer (1996) reported the presence of oncofetal fibronectin in the trophoctoderm from days 12 to 15. Fibronectins have been shown to be present on the day 5 blastocyst, while extracellular laminins do not appear on the conceptus until day 10 (Richoux et al., 1989). Integrin heterodimers known to bind fibronectin include $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, whereas only $\alpha_v\beta_1$ and $\alpha_v\beta_3$ bind vitronectin (Hynes et al., 1992). The interactions of integrins on the uterine epithelium with their ligands on the trophoctoderm, with peak expression during the time of implantation, suggest a role of integrins in the process of attachment in pigs.

Endometrial Secretions During the Estrous Cycle and Early Pregnancy

In species such as the pig, which have a diffuse, epitheliochorial type of placentation, uterine endometrial secretions, or histotroph, are important for the development of the conceptus (Roberts and Bazer, 1988). Areolae, specialized

chorionic structures, which form over the uterine glands around Day 30 of gestation, are thought to absorb the nutrients from the histotroph and transport it to the developing fetus (Brambel, 1933). Knight et al. (1974a; 1974b) demonstrated that the amount of uterine protein secreted is correlated primarily with levels of progesterone. During the estrous cycle, the protein content of uterine secretions remains relatively constant from Days 2-9, with an increase at Day 12. The maximum amounts are reached at Day 15, while under the influence of progesterone, followed by decreasing content after the regression of the CL, similar to that seen between Days 6-9 of the estrous cycle (Murray et al., 1972). It has also been demonstrated, through the use of superovulation and unilateral ovariectomy-hysterectomy, that the total amount of uterine protein secreted is related to the number and total weight of the corpora lutea present (Knight et al., 1973).

While examining the relationship between blastocyst development and uterine endometrial secretions, Geisert and coworkers (1982a) demonstrated that as estradiol concentration in uterine flushings increased, parallel to conceptus elongation, total uterine content of calcium, protein, acid phosphatase activity, $\text{PGF}_{2\alpha}$, and PGE_2 also increased. It is thought that endometrial calcium released may reflect the secretory activity of the uterine endometrium and glandular epithelium.

Geisert et al. (1982c) conducted another experiment to further demonstrate the role of conceptus estrogen synthesis on uterine secretory

activity. Estradiol valerate, injected on Day 11 of the estrous cycle, was shown to stimulate an increase in calcium, protein, $\text{PGF}_{2\alpha}$ and PGE within 24 hours, similar to the responses seen from Day 12 elongating blastocysts.

The uterine histotroph is composed of a large amount of uteroferrin, as well as plasmin inhibitor, riboflavin and lysozyme. Riboflavin appears as a yellow pigment (Moffatt et al., 1980) present in uterine flushings during Days 6-8 of the estrous cycle and pregnancy in response to estrogen and progesterone (Murray et al., 1980). The lysozyme present is assumed to function as an antibacterial agent in the uterus (see Roberts and Bazer, 1988). Uteroferrin is one of the most abundant proteins found in uterine flushings, making up approximately 15% of the total secreted protein (Roberts and Bazer, 1988). Uteroferrin, a protein with acid phosphatase activity (Bazer, 1975), gives flushings a deep purple color and has a molecular weight of 35,000 (Roberts and Bazer, 1988). It is secreted from the uterine glandular epithelium (Reneger, 1982), functions to carry iron to the developing fetus (Roberts and Bazer, 1988), and acts as a hematopoietic growth factor, stimulating proliferation and differentiation of erythroid and granulocyte-monocyte/macrophage cell lines (Bazer et al., 1991; Laurenz et al., 1997). Uteroferrin binds and transports two atoms of iron per molecule, with the excess iron being filtered through the fetal kidney, and then transported and stored in the allantoic fluid (Reneger, 1982).

Porcine embryos, when placed in an ectopic site, will invade the surrounding tissue forming a syncytium (Samuel and Perry, 1972). Protease

inhibitors, synthesized by the endometrium (see review Roberts et al., 1993), may help to protect the uterus from the invasive nature of the embryo. Plasmin inhibitor, found in uterine secretions during pregnancy and the estrous cycle (Stallings-Mann et al., 1994), is synthesized by not only uterine glandular epithelium, but surface epithelium as well (Fazleabas et al., 1985). The synthesis of plasmin inhibitor is progesterone dependent (Fazleabas et al., 1985), with maximum levels seen at Day 12 of pregnancy (Fazleabas et al., 1983). Fazleabas and coworkers (1983) demonstrated the release of plasminogen activator from Day 10-16 conceptuses and the presence of plasminogen in uterine flushings. Because of the presence of these two factors, conceptuses possess the ability to release plasmin, a substance that is shown to be highly proteolytic. Fazleabas et al. (1983) hypothesized that the release of plasmin inhibitor, stimulated at the time of elongation, functions to protect the uterine tissue from the proteolytic effects of plasmin.

Geisert and coworkers (1997) demonstrated the time- and steroid-dependent activity of cathepsin L in the uterine lumen of gilts during the estrous cycle and early pregnancy. Peak levels of the enzyme were seen on Day 15 of the cycle and pregnancy, corresponding to the time of placental attachment (King et al., 1982). Cathepsin L activity was shown to increase following 10 days of progesterone stimulation. Immunohistochemical analysis revealed that the surface and glandular epithelial cells of the uterus secrete cathepsin L. It has been suggested that this enzyme may be involved in the proteolysis of the

uterine glycocalyx, which occurs at the time of placental attachment (Geisert et al., 1997).

Another conceptus-derived factor likely to be involved in implantation in pigs is antileukoproteinase (ALP), also referred to as secretory leukocyte protease inhibitor (SLPI), a low molecular weight elastase/cathepsin G protease inhibitor (Badinga et al., 1994). Uterine expression of antileukoproteinase was shown to be highest during mid to late gestation (Badinga et al., 1994), due to the presence of the conceptuses, rather than progesterone or estrogen stimulation (Simmen et al., 1991). Reed and coworkers (1998) suggested that both a conceptus protein, such as $TGF\alpha$, and an inhibitory component coming from the maternal blood serum, might mediate SLPI gene expression. Antileukoproteinase synthesis seems to be correlated with the epitheliochorial type of placentation seen in species such as the cow, horse and pig (Badinga et al., 1994).

Menino and coworkers (1997) evaluated the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) in pig embryos and the uterus at the time of trophoblast attachment. Day 16 embryos expressed transcripts for TIMP-1, -2 and -3, with TIMP-1 and TIMP-3 being localized to the extraembryonic endoderm and the trophoblast, while TIMP-2 was found in all embryonic cells. Transcripts for the TIMP-1, -2 and -3 genes were seen in the uterine stroma as well. MMP-2 and MMP-9 transcripts were expressed in both the embryos and the uteri, however, no

specific localization was observed. The presence of these proteinases, along with their inhibitors, may explain the non-invasive behavior of pig embryos while in the uterine environment.

Another key player in the process of implantation and placentation is the plasma serine protease, kallikrein. Kallikrein gene expression has been detected in both the uterine endometrium and the conceptus (Vonnahme et al., 1999). Kallikrein enzymatic activity in uterine flushings increases between days 10 and 15 of the estrous cycle and pregnancy (Vonnahme et al., 1999). Along with its role in the regulation of kinin release (see review Margolius, 1996), kallikrein has been shown to cleave the 120 kDa glycoprotein, inter- α -trypsin inhibitor heavy chain 4 ($I\alpha IH4$), into 100 and 35 kDa fragments (Geisert et al., 1995). The 100 kDa fragment is then further cleaved into a 70 kDa fragment which releases the 30 kDa porcine endometrial glycoprotein pGP30 (Geisert et al., 1995). Kallikrein cleaving $I\alpha IH4$ could play a role in exposing conceptus attachment sites to the uterine surface (Geisert et al., 1998).

Inter- α -trypsin Inhibitor Family

Exogenous estradiol administration on days 9 and 10 of pregnancy stimulates the loss of the uterine epithelial glycocalyx (Blair et al., 1991), along with the loss of a basic (pI 7.9-8.9), 30 kDa glycoprotein (Gries et al., 1989). In 1995, Geisert and coworkers isolated a basic, 30 kDa endometrial glycoprotein,

pGP30, which has since been characterized as homologous to the C-terminal region of the inter- α -trypsin inhibitor heavy chain 4 (Geisert et al., 1998). This 30 kDa glycoprotein is now known to be a cleavage product of I α IH4 proteolysis (Geisert et al., 1997). Inter- α -trypsin inhibitor heavy chain 4 has been found in human (Saguchi et al., 1995) and pig liver (Hashimoto et al., 1996), as well as in pig endometrium (Geisert et al., 1998). Although the strongest cellular localization of the I α IH4 cleavage product, pGP30, is seen in the epithelium of the endometrium, it has been found in the oviduct as well (Geisert et al., 1995). Geisert et al. (1995) determined that the endometrial production of pGP30 is time and steroid dependent, as synthesis was increased in ovariectomized gilts treated with progesterone. Maximum expression of pGP30, detected by immunocytochemistry, was observed on day 12 of the estrous cycle and between days 12 and 18 of pregnancy (Geisert et al., 1995).

Endometrial cultures, analyzed with pGP30 antiserum, showed multiple bands, varying in molecular mass, depending on the stage of the cycle at which the tissue was obtained (Geisert et al., 1995). Geisert and coworkers (1995) reported that on day 0, also known as estrus, two bands, the 30 kDa and a 32 kDa, were present. Between days 5 and 10, with the absence of the 30 kDa band, three to four bands of molecular masses ranging from 35-40 kDa appear. The 30- and 32 kDa bands reappeared at day 12 and remained through day 18 of the estrous cycle. On day 10, and continuing through day 18 in the pregnant gilt, only the 30 kDa glycoprotein was detected. The presence of the 30 kDa glycoprotein during this stage of pregnancy, when elongation and trophoblastic

attachment are occurring, may suggest a role of pGP30 in conceptus attachment to the uterine surface and development (Geisert et al., 1995). pGP30 is related to the inter- α -trypsin inhibitor family of proteins, as it is a product of $\text{I}\alpha\text{H4}$ proteolysis (Geisert et al., 1997) and may be involved in the loss of the uterine epithelial glycocalyx.

The inter- α -trypsin inhibitor family consists of four heavy chains, 1, 2, 3, and 4, ($\text{I}\alpha\text{H1}$, $\text{I}\alpha\text{H2}$, $\text{I}\alpha\text{H3}$, $\text{I}\alpha\text{H4}$) and a light chain, bikunin. All $\text{I}\alpha\text{I}$ heavy chains contain a von Willebrand type A domain, which serves as a target for adhesive molecules such as integrins, collagen, proteoglycans and heparin (Colombatti and Bonaldo, 1991), and have been classified as acute phase proteins (Geisert et al., 1998). Acute phase proteins, coming from the liver, are molecules that increase rapidly during acute inflammation and infection (Roitt et al., 1998). In the reproductive tract, it has been suggested that acute phase proteins may help protect the uterus from inflammation that may occur during the process of conceptus attachment (Geisert et al., 1998). All inter- α -trypsin inhibitor heavy chains are homologous in their N-terminal regions; however, variations in the C-terminal regions are seen among the four proteins (Saguchi et al., 1995). Unlike the other heavy chains, $\text{I}\alpha\text{H4}$ does not possess a binding site for bikunin (Geisert et al., 1998) and, as mentioned earlier, is readily cleaved by kallikrein leaving 100 and 35 kDa fragments (Geisert et al., 1995). $\text{I}\alpha\text{H4}$ lacks the DPHFII consensus sequence, which encodes for the region of chondroitin sulphate attachment, thereby preventing $\text{I}\alpha\text{H4}$ from binding to the chondroitin sulfate chain of bikunin (Geisert et al., 1998). The N-terminal 600 residues of

α IH4 are highly homologous to those of the other α I heavy chains, whereas, the 300 C-terminal residues exhibit no homology (Saguchi et al., 1995). The cDNA encoding α IH4, previously referred to as inter- α -trypsin inhibitor heavy chain related protein (IHRP) (Hashimoto et al., 1996), was cloned from human liver cDNA libraries (Saguchi et al., 1995). The α IH4 gene is expressed in the endometrium of gilts during the estrous cycle (days 0-18) and early pregnancy (days 10-18) (Geisert et al., 1998). Gene expression is enhanced during the mid-luteal phase of the cycle and during trophoblastic attachment (Geisert et al., 1998), and is up-regulated during acute inflammation (see review Salier et al., 1996), suggesting that α IH4 is acting as an acute phase protein. It has been proposed that inter- α -trypsin inhibitor heavy chains play a role in stabilizing the extracellular matrix (Chen et al., 1994). Geisert and coworkers (1998) also suggested that α IH4 might stabilize the uterine glycocalyx, thereby preventing invasion by the conceptus.

Bikunin binds to α IH1, α IH2, and α IH3, via a chondroitin sulfate chain, forming either pre- α -inhibitor or inter- α -trypsin inhibitor (Blom et al., 1999; Thuveson and Fries, 1999). Pre- α -inhibitor, a positive acute-phase protein (Daveau et al., 1993), is composed of bikunin and heavy chain 3 linked together by a chondroitin 4-sulfate glycosaminoglycan (Enghild et al., 1991). This covalent linkage to the COOH-terminal amino acid residue of heavy chain 3 occurs while passing through the Golgi complex (Thuveson and Fries, 1999). Inter- α -trypsin inhibitor can also be a 180 kDa negative acute-phase protein

(Daveau et al., 1993) consisting of bikunin covalently linked to the C-terminal amino acid residues of $\alpha 1H1$ and $\alpha 1H2$ (Blom et al., 1999). The heavy chains of $\alpha 1$ appear as two large globular domains with the C-terminal ends of the polypeptides extending as thin flexible strands (Blom et al., 1999). Bikunin, present as a small globule, is found in the middle of the strands connecting the two heavy chains (Blom et al., 1999). The inter- α -trypsin inhibitor protein, along with functioning as a protease inhibitor, plays a role in the stabilization of the extracellular matrix (ECM) (see review Bost et al., 1998). The ECM allows for the interaction, adherence and migration of various cell types. A major component of the ECM is hyaluronic acid (HA), a non-sulfated glycosaminoglycan (GAG) that is synthesized in the plasma membrane by hyaluronan synthase (Prehm, 1989). Inter- α -trypsin inhibitor has been identified as a HA-binding protein in the oocyte (Chen et al., 1992) and inflamed synovial fluids (Sandson et al., 1965). Flahaut and coworkers (1998) reported the interaction of heavy chains 1 and 2 with hyaluronan, suggesting its role in stabilizing the ECM.

Bikunin, the light chain member of the inter- α -trypsin inhibitor family, is a 30 kDa serine protease inhibitor (see review Bost et al., 1998) with two Kunitz-type inhibitor domains (Xu et al., 1998). Kunitz-type inhibitors are known for having a low molecular mass, a basic isoelectric point, and one or more inhibitory domains to serine proteases (see review Salier et al., 1996). The inter- α -trypsin inhibitor light chain (ITIL) gene (see review Bost et al., 1998), also known as the AMBP gene (see review Salier et al., 1996), encodes for two proteins, $\alpha 1$ -

microglobulin and bikunin. The AMP-gene-encoded precursor polypeptide is cleaved, releasing the α 1-microglobulin and bikunin (see review Salier et al., 1996). The six exons encoding α 1-microglobulin are separated from the four exons encoding bikunin by a 7 kb intron (see review Salier et al., 1996). The AMBP gene, along with the four heavy chain genes, is transcribed in the liver, the primary source of all α 1 plasma proteins, with gene transcription being mediated by the cytokines, IL-1 and IL-6 (see review Salier et al., 1996). The enzymes targeted by the two Kunitz-type inhibitory domains of bikunin are trypsin, chymotrypsin, cathepsin G, leukocyte elastase, acrosin and plasmin (see review Salier et al., 1996), although plasmin inhibitors more effectively inhibit these proteases (see review Salier et al., 1990).

