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Abstract

A better understanding of the mechanisms mediating conceptus-reproductive tract interactions in pigs has important implications for breeding herd productivity and for understanding the effects of selection for reproductive merit on patterns of prenatal loss and associated changes in the uniformity and growth potential of offspring. Nutrition and metabolic state have an important impact on embryonic and fetal development, and there is extensive evidence for both direct and indirect effects of nutrition on the reproductive tract of pigs. Changes in progesterone status can be a key mediator of indirect effects on early embryonic development. Using the cyclic, unilaterally ovariectomized gilt model, we have initially confirmed steroid-dependent changes in the secretion of oviduct proteins (POSP 1-3) as one possible component of this effect. The identification of other genes that are differentially expressed in the oviduct in the peri-implantation period is progressing, using the DD-RT-PCR approach. At the uterine level, the study of the pattern of expression of matrix metalloproteinases (MMP) and their regulators during the peri-implantation period has contributed to our understanding of the regulation of the noninvasive type placental development in pigs. A failure of the blastocyst to express MMP-9 may be a key factor and seems to be dependent on local inhibitory regulation within the uterus. The coexpression of MMP-2, tissue inhibitor of MMP-2, and MMP-14 may be linked to the morphological development of the extraembryonic tissues. Changes in plasminogen activator expression are consistent with an estrogen-modulated role in tissue remodeling during embryogenesis. Studies of the integrin family of adhesion molecules also revealed key mediators of the adhesion and attachment process that is fundamental to the development of the diffuse, epitheliochorial placenta of pigs. The lack of the αv subunits on the apical surface of the trophoblast, associated with uterine epithelial expression of both $\alpha v\beta 1$ and $\alpha v\beta 3$, suggests that attachment is directed from the maternal side. From the perspective of the trophoblast, the low expression of the $\alpha v\beta 1$ subunit is consistent with noninvasive placentation. The temporal expression of other integrins is consistent with a role in the migation of both extraembryonic endoderm and mesoderm during embryogenesis.

Key Words; Pigs, Reproduction, Conceptus, Implantation, Oviducts, Uterus

Introduction

A uniform flow of weaned pigs into growing-finishing units is the foundation of a profitable production system. In North America, health advantages of segregated early weaning (**SEW**) often dictate the use of short lactations. In first-parity sows, this may limit sow productivity, whereas reducing lactation length in higher-parity sows may actually increase pigs weaned per sow per year (Foxcroft et al., 1995). Uniformity in birth weight and in weaning weight is very desirable in all-in/all-out systems, but little attention has been paid to factors that determine litter uniformity. The growth potential of weaned pigs is also clearly a key factor in the performance of growing-finishing units, and there is mounting evidence that in domestic farm species nutrition may have a major influence on embryonic and fetal development (see Robinson et al., 1999). Therefore, fully understanding how interactions between the conceptus and reproductive tract affect pregnancy outcomes, in terms of the size and uniformity of the litter born, may help to better meet the needs of production units.

Patterns of Embryonic Loss

Analysis of limited data from contemporary dam-line females suggests that the pattern of embryonic loss may be changing (Foxcroft, 1997). In higher-parity sows from "prolific" lines, increased ovulation rates of 25 to 30 can be associated with high embryonic survival through the implantation period (G. R. Foxcroft, J. A. Goller, and J. Willis, unpublished observations). Because litter size at farrowing in these higher-parity sows only increased to 12 to 13 pigs on average, these data suggest that a substantial proportion of developing conceptuses are now lost in the postimplantation stages of pregnancy. Given the implications of these data, we have recently further explored the patterns of embryonic loss in a gilt population. In this study, we established the relationships between the number of viable embryos in utero, placental size, and embryonic development. The results of this study indicate that increased ovulation rates in contemporary dam-line genotypes, associated with high embryonic survival through implantation, results in uterine crowding in early pregnancy and detrimental effects on placental development and embryo size (F. C. L. Almeida, S. Novak, and G. R. Foxcroft, unpublished observations). Because the eventual timing of embryonic or fetal loss is uncertain, the implica-

tions for fetal development cannot be predicted. However, a better match between uterine capacity and the number of conceptuses in the uterus would presumably counteract the detrimental effects on placental development and embryonic growth that are already measurable by d 28 of gestation. The data of Ford (1997) suggest that sows of Large White origin rely on continued placental growth, rather than increased placental vascularization, to meet the needs of fetal development in later pregnancy, so uterine crowding in early pregnancy may produce irreversible effects on fetal development. Further studies are needed to determine whether selection for prolificacy, based on litter size born over successive parities, has resulted in confounded effects on fetal development and the growth of slaughter generation pigs.

