

Regulation of conceptus development and attachment in pigs

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Implantation/placentation in domestic pigs is preceded by synthesis of oestrogen by the conceptus to maintain functional corpora lutea throughout pregnancy and a rapid morphological transformation of conceptuses from spherical to long filamentous thread-like structures. Initial conceptus expansion, reaching a metre in length, not only delineates the surface area for placental attachment, but also provides the mechanism for delivery of oestrogen to signal events necessary for placentation throughout the uterine horn. Timing for conceptus gene expression to induce trophoblast expansion and attachment in pigs is temporally associated with downregulation of progesterone receptors and increase in oestrogen receptors within the uterine epithelium. Within the confines of the uterine lumen, pig conceptuses normally do not erode or invade through the uterine epithelial surface. However, the pig conceptus possesses extensive proteolytic activity as it is highly invasive outside the uterine lumen of the pig. Initial release of oestrogen by the elongating pig conceptus induces endometrial release of cytokines and a variety of protease inhibitors. Recently, endometrial expression for the inter-trypsin inhibitor (α_1 I) family of protease inhibitors has been detected in the pig endometrium during conceptus elongation and attachment. It is possible that α_1 Is may function to inhibit trophoblast invasion and also serve as targets for adhesion molecules, such as integrins and heparin, to aid in placental attachment to the uterine epithelium.

Introduction

There are few mammalian species in which early embryonic development can compare with the rapid alteration in conceptus morphology that occurs during establishment of pregnancy in pigs. During the period of maternal recognition of pregnancy, pig conceptuses undergo a phenomenal morphological change from 10 mm spherical to tubular (20–40 mm) and finally filamentous (100 mm in length) shapes in less than 3–4 h (Anderson, 1978; Geisert *et al.*, 1982; Strobant and Van der Lende, 1990). Transformation from spherical to a filamentous thread-like morphology occurs through cytoskeletal reorganization which induces cellular modifications in shape and migration, rather than an increase in mitotic activity (Geisert *et al.*, 1982; Mattson *et al.*, 1990). Initial expansion of the pig trophoblast establishes boundaries for placental attachment and initial allotment of uterine space available to each conceptus to compete for nutrients necessary for growth and survival to term. It not only delineates the surface area for placental attachment, but it also provides the mechanism for delivery of conceptus oestrogen throughout the uterus to maintain functional corpora lutea during pregnancy (see reviews Bazer *et al.*, 1984; Geisert *et al.*, 1994a).

Inclusive of maternal recognition on days 10–12 of pregnancy, events critical for early pig embryonic survival include rapid trophoblast elongation, conceptus attachment to the uterine epithelial surface and inhibition of immune rejection by the maternal system. Uterine and conceptus factors involved with inducing rapid trophoblast elongation are of critical importance for embryonic survival. Trophoblast elongation involves many embryonic factors as well as presentation of uterine adhesion factors on the apical border of the endometrial surface epithelium. Initially, uterine

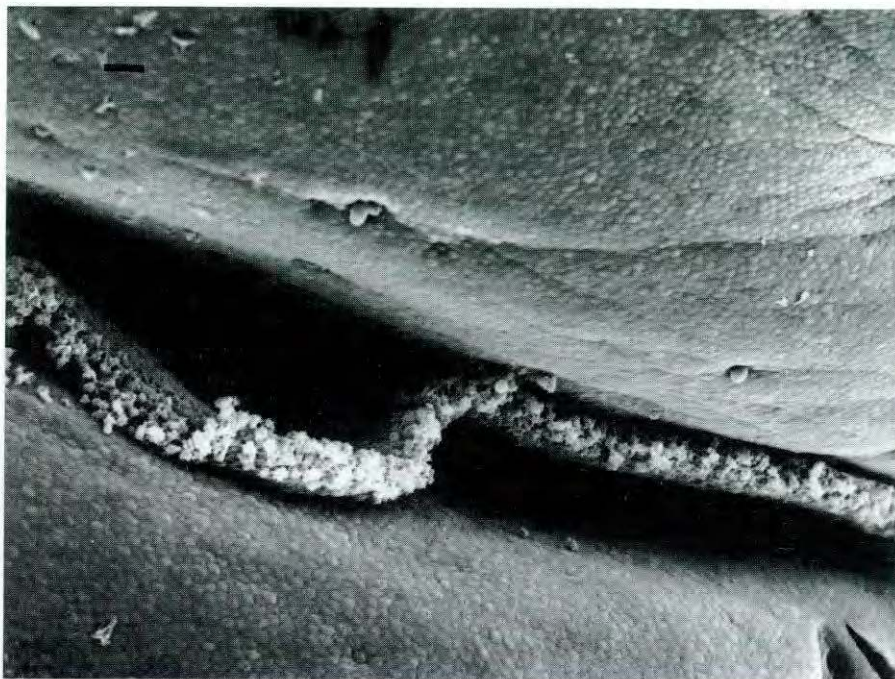


Fig. 1. Scanning electron micrograph showing contact of the elongated pig conceptus apposed to the uterine surface epithelium on day 14 of pregnancy. Scale bar represents 50 μm .

adhesion to the surface epithelium permits trophoblast elongation throughout the uterine lumen (Fig. 1) and then continuous adhesive attachment of the placenta throughout gestation (King *et al.*, 1982). The alterations within the uterine surface glycocalyx that allow trophoblast elongation and then permanent placental attachment are as critical to conceptus survival as the conceptus factors stimulating the morphological changes in the conceptus. Resolving the conceptus and uterine factors involved with trophoblast elongation and attachment within the confines of the pig uterine lumen would clarify our understanding of embryonic survival in pigs. The present paper will review recent literature concerning conceptus and uterine factors involved with trophoblast development and initial attachment to the uterine surface epithelium.

Conceptus Elongation within the Uterus

A critical period of embryonic loss occurs when the peri-implantation conceptus undergoes rapid differentiation and expansion of its trophoblastic membrane between days 11 and 12 of gestation (Geisert *et al.*, 1982; Barends *et al.*, 1989; Pope, 1994). Thus, trophoblast elongation is sensitive to changes in the uterine luminal environment that can be influenced by littermate embryos (Pope, 1994). On day 7–8 of pregnancy, the pig blastocyst is composed of an outer layer of polarized trophoblast, embryonic disc and an inner layer of endodermal cells (see Stroband and Van der Lende, 1990). By day 10, the pig conceptus expands to a 1–3 mm sphere before enlarging to a 3–8 mm ovoid shape at a rate of approximately 0.3 mm h^{-1} over the next 30 h. Once conceptus diameter reaches 9–10 mm, the conceptus rapidly (30–45 mm h^{-1}) undergoes a transition to a tubular (12–30 mm) and finally thin filamentous form measuring more than 100 mm in length (Geisert *et al.*, 1982). Perry (1981) compared elongation by the ovoid conceptus to rolling a ball of plasticene under your hand. He suggested that the rapidity of elongation would more likely be explained by deformation

than by cell division. As predicted by Perry, rapid morphological changes in the pig conceptus are not the result of cellular hyperplasia as proliferative activity, measured by DNA and mitotic index, significantly declines during transition from spherical to filamentous morphology (Geisert *et al.*, 1982; Pusateri *et al.*, 1990). Rather, trophoblastic elongation occurs through massive cellular remodelling of the trophoctoderm and endoderm.