Bikunin has been found in blood serum, urine and various tissues of humans and other species (Xu et al., 1998). Bikunin levels have been shown to increase during inflammation and such diseases as cancer (Xu et al., 1998). Yoshida and coworkers (1994) demonstrated the wide distribution of bikunin, by immunohistochemical analysis, in human brain tumors of various histological types. It has been suggested that bikunin may play a role in repairing tissue where destruction has occurred (Yoshida et al., 1994). Kobayashi et al., (1994a) described the use of bikunin for inhibiting tumor invasion in vitro and lung metastasis in vivo. Bikunin, present in the kidneys and urine, is known for being a potent inhibitor of calcium oxalate crystallization (Atmani et al., 1999), a process that leads to the formation of kidney stones. Gene expression is seen in renal epithelial cells, with bikunin being produced in response to substances such

as oxalate (Iida et al., 1999). Ebisuno and coworkers (1999) suggested that bikunin might regulate the adherence of crystals in the renal tubules during kidney stone formation.

More recently, bikunin has been identified in the reproductive tract. Marlor and coworkers (1997) identified and cloned bikunin from human placental tissue. Further studies characterized placental bikunin as being a potent inhibitor of plasma and tissue kallikreins, plasmin and factor XIa (Delaria et al., 1997). It is thought that placental bikunin may function as a regulator of contact activation and the intrinsic pathway of coagulation (Delaria et al., 1997).

Deiderich and coworkers (1997) identified message for bikunin in cyclic and pregnant porcine endometrium via RT-PCR. Although gene expression was seen in cyclic and pregnant endometrium on days 10 and 12, significantly greater levels were detected on days 15 and 18 of pregnancy, suggesting a possible role in conceptus development and implantation.

Statement of the Problem

Based on the previous results reported by Deiderich and coworkers (1997), the objective of this study was to determine whether bikunin protein was detectable during the estrous cycle and early pregnancy, and to further quantify and localize the gene expression of bikunin in the endometrium of the pig.

CHAPTER III

Bikunin protein and gene expression in the porcine endometrium during the estrous cycle and early pregnancy

Introduction

During early pregnancy, prior to the period of implantation, porcine conceptuses undergo a rapid, transformation from 10 mm spherical to greater than 100 mm filamentous morphology within a period of a few hours (Geisert et al., 1982b). This dramatic alteration in morphology, which occurs between day 11 to 12 of gestation, is temporally associated with conceptus synthesis and release of estrogen. Conceptus release of estradiol-17 β into the uterine lumen prolongs the normal lifespan of the corpora lutea, signaling the maternal system for recognition of pregnancy (Geisert et al., 1990). As the estrogen concentrations in uterine flushings increase, parallel to the time of conceptus elongation, uterine secretory activity is altered (Geisert et al., 1982a). Roberts and Bazer (1988) suggested that in species such as the pig, which have a diffuse, epitheliochorial type of placentation (King et al., 1982; Keys et al., 1990), uterine endometrial secretions are important for regulation of conceptus development and placentation.

Although highly invasive when placed in an ectopic site (Samuel and Perry, 1972), porcine conceptuses are noninvasive in utero. It has been hypothesized that secretion of various enzyme inhibitors by the surface and glandular uterine epithelium protect the endometrium from the invasive nature of the porcine embryo. Endometrial secretion of a variety of protease inhibitors regulates the uterine environment during attachment of the trophoblast to the uterine surface in the pig (see Roberts et al., 1993). The porcine endometrium synthesizes and releases a protease inhibitor to plasmin, chymotrypsin and trypsin (Fazleabas et al., 1983; Stallings-Mann et al., 1994), antileukoproteinase (Simmen et al., 1992), and a group of low molecular mass, basic proteins that are related to the "serpin family" of protease inhibitors (Malathy et al., 1990). Expression of the inter- α -trypsin inhibitor (α_1) family of protease inhibitors has also been detected within the endometrium during early pregnancy of the pig (Geisert et al., 1996).

Inter- α -trypsin inhibitor heavy chains contain a von Willebrand type A domain (Colombatti and Bonaldo, 1991) and have been classified as acute phase proteins (see review Salier et al., 1996). Geisert and coworkers (1998) demonstrated α_1 H4 gene expression in the endometrium of gilts during the estrous cycle (days 0-18) and early pregnancy (days 10-18). Gene expression is greatest during the mid-luteal phase of the cycle, and during trophoblastic attachment (Geisert et al., 1998). It has been hypothesized that α_1 H4 might assist in the conceptus attachment to the uterus by stabilizing the uterine epithelial surface glycocalyx (Geisert et al., 1998). Inter- α -trypsin inhibitor family

of serine protease inhibitors are composed of either a combination of two heavy chains, α 1H1 or α 1H2, and the light chain member of the family, bikunin (Salier et al., 1996). Bikunin can also form a complex with single heavy chains, α 1H2 and α 1H3, through binding to a chondroitin sulfate chain (Enghild et al., 1993). The serine protease inhibitory activity originates from bikunin, a 30 kDa serine protease inhibitor (see review Bost et al., 1998) with two Kunitz-type inhibitory domains (Xu et al., 1998). Through its tandemly arranged Kunitz domains, bikunin inhibits trypsin, cathepsin G, elastase and plasmin. The inter- α -trypsin inhibitor light chain (ITIL) gene (see review Bost et al., 1998), also known as the AMBP gene (see review Salier et al., 1996), encodes for two proteins, α 1-microglobulin and bikunin, which are separated through post-translational proteolytic cleavage. It has been suggested the bikunin may play a role in repairing tissue where proteolytic destruction has occurred (Yoshida et al., 1994). Certainly, bikunin could assist in the regulation of conceptus proteolysis of the uterine cellular surface and protein secretions. Therefore, the objective of this study was to determine whether bikunin protein was detectable during the estrous cycle and early pregnancy, and to further quantify and localize the gene expression of bikunin in the endometrium of the pig.

Materials and Methods

Animals

Cyclic, large white gilts of similar age (8-10 mo) and weight (100-130 kg) were checked twice daily for estrous behavior using intact boars. The onset of

estrus was considered Day 0 of the estrous cycle. Gilts assigned to be mated were bred naturally with fertile boars at the first detection of estrus and 12 h later.

Evaluation of uterine bikunin and gene expression in cyclic and pregnant gilts

Tissue Collection

Cyclic gilts (n=18) were hysterectomized on Days 0, 5, 10, 12, 15 and 18 of the estrous cycle, while pregnant gilts (n=12) were hysterectomized on Days 10, 12, 15 and 18 of gestation. Gilts were initially anesthetized with a 1.5 cc i.m. injection of a cocktail consisting of 2.5 cc Rompum (xylazine; 100 mg/ml) (Miles, Inc., Shawnee Mission, KS), and 2.5 cc Vetamine (ketamine HCl; 100 mg/ml) (Mallickrodt Veterinary, Mundelein, IL) in 500 mg of Telazol (tiletamine HCl and zolazepam HCl) (Fort Dodge, Syracuse, NE). Anesthesia was maintained with a closed-circuit system of halothane (Halocarbon Laboratories, Riveredge, NJ) and oxygen (1.0 liter/min). The uterine horns and ovaries were removed following midventral laparotomy. The incision site was closed using routine surgical procedures, and gilts were treated i.m. with procaine penicillin G (20,000 IU/kg BW). After surgical removal of the uterine horns as previously described (Gries et al., 1989), uterine flushings and endometrium were obtained by isolating one horn and flushing with 20 ml of PBS (pH 7.4). Uterine flushings were placed on ice until centrifugation (2500 x g, 10 min; 4°C). After flushing, the horn was cut along its antimesometrial border; endometrium was collected and snap frozen in liquid nitrogen, and tissue was stored at -80°C. The remaining uterine horn was immediately placed in a sterile container and transported on ice for use in explant

culture. Endometrium was removed from the mesometrial side and diced into 4x4-mm sections. A total of 0.5 g explant tissue was placed in 15 ml Dulbecco's modified Eagle's medium (MEM) (Gibco/Life Sciences, Gaithersburg, MD) and 2% (v:v) antibiotic-antimycotic (Gibco/Life Sciences). After 3 h, medium was replaced with fresh medium to remove serum leaching from the tissue.

Endometrial explant cultures were incubated in air on a rocking platform (4 cycles/min) for an additional 24 h in MEM at 37°C. Endometrial explant culture medium was centrifuged (2500 x g, 10 min; 4°C). Endometrial tissue, uterine flushings and endometrial explant medium were stored at -80°C until analyzed.

Western Blot Analysis

Uterine flushings and endometrial explant culture media were analyzed by Western blotting for the presence of immunoreactivity to antiserum against human bikunin (generously provided by Jean-Philippe Salier, Boisguillaume, France). Polypeptides in UTF and ECM (50 µg total protein) were separated by 12.5% one-dimensional SDS-PAGE (Laemmli, 1970) and immediately transferred to polyvinylidene fluoride membranes (Millipore Corporation, Bedford, MA) at 150 mA constant current for 35 minutes. After electroblotting, the membranes were washed in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) and incubated for 1 hour with the first blocking solution of 3% gelatin in TBS. After washing in Tween-TBS (TTBS; 20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5) for 10 minutes, membranes were incubated overnight with the first antibody (1:500 dilution) in 1% gelatin TTBS. The next day, membranes were washed

twice in TTBS and twice in TBS. Immunoreactive polypeptides were detected using the Bio-Rad Immuno-Blot kit (Bio-Rad, Hercules, CA) according to manufacturer's specifications.

RNA Extraction

Total RNA was isolated from endometrial tissue using TRIzol reagent (Gibco/Life Sciences, Gaithersburg, MD) according to manufacturer's specifications. Approximately 0.5 g of endometrial tissue was homogenized in 5 ml TRIzol reagent using a Virtishear homogenizer (Virtis Co. Inc., Gardiner, NY). After the addition of 1 ml of chloroform, samples were centrifuged at 4°C for 30 min at 3500 x g. The aqueous layer was removed and transferred into a new tube. Following the addition of 2.5 ml of isopropanol, samples were left at room temperature for 10 min to precipitate the RNA. Following centrifugation (3500 x g; 4°C) for 10 minutes, the supernatant was removed, RNA pellets were washed with 5 ml of 75% ethanol and centrifuged at 4°C for 4 min at 3500 x g. RNA pellets were rehydrated with 10 mM Tris, 1mM EDTA (pH 7.4) and stored at -80°C until further analysis. Total RNA was quantified spectrophotometrically at an absorbance of 260 nm, while the purity was determined based on 260/280 nm ratios. Integrity of the RNA was checked via gel electrophoresis.

Total RNA was reverse transcribed to cDNA in a Perkin Elmer Cetus (Norwalk, CT) DNA Thermal Cycler Model 480. The total reaction volume of 20 µl contained 200 U of Moloney murine leukemia virus reverse transcriptase-Rnase H- (M-MLV-RT) (Promega, Madison, WI), 1.0 µg of oligo(dT)₁₅ primer

(Promega, Madison, WI), 0.5 mM each of dATP, dCTP, dGTP, dTTP, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 20 U of Rnasin (Promega, Madison, WI), and 1.0 µg total RNA brought to volume with DEPC water. The reaction was incubated at 22°C for 15 min followed by a 30 min incubation at 42°C and a 5 minute incubation at 95°C. The samples were allowed to cool to 4°C and then placed in the -20°C freezer for long term storage. Quality and quantity of endometrial cDNA was checked by evaluating PCR expression of glyceraldehyde-3-phosphate dehydrogenase as previously described (Yelich et al., 1997).

Bikunin Primer Construction, Optimization and Sequencing

Figure 3.1 shows the complete mRNA sequence of the alpha1-microglobulin-bikunin precursor protein. Bikunin primers were designed from porcine cDNA sequence for α 1-microglobulin-bikunin precursor protein (Gebhard et al., 1990). The nucleotide sequence of porcine bikunin (509-903 base pairs) was utilized to construct the 5' AGGAAGGATCAGGAGCTGGACAA and 3' GGAAGTGTGTT CTCTTCAAC primers. To optimize the PCR conditions, cDNA from cyclic and pregnant endometrium of all days was pooled and amplified with 0.6 U of Taq DNA polymerase and its supplied MgCl₂-free buffer (Promega, Madison, WI) and a 3x2x3 factorial (see Table 3.1) combination of primer (50, 150, 250 nM), deoxynucleotide triphosphates (dNTPs) (50 or 100µM), and MgCl₂ (1.25, 2.50, or 3.75 mM). All samples were kept on ice until loaded directly into the heat block of the thermocycler, which was preheated to 95°C to minimize the

Figure 3.1. Porcine mRNA sequence for alpha1-microglobulin-bikunin precursor protein. Blue color indicates the portion of the mRNA that encodes for the alpha1-microglobulin protein. Red color indicates the portion of the mRNA that encodes for the bikunin protein. Bold red lettering indicates the portion of the bikunin sequence that PCR primers were constructed from.

Porcine mRNA for alpha1-microglobulin-bikunin precursor protein

```
1 gccgtgagcg ccagccctgt gctgacattg cccaatgaca tccaggtgca ggagaacttc
61 gacctgteta ggatctacgg gaaatggttc cacgtggccg tgggctccac ctgcccctgg
121 ctgaagaggt tcaaggacaa gatgacgatg ggcacgctga tgctgggaga gggggcgacg
181 gagagggaga tcagcgtgac caagactcac cggaggaaag gtatctgtga ggtgatctct
241 ggggcttatg agaaaacaag cactgatgga aagttcctct atcaciaatc caaatggaac
301 atcacatgg agtcctatgt ggtccacacc aactatgatg agtatgcoat atttctgacc
361 aagaagttca gccgcccca cggacccacc cttactgcca agctctacgg gcgggagccg
421 cagcttcggg aaagcctgct ggaggagttc agggaggttg ccctgggctg gggcatcccg
481 gaggactcca tctttacgat gcccgacaga ggggagtgtg tccttgggga gcaggagcct
541 gagcccacc tactctcaag agcccggcgg gccgtgctgc cccaggaaga ggaaggatca
601 ggagctggac aaccagtagc agatttcagc aagaaagaag attcctgcca gctgggctac
661 tccaaggcc cttgcctggg catgatcaag aggtatctct ataatggctc atccatggcc
721 tgcgagacct tccactatgg tggetgcatg gggaaaggca ataacttcgt ctcggaag
781 gagtgtctgc agacctgcc gactgtggag gectgcagtc tccccatcgt ctccggcccc
841 tgccgaggtt ttttccagct ctggggcttt gatgocgtgc aggggaagtg tgttctcttc
901 aactatgggg gctgccaggg caecggcaac cagttctact cggagaagga gtgcaaagag
961 tactgcggcg tccccggtga agaggatgaa gagctgctgc gctcctccaa ctgaccagcc
1021 cgcaggccac agggcagcag gagggccagc ccagegcctg cctggtgcc ccatggcagg
1081 ttccaataaa aaccaaatcg tagcctctg aaattccaag tectgactgt tcatcattaa
1141 gtgtaatgag atgggggagg ggaggggggg acaagctggg gtgggggccc gagtaacccc
1201 agcatcccca gaagtgaaaa atgtctgtgt tggaaatgta atagaactct cctccatag
1261 tgaaattggc tatgcaaatt atgaaacata aatcacctt ctgtataaaa tgg
```

Table 3.1. Conditions for PCR optimization.