Changing patterns of early embryonic development may also result from nutritional and other environmental effects on the development of the preovulatory follicle and the enclosed oocyte, and on the microenvironment of cleaving embryos (O'Callaghan and Boland, 1999; Robinson et al., 1999). Through such mechanisms, the pattern of embryo development may already be predetermined in the period before the expression of the embryonic genome, a phenomenon previously referred to as "nutritional imprinting" in pigs (Cosgrove and Foxcroft, 1996; Foxcroft, 1997). Nutrition may also act by affecting the expression of genes that participate in the regulation of placental and fetal development per se (Robinson et al., 1999). From both a biological and commercial standpoint, there is a need to better understand the physiological mechanisms that regulate embryonic and fetal development. Several different aspects of conceptus development merit consideration. As suggested previously (Pope, 1994), evaluation of the critical interaction between conceptus and dam may need to include the period of intrafollicular development of the oocyte, and interactions within the oviduct, as well as the more established studies on conceptus-endometrial interactions in the uterus.

In recent work, we have sought to achieve two objectives, first to contribute to a better understanding of the mechanisms that determine embryonic development and survival and, second, to use this knowledge to determine the extent to which interactions between nutrition and embryonic development contribute to overall sow productivity. In the remainder of this review, we will focus on three key topics: 1) evidence for progesterone as a key mediator of effects on embryonic survival, 2) recent data on the physiology of the oviduct, and 3) the molecular basis for the development of the "noninvasive" epitheliochorial placenta of pigs.

Progesterone as a Mediator of Effects on Embryonic Survival

Progesterone-Mediated Effects

There is both indirect (Ashworth, 1991; Pharazyn, 1992; Jindal et al., 1996) and direct (Ashworth, 1991; Jindal et al., 1997) evidence that reduced plasma progesterone concentrations in early pregnancy in gilts may mediate the detrimental effects of nutrition on embryonic survival. A positive association between plasma progesterone concentrations 72 h after the onset of estrus and embryo survival has also been observed in primiparous weaned sows, in which embryo survival was depressed by nutritionally induced catabolism in late lactation (Jindal et al., 1996). Furthermore, Clowes et al. (1994) reported an earlier rise in plasma progesterone concentrations in primiparous sows bred at second compared to first estrus after weaning, associated with an increase in litter size born. Finally, in studies of the endocrinology of the prolific Meishan breed, an earlier rise in plasma progesterone concentrations was observed compared to Large White breeds (Hunter et al., 1996). On the strength of these observations, it has been suggested that differences in plasma progesterone concentrations may be an important mediator of genotype and nutritional effects on embryonic survival (Foxcroft, 1997). Similar mechanisms have also been suggested in sheep (Parr et al., 1982, 1987).

Several mechanisms might contribute to nutritionally induced changes in progesterone secretion. Prime and Symonds (1993) reported effects of the plane of nutrition on hepatic portal blood flow and the metabolic clearance rate of progesterone in ovariectomized gilts, again supporting comparable observations in sheep (Parr et al., 1993a,b). It is also possible that increases in splanchnic circulation, as a consequence of increased feed intake, may divert blood away from the ovarian circulation and alter the dynamics of progesterone release from the corpora lutea. Even if progesterone secretion rates remain constant, changes in the perfusion rate of the utero-ovarian vasculature may dramatically affect the efficiency of the counter-current exchange mechanism discussed later in this review, and thus the distribution of steroids between the local and peripheral circulations. Finally, there is also the possibility that effects of nutrition on the steroidogenic competence of preovulatory follicles may translate into differences in the pattern of luteinization of these follicles in the periovulatory period. Available evidence suggests to us that the time at which critical thresholds for progesterone actions are reached in the pig, either locally or peripherally, may be functionally more important than the eventual peak luteal concentrations of progesterone.

Synchrony between embryonic and uterine development is a key factor in embryonic survival. Therefore, by delaying the time at which plasma progesterone reaches the threshold for stimulating the normal cascade of endometrial changes, high planes of nutrition may effectively deprive less-developed embryos of essential support and thus compromise their ability to survive the transitional stage of development. Because pigs are polyovulatory animals, the likelihood of plasma progesterone concentrations being below some essential threshold in established pregnancy is probably low, and luteal insufficiency is only likely to be a cause of infertility in early pregnancy. Thus, in pigs, the critical window of time during which progesterone-mediated effects of nutrition (or any other factor) become established is likely in the first 3 to 4 d of gestation. The consequences of a change in the timing of the switch from estrogen to progesterone dominance in the periovulatory period for events occurring in the oviduct are illustrated in Figure 1. In the most extreme situations that we

have recorded, it would be possible for sperm transport and capacitation, fertilization, and early embryonic cleavage to be completed in very different steroid environments. We have, therefore, attempted to establish the physiological consequences of such diverse situations and, in the next section, we present data to confirm the physiological effect of the steroid milieu on oviductal function. We will also highlight the physiological importance of the subovarian counter-current multiplier as a mechanism for magnifying the effects of ovarian steroid production at the level of the oviduct.