Conceptus elongation requires a mechanical force to generate the cellular restructuring necessary to transform its morphology rapidly as the trophoctoderm expands. Mattson *et al.* (1990) proposed that actin filaments may be involved with rearrangement of the trophoctoderm plasma membrane and initiate the force necessary for conceptus elongation. Modifications of filamentous-actin (f-actin) are consistent with alterations in trophoctoderm morphology for axial elongation and narrowing of the conceptus diameter. Polygonal trophoctodermal cells of tubular conceptuses vary in the distribution of f-actin in regions proximal to and distal to the embryonic disc. Numerous constricted regions have been observed along the length of the filamentous conceptus (Mattson *et al.*, 1990). Morphology of trophoctoderm cells within the constricted region and the distinct arrangement of f-actin suggest that these cells may generate a contractile force to elongate. Although changes in endodermal ultrastructure have been documented (Geisert *et al.*, 1982; Mattson *et al.* 1987), regulation of extraembryonic endoderm migration and function during trophoblast expansion have not been determined.

Currently, the factor(s) that initiates elongation and the cytoskeletal processing that dictates the morphological changes in the pig conceptus is coming under intensive study. It is clear that the force necessary for trophoblast elongation originates within the individual conceptus as a mixture of spherical, tubular and elongating conceptuses which can be found within the same litter (Heuser and Streeter, 1929; Anderson, 1978; Geisert *et al.*, 1982). Moreover, when oestrogen is administered to advance the uterine secretory response before conceptus elongation, conceptuses do not elongate until they have reached the 9–10 mm ovoid morphology (Morgan *et al.*, 1987). Conceptus elongation is, therefore, programmed through developmental cues rather than through direct stimulation by uterine secretions; however, uterine secretions do play a significant role in conceptus growth and survival. The importance of the 10 mm ovoid stage in conceptus development could be related to differentiation and expansion of the extraembryonic mesoderm (Patten, 1931; Geisert *et al.*, 1982; Gupta *et al.*, 1996). First detection of mesoderm is temporally associated with the initial capacity of the 5–6 mm conceptuses to synthesize oestrogens (Fischer *et al.*, 1985; Pusateri *et al.*, 1990; Wilson and Ford, 1997). Protein and gene expression for steroidogenic enzymes such as P450 17 α -hydroxylase and aromatase are consistent with the increase in conceptus oestrogen synthesis (Conley *et al.*, 1994; Ko *et al.*, 1994; Green *et al.*, 1995; Yelich *et al.*, 1997a; Wilson and Ford 1997). Later, expansion of the extraembryonic mesoderm between the trophoctoderm and endoderm at the 10 mm stage of development may direct cellular remodelling for trophoblast elongation. Certainly, there are many uterine and conceptus factors that contribute to the growth, differentiation and elongation of pig conceptuses.

Endometrial Contributions to Conceptus Development

Although rapid trophoblast elongation is programmed through developmental cues by the conceptus (Morgan *et al.*, 1987), uterine secretions play a significant role in conceptus growth and survival. A number of the major components of progesterone-stimulated uterine secretions during the oestrous cycle and following conceptus oestrogen release have been reviewed by Davis and Blair (1993) and Roberts *et al.* (1993a,b), and will only be briefly addressed here. Notably, endometrial synthesis of uteroferrin and retinol-binding protein (RBP) have been studied extensively in pigs. Uteroferrin was one of the earliest uterine proteins identified within the pig uterus (see Roberts *et al.*, 1993a). Although uteroferrin plays a role in iron transport to the fetus throughout pregnancy, evidence has indicated that uteroferrin may also serve as a haematopoietic stem cell growth factor during early conceptus development (Bazer *et al.*, 1991; Michel *et al.*, 1992).

Endometrial secretion of retinol-binding protein (RBP) may function in the transport and delivery of maternal plasma retinol to the developing conceptus (Trout *et al.*, 1992; Harney *et al.*,

1994a,b). Uterine secretion of retinol bound to RBP permits the uptake and cellular delivery of retinol to the conceptus through cellular RBP (CRBP) (Napoli *et al.*, 1991). Cytosolic retinol can then be metabolized to retinal and the most biologically active metabolite, retinoic acid (Ross, 1991). Retinoic acid binds directly to retinoic acid receptors (RAR) in the nucleus where it effects gene transcription (Chambon *et al.*, 1991). Harney *et al.* (1994a) indicated that the components for retinol transport, metabolism and receptor activation are present in endometrial and conceptus tissues during early pregnancy. Pig endometrial and conceptus tissues synthesize RBP, CRBP, RAR α and RAR γ during early conceptus development (Trout *et al.*, 1992; Harney *et al.*, 1994a). In fact, RBP is one of the earliest secretory proteins detected in the spherical conceptus on days 10–11 of gestation (Harney *et al.*, 1990). Both uterine and conceptus RBP deliver retinol to tissues, and buffer tissues from the teratogenic (Lammer *et al.*, 1985) and embryotoxic (Thompson *et al.*, 1993) effects of retinoids. Vallet *et al.* (1996) also proposed a role for RBP in protecting uterine and developing conceptus tissues from the lipid oxidizing activity of uteroferrin. Effects of retinoic acid on components of the extracellular matrix (see De Luca, 1991), cell surface adhesive molecules (Agura *et al.*, 1992), and expression of growth factors and their receptors (Roberts and Sporn, 1988) provide an attractive model for its possible involvement with rapid trophoblast elongation and conceptus development in pigs.