Lane	Primer (5 μ M stock)	dNTPs (10 mM stock)	10 X MgCl
1	0.5 μ l	0.5 μ l	1.25 μ l
2	0.5	0.5	2.50
3	0.5	0.5	3.75
4	0.5	1.0	1.25
5	0.5	1.0	2.50
6	0.5	1.0	3.75
7	1.5	0.5	1.25
8	1.5	0.5	2.50
9	1.5	0.5	3.75
10	1.5	1.0	1.25
11	1.5	1.0	2.50
12	1.5	1.0	3.75
13	2.5	0.5	1.25
14	2.5	0.5	2.50
15	2.5	0.5	3.75
16	2.5	1.0	1.25
17	2.5	1.0	2.50
18	2.5	1.0	3.75

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time required to reach the denaturation temperature. The first cycle used a denaturation temperature of 95°C for 2 min, an annealing temperature of 57°C for 1 min, and an extension temperature of 72°C for 2 min. This was followed by 29 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and a 2 min extension at 72°C. The PCR reaction was completed by a 9 min extension at 72°C followed by a final, holding temperature of 4°C. The PCR products were resolved on a 3% agarose gel at 95 volts for 45 minutes. The gel was stained for 45 minutes in ethidium bromide (1.0 mg/ml). The optimal conditions for bikunin gene amplification were 25 mM MgCl₂, 50 μM dNTPs, and 250 nM primers. To verify the PCR product as endometrial bikunin, pooled cDNA was amplified with the previously described optimal conditions, run on a 3% agarose gel, stained with ethidium bromide (0.5 g/ml) and destained in water for 30 min; bands were cut from the gel with a razor blade. The single band of the PCR product was extracted using the Qiaquick (Qiagen, Santa Clarita, CA) and sequenced by the Recombinant DNA/Protein Research Facility at Oklahoma State University. The 314 bp product was 100 % homologous to the region (519-903 bp) of the gene encoding for bikunin in the cDNA sequence for porcine alpha1-microglobulin-bikunin precursor protein (GenBank accession number X53685). Endometrial cDNA (1 μg) was amplified using the optimum conditions previously described to determine bikunin gene expression in the uterus during the estrous cycle (days 0, 5, 10, 12, 15, and 18) and early pregnancy (days 10, 12, 15, and 18). The PCR products were resolved on a 3% agarose gel at 84V for 1 h followed by staining with ethidium bromide. Agarose gel was exposed to ultraviolet light and

photographed with an MP4 Polaroid Camera System (Fotodyne, Inc., Hartland, WI). Molecular standards were obtained from Boehringer Mannheim VIII (Indianapolis, IN).

Quantitative RT-PCR and in situ hybridization of endometrial bikunin gene expression

Tissue collection

Endometrial tissue was collected from gilts on days 0, 5, 10, 12, 15 and 18 of the estrous cycle (n=24) and days 10, 12, 15 and 18 of pregnancy (n=16) as previously described. Following hysterectomy, the uterine horn was opened along its anti-mesometrial border and endometrium removed from the underlying myometrium using sterile scissors. Multiple sections of endometrium (~0.5 cm) from the mesometrial region of the uterine horn were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). Additional endometrial tissue was removed, snap-frozen in liquid nitrogen and stored at -80°C until processed for RNA extraction as described previously. Fixed tissue was transported on ice to the laboratory where samples were trimmed into 5 x 5 mm pieces, transferred to fresh 4% paraformaldehyde and placed on a rocking platform overnight. The following day, paraformaldehyde was removed and tissues were stored in PBS. Tissues were dehydrated and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Quantification of endometrial bikunin gene expression was evaluated using the one-step RT-PCR reaction following manufacturers recommendations for the TaqMan[®] Gold RT-PCR Kit (P/N N808-0233) (PE Biosystems, Foster City, CA). The TaqMan[®] Gold RT-PCR kit is designed to reverse transcribe and amplify target RNA that results in alteration of fluorescence from the bikunin specific probe permitting a quantitative measure of gene expression. The TaqMan probe contained a 5' reporter dye (TET) and a 3' quencher dye (TAMRA). Cleavage of the probe by the endogenous 5' nuclease activity of AmpliTaq Gold DNA polymerase, during each cycle of PCR, results in increased fluorescence of the reporter dye, which is quantified at each cycle. The total reaction volume of 50 μ l contained TaqMan Buffer A, 5.5 mM MgCl₂, 300 μ M deoxyATP, 300 μ M deoxyCTP, 300 μ M deoxyGTP, 600 μ M deoxyUTP, 200 nM bikunin forward primer (bp 804-822) TGTGGAGGCCTGCAGTCTC, 200 nM bikunin reverse primer (bp 856-874) CATCAAACGCCAGAGCTG, 100 nM TET labeled bikunin probe (bp 825-844) CATCGTCTCCGGCCCCTGCC designed from the porcine mRNA sequence for α 1-microglobulin-bikunin precursor protein (Gebhard et al., 1990), 0.025 U/ μ l AmpliTaq Gold DNA polymerase, 0.25 U/ μ l MultiScribe reverse transcriptase, 1.25 U/ μ l recombinant Moloney murine leukemia virus (MuLV) reverse transcriptase, 0.4 U/ μ l Rnase Inhibitor and 100 ng of total RNA brought to volume with Rnase-free water. The PCR amplification was carried out in the ABI PRISM[®] 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Thermal cycling conditions were 50°C for 2 min,

95°C for 10 min followed by 40 repetitive cycles of 95°C for 15sec and 60°C for 1 min. The ribosomal 18S RNA control kit (43108993E, Applied Biosystems, Foster City, CA), was used to quantify 18S rRNA as a normalization control for RNA loading.

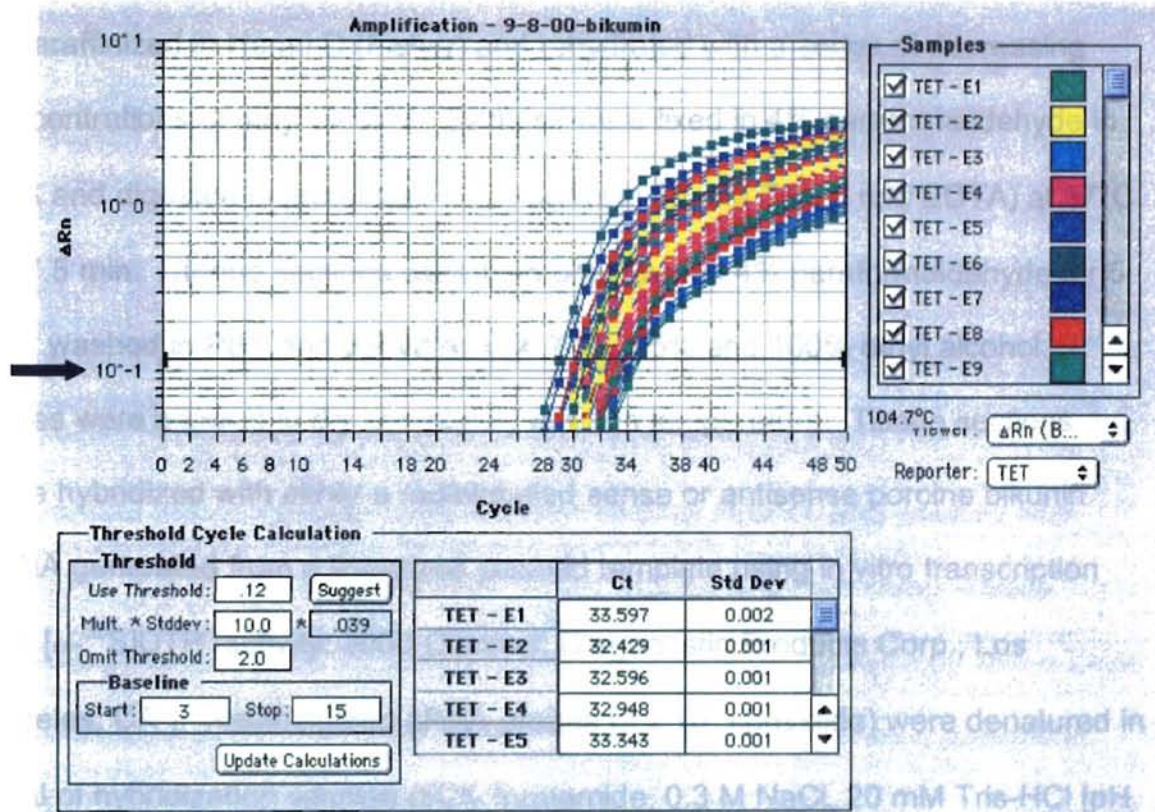
Quantitation of gene amplification was made following RT-PCR by setting the threshold on the TET layer in the geometric region of the plot after examining the semi-log view of the amplification plot (Figure 3.2). The relative quantitation of bikunin gene expression was evaluated using the comparative C_T (threshold cycle) method (PE Biosystems, Foster City, CA). The ΔC_T value is determined by subtracting the bikunin C_T of each sample from the ribosomal 18S C_T value. Calculation of $\Delta\Delta C_T$ involves using the highest sample ΔC_T value as an arbitrary constant to subtract from all other ΔC_T sample values. Fold change in gene expression of bikunin is then determined by evaluating the expression, $2^{-\Delta\Delta C_T}$

In Situ Hybridization Analysis

A partial porcine bikunin cDNA was generated by RT-PCR using the primers and method described previously. The RT-PCR product was excised from 3% agarose gel and extracted utilizing the QIAEX II agarose gel extraction protocol (QIAGEN Inc., Valencia, CA). The 314-bp RT-PCR product was subcloned into pCR II[®]-TOPO[®] (Invitrogen, San Diego, CA) and fully sequenced to confirm identity and directionality. Antisense cRNA probes were generated by linearizing the pCR[®]II TOPO[®] bikunin plasmid with BamHI and in vitro

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Figure 3.2. Quantitative RT-PCR cycle amplification of bikunin using the TET labeled bikunin probe. Arrow indicates cycle threshold (ct) level.



transcription with SP6 RNA polymerase. Sense cRNA probes were generated using EcoRV and T7 RNA polymerase.

Paraffin embedded endometrial tissue was sectioned (5 μm) using a Rotary Microtome Model 1820 (American Optical, Buffalo, New York), and mounted on positively charged microscope slides. Tissue sections were deparaffinized in Hemo-D (Fisher) and rehydrated with a series of decreasing concentrations of ethyl alcohol. Sections were fixed in 4% paraformaldehyde in PBS and digested in proteinase K (20 $\mu\text{g}/\text{ml}$ in 50 mM Tris, 5 mM EDTA) at 37°C for 7.5 min. Uterine sections were then post fixed in 4% paraformaldehyde for 5 min, washed in PBS and dehydrated in 70%, 95% and 100% ethyl alcohol. Slides were allowed to dry completely at room temperature. Tissue sections were hybridized with either a radiolabeled sense or antisense porcine bikunin cRNA generated from a linearized plasmid template using in vitro transcription with [α -³⁵S]UTP (activity: 3000 Ci/mmol; Diagnostic Products Corp., Los Angeles, CA). Radiolabeled cRNA probes (3×10^6 cpm/slide) were denatured in 75 μl of hybridization solution (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl [pH 8.0], 5 mM EDTA [pH 8.0], 10 mM sodium phosphate [pH 8.0], Denhardt's solution, 10% dextran sulfate, 0.5 mg/ml yeast tRNA, 100 mM dithiothreitol [DTT]) at 70°C for 10 min. Hybridization solution was added to the middle of the slide and a cover slip was placed on top. The slides were hybridized overnight at 55°C in a humidified chamber containing Whatman 3MM paper soaked in 50% formamide/5X SSC. The hybridized slides were washed in 5X SSC/10 mM β -mercaptoethanol (βME) for 30 min at 55°C to remove the coverslips. Slides were

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then washed in the following conditions: 50% formamide/2X SSC/50 mM β ME for 20 min at 65°C; TEN (0.5 M NaCl/10 mM Tris (pH 8.0)/5 mM EDTA) at room temperature for 10 min; three 10 min washes in TEN at 37°C; Rnase (10 μ g/ml) in TEN for 30 min at 37°C to remove non-specifically bound probe; TEN for 15 min at 37°C; 50% formamide/2X SSC/50 mM β ME for 20 min at 65°C; 2X SSC for 15 min at r.t.; 0.1X SSC for 12 min ; two washes of 70% EtOH/0.3 M ammonium acetate for 5 min; 95% EtOH/0.3 M ammonium acetate for 1 min; twice in 100% EtOH for 1 min. Liquid film autoradiography was carried out using Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY) at 42°C. Slides were stored for 3 weeks at r.t. in a light tight box containing dessicant. Slides were developed in Kodak D-19 Developer, fixed, counterstained with hematoxylin, dehydrated with increasing concentrations of ethyl alcohol, and washed with H.emo-D. Coverslips were mounted on the slides using Permount. Tissue sections were evaluated by both brightfield and darkfield microscopy with a Zeiss Photomicroscope III (Carl Zeiss, New York, NY), and images were assembled using Adobe Photoshop 5.0 (Adobe Systems, Seattle, WA).

Statistical Analysis

Data were analyzed by least-squares analysis of variance using the Proc Mixed of SAS (SAS, 1999). Quantitative RT-PCR $\Delta\Delta$ CT values were analyzed with a model that included the effects of day, reproductive status, and the day x reproductive status interaction.

Manuscript

Results

Western Blot Analysis of Endometrial Bikunin

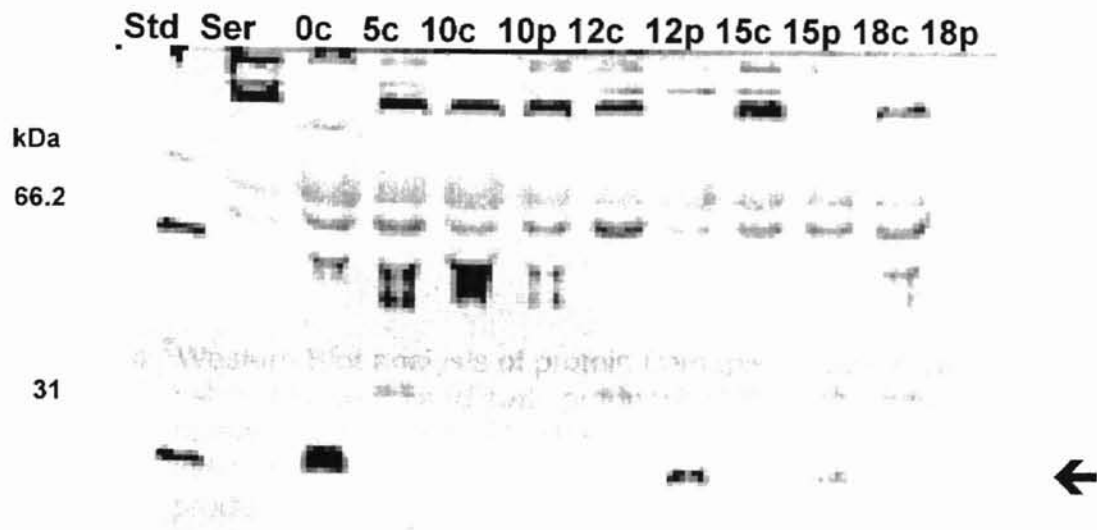
Antiserum to human bikunin detected an approximate 30 kDa immunoreactive product in ECM (Figure 3.3) and UTF (Figure 3.4) that is consistent with reported M_r for free-bikunin (see review Bost et al., 1998). A strong immunoreactive 30 kDa product was detected in ECM on Days 12, 15 and 18 of pregnancy. However, with the exception of Day 0 (estrus), the 30 kDa product was not detected in ECM on any of the other days of the estrous cycle evaluated. A larger M_r reactive product detected on the blot may represent the larger 100 to 125 kDa forms of $I\alpha I$ that consist of the various combinations of inter- α -trypsin inhibitor heavy chains 1, 2, 3 and bikunin (Bost et al. 1998). Strong immunostaining of the 30 kDa immunoreactive product was detected in UTF on Days 12, 15 and 18 of both cyclic and pregnant gilts. A less intense 30 kDa band was present at estrus with no similar size reactive product detected on Days 5 and 10.