Recent Studies on the Physiology of the Pig Oviduct

Role of the Subovarian Counter-Current Multiplier System

Kryzmowski et al. (1990) presented an extensive review of evidence for an effective subovarian counter-current mechanism in pigs that is able to provide a unique endocrine milieu in the ovarian and oviductal vasculature. This subovarian counter-current multiplier is highly efficient, retaining up to 85% of the steroids being released by the ovary and redirecting them back into the ovarian and oviductal circulation. Consequently, as confirmed by Pharazyn et al. (1991), at a time when progesterone concentrations are already very high in the blood draining the oviduct and ovary, they are minimal in the jugular vein (Figure 2). In more recent studies, in which the time of the preovulatory LH surge was used to more precisely stage changes in plasma progesterone in the immediate postovulatory period, we have confirmed the unique steroid environment of the oviduct (S. Novak, W. T. Dixon, and G. R. Foxcroft, unpublished observations).

Secretory Activity of the Oviduct

Acting in part via the subovarian counter-current multipier, ovarian steroids regulate the oviductal and uterine environment by inducing the expression and secretion of proteins that play a part in the complex regulation of gamete and embryonic development. Although the oviduct has traditionally been thought of as a passive conduit, evidence is accumulating to suggest that it plays an active role in the control of early embryonic development, and it may also be an important mediator of embryonic survival in pigs.

Gandolfi and Moor (1987) showed that in vitro coculture of sheep embryos with oviductal cells improved the proportion of embryos developing to the blastocyst stage. The possibility that oviductal proteins may be differentially expressed and secreted, and thus affect subsequent embryonic viability, sparked research into the composition and pattern of secretion of oviductal proteins. Two types of proteins are secreted into the oviduct lumen, those of a serum origin and those that are synthesized *de novo* in the oviduct cells and secreted into the oviduct in the periestrous period (Buhi et al., 1989). Porcine oviductal secretory proteins (**POSP**) 1–3 have been described as two 100-kDa and one 85-kDa glycoprotein secreted into the isthmus (Buhi et al., 1990). Counterparts to the 85-kDa protein have been characterized in cows (Sendai et al., 1994), sheep (DeSouza and Murray, 1995), mice (Sendai et al., 1995), and humans (Arais et al., 1994) and exhibit a high degree of homology within conserved sequences (Buhi et al., 1996). In cows (Boice et al., 1992), mice (Kim et al., 1996), and pigs (Buhi et al., 1993), these proteins have been shown to bind to the zona pellucida, oocyte membrane, and perivitelline membrane and have also been located within the perivitelline space. Oviduct-derived proteins have been shown to facilitate embryonic development past the species-associated block (Nancarrow and Hill, 1995), to improve oocyte maturation (Nagai and Moor, 1990), to remain associated with the embryo until the hatched blastocyst stage in pigs (Buhi et al., 1993), and to improve implantation rates in humans (Yeung et al., 1996), providing evidence for an active role of oviduct proteins in fertilization and embryonic survival.

The POSP appear in the oviductal fluid at the time of estrus, and their secretion is induced by estrogen in pigs (Buhi et al., 1992); however, there is no rapid amplification of gene expression in in vitro studies consistent with traditional steroid function. This suggests that we do not fully understand how these proteins are synthesized and secreted. Previous studies have concentrated on defining relationships between peripheral steroid concentrations and the oviduct environment. However, from the material reviewed in the previous section, we conclude that peripheral steroid concentrations may not necessarily reflect the steroid concentrations present in the subovarian counter-current circulation in the periestrous period. Furthermore, to date, research on oviduct protein secretion has related secretion to day of the estrous cycle, as defined by the time of the onset of estrus. We believe that this creates a degree of imprecision that makes it difficult to relate changes in the steroid status of the animal to the precise events occurring within the oviduct. Therefore, in our recent studies, we have defined oviduct protein secretion in relation to the time of ovulation determined with real-time ultrasonography. This technique enables us to define the time of ovulation and thus better interpret the results obtained in terms of steroid concentrations, oviduct protein secretion, and associations with embryonic survival.

In these studies we have used cyclic, unilaterally ovariectomized gilts to extend previous information on the physiological relevance of the counter-current multiplier system. In one component of the study, we first sought to establish the relationship between local steroid concentrations and the secretion of oviductal proteins. Using an antibody against POSP 1–3 (gift of W. C. Buhi) and Western blot analysis, we were able to confirm that the elevated steroids in the oviductal vasculature, ipsilateral to the remaining ovary, were associated with greater concentrations of POSP in oviduct flushings (Figure 3). With data from a wider selection of intact gilts, we were also able to demonstrate the discrete window of time over which POSP appear in the oviductal fluid (Figure 4). We are presently assembling a panel of specific antibodies with which to describe qualitative and quantitative changes in other oviductal proteins that seem to be regulated by the steroid milieu of the oviduct in the periestrous period. In a second component of the study,

Northern analysis of oviductal mRNA extracted from oviductal tissues, recovered at the time of oviduct flushing, is being used to screen the expression of candidate genes that may regulate the secretory activity of the oviduct.