In addition to uteroferrin and RBP, the pig endometrium contains many enzymes such as lysozyme, leucine-aminopeptidase (Roberts *et al.*, 1976; Hansen *et al.*, 1985), β -hexosaminidase (Hansen *et al.*, 1985), cathepsins B, D, E (Roberts *et al.*, 1976) and L (Geisert *et al.*, 1997). Secretion of enzymes such as lysozyme can serve a bactericidal function and for selective proteolysis of proteins for conceptus uptake (Roberts *et al.*, 1993a). Cathepsins are lysosomal cysteine proteases that have been implicated as modulators of invasive implantation of rats (Elangovan and Moulton, 1980) and cats (Li *et al.*, 1992). Cathepsin L activity in the pig uterus is induced by progesterone and increases at the time of trophoblast elongation with peak activity on day 15 of pregnancy (Geisert *et al.*, 1997). Although the pig forms a diffuse, epitheliochorial type of placental attachment (King *et al.*, 1982), the high affinity of cathepsin L for collagen (Kirschke *et al.*, 1982) and elastin (Mason *et al.*, 1982) suggests that it may play a role in placental attachment on day 13–18 of gestation through limited proteolysis of the uterine epithelial surface glycocalyx. Uterine growth and expansion during early pregnancy involves elastase activity and collagen remodelling (Renegar, 1982) in which cathepsin L could play a role in both uterine and placental development.

The pig conceptus is normally noninvasive within the confines of the uterine lumen. However, the pig conceptus possesses extensive invasive activity outside the uterine lumen (Samuel and Perry, 1972). Trophoblast secretion of plasminogen activator at the time of elongation can stimulate the release of plasmin through cleavage of plasminogen which is present in the uterine lumen at the time of implantation/placentation (Fazleabas *et al.*, 1983). Generation of plasmin by the pig conceptuses can activate latent forms of other proteases involved with regulation of the cell basement membrane and extracellular matrix (Werb *et al.*, 1980). Association of plasminogen activator with cellular migration and remodelling of various tissues (Bode and Dziadek, 1979) suggests a primary role for plasmin in the remodelling of the conceptus (Fazleabas *et al.*, 1983). The release of plasmin into the uterine lumen would represent a by-product of conceptus development which must be controlled to avoid damage to the uterine luminal epithelium.

Endometrial secretion of a variety of protease inhibitors regulates the microenvironment during placental attachment in pigs (see Roberts *et al.*, 1993a). The pig endometrium secretes a protease inhibitor specific for plasmin, chymotrypsin and trypsin during the period of trophoblast elongation and placentation (Fazleabas *et al.*, 1983). This inhibitor contains a kunitz domain which provides inhibitory activity against serine proteases (Stallings-Mann *et al.*, 1994). Expression of a uterine elastase/cathepsin G protease inhibitor, antileukoprotease, during pregnancy in pigs may also support and maintain the noninvasive, epitheliochorial placenta throughout pregnancy (Simmen *et al.*, 1992a). In addition, the pig uterus also secretes a group of low molecular mass, basic proteins that are related to the 'serpin family' of protease inhibitors (Murray *et al.*, 1989; Malathy *et al.*, 1990).

Recently, expression of the inter-trypsin inhibitor (α 1) family of protease inhibitors was detected in the pig endometrium during conceptus elongation and attachment (Geisert *et al.*, 1996; Diederich

et al., 1997). Inter-trypsin inhibitors are plasma serine protease inhibitors that have been described during the acute phase reaction to cardiogenic shock (see Salier *et al.*, 1996). The family of inter-trypsin inhibitors are interesting in that they are synthesized as precursor polypeptides that give rise to mature chains with distinct functions and form inter-chain glycosamino-glycan bonds with various molecules. The inter-trypsin inhibitor family of serine protease inhibitors can consist of either a combination of two heavy chains αIH1 , αIH2 and a single light chain known as bikunin, αIH3 and bikunin or αIH2 and bikunin (see review Salier *et al.*, 1996). Heavy chains of αIH1 , αIH2 and αIH3 form the various complexes with bikunin through binding to a chondroitin sulfate chain (Enghild *et al.*, 1993). All the serine protease inhibitory activity is attributed to bikunin, as it contains two Kunitz-type serine protease inhibitor domains (Hochstrasser *et al.*, 1981). Bikunin originates from a separate mRNA in which α -1-microglobulin and bikunin are synthesized as single proteins which undergo proteolytic cleavage (Kaumeyer *et al.*, 1986). However, the αI heavy chains are translated from separate mRNAs (Diarra-Mehrpour *et al.*, 1989). Through its tandemly arranged kunitz domains, bikunin can target inhibition of trypsin, cathepsin G, elastase and plasmin. The biological role of the αI family as protease inhibitors is under investigation (Salier *et al.*, 1996). Previously, it was proposed that synthesis of the inter-trypsin inhibitors was restricted to the liver (Saguchi *et al.*, 1995). Uterine synthesis of inter-trypsin inhibitors could regulate conceptus attachment and limit proteolysis. Certainly, bikunin can assist with regulation of endometrial invasion by the pig trophoblast as endometrial bikunin gene and protein expression are detected from day 12 to day 18 of pregnancy (Diedrich *et al.*, 1997). However, it is possible that the heavy chains of αI may function in the initial attachment of the conceptus to the uterine epithelial surface as will be addressed later.

Continuous growth, development and differentiation of pig conceptuses are highly dependent upon the timing and quantitative amounts of growth factors secreted into the uterine lumen. Insulin-like growth factor I (IGF-I) is one of the earliest and most completely characterized of the growth factors identified in uterine secretions of pigs (Simmen *et al.*, 1993). As endometrial IGF-I expression reaches peak values during conceptus elongation and oestrogen release on day 12 of gestation (Letcher *et al.*, 1989; Simmen *et al.*, 1992b), uterine secretion of IGF-I may enhance conceptus oestrogen synthesis (Hofig *et al.*, 1991), possibly through the enhancement of P450 aromatase gene expression (Ko *et al.*, 1994; Green *et al.*, 1995). The IGF-I receptor mRNA is constitutively expressed during early conceptus development (Green *et al.*, 1995); however, trophoctoderm IGF-I receptor protein content is low (Chastant *et al.*, 1994). Uterine secretion of IGF-I may play more of an autocrine role in uterine growth and development as the endometrium contains an abundance of IGF-I receptors (Simmen *et al.*, 1992b; Chastant *et al.*, 1994). Trophoctoderm of developing conceptuses expresses IGF-II/mannose 6-phosphate receptor which may stimulate conceptus growth and differentiation (Chastant *et al.*, 1994). However, expression of endometrial IGF-II mRNA increases on day 15 (Simmen *et al.*, 1992b), which is several days after conceptus elongation. It is possible that uterine IGF-I effects early conceptus growth and development by binding to the IGF-II receptor (Czech, 1986). Targeted mutagenesis of the IGF-II/mannose-6-phosphate receptor gene in the mouse has demonstrated its importance in early embryonic survival (Barlow *et al.*, 1991). Since mutation of either IGF-I, IGF-II or IGF-I receptor only reduce prenatal growth (DeChiara *et al.*, 1990; Liu *et al.*, 1993), it is possible that one receptor can substitute for loss of another. Newton *et al.* (1994) indicated that the oestrogen receptor can be transcriptionally activated through IGF-I intracellular signalling suggesting that the oestrogen receptor is one of the nuclear factors involved with growth stimulation. It is possible that conceptus oestrogen and uterine IGF-I coordinate uterine growth and development during trophoblast elongation.