RT-PCR Analysis of Endometrial Bikunin mRNA Expression

A 314 bp product representing endometrial gene expression of bikunin was amplified on days 0, 5, 10, 12, 15 and 18 of the estrous cycle and days 10, 12, 15 and 18 of pregnancy (Figure 3.5). Bikunin was expressed throughout the estrous cycle and expression did not appear to vary greatly. Although PCR procedures utilized in this study are only semi-quantitative, expression of the 314 bp product was low on days 10 and 12 of pregnancy, similar to the estrous cycle,

Figure 3.3. Western Blot analysis of protein from endometrial explant culture media during the estrous cycle (c) and early pregnancy (p) using antiserum against human bikunin. Std indicates molecular weight standard lane. Ser indicates pig serum lane. Arrow indicates 30 kDa immunoreactive product.

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Figure 3.4. Western Blot analysis of protein from uterine flushings during the estrous cycle (c) and early pregnancy (p) using antiserum against human bikunin. Std indicates molecular weight standard lane. Ser indicates pig serum lane. Arrow indicates 30 kDa immunoreactive product.

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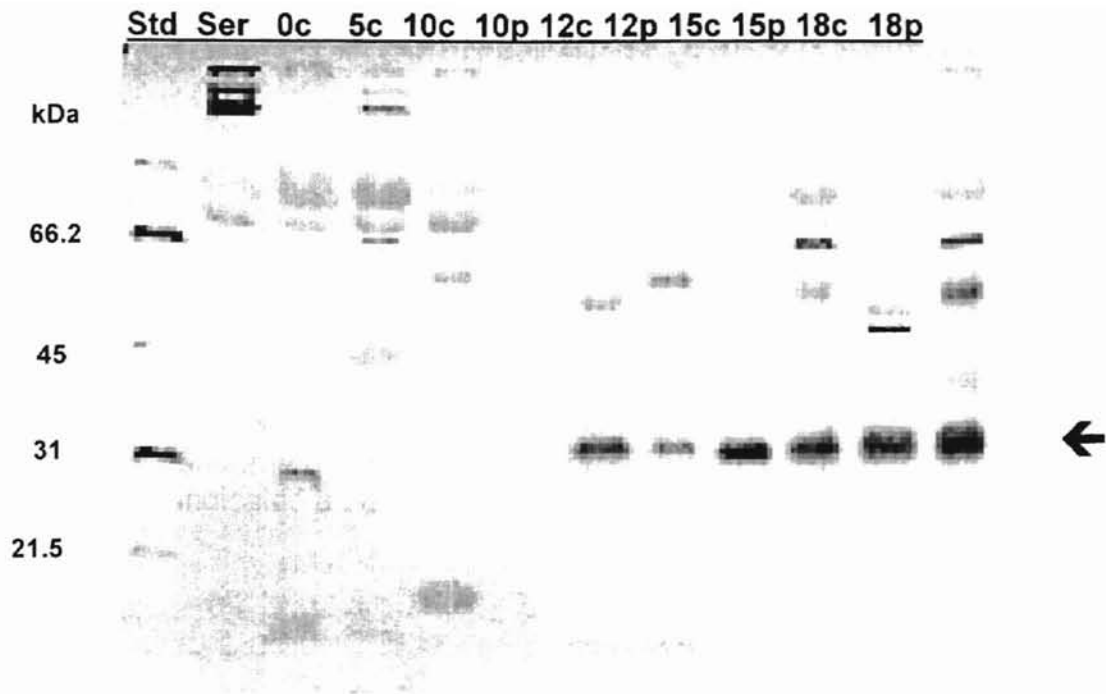
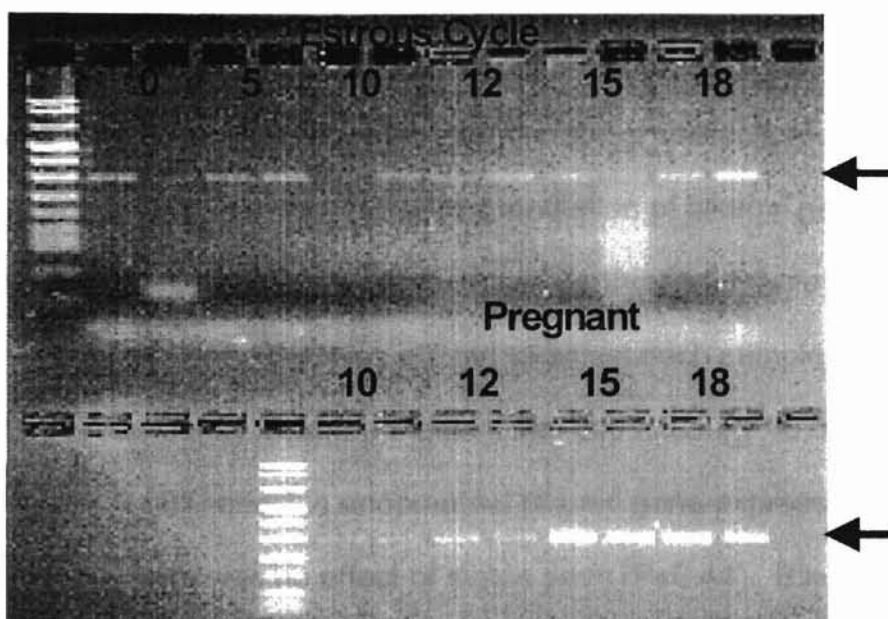


Figure 3.5. Photograph of an ethidium bromide stained 3% agarose gel with endometrial PCR products produced with bikunin primers. Arrow indicates a band size of 314 base pairs representing porcine bikunin.

Bikunin



but expression of bikunin was notably more pronounced on Day 15 and 18 of pregnancy.

Quantitative RT-PCR Analysis of Endometrial Bikunin mRNA Expression

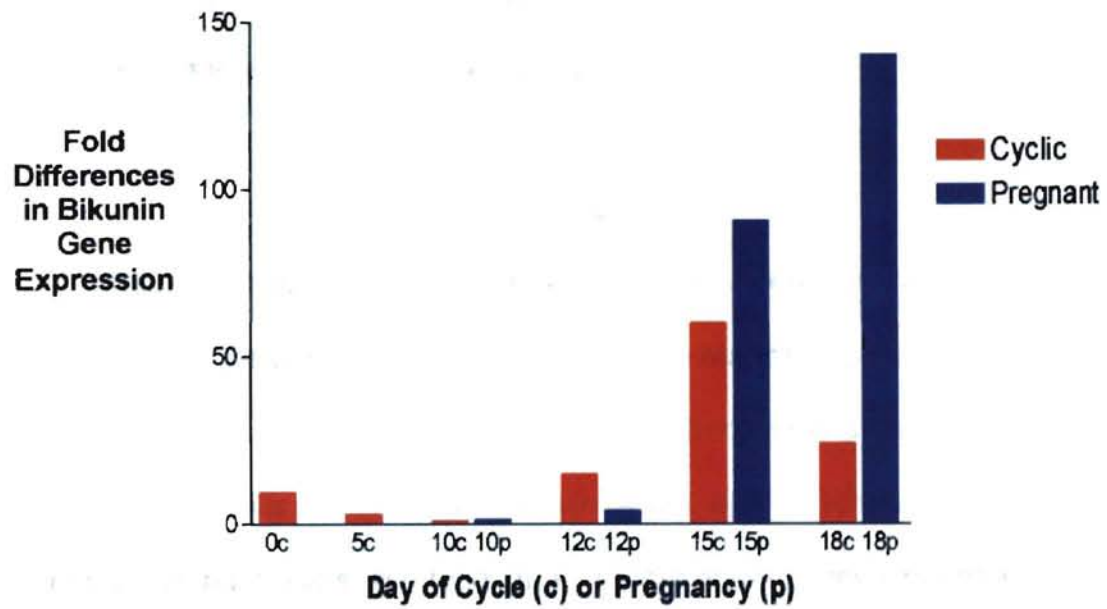
The mRNA expression of bikunin was quantified using the ABI PRISM[®] 7700 Sequence Detection System (PE Biosystems, Foster City, CA). Specific primers designed to porcine bikunin amplified RNA in all endometrial samples with alteration of probe fluorescence detected within 30 cycles (Figure 3.2). The threshold cycle was set on the geometric region of the amplification curve of all samples for comparative analysis. Relative quantitation of bikunin gene expression was evaluated using the comparative C_T (threshold cycle) method (Table 3.2). Ribosomal 18S RNA was utilized to adjust each sample for variation in RNA loading. A tendency toward a status x day interaction ($P=0.06$), and a significant day ($P < 0.001$) effect in endometrial bikunin gene expression was detected; however, there was no effect of status seen ($P=0.44$). Bikunin gene expression decreased 3-fold from Day 0 to 5 of the estrous cycle. Gene expression was lowest on Day 10 of the estrous cycle and pregnancy (Figure 3.6) followed by an approximate 4 to 14-fold increase at Day 12. There was a 60-fold increase in bikunin gene expression at Day 15 of the estrous cycle with an approximate 1 to 6 fold increase observed in pregnant gilts compared to cyclic females on Days 15 and 18 of the estrous cycle. Gene expression of bikunin decreased on Day 18 of the estrous cycle, in contrast to the dramatic increase in expression during early pregnancy.

Table 3.2 Relative Quantitation Using the Comparative C_T Method for Bikunin (BKUN)

Date & Status	BKUN Average C _T	18S-Ribo Average C _T	ΔC _T BKUN-18S-Ribo	ΔΔC _T ΔC _T -ΔC _{T, 10C}	BKUN Rel. to 10C
D-0C	36.40 ± 0.92	17.91 ± 0.22	18.49 ± 1.11	-3.25 ± 1.11	9.51
D-5C	37.30 ± 0.30	17.09 ± 0.30	20.21 ± 0.41	-1.53 ± 0.41	2.89
D-10C	38.79 ± 1.81	17.05 ± 0.25	21.74 ± 1.92	0.00 ± 1.92	1.00
D-10P	38.76 ± 2.53	17.57 ± 0.16	21.20 ± 2.69	-0.54 ± 2.69	1.45
D-12C	35.29 ± 1.49	17.43 ± 0.15	17.85 ± 1.35	-3.89 ± 1.35	14.83
D-12P	37.39 ± 2.69	17.64 ± 0.32	19.75 ± 2.83	-1.99 ± 2.83	3.97
D-15C	33.82 ± 0.92	17.99 ± 0.40	15.83 ± 0.67	-5.91 ± 0.67	60.13
D-15P	32.51 ± 1.01	17.27 ± 0.89	15.24 ± 0.96	-6.50 ± 0.96	90.51
D-18C	34.82 ± 0.91	17.67 ± 0.49	17.15 ± 0.73	-4.59 ± 0.73	24.08
D-18P	32.13 ± 1.10	17.52 ± 0.57	14.61 ± 1.22	-7.13 ± 1.22	140.07

Figure 3.6. Diagrammatical representation of fold differences in bikunin mRNA expression detected through quantitative RT-PCR analysis.

Relative Quantitation Using the Comparative C_T Method for Bikunin



In Situ Hybridization Analysis of Bikunin mRNA in the Porcine Endometrium

In situ hybridization of bikunin mRNA during Days 0 to 5 of the estrous cycle showed little accumulation of gene expression in the endometrial tissue (Figure 3.7). Expression of bikunin mRNA was prevalent around the uterine glands on Days 15 and 18 of the estrous cycle (Figure 3.8). Bikunin gene expression was observed to be scattered in the stroma, but localized mostly to the deep glandular epithelium. Endometrial bikunin gene expression appeared to be more intense in pregnant compared to cyclic females between days 15 to 18 (Figure 3.9). Little or no gene expression was noted in the uterine surface epithelium on any of the days investigated.

Discussion

Following rapid elongation of the trophoblast between days 10 and 12 of gestation, porcine conceptuses initiate the early stages of placental attachment to the uterine surface epithelium (see review, Stroband and Van der Lende, 1990). The pig is a species with a non-invasive, epitheliochorial type of placentation that begins with focal attachments of the newly elongated, filamentous conceptus on Day 13 of gestation (King et al., 1982). Full apposition and adhesion of the maternal and conceptus trophoctodermal microvilli throughout the uterine lumen occurs with growth and fluid expansion of the allantois from the embryonic hindgut between days 13 and 18 of pregnancy (see Stroband and Van der Lende, 1990).

Timing of trophoblast attachment to the uterine surface appears to be temporally associated with the decreased expression of Muc-1 during the

Figure 3.7. Brightfield and darkfield images illustrating in situ hybridization of porcine endometrial tissue sections from days 0 (A,B), 5 (C,D), and 12 (E,F) cyclic gilts using a radiolabeled cRNA probe specific for porcine bikunin mRNA. Magnification = 40X.

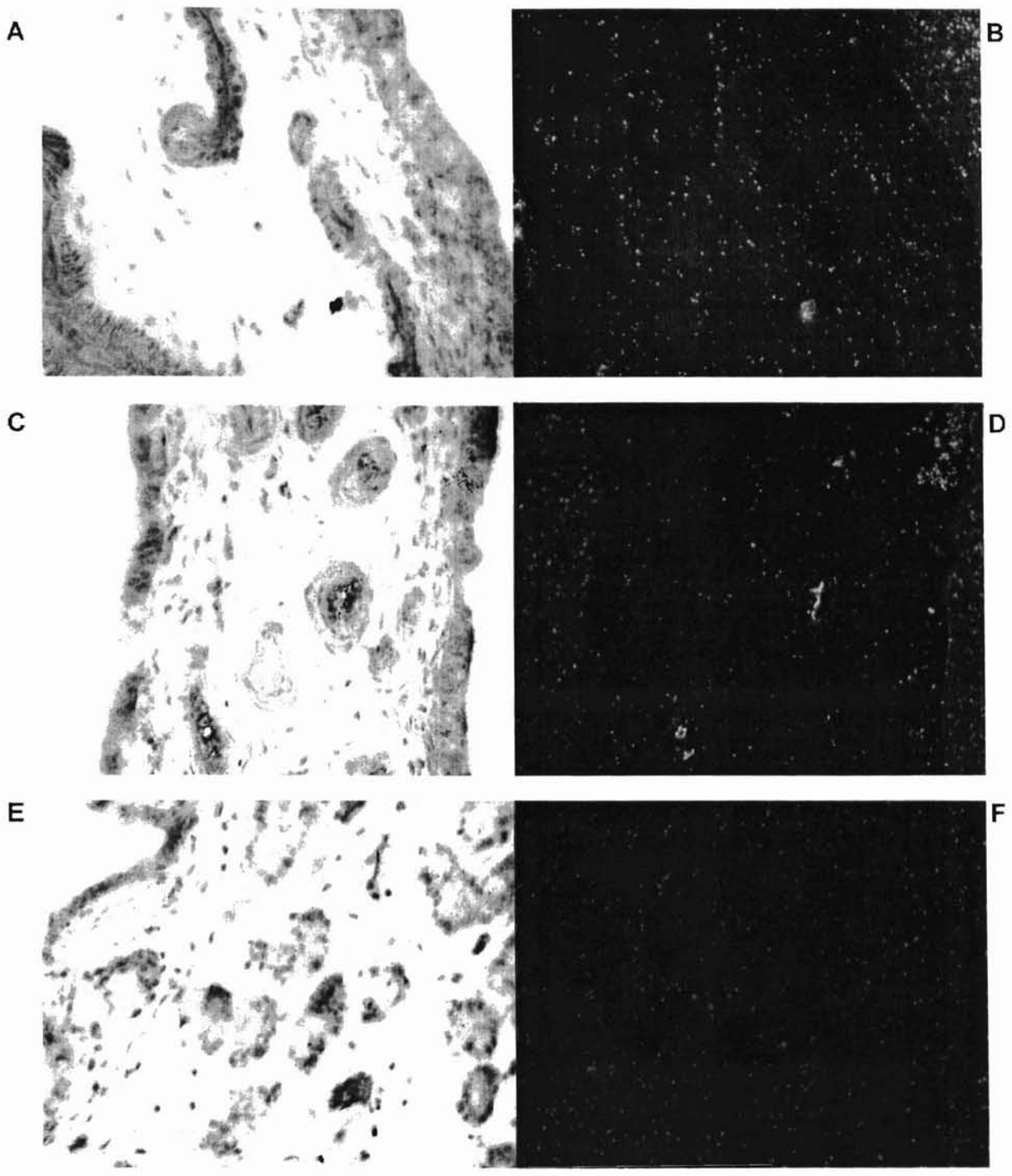


Figure 3.8. Brightfield and darkfield images illustrating in situ hybridization of porcine endometrial tissue sections from day 15 (A,B) and 18 (C,D) cyclic, and 12 pregnant (E,F) gilts using a radiolabeled cRNA probe specific for bikunin mRNA. Magnification = 40X.