In a third component of the study, the DD-RT-PCR technique has been used to identify oviductal genes whose expression seems to be regulated by the local endocrine environment. Results in Figure 5 indicate a number of genes that seem to be differentially regulated during the periestrous period, and we are presently identifying, sequencing, and developing probes for these genes. We plan to study their differential expression in oviductal tissues recovered from gilts in which the steroid milieu of the oviduct was affected by previous nutritional treatment. In this way we hope to provide evidence for direct, and for indirect and steroid-mediated, mechanisms through which nutrition can affect early embryonic development within the oviduct (Figure 6).

Molecular Interactions at the Conceptus-Endometrial Interface *In Utero*

A difference exists in estimates of embryonic mortality made between d 25 to 30 (30%) and d 12 (5 to 10%) in pigs, suggesting that considerable losses occur between d 13 to 20 (Pope, 1994). This period of high embryonic mortality is coincident with blastocyst elongation and attachment in pigs (Perry and Rowlands, 1962), and these processes are regulated by steroid and protein factors secreted from the endometrium and the embryo. Therefore, elucidating the mechanisms regulating blastocyst-endometrial interactions during blastocyst elongation and attachment will lend insight into aberrant processes of embryogenesis that may contribute to embryonic death.

The Implantation Process in Pigs

A schematic representation of the key events in early embryonic development in pigs is shown in Figure 7. Blastocyst expansion and elongation occurs between d 9 and 14 of gestation (Perry and Rowlands, 1962; Anderson, 1978). During this period, porcine blastocysts transform from a sphere of approximately 650 μm in diameter to a filamentous form that is up to 100 cm in length. The capacity to synthesize estrogen is acquired when porcine blastocysts expand to 5 to 6 mm in diameter, and it is temporally associated with the formation of embryonic mesoderm (Fischer et al., 1985; Pusateri et al., 1990; Wilson and Ford, 1997). Blastocyst elongation seems to be programmed by endogenous developmental cues in the embryo, because blastocysts only seem to elongate after they have reached approximately 10 mm in diameter (Morgan et al., 1987a,b). The 10-mm stage in conceptus development is coincident with the differentiation and expansion of extraembryonic mesoderm (Geisert et al., 1982a; Gupta et al., 1996) and is thought to be the driving force for the cellular remodeling that permits blastocyst elongation (Geisert and Yelich, 1997). Transition from spherical to tubular and filamentous forms precedes the initial attachment of the blastocyst to the endometrium. During the initial elongation of pig blastocysts from d 10 to 12, it seems that cellular remodeling, rather than cellular hyperplasia, is responsible for morphological changes (Geisert et al., 1982a), and the actin cytoskeleton plays an important role in the modification of trophoblast cell shape in elongating porcine embryos (Mattson et al., 1990). Along with the elongation process, the biochemical and physiological activities of blastocysts are increased, evident from the onset of estrogen (Gadsby et al., 1980; Fischer et al., 1985) and protein synthesis (Godkin et al., 1982). The onset of embryonic estrogen synthesis is followed by an increase in intraluminal calcium content and a rapid release of secretory vesicles from uterine epithelial cells into the uterine lumen (Geisert et al., 1982b; Stroband and Van der Lende, 1990).

Implantation is the result of a series of complex interactions between the embryo and the uterus, which begins with the apposition of the blastocyst to the uterine epithelium and ends with the formation of a definitive placenta. During implantation, porcine embryos do not normally penetrate into the uterine tissues, and placentation involves a process of extensive attachment to the uterine epithelium, as in other domestic species with an epitheliochorial type of placentation (Dantzer, 1985; Guillomot et al., 1981; Keys and King, 1990). In this situation, attachment of embryos to the uterus involves a progressive interdigitation of microvilli between the trophoblast and the uterine epithelium that occurs in two phases, apposition and adhesion. Adhesion between the two surfaces develops as the apposition phase progresses, and the trophoblast and the uterine epithelium become closely apposed to each other as soon as blastocyst elongation is complete (Flood, 1991). The process of adhesion, which begins around d 14, is initiated in the region of the embryonic disc and progresses toward the trophoblastic tips (Dantzer, 1985; Keys and King, 1990). The morphological observations of these authors suggested that the attachment between the blastocyst and the uterus is facilitated by the development of epithelial protrusions in the endometrium enclosed by trophoblastic caps, and the formation of such structures is believed to serve to immobilize the elongated blastocysts. Adhesion between maternal and fetal epithelial cells is evident by the microvillous interdigitation between the uterine epithelium and the trophoblast by d 16. This process of attachment is accomplished over the entire surface of the embryo/conceptus by d 26 of pregnancy (Amoroso, 1952).