Interest in the role of growth factors in pig conceptus and uterine development has led to identification of a host of growth factors within the uterus (Table 1). Epidermal growth factor (EGF), heparin-binding EGF (HB-EGF), transforming growth factor α (TGF- α) and amphiregulin, all of which bind and activate the EGF receptor (Prigent and Lemoine, 1992), are expressed by the pig endometrium during early pregnancy (Brigstock *et al.*, 1990; Kennedy *et al.*, 1994; Kim *et al.*, 1995; Brigstock *et al.*, 1996a,b). Both EGF and HB-EGF have been immunolocalized to the surface and glandular epithelium of the endometrium (Kennedy *et al.*, 1994; Kim *et al.*, 1995) and the luminal

Table 1. Growth factor and receptor expression by the pig endometrium during early conceptus development and placentation

Expression	Developmental process affected	Reference
Insulin-like growth factors (IGF)	Regulation of cellular proliferation and differentiation	Letcher <i>et al.</i> , 1989 Green <i>et al.</i> , 1995 Simmen <i>et al.</i> , 1992
Insulin-like growth factor receptor	Receptor activation for cellular proliferation and differentiation	Green <i>et al.</i> , 1995
Insulin-like growth factor binding protein 2	Regulation of IGF-I and IGF-II activity	Simmen <i>et al.</i> , 1992
Epidermal growth factor (EGF)	Regulation of cellular proliferation and differentiation	Kennedy <i>et al.</i> , 1994
Heparin binding-epidermal growth factor (HB-EGF)	Regulation of cellular proliferation and differentiation	Brigstock <i>et al.</i> , 1990 Kim <i>et al.</i> , 1995
Epidermal growth factor receptor	Receptor activation by EGF, HB-EGF, TGF and amphiregulin	Zhang <i>et al.</i> , 1992 Kennedy <i>et al.</i> , 1994
Transforming growth factor- α (TGF α)	Regulation of cellular proliferation and differentiation	Vaughan <i>et al.</i> , 1992 Kennedy <i>et al.</i> , 1994
Transforming growth factor- β 3	Chemoattractant regulates cellular differentiation and morphogenesis Collagen and fibronectin gene expression	Sun <i>et al.</i> , unpublished results
Keratinocyte growth factor	Heparin-binding growth factor Mediates effects of progesterone and androgen on epithelium	Liu <i>et al.</i> , 1995
Keratinocyte growth factor receptor	Receptor activation for stromal function	Tuo <i>et al.</i> , 1996
Retinoic acid receptor- α , - β , - γ	Retinoic acid receptor for uterine development	Harney <i>et al.</i> , 1994 Sun <i>et al.</i> , unpublished results
Interleukin 6	Uterine immunological stimulation Uterine hyperaemic response	Mathialagan <i>et al.</i> , 1992 Anegon <i>et al.</i> , 1994
Fibroblast growth factors	Regulation of cellular proliferation and differentiation	Brigstock <i>et al.</i> , 1989
Leukaemia inhibitory factor	Regulation of embryo differentiation	Anegon <i>et al.</i> , 1994
Colony stimulating factor 1	Haematopoietic growth factor Cellular proliferation and differentiation	Tuo <i>et al.</i> , 1995
Pleiotrophin	Neurotrophic factor	Brigstock <i>et al.</i> , 1996

content of EGF in uterine secretions is increased on day 12 of pregnancy followed by a decline to day 16 (Diehl *et al.*, 1994). Endometrial and conceptus tissues express EGF receptor (Zhang *et al.*, 1992a,b; Kennedy *et al.*, 1994) which indicates that uterine secretion of EGF and other ligands for EGF receptor could regulate uterine and conceptus development. Although expression of these growth factors, as well as basic fibroblast growth factor (Brigstock *et al.*, 1989) and pleiotrophin (Brigstock *et al.*, 1996a), has been demonstrated, their relationships to conceptus development have not been defined.

In addition to pleiotrophin, several haematopoietic cytokines have been detected within the pig endometrium and uterine secretions during early conceptus development. Given its role as a haematopoietic regulator involved in cellular differentiation and cellular growth, leukaemia inhibitory factor (LIF) is one of the most notable cytokines secreted by pig endometrium at the time

of conceptus elongation (Anegon *et al.*, 1994). Endometrial gene expression and secretion of LIF into the uterine lumen are maximal on days 11–12 of pregnancy in pigs (Anegon *et al.*, 1994). The essential role of LIF in blastocyst growth and implantation in mice (see Stewart, 1994) implies that LIF may serve a vital function in conceptus development and implantation in pigs. The importance of conceptus growth and differentiation, and possibly of expansion of the extraembryonic mesoderm, provides an attractive model for studies of endometrial LIF regulation of trophoblast elongation and implantation in pigs.

LIF is related to and binds to a receptor common to interleukin 6 (IL-6), and colony-stimulating factor 1 (CSF-1) (Bazan, 1991). Pig endometrium contains mRNA for IL-6 and IL-6 is present within the uterine secretions during the oestrous cycle and early pregnancy (Anegon *et al.*, 1994). However, changes in mRNA and protein are not associated with changes in conceptus development on days 11–12 of pregnancy as observed for LIF. Gene expression and tissue production of CSF-1 have been detected in the pig endometrium and conceptus (Tuo *et al.*, 1995). Immunoreactive CSF-1 is localized in the uterine surface and glandular epithelium on day 10 of pregnancy, but the greatest tissue content and mRNA expression occur after day 30 of gestation. Temporal changes in CSF-1 mRNA and protein in the endometrium and placenta of pigs suggest that CSF-1 may influence placental and fetal growth following implantation/placentation (Tuo *et al.*, 1995).