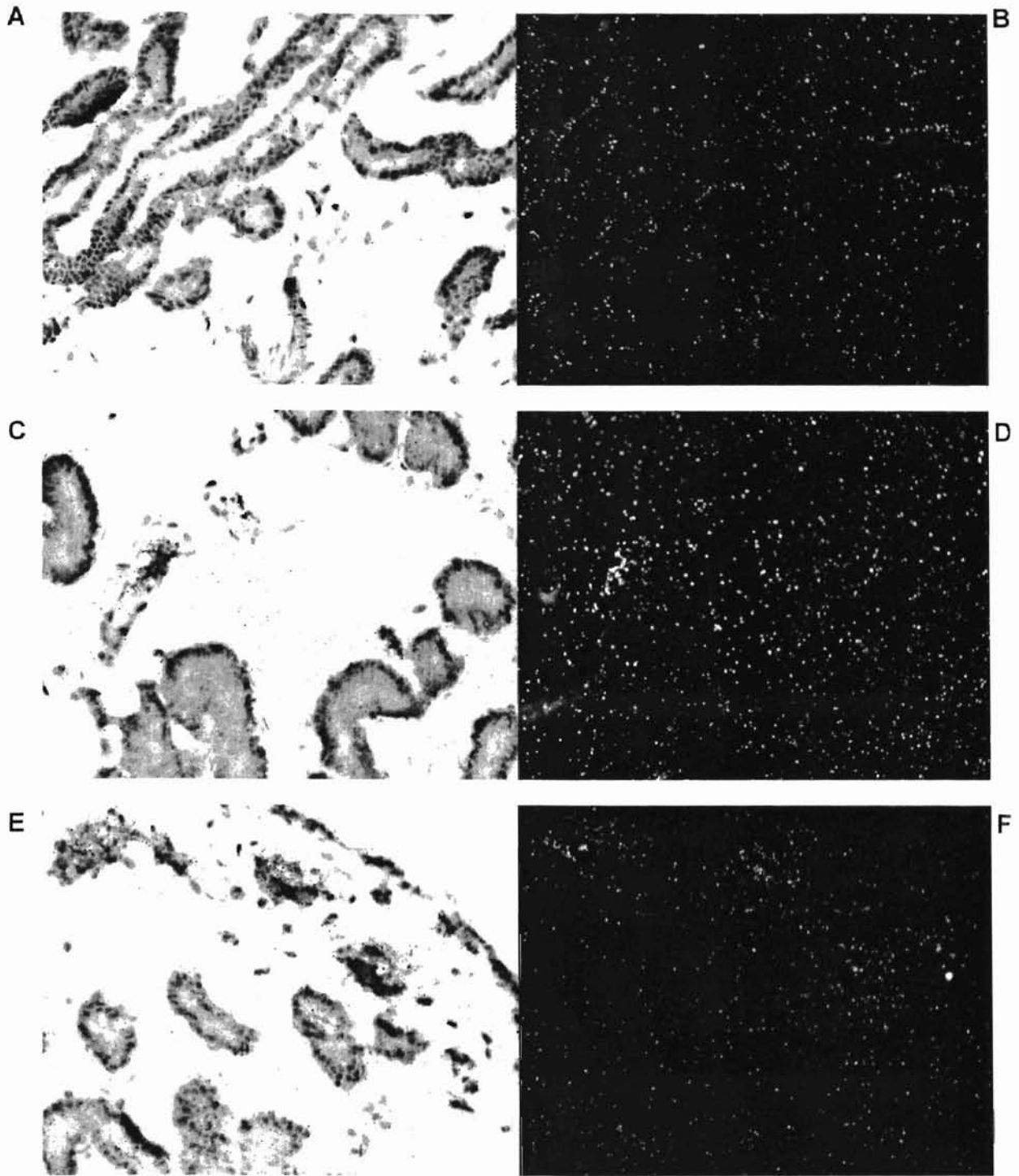
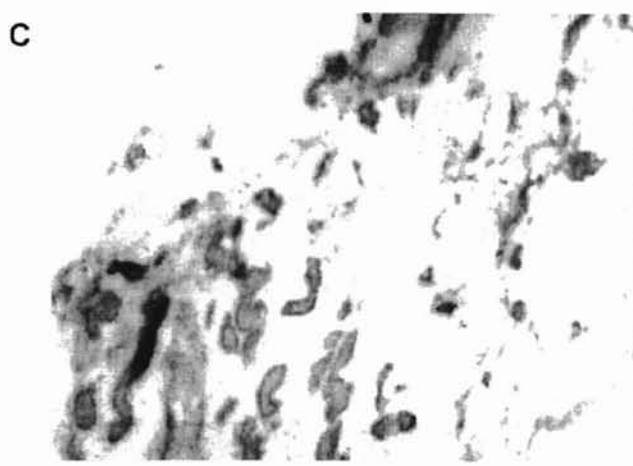
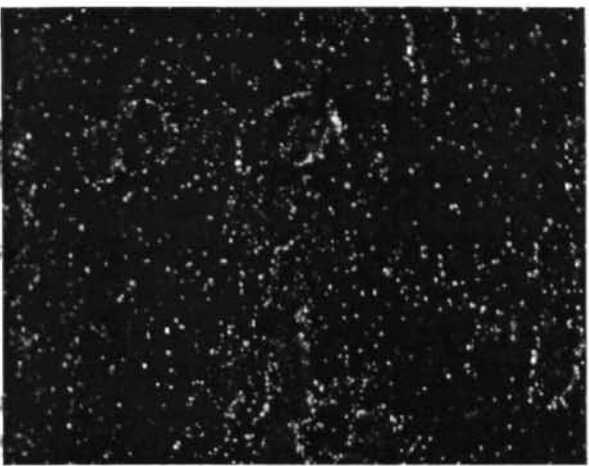
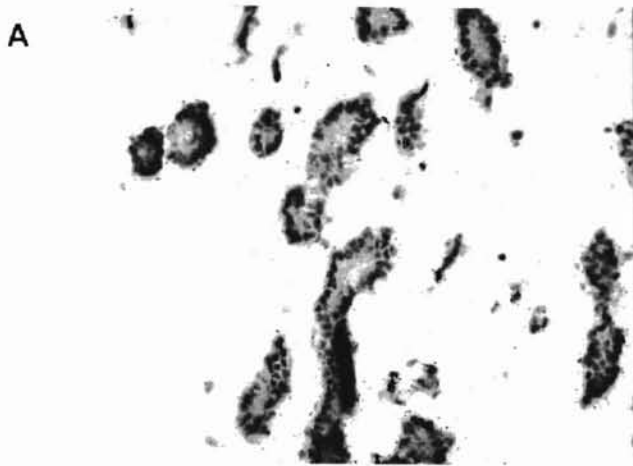


Figure 3.9. Brightfield and darkfield images illustrating in situ hybridization of porcine endometrial tissue sections from day 15 (A,B) and 18 (C,D) pregnant, and sense control (E) using a radiolabeled cRNA probe specific for bikunin mRNA. Magnification = 40X.



transition of the porcine uterus from the prereceptive to receptive state (Bowen et al., 1996). A reduction in Muc-1 expression on the luminal and glandular epithelial surface is associated with events following down-regulation of progesterone receptor in the uterine epithelium (Geisert et al., 1994). The loss of Muc-1 allows the conceptus to interact with such adhesive molecules as integrins, proteoglycans and heparin (Burghardt et al. 1997).

Loss of the heavily glycosylated transmembrane protein expression prior to the period of conceptus placental attachment not only permits interaction of the adhesive factors for attachment of the trophoblast but also exposes the epithelium to proteolytic attack by the conceptus. Porcine conceptuses are highly invasive when placed in an ectopic site (Samuel and Perry, 1972) and therefore have the enzymatic potential to erode into the uterine tissue. However, loss of Muc-1 and the initiation of the receptive state to uterine attachment in the pig are tightly coupled with endometrial synthesis and release of several protease inhibitors (see review Roberts et al., 1993). These inhibitors serve a major function in protecting the uterine epithelium from the vast proteolytic activity of developing porcine conceptuses. The porcine endometrium is the source of several protease inhibitors such plasmin/trypsin inhibitor (Fazleabas et al., 1983), antileukoproteinase (Badinga et al., 1994), tissue inhibitors of metalloproteinases (Menino et al., 1997), and low Mr, basic proteins related to the serpin family of protease inhibitors (Malathy et al., 1990). Uterine expression and luminal secretion of these protease inhibitors is consistent with their

possible role in neutralization of the proteolytic activity of the developing porcine placenta.

The present study adds bikunin to the growing list of uterine protease inhibitors synthesized and released during the critical period of conceptus attachment in the pig. Bikunin is a component of the inter- α -trypsin inhibitor proteoglycan family (α 1) that are proposed to function in binding and stabilizing the extracellular matrix (Bost et al. 1998). Bikunin, the light chain component of the α 1 family, is a 30 kDa serine protease inhibitor (see review Bost et al., 1998) that is a major member of Kunitz-type protease inhibitors (Salier et al. 1990). Kunitz type inhibitors are characterized as having a low M_r , basic isoelectric point and containing one or several enzymatic inhibitory domains against a large number of serine proteases such as trypsin, chymotrypsin, cathepsin G, leukocyte elastase, and plasmin (Salier et al., 1990). Stallings-Mann et al. (1994) previously isolated a 14 kDa, basic, progesterone stimulated porcine uterine plasmin/trypsin inhibitor containing a single Kunitz domain at its amino terminus. Bikunin, as its name implies, contains two tandemly arranged Kunitz-type inhibitory domains (Hochstrasser et al., 1981; Diarra-Mehrpour et al., 1990). Evaluation of uterine bikunin with antiserum to human bikunin identified a major reactive product of 30 kDa. The 30 kDa product was first detectable in uterine flushings on Day 12 of the estrous cycle and early pregnancy. The release of bikunin is temporally associated with the period following the loss of Muc-1 from the uterine epithelium and the stage of conceptus synthesis of plasminogen activator for conversion of uterine plasminogen to plasmin during rapid

elongation of trophoblast (Fazleabas et al., 1983; Bowen et al., 1996). Uterine release of the uterine plasmin/trypsin inhibitor has been suggested to be closely associated with conceptus estrogen release during elongation (Fazleabas et al. 1983). The bikunin reactive product was detected in UTF in both cyclic and pregnant females on Days 12 through 18 in the present study. However, absence of the 30 kDa reactive product in culture media from cyclic gilts would indicate that uterine exposure to the elongating conceptuses alters bikunin release from the α_1 complex at least as evaluated *in vitro*. It is possible that conceptus estrogen stimulates bikunin release as the 30 kDa product is also detectable in ECM of endometrium from gilts during estrus.

The approximate 100 to 120 kDa products detected in ECM with antiserum to bikunin, represents the variable 95-200 kDa forms of the α_1 family. The lack of detection of the bands in UTF suggests that bikunin is free and not associated with the heavy chains in the uterine lumen. The presence of the larger M_r forms in ECM without detection of the free 30 kDa bikunin in cyclic gilts further demonstrates an importance of conceptus interaction for bikunin release. Bikunin interacts with various combinations of the inter- α -trypsin inhibitor heavy chains (α_1 IH) to form members of the α_1 family (Salier et al., 1990). Covalent crosslinking of bikunin to α_1 IH occurs through a chondroitin 4-sulfate glycosaminoglycan bond (Enghild et al. 1991). The α_1 family of serine protease inhibitors can consist of a combination of α_1 IH2, α_1 IH3 and bikunin, α_1 IH1, α_1 IH2, and bikunin, or single chains of each α_1 IH covalently linked to bikunin (Bost et al. 1998). We have previously reported the presence and synthesis of α_1 IH4 by the

porcine endometrium (Geisert et al., 1997). However, unlike the other three α I heavy chains, α IH4 does not possess a binding site for the bikunin (Hashimoto et al., 1996). α IH1 has been detected in the porcine uterus and endometrial α IH1 gene expression demonstrated by RT-PCR (Geisert unpublished results). Therefore, it appears that components of α I are present within the endometrium, however, the appearance of the various α IH during the estrous cycle and early pregnancy need to be investigated.

Since the liver is a major source of α IH and bikunin (Bost et al. 1998), presence of bikunin within the uterus could result in transfer of plasma components. However, the present study demonstrates endometrial bikunin gene expression during the estrous cycle and early pregnancy. The 140-fold increase in endometrial bikunin gene expression between Day 10 to 18 of the estrous cycle and pregnancy is consistent with the detection of free bikunin in the UTF and localization of bikunin mRNA in glandular epithelium of the endometrium. Increase in gene expression and protein within the uterus on Days 12 through 18, suggests that bikunin is possibly regulated by progesterone as previously indicated for the uterine plasmin/trypsin inhibitor (Stallings-Mann et al., 1994).

Although bikunin is denoted as member of the α I family, it is not related to the α IH genes. Bikunin, inter- α -trypsin inhibitor light chain, is part of the gene designated α 1-microglobulin/bikunin precursor (AMBP), which encodes for two proteins, α 1-microglobulin and bikunin (Gebhard et al., 1990). Following glycosylation and sulphation of bikunin, the larger AMBP polypeptide is cleaved

in the golgi, possibly by the pro-protein processing activity of furin, releasing α 1-microglobulin and bikunin (see review Salier et al., 1996). Sulphation of bikunin permits the assembly of the α 1H with bikunin prior to AMBP cleavage. Presently, there is no functional relationship known for the evolutionary fusion of the two genes that are separated by a 7 kb intron (Salier et al. 1996). Role for the release of α 1-microglobulin in the porcine uterus is unknown. It belongs to the lipocalin family of hydrophobic ligand carriers and can bind to IgAs and fibronectin (Chan et al., 1994).