Mechanisms Regulating Blastocyst Elongation and Implantation in Pigs

The Expression of Matrix Metalloproteinases and Their Regulators During the Peri-implantation Period. Matrix metalloproteinases (MMP), or matrixins, are a group of at least 16 endopeptidases that can degrade extracellular matrix (ECM) components such as collagen(s), laminin, fibronectin, and vitronectin (Benbow and Brinckerhoff, 1997; Nagase, 1997; Parsons et al., 1997). Most of the MMP are secreted as zymogens (proMMP), and, as an example, activation of proMMP-2 at the cell surface can be mediated in vivo by membrane type-1 MMP (MMP-14 or Mt1-MMP) that is

abundantly expressed in the placenta (Will and Hinzmann, 1995; Corcoran et al., 1996; Parsons et al., 1997). Zymogens of other MMP can be activated through the plasmin/urokinase-type plasminogen activator (uPA) system (Parsons et al., 1997). The MMP play a major role in tissue remodeling in a variety of physiological processes, which include embryo development, morphogenesis, angiogenesis, and tissue involution, as well as pathological processes such as tissue ulceration, arthritis, and cancers (Woessner, 1994; Damski et al., 1997). The MMP have also been implicated in the subtle modulation of cell-matrix interactions governing processes as diverse as cellular differentiation and migration (Damski et al., 1997). Tissue inhibitors of metalloproteases (TIMP) are the specific inhibitors for MMP; TIMP-1 and TIMP-2 are present in a soluble form and play a key role in maintaining the balance between ECM deposition and degradation in different physiological processes (Gomez et al., 1997). The TIMP-3 protein is insoluble and is the only member of the TIMP family found exclusively in the ECM (Leco et al., 1994). The TIMP-4 protein is mainly expressed in the adult heart, with low levels in the placenta (Greene et al., 1996). The role of MMP and TIMP during embryo implantation has been extensively studied in mice and humans (Alexander et al., 1996; Bass et al., 1997; Damsky et al., 1997). In pigs, a previous study on the localization and expression of MMP and TIMP at the time of embryo-uterine contact was carried out by Menino et al. (1997).

Although implantation in pigs is a noninvasive process, when porcine embryos were transplanted to ectopic sites the trophoblast cells seemed to be invasive (Samuel, 1971, 1972; Samuel and Perry, 1972). This suggests that either the environmental cues present at the ectopic site caused these cells to become invasive or, alternatively, that the intrinsically invasive nature of these cells must be regulated in the uterus in vivo during normal conceptus development. If the latter is correct, then the balance between ECM degradation and deposition in the embryo, in the endometrium, and at the interface between the trophoblast and the uterine epithelium must be tightly controlled during the peri-implantation period, such that implantation and early embryogenesis can proceed appropriately.

One objective of our recent studies was to characterize the temporal expression of MMP and their regulators in blastocysts and the endometrium during the peri-implantation period using RT-PCR and zymography. During this period, the expression for MMP-2 transcripts was up-regulated in blastocysts (Figure 8A). The proenzyme form of MMP-2 (proMMP-2) was detected in uterine flushings with increased activity on d 14 of pregnancy (Figure 9). In contrast, MMP-9 transcripts were undetectable in blastocysts by RT-PCR, even when 45 cycles were used for PCR amplification (Figure 8B). ProMMP-9 was not detected in uterine flushings obtained between d 9 and 14 of pregnancy, although it was present in uterine fluids on d 3 and 7 of pregnancy (Figure 9). There is convincing evidence that the invasive properties of murine and human trophoblast cells during pregnancy are mainly due to their expression and secretion of MMP-9 (Salamonsen, 1999). Therefore, it is not surprising that MMP-9 transcripts and proteins were not observed in implanting porcine blastocysts during normal pregnancy. However, a weak level of MMP-2 and MMP-9 transcripts was detected in cultured trophoblast (Jag-1) cells, and significant levels of both proMMP-2 and proMMP-9 were also detected in conditioned media of Jag-1 cells (L. Jiang, unpublished data). In addition, the Jag-1 cell line was able to invade matrigel (artificial basement membrane) in vitro in our laboratory (R. Chai, unpublished results). These data suggest that porcine trophoblast cells are invasive in vitro, which provides a reasonable explanation at the molecular level for previous histological observations on the invasiveness of porcine trophoblast cells at ectopic sites (Samuel, 1971, 1972; Samuel and Perry, 1972). However, it is not known whether this invasiveness of porcine trophoblast cells in vitro is caused by their production of MMP-2 or MMP-9, because production of MMP-2 is the major factor for the invasive behavior of some tumor cells (Hamasaki et al., 1998; Parsons et al., 1998; Rha et al., 1998). The differential behavior of porcine trophoblast cells under in vivo and in vitro conditions suggests that negative regulatory factor(s), possibly in the uterine fluid and(or) within the blastocyst itself, might suppress the transcription of MMP-9 gene in trophoblast cells of pig blastocysts.