The pig endometrium has recently been shown to be a source of relaxin (Knox *et al.*, 1994) and oxytocin (Trout *et al.*, 1995). Although the pig corpus luteum has long been known to be an endocrine source of relaxin (Sherwood, 1994), relaxin has now been localized in the uterine surface and glandular epithelium of gilts during the oestrous cycle and early pregnancy (Knox *et al.*, 1994). Intensity of relaxin immunostaining is weak on day 12 but increases to day 16 and expression is maintained to day 20 in pregnant gilts. Detection of mRNA encoding relaxin demonstrated that the endometrium is the source of relaxin in the uterine epithelium (Knox *et al.*, 1994). Relaxin has a definite uterotrophic effect (Galvin *et al.*, 1991) which could stimulate growth of the uterus to accommodate expansion of the allantochorionic membranes from days 18–30 of pregnancy (see Bazer *et al.*, 1981). Oestrogen stimulation of uterine secretions by the elongating conceptus at the time of maternal recognition of pregnancy initiates release of endometrial oxytocin into the uterine lumen (Trout *et al.*, 1995). Endometrial oxytocin mRNA reaches peak values on day 12 of the oestrous cycle or pregnancy (S. Sun, T. Yelich and R. Geisert, unpublished results). Lack of oxytocin receptor mRNA in day 10–12 conceptus tissue (Yelich *et al.*, 1997a) suggests that uterine oxytocin release may have an autocrine function in uterine contraction and closure of the uterine lumen surrounding the developing conceptus, rather than a direct effect on the developing conceptus. High luminal content of oxytocin may also play a role in regulating the movement of prostaglandin $F_{2\alpha}$ during maternal recognition of pregnancy (see Bazer *et al.*, 1984).

As described previously, there is a great deal of information on the presence of uterine growth factors which may effect conceptus development, many of which are steroid regulated (IGF-I, IGF-II, LIF, RBP). Timing of uterine growth factor expression and subsequent protein release relative to conceptus development needs to be tightly regulated as the uterine environment is not tolerant of asynchrony in conceptus development (see Pope, 1994). The increase in concentrations of plasma progesterone from the corpora lutea is certainly the primary director of uterine development and secretion. However, the marked changes in uterine epithelial secretions and conceptus elongation in pigs are actually temporally related to downregulation of progesterone receptors in the surface and glandular epithelium from day 10 of the oestrous cycle or pregnancy (Geisert *et al.*, 1994b) and the increase in epithelial oestrogen receptors (Geisert *et al.*, 1993). Thus, the loss of progesterone receptors from the uterine epithelium may play an essential role in the timing of growth factor secretion for the developing conceptus and the responsiveness of uterine epithelium to conceptus oestrogens during trophoblastic elongation and implantation. Stromal cell progesterone receptors are maintained during this period and may stimulate the uterine epithelium by secretion of keratinocyte growth factor (KGF) in response to progesterone (Liu *et al.*, 1995). KGF receptors are expressed constitutively by the uterine epithelium (Liu *et al.*, 1996). Although there are now clues to the regulation of uterine growth factor secretion during early conceptus development, definitive roles for each growth factor in conceptus development need to be established.

Table 2. Growth factor and receptor expression by the pig conceptus before and during elongation

Expression	Developmental process affected	Reference
Brachyury	Mesoderm formation	Yelich <i>et al.</i> , 1997a
Cytochromes	Steroidogenesis	Conley <i>et al.</i> , 1994
P450 _{17α}		Green <i>et al.</i> , 1995
P450 _{11β}		Corbin <i>et al.</i> , 1996
P450 _{3β}		
Insulin-like growth factor I	Regulation of cellular proliferation and differentiation	Letcher <i>et al.</i> , 1989 Green <i>et al.</i> , 1995
Insulin-like growth factor I receptor	Regulation of cellular proliferation and differentiation	Green <i>et al.</i> , 1995
Insulin-like growth factor II	Regulation of cellular proliferation and differentiation	Simmen <i>et al.</i> , 1992
Insulin-like growth factor II receptor	Regulation of cellular proliferation and differentiation	Chastant <i>et al.</i> , 1994
Epidermal growth factor	Regulation of cellular proliferation and differentiation	Vaughan <i>et al.</i> , 1992
EGF receptor	Regulation of cellular proliferation and differentiation	Vaughan <i>et al.</i> , 1992 Corps <i>et al.</i> , 1990 Zhang <i>et al.</i> , 1992
Transforming growth factor α	Regulation of cellular morphogenesis	Vaughan <i>et al.</i> , 1992
Transforming growth factor β -1, β -2, β -3	Chemoattractant. Regulation of cellular differentiation and morphogenesis. Collagen and fibronectin expression	Yelich <i>et al.</i> , 1997a Gupta <i>et al.</i> , 1996
Retinol-binding protein	Retinol transport to conceptus	Harney <i>et al.</i> , 1990, 1994b Trout <i>et al.</i> , 1992 Yelich <i>et al.</i> , 1997b
Retinoic acid receptor- α , - β , - γ	Receptor for retinoic acid for embryo morphogenesis	Harney <i>et al.</i> , 1994a Yelich <i>et al.</i> , 1997b
Leukaemia inhibiting factor receptor	Regulation of cellular proliferation and differentiation	Yelich <i>et al.</i> , 1997
Interleukin 1 β	Uterine immunological stimulation Haematopoiesis	Tuo <i>et al.</i> , 1996
Interleukin 6	Uterine immunological stimulation	Mathialagan <i>et al.</i> , 1992 Anegon <i>et al.</i> , 1994
Colony stimulating factor 1	Cellular proliferation and differentiation	Tuo <i>et al.</i> , 1995
Interferons	Immunological regulation	Lefevre and Boulay, 1993 Cross and Roberts, 1989

Conceptus Regulation of Trophoblastic Elongation

As previously discussed, rapid elongation of the conceptus on days 10–12 of gestation appears to be programmed by endogenous developmental cues. Since conceptuses elongate only after they have reached the 10 mm morphology (Morgan *et al.*, 1987), the critical period of development may prepare the early spherical (1–3 mm) and late-spherical (9–10 mm) conceptuses for elongation. This

period is characterized by enhanced conceptus gene expression that orchestrates the sequence of cellular events necessary to initiate and allow trophoblast elongation to occur (Table 2).

Synthesis and secretion of oestrogen by the developing pig conceptus is the fundamental marker of trophoblast elongation (see Geisert *et al.*, 1990). Two key enzymes involved in the steroidogenic pathway for oestrogen synthesis in the conceptus are the cytochromes P450 17 α -hydroxylase (P450_{17 α}) and aromatase (P450_{arom}). Although low, initial expression of P450_{17 α} is detected in conceptuses of less than 6 mm diameter, and increases as conceptuses approach the large spherical stages (10 mm) just before elongation. Conceptus P450_{arom} gene expression follows a similar pattern that is greatly enhanced at the time of elongation (Conley *et al.*, 1992, 1994; Ko *et al.*, 1994; Green *et al.*, 1995; Yelich *et al.*, 1997a). Immediately after elongation of the trophoblast, gene expression for both enzymes decreases markedly (King and Ackerley, 1985; Conley *et al.*, 1992; Ko *et al.*, 1994; Green *et al.*, 1995). Oestrogen production by the conceptus has a direct effect on uterine function as evidenced by an abundance of oestrogen receptors in the uterine epithelium at the time of trophoblast elongation (Geisert *et al.*, 1993) which, when activated, result in the secretion of numerous uterine proteins as previously discussed in this review. Whether there is a direct effect of oestrogen produced by the conceptus on trophoblast elongation is unclear, although it appears unlikely since oestrogen receptors cannot be detected in the early conceptus by either immunocytochemistry or RT-PCR (R.D. Geisert, unpublished results; Yelich *et al.*, 1997a).