Several biological functions can be proposed for endometrial synthesis of bikunin in the pig. Bikunin functions as a serine protease inhibitor that is most noted for its status as an acute phase α 1 protein for regulation of uncontrolled proteolysis following trauma, disease and inflammation (Romisch et al., 1996; Salier et al., 1996). Certainly, release of bikunin would contribute to the uterine proteinase inhibitors released to regulate the enzymatic activity of the developing porcine conceptuses *in utero*, as well as protecting uterine glandular secretions from proteolysis. Bikunin could serve a role independent of its function as a protease inhibitor. The α 1H have recently been established as hyaluronic-binding proteins, which are involved with stabilization of the extracellular matrix (see Bost et al., 1998). Current evidence in expansion of the cumulus oocyte complex (Chen et al., 1992; Camanioni et al., 1993) supports a principal role for α 1, namely α 1H, in matrix stabilization through its interaction with hyaluronic acid (HA). Bost and coworkers (1998) have suggested that initial ionic binding of HA with α 1H allows covalent substitution of the chondroitin 4-sulfate linkage of

bikunin with HA to form a stable α IH-HA complex. This interaction would result in not only the stabilization of the extracellular matrix, but the release of bikunin into the cellular microenvironment. Need for stabilization of the uterine epithelial surface glycocalyx during placental attachment in pigs (Geisert et al. 1990) and release of bikunin observed in ECM of pregnant gilts in the present study, suggest α I may serve a major function with uterine attachment and the establishment of pregnancy in pigs. Further investigation of the α I family's role in uterine function during establishment of pregnancy in swine is warranted.

CHAPTER IV

SUMMARY AND CONCLUSIONS

In species such as the pig, which have a diffuse, epitheliochorial type of placentation, uterine endometrial secretions, or histotroph, are important for the development of the conceptus (Roberts and Bazer, 1988). Because the porcine embryo is normally invasive, the uterine endometrium synthesizes and secretes protease inhibitors (see review Roberts et al., 1993), which may help to protect the uterus from the invasive nature of the embryo.

Plasmin inhibitor, synthesized by the uterine surface and glandular epithelium, is progesterone dependent (Fazleabas et al., 1985). Maximum levels of plasmin inhibitor are seen at Day 12 of pregnancy (Fazleabas et al., 1983). Fazleabas and coworkers (1983) hypothesized that the release of plasmin inhibitor, stimulated at the time of elongation, may function to protect the uterine tissue from the proteolytic effects of plasmin.

Another protease inhibitor likely to be involved in implantation in pigs is antileukoproteinase (ALP), also referred to as secretory leukocyte protease inhibitor (SLPI) (Badinga et al., 1994). Antileukoproteinase, a low molecular

weight elastase/cathepsin G protease inhibitor (Badinga et al., 1994), shows the greatest amount of expression in the uterus during mid to late gestation (Badinga et al., 1994) in response to the presence of a conceptus, rather than progesterone or estrogen stimulation (Simmen et al., 1991). Antileukoproteinase synthesis seems to be correlated with the epitheliochorial type of placentation (Badinga et al., 1994).

The expression of matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) has been demonstrated in porcine embryos and the uterus during the time of trophoblastic attachment (Menino et al., 1997). Uterine tissue and Day 15 embryos expressed transcripts for TIMP-1, -2 and -3 and MMP-2 and -9 (Menino et al., 1997). The presence of these proteinases, along with their inhibitors, may explain the non-invasive behavior of pig embryos while in the uterine environment.

Epitheliochorial placentation is associated with interdigitation of the trophoblastic and uterine microvilli and changes in the uterine glycocalyx (Dantzer, 1985). Exogenous estradiol administration on days 9 and 10 of pregnancy stimulates the loss of the uterine epithelial glycocalyx (Blair et al., 1991), along with the loss of a basic (pI 7.9-8.9), 30 kDa glycoprotein (Gries et al., 1989). A 30 kDa endometrial glycoprotein, pGP30, has since been isolated and characterized as homologous to the C-terminal region of the inter- α -trypsin inhibitor heavy chain 4 (Geisert et al., 1998). The production of this 30 kDa glycoprotein, known to be a cleavage product of $I\alpha$ LH4 proteolysis (Geisert et al.,

1997), is time and steroid dependent with maximum expression observed on day 12 of the estrous cycle and days 12 and 18 of pregnancy (Geisert et al., 1995). The presence of pGP30 during this stage of pregnancy, when elongation and trophoblastic attachment are occurring, may suggest its role in conceptus attachment to the uterine surface and development (Geisert et al., 1995).

The inter- α -trypsin inhibitor family of protease inhibitors consists of four heavy chains, 1, 2, 3 and 4, (α 1H1, α 1H2, α 1H3, α 1H4) and a light chain, bikunin. All α 1 heavy chains, classified as acute phase proteins (Geisert et al., 1998), contain a von Willebrand type A domain, which serves as a target for adhesive molecules such as integrins, collagen, proteoglycans and heparin (Colombatti and Bonaldo, 1991). Geisert and coworkers (1998) suggested that acute phase proteins protect the uterus from inflammation that may occur during trophoblastic attachment. The α 1H has recently been recognized as hyaluronic-binding proteins (see Bost et al., 1998). Hyaluronic acid, a glycosaminoglycan (GAG), is a major component of the extracellular matrix (see Bost et al., 1998). Bost and coworkers (1998) have reported that the binding of heavy chains 1 and 2 with hyaluronic acid, forming a stable α 1H-HA complex, releases bikunin into the cellular microenvironment and results in the stabilization of the extracellular matrix. α 1H4, unlike the other heavy chain members, does not bind bikunin (Geisert et al., 1998), and is readily cleaved by kallikrein (Geisert et al., 1995). α 1H4 is expressed in the porcine uterus during the estrous cycle and early pregnancy (days 0-18), with enhanced gene expression seen during the mid-luteal phase of the cycle and during trophoblastic attachment (Geisert et al.,

1998). It has been suggested that α 1H4 might stabilize the uterine glycocalyx, thereby preventing invasion by the conceptus (Geisert et al., 1998).

Bikunin binds to α 1H1, α 1H2, and α 1H3, via a chondroitin sulfate chain, forming either pre- α -inhibitor or inter- α -trypsin inhibitor complexes (Blom et al., 1999; Thuveson and Fries, 1999). The serine protease inhibitory activity of these complexes comes from bikunin, as it contains two Kunitz-type inhibitory domains (Xu et al., 1998). Bikunin can inhibit trypsin, cathepsin G, elastase and plasmin. The inter- α -trypsin inhibitor light chain (ITIL) gene (see review Bost et al., 1998), also known as the AMBP gene (see review Salier et al., 1996), encodes for two proteins, α 1-microglobulin and bikunin, which are separated through post-translational proteolytic cleavage. Bikunin has been found in blood serum, urine and various tissues of humans and other species (Xu et al., 1998) and is thought to play a role in repairing tissue where proteolytic destruction has occurred (Yoshida et al., 1994).

The present study detected bikunin protein and gene expression during the estrous cycle and early pregnancy. There was a tendency towards a reproductive status by day interaction, as well as a significant day effect on gene expression. During the estrous cycle, bikunin gene expression increased at days 12 and 15 followed by a decrease at day 18. During early pregnancy, levels were high at days 12 and 15 and further increased at day 18. This pattern of expression suggests that bikunin gene expression may be regulated by progesterone.

The presence of bikunin in the uterus during the estrous cycle and early pregnancy could imply many possible roles of this protease inhibitor in conceptus attachment to the uterine surface and development. The first possibility could be that bikunin serves to protect the uterus from invasion by the conceptus. Kobayashi and coworkers (1994a) described the use of bikunin for inhibiting tumour invasion *in vitro* and lung metastasis *in vivo*. Because of these previous findings in other tissue and organ systems, and bikunin's localization to the deep uterine glands in pigs, it may be serving as a secondary, back-up mechanism, to prevent conceptus invasion if the protease inhibitors on the surface epithelium fail. Another possible role, based on the localization of bikunin, is that it protects uterine secretions from proteolysis on their way from the glands to the areolae, where they are absorbed by the conceptus. Species with an epithelialchorial type of placentation rely on uterine secretions for conceptus development (Roberts and Bazer, 1988); therefore proteolysis of these nutrients could be detrimental to the developing conceptus. The presence of bikunin during these critical stages of development suggests that it may be involved in regulating the biological processes that are occurring during conceptus attachment and development.

APPENDIX

Western Blot Analysis

1. After electrophoresis, carefully remove the gel from the glass plates by prying off the top glass with a spacer. Cut off the stacking gel and notch the left hand corner of the gel to mark the location of the molecular weight standards. Place the gel in transfer buffer on a rocking platform for 15-20 minutes.
2. While the gel is in the transfer buffer prepare the PVDF membrane by soaking it in:
 - a. Methanol, 1-3 seconds
 - b. Millipore water (dH₂O), 1-2 minutes
 - c. Transfer buffer, 2 minutes

*Handle the dry and wet membrane with forceps at one corner. After wetting do not allow the membrane to dry out.
3. Prepare the transfer unit on the semi-dry electroblotting (SDE) apparatus. Put the sandwich together on the anode base as follows:
 - a. 1 sheet 3MM paper soaked in anode buffer #1
 - b. 2 sheets 3MM paper soaked in anode buffer #2
 - c. PVDF membrane soaked in transfer buffer; center the gel and place the notch in the upper left corner of the sandwich
 - d. Gel soaked in transfer buffer; center the gel and place the notch in the upper left corner of the sandwich; roll out the air bubbles using cathode buffer
 - e. 3 sheets 3MM paper soaked in cathode buffer

*Wear gloves while preparing the sandwich. Roll out air bubbles from each layer with a gloved finger or a glass stirring rod. Don't allow carbon particles from the anode base to get on the membrane or gel. Make sure that the filter papers, gel and membrane have the same dimensions and are stacked evenly.
4. Place the lid (cathode) on top of the sandwich. Loosely attach the screws to prevent the filter papers from drying out.
5. Electroblot for 35 minutes at 100 mA (constant current) for one sandwich and 150 mA for two; allow a maximum of 2.5 mA/cm² gel.

Immunostaining

**For immunostaining use a plastic container to incubate the membrane. Use a rocking platform for all incubations and washes. Handle the membrane with forceps at one corner. Do not allow the membrane to dry out.

1. After electroblotting, immerse the membrane in TBS for 2 minutes. Place the gel in gel fixative and later stain the gel with Coomassie blue. All washes contain 30 ml of wash solution.
2. Pour off the TBS and add 30 ml of the first blocking solution (3% gelatin in TBS). Incubate for 1 hour.
3. Wash the membrane in TTBS one time for 10 minutes.
4. Pour off the TTBS and add the first antibody solution. Incubate overnight. The first antibody is bikunin antibody. It is diluted 1/330 in 25 ml of antibody buffer (1% gelatin in TTBS).
5. Wash the membrane in TTBS three times for five minutes each.
6. Pour off the TTBS and add the second antibody solution. Incubate for 2 hours.
7. During the second antibody incubation measure 33 ml of HRP color development buffer and allow it to reach room temperature. Thaw and measure 6.67 ml of HRP color reagent A and protect from light.
8. Wash the membrane in TTBS two times for five minutes each.
9. Prepare the color development solution for horseradish peroxidase. Add 200 μ l of HRP color reagent B to 33 ml of HRP color development buffer at room temperature. Then add 6.67 ml of HRP color reagent A at room temperature immediately prior to use.
10. Wash the membrane in TBS two times for five minutes each.
11. Pour off the TBS and add the color development solution. Color development incubation will last about 15 minutes. Cover the incubation container to prevent fading.
12. Pour the color development solution into the waste bottle. Wash the membrane in Millipore water 2 times for 5 minutes each.
13. Store the membrane in water at 4°C protected from light.

Solutions

**Use Millipore water for all solutions. Use electrophoresis grade Tris and glycine. Use blotting grade Tween-20 and gelatin.

10% Methanol

40 ml MeOH, QS to 400 ml with dH₂O

20% Methanol

120 ml MeOH, QS to 600 ml with dH₂O

Transfer Buffer

25 mM Tris, 192 mM glycine in 20% (v/v) methanol, pH 8.2-8.3.

Anode Buffer 1

0.3 M Tris in 10% (v/v) methanol, pH 10.4

200 ml contains 7.27 g Tris

Anode Buffer 2

0.025 M Tris in 10% (v/v) methanol, pH 10.4

200 ml contains 0.6055 g Tris

Cathode Buffer

0.025 M Tris, 40 mM glycine in 20% (v/v) methanol, pH 9.4

200 ml contains 0.6055 g Tris, 0.6006 g glycine

Tris Buffered Saline (TBS)

20 mM Tris, 500 mM NaCl, pH 7.5

400 ml contains 0.9691 g Tris, 11.688 g NaCl

Wash Solution (TTBS)

20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5

250 ml Tris, 125 μ l Tween-20

First Blocking Solution

3% gelatin in TBS

30 ml TBS contains 0.9278 g gelatin

Antibody Buffer

1% gelatin in TTBS

60 ml TTBS contains 0.6061 g gelatin

First Antibody Solution

1:330 antibody (stored at -20°C) in 25 ml antibody buffer

Second Antibody Solution

10 ul (1:3000) Goat Anti-Rabbit IgG HRP Conjugate (stored at 4°C), 10 ul (1:3000) avidin HRP conjugate (stored at 4°C) in 30 ml antibody buffer

Color Development Solution for Horseradish Peroxidase

33 ml HRP color development buffer (20 mM Tris, 500 mM NaCl, pH 7.5) at room temperature, 200 µl HRP color reagent B (3% hydrogen peroxide), 6.67 ml HRP color reagent A (0.3% 4-chloro-1-naphthol in diethylene glycol) at room temperature. Prepare just before use.

**All solutions are enough to process one PVDF membrane.

Coomassie Staining Procedure

1. Fix gels in 7% acetic acid and 40% EtOH overnight.
2. Coomassie blue stain:
1.25 g Coomassie blue in 400 ml 95% EtOH, stir several hours then add: 70 ml conc. acetic acid, 530 ml distilled water. Filter with filter paper.
3. Stain gel for minimum of 2 hours at room temperature.
4. Destain gel in 7% acetic acid and 10% EtOH. Will need several changes.

General Procedures for Polymerase Chain Reaction (PCR)

For each new gene, the optimal conditions for its amplification must be determined. The total reaction volume in each tube is 25 μ l. The amounts of Taq polymerase (0.175 μ l/tube) and 10X Buffer (2.5 μ l/tube) remain constant for every reaction. QS the volume of each tube to 25 μ l with PCR water.

Lane	Primer (5 μ M stock)	dNTPs (10 mM stock)	10 X MgCl
1	0.5 μ l	0.5 μ l	1.25 μ l
2	0.5	0.5	2.50
3	0.5	0.5	3.75
4	0.5	1.0	1.25
5	0.5	1.0	2.50
6	0.5	1.0	3.75
7	1.5	0.5	1.25
8	1.5	0.5	2.50
9	1.5	0.5	3.75
10	1.5	1.0	1.25
11	1.5	1.0	2.50
12	1.5	1.0	3.75
13	2.5	0.5	1.25
14	2.5	0.5	2.50
15	2.5	0.5	3.75
16	2.5	1.0	1.25
17	2.5	1.0	2.50
18	2.5	1.0	3.75

After the determination of the optimal conditions, a regular PCR reaction is carried out.

A PCR reaction sheet should be filled out prior to every reaction.

Prior to use, the PCR pipettors, tube opener and work area should be cleaned thoroughly with a 10% bleach solution.

Use only tubes and pipettor tips that are designated for PCR use.

Keep all ingredients and tubes on ice. Taq polymerase should remain in the -20°C freezer until use.

Prepare a Master Mix (MM) including all of the ingredients except the cDNA.

Each reaction should include a negative control to check for any DNA contamination. The negative control includes the Master Mix with PCR water in the place of the cDNA. In the case of any contamination, discard all of the PCR reaction ingredients, with the exception of the Taq polymerase and the cDNA, and redo the PCR reaction using new ingredients.

20 μ l of PCR oil should be placed on top of the contents of each tube when using a thermocycler without a heated lid.

Store PCR products at 4°C until run on an agarose gel.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction

For each reaction, primers and a probe must be constructed based on the sequence of the target gene. The primers and fluorescent probe can be ordered from PE Applied Biosystems online at <http://oligos.pebio.com>. Upon arrival, the primers, probe and TaqMan Gold RT-PCR kit need to be stored at -20°C.