The coexpression of MMP-2, TIMP-2, and MMP-14 (or Mt1-MMP) coincides with the extraembryonic mesodermal formation and cell migration in blastocysts during the peri-implantation period. The presence of the three molecules in implanting blastocysts could potentially form a trimolecular complex of MMP-14, MMP-2, and TIMP2, which will result in the cell-surface activation of proMMP-2. This process has been considered to be critical for cell migration and invasion (Nagase, 1998). More interestingly, three groups of investigators have recently shown that MMP-2 is coexpressed temporally and spatially with its activator MMP-14 and its inhibitor TIMP-2 in mouse tissues during embryogenesis and in human fetal membranes, respectively (Apte et al., 1997; Fortunato et al., 1998; Kinoh et al., 1996). Therefore, the coexpression of MMP-2, TIMP-2, and MMP-14 in blastocysts during the peri-implantation period might contribute to the spatially regulated extracellular proteolysis required for extraembryonic mesoderm migration in pigs.

Evidence for the biphasic expression pattern of uPA transcripts in porcine blastocysts during the peri-implantation period is consistent with a previous observation by Fazleabas et al. (1983) on uPA enzyme activities released by porcine blastocysts isolated from pregnant pigs at the same stages of pregnancy. Interestingly enough, expression of uPA in blastocysts is temporally correlated with embryonic estrogen synthesis, which has been shown by several groups to peak around d 11 and 16 of pregnancy (Zavy et al., 1980; Heap et al., 1981; Geisert et al., 1987). Because there is evidence that estrogen can decrease the expression of uPA transcripts in a dose-dependent fashion in human breast cancer cells (Levenson et al., 1998), it is likely that the biphasic pattern of uPA expression in blastocysts is due to the biphasic production of embryonic estrogen during the peri-implantation period. Furthermore, Menino et al. (1997) demonstrated that uPA

transcripts were mainly localized to the extraembryonic endoderm of implanting blastocysts, with a low level in trophoblast cells. Through the action of plasmin, uPA could activate zymogens of other MMP (Parsons et al., 1997). Collectively, these data suggest that the proteolytic cascade driven by uPA through plasmin might be required for tissue remodeling occurring during early porcine embryogenesis. An overall summary of existing data on the role of matrix metalloproteinases and their regulators during the periimplantation period in pigs is presented in Figure 10.

Expression of Integrin Transcripts in Blastocysts and Endometrium During Early Gestation. Early embryogenesis and attachment/implantation in pigs involves extensive cell-cell and cell-extracellular matrix interactions. These cellular interactions are mediated by several classes of adhesion molecules, of which the most important is the integrin family of cell surface adhesion receptors (Albelda and Buck, 1990; Hynes, 1992; Gille and Swerlick, 1996). Integrins function as transmembrane heterodimeric proteins consisting of α and β subunits, linking cytoplasmic components of the cytoskeleton with the extracellular matrix (Juliano, 1996; Yurochko, 1997). At least 24 integrin heterodimers can be formed from the 16 α subunits and 9 β subunits known, and nine of these have been detected in human uterine epithelium (Aplin, 1997). Integrins are modulated in the uterus during the reproductive cycle and early pregnancy in humans and rats (Nishida et al., 1991; Lessey et al., 1992; Tabibzadeh, 1992; Lessey, 1997), and human and mouse embryos express specific integrins on their surface during preimplantation development (Sutherland et al., 1993; Campbell et al., 1995). The critical roles of integrins in differentiation, migration, and invasion of trophoblast in both humans and mice have been reviewd by Damsky et al. (1993). Recently, Bowen and Hunt (1999) showed that integrins (α4, αv, β1, and β3) were also expressed within developing murine placenta. As a first step in understanding the function of integrins during pregnancy in pigs, it is necessary to examine the expression and distribution of integrins in both the developing conceptus and in the female reproductive tract during pregnancy. Using indirect immunocytochemical techniques, the expression of selected integrin protein subunits was previously localized in porcine uterine epithelium and trophoblast in vivo and in vitro by Bowen et al. (1996, 1997). Expression of integrin β1 subunit transcripts was previously examined in the early developing porcine conceptus by Yelich et al. (1997b). Due to limitations in the amount of conceptus tissue during early embryogenesis, examination of the expression of gene transcripts in the whole embryo at this developmental stage has relied on the use of sensitive detection methods such as reverse-transcription polymerase chain reaction (RT-PCR) and in situ hybridization (Rappolee et al., 1989; Yelich et al., 1997a,b).