Initial synthesis of oestrogen and elongation of the conceptus is closely associated with first detection of mesoderm differentiation in 5 mm spherical conceptuses that may be the key to the programming of trophoblast elongation (see reviews by Geisert *et al.*, 1990; Stroband and Van der Lende, 1990). The appearance of mesoderm within the embryoblast corresponds to initial expression of the brachyury gene in conceptuses (Yelich *et al.*, 1997a). Brachyury gene expression encodes a transcription factor necessary for mesodermal differentiation in mice (Herrmann *et al.*, 1990), as homozygous mutants for the gene are lethal (Conlon *et al.*, 1995). Brachyury gene expression coincides with and parallels P450_{17 α} and P450_{arom} gene expression in early spherical conceptuses (Yelich *et al.*, 1997a). Whether or not expression of brachyury, or the subsequent differentiation of mesoderm, initiates steroidogenesis is debatable. Absence of P450_{17 α} and P450_{arom} gene expression in the mesoderm (Conley *et al.*, 1994) suggests that effects of the mesoderm, if any, on oestrogen synthesis are indirect.

Expression of receptors for LIF, IGF-II and EGF have been described in early developing conceptuses (see Table 2). As previously discussed, the uterus secretes IGF-I, EGF, HB-EGF and CSF-1 during conceptus elongation, but these growth factors appear to play more of a supportive role in growth and differentiation rather than in cellular remodelling. Initial expression of LIF receptor is observed in 2 mm spherical conceptuses, with a marked increase to a peak at the 7 mm spherical conceptus stage, which is maintained throughout trophoblast elongation (Yelich *et al.*, 1997a). Uterine secretion of LIF at the time of conceptus elongation suggests that LIF may serve a vital function in conceptus development, possibly expansion of the extraembryonic mesoderm, which initiates signalling for remodelling of the trophoctoderm. LIF could direct remodelling by enhancing production of conceptus proteases and this is supported by the observation that LIF regulates protease activity in the expanding mouse blastocyst (Harvey *et al.*, 1995). Proteases serve as modifiers of the extracellular matrix (Brenner *et al.*, 1989; Alexander and Werb, 1991).

Expression of mRNA for the EGF receptor has also been detected equally across all stages of pre-elongated conceptuses (Vaughan *et al.*, 1992). Specific binding of EGF to its receptor was also detected (Zhang *et al.*, 1992b), although binding increased temporally from day 10 to day 13 and increased sixfold by day 15 after elongation. The EGF receptor also binds transforming growth factor α (TGF- α). This may be significant because expression of conceptus TGF- α mRNA is maximal before and during elongation, and declines during the post-elongation period (Vaughan *et al.*, 1992). Vaughan *et al.* (1992) speculated that TGF- α plays a role in fluid transport during elongation, and this is supported by the observation that exogenous TGF- α enhances fluid uptake and subsequent blastocoel expansion in the peri-implantation mouse conceptus (Dardick and Schultz, 1991). Whatever role TGF- α may have in conceptus elongation, increased expression of TGF- α could initiate events necessary for rapid trophoblastic elongation, while conceptus EGF gene expression may regulate

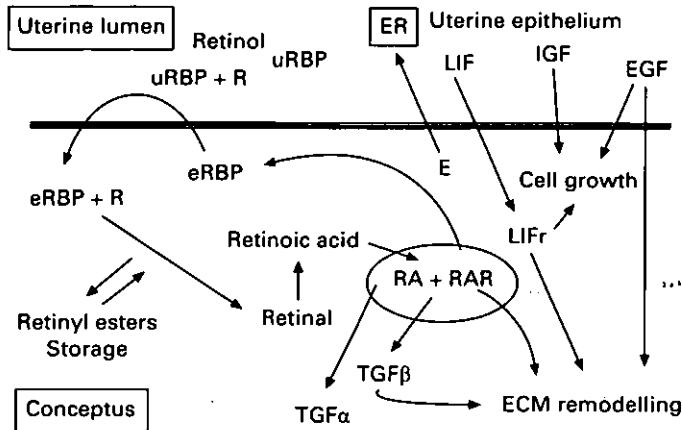


Fig. 2. Proposed simplified model of events associated with rapid trophoblastic elongation in pigs. Oestrogen (E), synthesized by the conceptus, stimulates endometrial oestrogen receptors (ER) which initiate the release of uterine retinol-binding protein (uRBP). As uRBP accumulates in the uterine lumen it transfers retinol (R) to the conceptus either directly or indirectly by exchange with embryonic retinol-binding protein (eRBP). Free R in the conceptus cytoplasm is converted to retinoic acid (RA) which activates retinoic acid receptors (RAR). The RAR may serve as initiators of the extracellular matrix (ECM) remodelling necessary for cell migration and trophoblast elongation either directly or indirectly through activation of the transforming growth factor β s (TGF β) and other morphogens. Uterine secretion of leukaemia inhibitory factor (LIF) would stimulate conceptus LIF receptors (LIFr) which may initiate the production of proteases that assist in ECM remodelling. Conceptus expression of transforming growth factor α (TGF α) is maximal before and during elongation and it may play an active role in fluid transport during elongation. Other cytokines like the insulin-like growth factors (IGFs) and epidermal growth factor (EGF) may regulate cell proliferation through receptors associated with the trophoblast before, during and following the period of elongation.

development of the embryo during early placentation. Recently, several members of the transforming growth factor β (TGF- β) superfamily and their receptors have been detected in peri-implantation pig conceptuses. Yelich *et al.* (1997b) observed increased expression of mRNA encoding TGF β -3 during the period of conceptus elongation, but did not detect expression of TGF β -2. In a comprehensive study Gupta *et al.* (1996) found that TGF β -1, TGF β -2 and TGF β -3 were immunohistochemically localized in peri-implantation pig conceptuses as were type I and type II TGF β receptors. Gupta *et al.* (1996) concluded that the TGF β s may be involved in induction or advancement of mesoderm migration in developing conceptuses, possibly through regulation of expression of extracellular matrix proteins (Igotz and Massague, 1986). Laminin and fibronectin are both produced in the early developing pig conceptus (Richoux *et al.*, 1989; Tuo and Bazer, 1996; Bowen *et al.*, 1997).