RNA samples were diluted to a concentration of 20 ng/μl in DEPC water. Reactions were set up using both 100 ng and 200 ng of RNA.

A 50 μl RT-PCR reaction was set up using the following components:

Component	Volume/Tube (μl)	Final Concentration
RNA	5 or 10	100 ng or 200 ng
Rnase-free water	18.5 or 13.5	--
10X TaqMan Buffer A	5	1X
25 mM Magnesium Chloride	11	5.5mM
10 mM deoxyATP	1.5	300 μM
10 mM deoxyCTP	1.5	300 μM
10 mM deoxyGTP	1.5	300 μM
20 mM deoxyUTP	1.5	600 μM
10 μM Forward Primer	1.0	200 nM
10μM Reverse Primer	1.0	200 nM
5 μM Fluorescent Probe	1.0	100 nM
AmpliTaq Gold DNA Polymerase (5.0 U/μl)	0.25	0.025 U/μl
MultiScribe Reverse Transcriptase (50 U/μl)	0.25	0.25 U/μl
Rnase Inhibitor	1.0	0.4 U/μl

A Master Mix (MM) should be made up (minus the RNA) for the number of samples being run plus 10% for pipetting errors.

Pipette the RNA into .5 ml microcentrifuge tubes. Gloves should be changed after handling RNA to prevent any sample cross contamination. Following addition of the RNA, the Master Mix should be distributed to the tubes.

After vortexing the samples, they are transferred into a MicroAmp Optical 96-well Reaction Plate and the wells were capped using MicroAmp Optical Caps.

Transfer the plates to the thermal cycler block of the ABI PRISM 7700 Sequence Detection System.

The following thermal cycling parameters were used to amplify the bikunin gene:

Step	Reverse Transcription Hold	AmpliTaq Gold Activation Hold	PCR	
			Cycle (40 cycles)	
			Denature	Anneal/ Extend
Time	30 min	10 min	15 sec	1 min
Temperature	48°C	95°C	95°C	60°C

Results are analyzed using the software provided.

In Situ Hybridization

*Plasmids must be linearized for in vitro transcription (IVT)

1. Digest 20 μ g of DNA with appropriate restriction enzyme for >2 hours at appropriate temperature. (With TOPO plasmid use EcoRV and BamHI at 37°C)
2. Extract once with PCI (25 Phenol: 24 Chloroform: 1 Isoamyl alcohol) and back extract with water. Extract once again with chloroform.
3. Precipitate DNA with 3 volumes 100% EtOH, 1/10 volume 3M NaOAc.
4. Place at -80°C for 15 minutes. Spin down for 10 minutes at maximum speed at room temperature.
5. Remove EtOH and wash pellet with 150 μ l of 70% EtOH.
6. Remove all 70% EtOH and resuspend pellet in 40 μ l of Rnase-free water. (Volume of water depends on the size of the pellet)

Preparation of Probe

1. Set up probe synthesis reaction in 1.5 ml tube:

<u>Solution</u>	<u># μl</u>
DNA	2 (~1-2 μ g)
5X Tsc Buffer	5 (Promega)
100 mM DTT	2.5 (Promega)
2.5 mM rACG	2.5 (Promega)
Radiolabeled UTP	5 (20 mCi/ml)
Rnasin	1 (Promega)
Water	5.5
T7 or SP6 Polymerase	<u>1.5</u> (Promega)

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2. Incubate 2 hours at 37°C. During the 2 hour incubation prepare the slides for hybridization.
3. Add 3 μ l RQ1-Dnase I (Promega) and 0.5 μ l Rnasin. Incubate at 37°C for 15 minutes.

4. Add 8 μ l yeast tRNA (25 mg/ml) and 60 μ l PCI. Spin for 5 minutes and back extract with 20 μ l water. Extract again with 100 μ l chloroform.
5. Remove unincorporated nucleotides using a Sephadex G-50 column.
6. Precipitate by adding 60 μ l 3 M NaOAc (pH 5.0), 1 μ l yeast tRNA, and 300 μ l EtOH.
7. Leave at -80°C for 15-20 minutes. Spin down at maximum speed for 10 minutes at 4°C.
8. Wash the pellet with 70% EtOH and redissolve pellet in 50 μ l of 100 mM DTT.
9. Count 2 μ l on beta counter.

Preparation of Slides for Hybridization

SOLUTION	TIME (min)	# OF CHANGES	COMMENTS
Xylene or Substitute	10	2	Agitate every 2-3 minutes
100% EtOH	3	3	
95% EtOH	3	1	
70% EtOH	5	1	
PBS	5	2	Can hold here a short time
Fresh 4% Paraformaldehyde	20	1	Reuse for later PAF step
PBS	5	2	
Proteinase K (at 37°C)	7.5	1	20 μ g/ml Prot. K in 50 mM Tris, 5 mM EDTA
Fresh 4% PAF	5	1	
DEPC Water	1	1	
PBS	5	2	Can hold here a short time
70% EtOH	5	2	
95% EtOH	2	1	
100% EtOH	2	2	

Air dry slides at room temperature.

Hybridization

1. Denature radiolabeled cRNA probe (5×10^6 CPM/slide) at 70°C for 10 minutes in hybridization solution containing 100 mM DTT. Need ~ 75 μ l of hybe solution per slide.
2. Add hybe probe solution to middle of slide.
3. Gently place coverslip on each slide. Avoid air bubbles!!
4. Hybridize overnight at 55°C in a humidified chamber containing Whatman 3 MM paper wetted with 50% formamide/5X SSC.

Washing

*Wash the hybridized slides using the following protocol:

WASH SOLUTIONS	TIME (min)	# OF CHANGES	TEMPERATURE
5X SSC/10 mM β ME	30	1	55°C
50% Formamide/2X SSC/50 mM β ME	20	1	65°C
TEN	10	1	RT
TEN	10	3	37°C
RnaseA (10 μ g/ml) in TEN	30	1	37°C
TEN	15	1	37°C
50% Formamide/2X SSC/50 mM β ME	20	1	65°C
2X SSC	15	1	RT
0.1X SSC	12	1	RT
70% EtOH/0.3M Ammonium Acetate	5	2	RT
95% EtOH/0.3M Ammonium Acetate	1	1	RT
100% EtOH	1	2	RT

Air dry slides completely and expose to film overnight to estimate autoradiography time.

Roughly:

Shadow - ~2 weeks

Clearly visible but faint - ~1 week

Obviously dark - 2-3 days

Autoradiography

1. Thaw Kodak NTB2 emulsion at 42°C in a light tight container.
2. Using a Kodak Safelight filter, mix the emulsion (1:1) with an equal volume of 42°C water.
3. Pour into a slide holder, dip slide, and wipe off excess emulsion on slide back.
4. Place slides in a light tight box containing dessicant.
5. Wrap the box in foil and store at room temperature for appropriate amount of time.

Develop and stain the slides using the following protocol:
(Begin in the dark room!!)

SOLUTION	TIME (min)	# OF CHANGES	COMMENTS
Kodak D-19 Developer	4	1	Dilute 1:1 and chill to 15°C on ice
Water	0.5	1	
Fixer	5	1	Use fixer with hardener
Water	5	2	Can turn on light
Hematoxylin	1	1	
Water	0.5	Until clear	
50% EtOH	5	1	
70% EtOH	5	1	
95% EtOH	3	2	
100% EtOH	3	3	
Xylene or substitute	1	3	
Permunt			Use to mount coverslip

Literature Cited

- Adams KL, Bazer FW, Roberts RM. Progesterone-induced secretion of a retinol-binding protein in the pig uterus. *J Reprod Fert* 1981; 62:39-47.
- Agura ED, Howard M, Collins SJ. Identification and sequence analysis of the promoter for the leukocyte integrin β -subunit (CD18): A retinoic acid-inducible gene. *Blood* 1992; 79(3):602-609.
- Anegon I, Cuturi MC, Godard A, Moreau M, Terqui M, Martinat-Botte F, Soullillou JP. Presence of leukaemia inhibitory factor and interleukin 6 in porcine uterine secretions prior to conceptus attachment. *Cytokine* 1994; 6:493-499.
- Atmani F, Khan SR. Role of urinary bikunin in the inhibition of calcium oxalate crystallization. *J Am Soc Nephrol* 1999; Suppl 14:S385-S388.
- Badinga L, Michel FJ, Fields MJ, Sharp DC, Simmen RCM. Pregnancy-associated endometrial expression on antileukoproteinase gene is correlated with epitheliochorial placentation. *Mol Reprod Dev* 1994; 38:357-363.
- Bazer FW. Uterine protein secretions: Relationship to development of the conceptus. *J Anim Sci* 1975; 41(5):1376-1382.
- Bazer FW, Thatcher WW. Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin $F2\alpha$ by the uterine endometrium. *Prostaglandins* 1977; 14(2):397-401.
- Bazer FW, Worthington-White D, Fliss MF, Gross S. Uteroferrin: A progesterone-induced hematopoietic growth factor of uterine origin. *Exp Hematol* 1991; 19(9):910-915.
- Blair RM, Geisert RD, Zavy MT, Yellin T, Fulton RW, Short EC. Endometrial surface and secretory alterations associated with embryonic mortality in gilts administered estradiol valerate on days 9 and 10 of gestation. *Biol Reprod* 1991; 44:1063-1079.

- Blom AM, Morgelin M, Oyen M, Jarvet J, Fries E. Structural characterization of inter- α -inhibitor. *J Biol Chem* 1999; 274(1):298-304.
- Blomhoff R, Green MH, Berg T, Norum KR. Transport and storage of vitamin A. *Nature* 1990; 250:399-404.
- Bost F, Diarra-Mehrpour M, Martin JP. Inter- α -trypsin inhibitor proteoglycan family: A group of proteins binding and stabilizing the extracellular matrix. *Eur J Biochem* 1998; 252:339-346.
- Bowen JA, Bazer FW, Burghardt RC. Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm in vivo. *Biol Reprod* 1996; 55:1098-1106.
- Bowen JA, Bazer FW, Burghardt RC. Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm in vitro. *Biol Reprod* 1997; 56:409-415.
- Braga VMM, Gendler SJ. Modulation of Muc-1 mucin expression in the mouse uterus during the estrus cycle, early pregnancy and placentation. *J Cell Sci* 1993; 105:397-405.
- Brambel CE. Allantochorionic differentiation of the pig studied morphologically and histochemically. *Am J Anat* 1933; 52:397-459.
- Chastant S, Monget P, Terqui M. Localization and quantification of insulin-like growth factor-I (IGF-I) and IGF-II/mannose-6-phosphate (IGF-II/M6P) receptors in pig embryos during early pregnancy. *Biol Reprod* 1994; 51:588-596.
- Chen L, Mao STJ, Larsen WJ. Identification of a factor in fetal bovine serum that stabilizes the cumulus extracellular matrix: a role for a member of the inter-alpha-trypsin inhibitor family. *J Biol Chem* 1992; 267:12380-12386.
- Chen L, Mao SJT, McLean LR, Powers RW, Larsen WJ. Proteins of the inter- α -trypsin inhibitor family stabilize the cumulus extracellular matrix through direct binding with hyaluronic acid. *J Biol Chem* 1994; 269:28282-28287.
- Christenson RK, Leymaster KA, Young LD. Justification of unilateral hysterectomy-ovariectomy as a model to evaluate uterine capacity in swine. *J Anim Sci* 1987; 65:738-744.
- Colombatti A, Bonaldo P. The superfamily of proteins with von Willebrand factor type A-like domains: One theme common to components of extracellular matrix, hemostasis, cellular adhesion, and defense mechanisms. *Blood* 1991; 77(11):2305-2315.

- Conley AJ, Christenson RK, Ford SP, Geisert RD, Mason JI. Steroidogenic enzyme expression in porcine conceptuses during and after elongation. *Endocrinology* 1992; 131:896-902.
- Corner GW. The problem of embryonic pathology in mammals, with observations upon intrauterine mortality in the pig. *Am J Anat* 1923; 31:523-545.
- Croy BA, Wood W, King GJ. Evaluation of intrauterine immune suppression during pregnancy in a species with epitheliochorial placentation. *J Immunol* 1987; 139:1088-1095.
- Dantzer V. Electron microscopy of the initial stages of placentation in the pig. *Anat Embryol* 1985; 172:281-293.
- Daveau M, Roucet P, Scotte M, Faye L, Hiron M, Lebreton JP, Salier JP. Human inter- α -inhibitor family in inflammation: Simultaneous synthesis of positive and negative acute-phase proteins. *Biochem J* 1993; 292:485-492.
- Davis AJ, Fleet IR, Harrison FA, Maule Walker FM. Pulmonary metabolism of prostaglandin F_{2 α} in the conscious nonpregnant ewe and sow. *J Physiol (London)* 1979; 301:86.
- De Luca LM. Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. *FASEB J* 1991; 5:2924-2933.
- Diederich MJ, Yelich JV, Geisert RD. Gene and protein expression of the inter- α -trypsin inhibitor, bikunin, in porcine conceptus and endometrium. *J Anim Sci* 1997; 75 (Suppl 1) 83.
- Dhindsa DS, Dzuik PF. Effects of pregnancy in the pig after killing embryos or fetuses in one uterine horn in early pregnancy. *J Anim Sci* 1968;27:122-126.
- Dziuk PJ. Effect of number of embryos and uterine space on embryo survival in the pig. *J Anim Sci* 1968; 27:673-676.
- Ebisuno S, Nishihata M, Inagaki T, Umehara M, Kohjimoto Y. Bikunin prevents adhesion of calcium oxalate crystal to renal tubular cells in human urine. *J Am Soc Nephrol* 1999; Suppl 14:S436-S440.
- Enghild JJ, Salvesen G, Hefta SA, Thogersen IB, Rutherford S, Pizzo SV. Chondroitin 4-sulfate covalently cross-links the chains of the human blood protein pre- α -inhibitor. *J Biol Chem* 1991; 266:747-751.

- Fazleabas AT, Bazer FW, Hansen RJ, Geisert RD, Roberts RM. Differential patterns of secretory protein localization within the pig uterine endometrium. *Endocrinology* 1985; 116:240-245.
- Fazleabas AT, Geisert RD, Bazer FW, Roberts RM. Relationship between release of plasminogen activator and estrogen by blastocysts and secretion of plasmin inhibitor by uterine endometrium in the pregnant pig. *Biol Reprod* 1983; 29:225-238.
- Fischer HE, Bazer FW, Fields MJ. Steroid metabolism by endometrial and conceptus tissues during early pregnancy and pseudopregnancy in gilts. *J Reprod Fert* 1985; 75:69-78.
- Ford SP, Christenson LK. Direct effects of oestradiol-17 β and prostaglandin E-2 in protecting pig corpora lutea from a luteolytic dose of prostaglandin F-2 α . *J Reprod Fert* 1991; 93:203-209.
- Ford SP, Christenson RK. Blood flow to uteri of sows during the estrous cycle and early pregnancy: Local effect of the conceptus on the uterine blood supply. *Biol Reprod* 1979; 21:617-624.
- Frank M, Bazer FW, Thatcher WW, Wilcox CJ. A study of prostaglandin F_{2 α} as the luteolysin in swine. III. Effects of estradiol valerate on prostaglandin F, progesterone, estrone and estradiol concentration in the utero-ovarian vein of non-pregnant gilts. *Prostaglandins* 1977; 14:1183-1196.
- Gadsby JE, Heap RB, Burton RD. Oestrogen production by blastocyst and early embryonic tissue of various species. *J Reprod Fert* 1980; 60:409-417.
- Gardner ML, First ML, Casida LE. Effect of exogenous estrogens on corpus luteum maintenance in gilts. *J Anim Sci* 1963; 22:132-134.
- Geisert RD, Blair RM, Pratt T, Zavy MT. Characterization and proteolytic activity of a cathepsin L-like polypeptide in endometrium and uterine flushings of cycling, pregnant and steroid-treated ovariectomized gilts. *Reprod Fertil Dev* 1997; 9:395-402.
- Geisert RD, Brookbank JW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig: II. Cellular remodeling of the porcine blastocysts during elongation on day 12 of pregnancy. *Biol Reprod* 1982b; 27:941-955.
- Geisert RD, Dixon MJ, Pratt T, Schmitt RAM, Lessley BA, McCann JP. Isolation and characterization of a 30-kDa endometrial glycoprotein synthesized during the estrous cycle and early pregnancy of the pig. *Biol Reprod* 1995; 53:1038-1050.