In our recent studies, the RT-PCR approach was used to characterize the expression patterns of transcripts for eight integrin subunits (α1, α2, α3, α4, α5, αv, β1, and β3) at the mRNA level in blastocysts during early embryogenesis, in d-28 placental tissue, in cultured trophoblast cells (Jag-1), and in endometrium during early gestation in pigs. Our results support an important role for integrins in the processes of early embryogenesis, embryo implantation, and placentation in pigs (Figure 11). Expression of α_1 transcripts was not detected in porcine blastocysts until d 14 of pregnancy, when the attachment between the trophoblast and the uterine epithelium is initiated in pigs. Expression of α_1 transcripts was higher in endometrial tissues at the time of embryo-uterine contact than during other developmental stages (data not shown). These results extend previous observations on the contact-inductive nature of α_1 expression during embryo implantation in humans and mice, in which α_1 expression on the trophoblast is a response to trophoblast contact with ECM in the uterus (Damsky et al., 1992; Sutherland et al., 1993).

Five integrin subunits (α2, α3, αv, β1, and β3) showed similar patterns of expression and were developmentally regulated in blastocysts, with high expression in smaller, 1- to 2-mm, spherical blastocysts (Lane S1 in Figure 11), low expression in larger, 5- to10-mm, spherical blastocysts (Lanes S2 and 3), and variable expression in elongated blastocysts (Lanes F1 to F4). Our observation on the progressive increase in the expression of α2, α3, αv, β1, and β3 in elongated blastocysts obtained between d 11 and 16 is again temporally correlated with the increased physiological activities of blastocysts during this stage of development, as evidenced by the onset of embryonic estrogen and protein synthesis (Gadsby et al., 1980; Godkin et al., 1982; Fischer et al., 1985). Our data on the expression profile of β1 transcripts in blastocysts extended a previous observation in which expression of β1 transcripts tended to increase from spherical to filamentous blastocysts obtained from d 10 to 12 of pregnacy (Yelich et al., 1997b). Overall, the temporal expression patterns of these integrin subunits are coincident with the extraembryonic endodermal and mesodermal development in blastocysts, which suggests a role of integrins during early embryogenesis in the pig.

Integrin $\alpha_3\beta_1$ is a cell surface receptor for collagen, laminin, and fibronectin (FN) (Lessey, 1997), all of which have been shown to be present in the ECM underlying the trophoblast (Richoux et al., 1989; Bowen et al., 1996; Burghardt et al., 1997). High levels of α_3 and β_1 transcripts were observed in small spherical blastocysts (1 to 2 mm in diameter) and in elongated blastocysts obtained between d 11 and 16, which correlates with the initiation and migration of extraembryonic mesoderm in pigs. However, α_3 subunit proteins were only localized in the endoderm and not in the trophoblast of blastocysts during early pregnancy in pigs (Bowen et al., 1996). Therefore, integrin $\alpha_3\beta_1$ might be directly involved in cellular interactions during extraembryonic endodermal and mesodermal cell migration in pigs.

The interactions between integrins and their ligands on the apical surfaces of both the trophoblast and the uterine epithelium could play a major role in the attachment between these two cell layers during implantation in pigs. High levels of α_v and β_3 transcripts have been observed in implanting blastocysts and in the endometrium at the interface between

embryo and uterus. Our data are consistent with the previous observation of α_v and β_3 protein expression at implantation sites by Bowen et al. (1996). Integrin $\alpha_v\beta_3$ is a receptor for both FN and vitronectin (VN) (Hynes, 1992; Aplin, 1997), both of which have been shown to be present on apical surfaces of the trophoblast and the uterine epithelium during early pregnancy in pigs (Bowen et al., 1996). However, both α_v and β_3 subunits were only localized on the apical surface of the uterine epithelium and not on the trophoblast at the site of implantation in pigs (Bowen et al., 1996). These results suggest that interactions between the integrin $\alpha_v\beta_3$ on the uterine epithelium and its ligands (FN and VN) on the trophoblast might play a major role during embryo attachment and implantation in pigs. Because conceptus and fetal development in pigs relies on a noninvasive epitheliochorial placenta, firm adhesion between the trophoblast and the uterine epithelium is essential to establish and sustain an efficient means for nutrient and waste exchange with the maternal system.