Several conceptus cytokines may regulate immunofunction within the pig uterus. Interleukin 1 β (IL-1 β) expression by the early pig conceptus is temporally associated with maternal recognition of pregnancy (Tuo *et al.*, 1996). Conceptus synthesis of IL-1 β may play a role in the interplay of the trophoblast and uterus for the establishment of pregnancy through its influence on conceptus remodelling and stimulation of prostaglandin E release. There may be an interaction between oestrogen, PGE, IL-1 β and interferon- γ (INF- γ), as conceptus INF- γ increases on day 13 (see La Bonnardiére, 1993) when IL-1 β expression has declined (Tuo *et al.*, 1996).

One of the most promising candidates for activation of trophoctoderm remodelling is the cellular morphogen, retinoic acid (Harney *et al.*, 1990). Transport of retinol by RBP into the uterine lumen, as described previously, provides the substrate for retinoic acid to the developing conceptus. Transcripts for RBP have been detected in day 11 pig conceptuses (Trout *et al.*, 1991; Harney *et al.*, 1994b; Yelich *et al.*, 1997b) consistent with the presence of immunoreactive RBP in early conceptuses (Harney *et al.*, 1990). Gene expression for RBP is initially observed in 2 mm conceptuses and increases temporally with peak expression just before the initiation of trophoblast elongation (Yelich *et al.*, 1997b). In contrast, uterine expression of mRNA encoding RBP is very low on days 10 and 11 of pregnancy (Harney *et al.*, 1994b), which is consistent with low uterine content of retinol during this period (Trout *et al.*, 1992). The initial rise in conceptus RBP gene expression could serve to protect the developing conceptus from excessive concentrations of retinol released into the uterine lumen in response to conceptus oestrogen secretion (Harney *et al.*, 1990), and to assist in the distribution of retinol to target tissues in the conceptus (Trout *et al.*, 1991) to stimulate cellular remodelling and elongation. Retinoic acid alters embryonic development in mammals (Tickle *et al.*, 1985), affects gene transcription (Chiocca *et al.*, 1989), influences production of extracellular matrix components (see De Luca, 1991) and cell surface adhesive molecules (Agora *et al.*, 1992), and can induce the expression of several peptide growth factors (Roberts and Sporn, 1988) and their receptors (Jetten, 1980). Retinoids exert their biological effects through RAR within the cell. Harney *et al.* (1994a) first described the presence of RAR α and RAR γ mRNA and protein in day 15 conceptuses. Yelich *et al.* (1997b) demonstrated the presence of RAR α , - β and - γ mRNA during both the pre- and post-elongation periods of conceptus development. Gene expression for RAR α was activated before and during conceptus elongation, in a similar way to TGF β -3 gene expression (Yelich *et al.*, 1997b), whereas RAR γ expression was more pronounced at the time of trophoblast elongation. It would be expected that RAR would be localized in the trophoblast where RBP is concentrated (Harney *et al.*, 1990; Trout *et al.*, 1992). It is evident that both RBP and RAR may have a role in the initiation or eventual remodelling of the trophoblast during elongation (see model, Fig. 2). Retinoic acid activates expression of laminin β 1 (Ross *et al.*, 1994), and integrin β -1 expression (Ross *et al.*, 1994) while it also stimulates gene expression of TGF- β s (Roberts and Sporn, 1988). The TGF- β s are major modifiers of the ECM, particularly in the case of integrins which bind to numerous ECM proteins (Igotz and Massague, 1986). The ECM may also be modulated through retinoic acid induced activation of conceptus proteases. Retinoic acid influences the production of the protease urokinase-type plasminogen activator and its respective inhibitor (Tienari *et al.*, 1991), both of which are involved in ECM breakdown. In addition, Adler *et al.* (1990) suggested that the expression of ECM-degrading metalloproteinases and their inhibitors are developmentally regulated during the differentiation and spreading of the endoderm in embryonal carcinoma cells stimulated to differentiate with retinoic acid.

Kallikrein – a Key to Conceptus–Endometrial Interaction

Since the time that conceptus oestrogen synthesis was first shown to be involved in establishment of pregnancy in pigs, many alterations in protein secretion, prostaglandin movement, blood flow and changes in uterine morphology have been described (see Geisert *et al.*, 1990). However, the factor(s) induced by conceptus oestrogen to orchestrate these biological changes has not been elucidated. The discovery of the I α I family and a novel inter-trypsin inhibitor H4 produced by the pig endometrium has provided insights to the mechanism whereby conceptus oestrogens regulate uterine function during placentation. Geisert *et al.* (1995) described changes in an endometrial glycoprotein (GP30) which is associated with the time of conceptus attachment to the uterine surface and conceptus survival. GP30 is homologous to the C-terminal region of a larger (120 kDa) pig plasma glycoprotein, I α IH4 (Geisert *et al.*, 1996; Hashimoto *et al.*, 1996). Human and pig I α IH4 are different from other inter-trypsin inhibitor heavy chains, as the consensus DPHFII sequence for binding to bikunin is absent (Saguchi *et al.*, 1995; Hashimoto *et al.*, 1996). Because I α IH4 does not contain protease inhibitory activity or bind other I α IH chains, I α IH4 must have a biological