- Geisert RD, Morgan GL, Zavy MT, Blair RM, Gries LK, Cox A, Yellin T. Effect of asynchronous transfer and oestrogen administration on survival and development of porcine embryos. *J Reprod Fert* 1991; 93:475-481.
- Geisert RD, Pratt TN, Bazer FW, Mayes JS, Watson HG. Immunocytochemical localization and changes in endometrial progesterin receptor protein during the porcine oestrous cycle and early pregnancy. *Reprod Fertil Dev* 1994; 6:749-760.
- Geisert RD, Renegar RH, Thatcher WW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig: I. Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. *Biol Reprod* 1982a; 27:925-939.
- Geisert RD, Thatcher WW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig: III. Endometrial secretory response to estradiol valerate administered on day 11 of the estrous cycle. *Biol Reprod* 1982c; 27:957-965.
- Geisert RD, Yelich JV. Regulation of conceptus development and attachment in pigs. *J Reprod Fert Suppl* 1997; 52:133-149.
- Geisert RD, Yelich JV, Pratt T, Pomp D. Expression of an inter- α -trypsin inhibitor heavy chain-like protein in the pig endometrium during the oestrous cycle and early pregnancy. *J Reprod Fert* 1998; 114:35-43.
- Geisert RD, Zavy MT, Moffatt RJ, Blair RM, Yellin T. Embryonic steroids and the establishment of pregnancy in pigs. *J Reprod Fert Suppl* 1990; 40:293-305.
- Gupta A, Bazer FW, Jaeger LA. Differential expression of beta transforming growth factors (TGF β 1, TGF β 2, and TGF β 3) and their receptors (Type I and Type II) in peri-implantation porcine conceptuses. *Biol Reprod* 1996; 55:796-802.
- Guthrie HD, Lewis GS. Production of prostaglandin F $_{2\alpha}$ and estrogen by embryonal membranes and endometrium and metabolism of prostaglandin F $_{2\alpha}$ by embryonal membranes, endometrium and lungs from gilts. *Domest Anim Endocrinol* 1986; 3:185-198.
- Harney JP, Miranda MA, Smith LC, Bazer FW. Retinol-binding protein: A major secretory product of the pig conceptus. *Biol Reprod* 1990; 42:523-532.
- Hashimoto K, Tobe T, Sumiya J, Sano Y, Choi-Miura N, Ozawa A, Yasue H, Tomita M. Primary structure of the pig homologue of human IHRP: inter-

- α -trypsin inhibitor family heavy chain-related protein. *J Biochem* 1996; 119:577-584.
- Hofig A, Michel FJ, Simmen FA, Simmen RCM. Constitutive expression of uterine receptors for insulin-like growth factor-I during the peri-implantation period in the pig. *Biol Reprod* 1991; 45:533-539.
- Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 1992; 69:11-25.
- Iida S, Peck AB, Byer KJ, Khan SR. Expression of bikunin mRNA in renal epithelial cells after oxalate exposure. *J Urol* 1999; 162(4):1480-1486.
- Keys JL, King GL. Microscopic examination of porcine conceptus-maternal interface between days 10 and 19 of pregnancy. *Am J Anat* 1990; 188:221-238.
- King GJ. Reduction in uterine intra-epithelial lymphocytes during early gestation in pigs. *J Reprod Immunol* 1988; 14:41-46.
- King GJ, Atkinson BA, Robertson HA. Implantation and early placentation in domestic ungulates. *J Reprod Fert* 1982; 31:17-30.
- Knight JW, Bazer FW, Wallace HD. Effect of progesterone induced increase in uterine secretory activity on development of the porcine conceptus. *J Anim Sci* 1974a; 39(4):743-746.
- Knight JW, Bazer FW, Wallace HD. Effects of superovulation and unilateral ovariectomy-hysterectomy on porcine uterine protein secretions. *J Anim Sci* 1973; 36(1):61-65.
- Knight JW, Bazer FW, Wallace HD, Wilcox CJ. Dose-response relationships between exogenous progesterone and estradiol and porcine uterine protein secretions. *J Anim Sci* 1974b; 39(4):747-751.
- Ko Y, Choi I, Green ML, Simmen FA, Simmen RCM. Transient expression of the cytochrome P450 aromatase gene in elongating porcine blastocysts is correlated with uterine insulin-like growth factor levels during peri-implantation development. *Mol Reprod Dev* 1994; 37:1-11.
- Kobayashi H, Fujie M, Shinohara H, Ohi H, Sugimura M, Terao T. Effects of urinary trypsin inhibitor on the invasion of reconstituted basement membranes by ovarian cancer cells. *Int J Cancer* 1994; 57:378-384.
- Laurenz JC, Hadjisavas M, Schuster D, Bazer FW. The effect of uteroferrin and recombinant GM-CSF on hematopoietic parameters in normal female

- pigs (*Sus scrofa*). *Comp Biochem Physiol B Biochem Mol Biol* 1997; 118(3):579-586.
- Letcher R, Simmen RCM, Bazer FW, Simmen FA. Insulin-like growth factor-I expression during early conceptus development in the pig. *Biol Reprod* 1989; 41:1143-1151.
- Margolius HS. Kallikreins and kinins: Molecular characteristics and cellular and tissue responses. *Diabetes* 1996; 46:S14-S19.
- Marlor CW, Delaria KA, Davis G, Muller DK, Greves JM, Tamburini PP. Identification and cloning of human placental bikunin, a novel serine protease inhibitor containing two kunitz domains. *J Biol Chem* 1997; 272(18):12202-12208.
- Mattson BA, Overstrom EW, Albertini DF. Transitions in trophectoderm cellular shape and cytoskeletal organization in the elongating pig blastocyst. *Biol Reprod* 1990; 42:195-205.
- Menino AR Jr, Hogan A, Schultz GA, Novak S, Dixon W, Foxcroft GH. Expression of proteinases and proteinase inhibitors during embryo-uterine contact in the pig. *Dev Genet* 1997; 21(1):68-74.
- Moeljono MPE, Bazer FW, Thatcher WW. A study of prostaglandin $F_{2\alpha}$ as the luteolysin in swine: I. Effect of prostaglandin $F_{2\alpha}$ in hysterectomized gilts. *Prostaglandins* 1976; 11:737-743.
- Moffat RJ, Murray FA, Grifo AP, Jr., Haynes LW, Kinder JE, Wilson GR. Identification of riboflavin in porcine uterine secretions. *Biol Reprod* 1980; 23:331-335.
- Morgan GL, Geisert RD, Zavy MT, Fazleabas AT. Development and survival of pig blastocysts after oestrogen administration on day 9 or days 9 and 10 of pregnancy. *J Reprod Fert* 1987b; 80:133-141.
- Morgan GL, Geisert RD, Zavy MT, Shawley RV, Fazleabas AT. Development of pig blastocysts in a uterine environment advanced by exogenous oestrogen. *J Reprod Fert* 1987a; 80:125-131.
- Murray FA, Jr., Bazer FW, Wallace HD, Warnick AC. Quantitative and qualitative variation in the secretion of protein by the porcine uterus during the estrous cycle. *Biol Reprod* 1972; 7:314-320.
- Murray FA, Moffatt RJ, Grifo AP, Jr. Secretion of riboflavin by the porcine uterus. *J Anim Sci* 1980; 50(5):926-929.

- Perry JS, Heap RB, Burton RD, Gadsby JE. Endocrinology of the blastocyst and its role in the establishment of pregnancy. *J Reprod Fert* 1976; 28:85-104.
- Perry JS. The mammalian fetal membranes. *J Reprod Fert* 1981; 62:321-335.
- Polge C, Rowson LEA, Chang MC. The effect of reducing the number of embryos during early stages of gestation on the maintenance of pregnancy in the pig. *J Reprod Fert* 1966; 12:395-397.
- Pope WF, First NL. Factors affecting the survival of pig embryos. *Theriogenology* 1985; 23:91-105.
- Pope WF, Xie S, Broermann DM, Nephew KP. Causes and consequences of early embryonic diversity in pigs. *J Reprod Fert Suppl* 1990; 40:251-260.
- Prehm P. Identification and regulation of the eucaryotic hyaluronate synthase in the biology of hyaluronan. *Ciba Found Symp* 1989; 143:21-40.
- Pusateri AE, Rothschild MF, Warner CM, Ford SP. Changes in morphology, cell number, cell size and cellular estrogen content of individual littermate pig conceptuses on days 9 to 13 of gestation. *J Anim Sci* 1990; 68:3727-3735.
- Reed KL, Blaeser LL, Dantzer V, Green ML, Simmen RCM. Control of secretory leukocyte protease inhibitor gene expression in the porcine periimplantation endometrium: A case of maternal-embryo communication. *Biol Reprod* 1998; 58:448-457.
- Reneger RH, Bazer FW, Roberts RM. Placental transport and distribution of uteroferrin in the fetal pig. *Biol Reprod* 1982; 27:1247-1260.
- Richoux V, Darribere R, Boucaut JC, Flechon JE, Thiery JP. Distribution of fibronectins and laminin in the early pig embryo. *Anatomical Record* 1989; 223:72-81.
- Roberts AB, Sporn MB. The transforming growth factor β s. In: Sporn MB, Roberts AB (eds.), *Peptide Growth Factors and Their Receptors- Handbook of Experimental Pharmacology*. Heidelberg: Springer-Verlag; 1990:419-472.
- Roberts RM, Bazer FW. The functions of uterine secretions. *J Reprod Fert* 1988; 82:875-892.
- Roberts RM, Trout WE, Mathialagan N, Stallings-Mann M, Ling P. Uterine secretory activity and embryonic development. 1993; In: *Preimplantation Embryo Development*, pp 229-243. Ed. BD Bavister. Springer-Verlag, New York.

- Saguchi K, Tobe T, Hashimoto K, Sano Y, Nakano Y, Miura NH, Tomita M. Cloning and characterization of cDNA for inter- α -trypsin inhibitor family heavy chain-related protein (IHRP), a novel human plasma glycoprotein. *J Biochem* 1995; 117:14-18.
- Salier JP. Inter-alpha-trypsin inhibitor: Emergence of a family within the Kunitz-type protease inhibitor superfamily. *Trends Biochem* 1990; 15:435-439.
- Salier JP, Rouet P, Raguenez G, Daveau M. The inter- α -inhibitor family: From structure to regulation. *Biochem J* 1996; 315:1-9.
- Samuel CA. The development of pig trophoblast in ectopic sites. *Proc Soc Study Fert* 1971; 494-495.
- Samuel CA, Perry JS. The ultrastructure of the pig trophoblast transplanted to an ectopic site in the uterine wall. *J Anat* 1972; 113:139-149.
- Sandson PJ, Weeke B, Johansson BG. Altered properties of pathological hyaluronate due to a bound inter-alpha-trypsin inhibitor. *Trans Assoc Am Physicians* 1965; 78:304-313.
- Short RV. Implantation and the maternal recognition of pregnancy. 1969. In *Foetal Autonomy* (Ciba Fdn Symp. No. 111), pp. 2-26. Eds G.E.W. Wolstenholme & M. O'Connor. J. & A. Churchill Ltd, London.
- Simmen RCM, Simmen FA, Bazer FW. Regulation of synthesis of uterine secretory proteins: Evidence for differential induction of porcine uteroferrin and antileukoprotease gene expression. *Biol Reprod* 1991; 44:191-200.
- Stallings-Mann ML, Burke MG, Trout WE, Roberts RM. Purification, characterization, and cDNA cloning of a kunitz-type proteinase inhibitor secreted by the porcine uterus. *J Biol Chem* 1994; 269:24090-24094.
- Stice SL, Ford SP, Rosazza JP, Van Orden DE. Interaction of 4-hydroxylated estradiol and potential-sensitive Ca^{2+} channels in altering uterine blood flow during the estrous cycle and early pregnancy in gilts. *Biol Reprod* 1987a; 36:369-375.
- Stice SL, Ford SP, Rosazza JP, Van Orden DE. Role of 4-hydroxylated estradiol in reducing Ca^{2+} uptake of uterine arterial smooth muscle cells through potential sensitive channels. *Biol Reprod* 1987b; 36:361-368.
- Surveyor GA, Gendler SJ, Pemberton L, Das SK, Chakraborty I, Julian J, Pimental RA, Wegner CC, Dey SK, Carson DD. Expression and steroid

- hormonal control of Muc-1 in the mouse uterus. *Endocrinology* 1995; 136:3639-3647.
- Tavakkol A, Simmen FA, Simmen RCM. Porcine insulin-like growth factor-I (pIGF-I): Complementary deoxyribonucleic acid cloning and uterine expression of messenger ribonucleic acid encoding evolutionarily conserved IGF-I peptides. *Mol Endocrinol* 1988; 2:674-681.
- Thuveson M, Fries E. Intracellular proteolytic processing of the heavy chain of rat pre- α -inhibitor: The COOH-terminal propeptide is required for coupling to bikunin. *J Biol Chem* 1999; 274(10):6741-6746.
- Trout WE, Hall JA, Stallings-Mann ML, Galvin JM, Anthony RV, Roberts RM. Steroid regulation of the synthesis and secretion of retinal-binding protein by the uterus of the pig. *Endocrinology* 1992; 130:2557-2564.
- Trout WE, McDonnell JJ, Kramer KK, Baumbach GA, Roberts RM. The retinol-binding protein of the expanding pig blastocyst: Molecular cloning and expression in trophoblast and embryonic disc. *Mol Endocrinol* 1991; 5:1533-1540.
- Tuo W, Bazer FW. Expression of oncofetal fibronectin in porcine conceptuses and uterus throughout gestation. *Reprod Fertil Dev* 1996; 8:1207-1213.
- Tuo W, Harney JP, Bazer FW. Colony-stimulating factor-I in conceptus and uterine tissues in pigs. *Biol Reprod* 1995; 53:133-142.
- Vonnahme KA, Malayer JR, Spivey HO, Ford SP, Clutter A, Geisert RD. Detection of kallikrein gene expression and enzymatic activity in porcine endometrium during the estrous cycle and early pregnancy. *Biol Reprod* 1999; 61:1235-1241.
- Xu Y, Carr PD, Guss JM, Ollis DL. The crystal structure of bikunin from the inter- α -inhibitor complex: A serine protease inhibitor with two kunitz domains. *J Mol Biol* 1998; 276:955-966.
- Yelich JV, Pomp D, Geisert RD. Detection of transcripts for retinoic acid receptors, retinal-binding protein, and transforming growth factors during rapid trophoblastic elongation in the porcine conceptus. *Biol Reprod* 1997a; 57:286-294.
- Yelich JV, Pomp D, Geisert RD. Ontogeny of elongation and gene expression in the early developing porcine conceptus. *Biol Reprod* 1997b; 57:1256-1265.

Yoshida E, Maruyama M, Sugiki M, Mihara H. Immunohistochemical demonstration of bikunin, a light chain of inter- α -trypsin inhibitor, in human brain tumors. *Inflammation* 1994; 18(6):589-596.

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