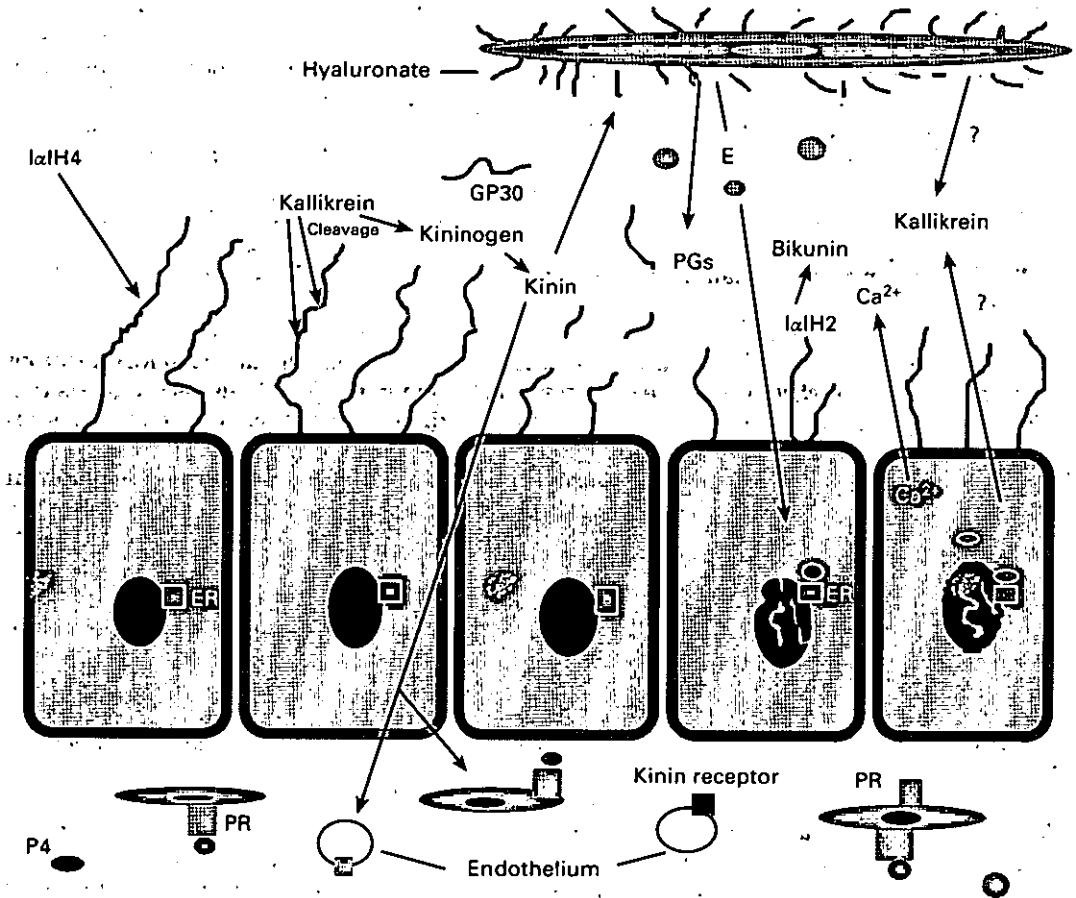


Fig. 3. Proposed model for initial attachment of the conceptus to the uterine epithelium. Release of conceptus oestrogen (E) may stimulate release of uterine kallikrein which acts to cleave inter-trypsin inhibitor heavy chain 4 (αIiH4) present on the uterine epithelial surface. Cleavage of αIiH4 releases a 30 kDa glycoprotein (GP30) the function of which is unknown and a smaller polypeptide fragment. The N-terminal region of αIiH4 , which contains binding sites for hyaluronate and integrins, remains on the uterine epithelial surface to bind to the conceptus. Presence of kallikrein may also activate cleavage of kininogen to kinin for stimulation of uterine bloodflow, calcium release and prostaglandin synthesis. Detection of αIiH2 and bikunin in the endometrium indicates that the αI family of acute phase proteins may regulate uterine inflammation and conceptus invasion through the uterine epithelial surface.

function unrelated to protease inhibition. In contrast to the other αI heavy chains, plasma human and pig αIiH4 serve as substrates for the plasma serine protease, kallikrein (Nishimura *et al.*, 1995). Plasma kallikrein first cleaves the 120 kDa αIiH4 glycoprotein into 100 and 35 kDa fragments, and then further cleaves the 100-kDa protein to a 70 kDa fragment which can release GP30, as observed in the pig endometrium (Geisert *et al.*, 1995).

Heavy chains of inter-trypsin inhibitors contain calcium-binding sites, potential reactive sites as thiol-protease inhibitors (Salier *et al.*, 1987, 1996) and most importantly, associate with hyaluronan (Zhao *et al.*, 1995). Numerous studies have established the relevance of hyaluronic acid (HA) binding to αI heavy chains with cell types that display an HA-containing coat (see Salier *et al.*, 1996). All αI heavy chains possess a von Willebrand type A domain that functions as a target for adhesion molecules such as integrins, collagen, proteoglycans and heparin (see Salier *et al.*, 1996). It has been proposed that inter-trypsin inhibitor heavy chains stabilize the extracellular matrix (Chen *et al.*, 1994;

Jessen *et al.*, 1994). In arthritis, binding of inter-trypsin inhibitor heavy chains to hyaluronate may protect the joint from inflammatory damage possibly caused by free oxygen radicals (Hutadilok *et al.*, 1988). Hyaluronan, a ubiquitous structure within the tissue extracellular matrix serves many roles in tissue morphogenesis, cell proliferation and cell migration. Therefore, in addition to the possible role of pig endometrial α IH4 in trophoblast attachment during pregnancy, the multipolypeptide chain of the pig α IH4 could also serve to stabilize the epithelial glycocalyx and protect it from free radical damage. Alteration in α IH4 may not be the only factor involved with trophoblast attachment; however, cleavage of α IH4 could induce local alterations in receptivity to the conceptus that permits the conceptus to contact integrins for firm attachment to the uterine epithelium (Bowen *et al.*, 1997). Kallikrein cleaves α IH4 to allow interaction of the 55 kDa fragment of α IH4 with integrins and HA for placental attachment.

Kallikrein protease activity increases during conceptus elongation and oestrogen secretion (Vonnahme and Geisert, unpublished results), which is associated with alterations in α IH4 during early pregnancy (Geisert *et al.*, 1995). The presence of kallikrein in the pig uterus also suggests that the pig uterus may have a functional kallikrein-kininogen-kinin system. A number of physiological responses that occur during early pregnancy in pigs are consistent with functions of bioactive kinins (Margolius, 1996). The pig uterus is responsive to oestrogen during the period of placental attachment and oestrogen stimulates increases in uterine blood flow, vasodilatation of the capillaries surrounding the elongated conceptus, contraction of the myometrium, and endometrial release of calcium, protein and histamine (see Geisert *et al.*, 1990). Kinins have a high affinity for specific membrane receptors on diverse cell types that regulate tissue blood flow, ion transport and smooth muscle contractions (see Bhoola *et al.*, 1992; Rusiniak and Black, 1995; Margolius, 1996). Several studies have demonstrated that the presence of tissue kallikrein in the rat uterus is steroid regulated and correlated with the timing of implantation (Corthorn and Valdes, 1994; Brann *et al.*, 1995; Valdes *et al.*, 1996). These studies suggest that the kallikrein-kininogen-kinin system plays a major role in the induction of the prostaglandin and histamine cascade involved with endometrial permeability and decidual transformation in rats following oestrogen stimulation.

Studies demonstrating a biologically active kallikrein-kininogen-kinin system in the pig uterus during early pregnancy have not been completed; however, we propose that conceptus elongation and oestrogen release initiates a cascade of uterine changes through kallikrein that influence implantation/placentation (see model, Fig. 3). Activation of kallikrein within the uterine lumen would cleave endometrial α IH4 and permit conceptus trophoblast adhesion to the uterine surface epithelium during and following conceptus elongation. In addition, kallikrein cleaves kininogen into kinin which functions to increase calcium, protein and proteinase inhibitor release locally into the uterine lumen as well as to increase uterine blood flow and production of postaglandins by the conceptus during trophoblast elongation. The localized stimulation of α IH4 and the short half-life of kinins would permit regulation of the uterine environment by each developing conceptus within the uterine horn.

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