In d-28 placental tissue, moderate expression of α1, α3, αv, and β3 and weak expression of α2 and β1 were observed. Cultured trophoblast cells (Jag-1), derived from d-14 blastocysts (Ramsoondar et al., 1993), expressed high levels of α1, α2, α3, αv, and β3 and low levels of α5 and β1. Expression of α4 was not observed at any stage of blastocyst development, in placental tissue, or in Jag-1 cells. Interestingly, relative expression of both β1 and β3 in the placental tissues and Jag-1 cells was similar, with β3 levels being higher in both cases. In contrast, Bowen and Hunt (1999) showed that expression of β1 at the mRNA and protein levels was higher than that of β3 in the invasive mouse placenta. In addition, expression of the β1 subunit was shown to be upregulated during trophoblast invasion in the human uterus (Aplin, 1997). Furthermore, β1-knockout mouse embryos develop normally to the blastocyst stage but fail to sustain implantation (Fassler and Meyer, 1995; Stephens et al., 1995). Collectively, these results are consistent with a role for the β1 subunit in the invasive type of implantation seen in humans and mice. Therefore, low-level expression of β1 in the porcine placenta might be partially responsible for the noninvasive type of implantation seen in pigs. Because β3 proteins were restricted to the intercellular borders between trophoblast cells (Bowen et al., 1996), high levels of β3 might be required for the adhesion between trophoblast cells in implanting porcine blastocysts. As such, an intact layer of trophoblast cells could be maintained to ensure the integrity of the noninvasive epitheliochorial placenta in pigs. The potential role of the integrins in early embryogenesis, attachment, and implantation in pigs is summarized in Figure 12.

Conclusions

This review has provided an overview of relevant literature and recent data from our laboratory that provide new insights into conceptus-reproductive tract interactions in pigs. We have emphasized the broad window of time over which such interactions may occur, even to the extent of considering events in the preovulatory period that may affect subsequent embryonic development. The unique environment of the oviduct has been a specific interest in our research, and a growing body of evidence suggests that differences in the oviductal function are critical for early embryonic development. We have also focused on a description of key mechanisms by which nutrition may affect embryonic and fetal development, and in particular on an important role for progesterone-mediated events. Collectively, the data reported offer exciting prospects for defining both direct and indirect mechanisms by which nutrition and metabolic state affect embryonic survival. Extensive data from other species suggest that the same mechanisms may also regulate placental development and have a critical influence on the size, uniformity, and developmental potential of the fetus at term.

Implications

Complex interactions between the developing conceptus and the reproductive tract in pigs mediate detrimental effects on embryonic loss and early embryo development. In part, these effects are environmental, such as nutrition and metabolic state. By describing the mediators and timing of these effects on embryo development, it will be possible to develop optimal management strategies for the breeding herd. For example, evidence that nutritional effects during preovulatory follicular growth may have latent, and deterimental, effects on steroid production in the immediate postovulatory period, and thereby increase embryonic loss, emphasizes the importance of nutrition in the proestrous period. High embryonic survival, but relatively limited uterine capcity for subsequent fetal develpment, seems to be affecting conceptus development in modern genotypes. A better understanding of these interactions should indicate breeding and management strategies to address this problem.

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Notes

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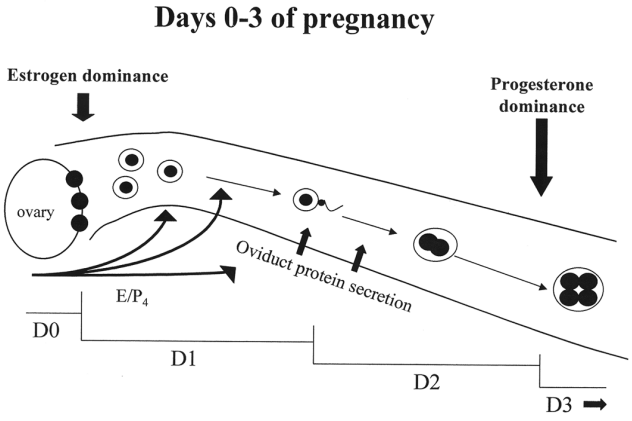

Figure 1. A summary of the key events occurring in the oviduct in the peri-ovulatory period that are likely influenced by the steroid milieu of the oviductal vasculature. D0 represents the onset of standing estrus. E, estradiol; P_4, progesterone.

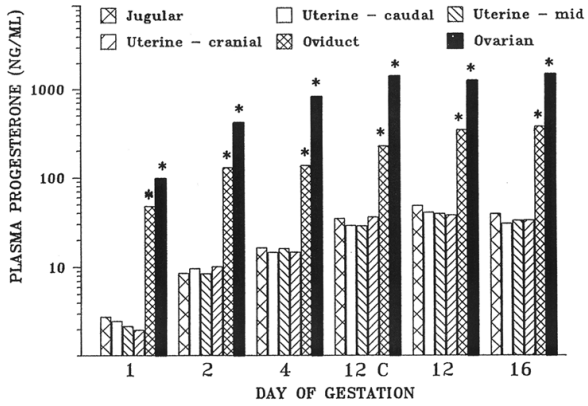

Figure 2. Plasma progesterone concentrations in peripheral plasma and in the oviduct and subovarian vasculature in early pregnancy in pigs. 12C indicates samples from non-pregnant cyclic gilts on d 12 of the cycle. Note the log scale (from Parazyn, 1992).

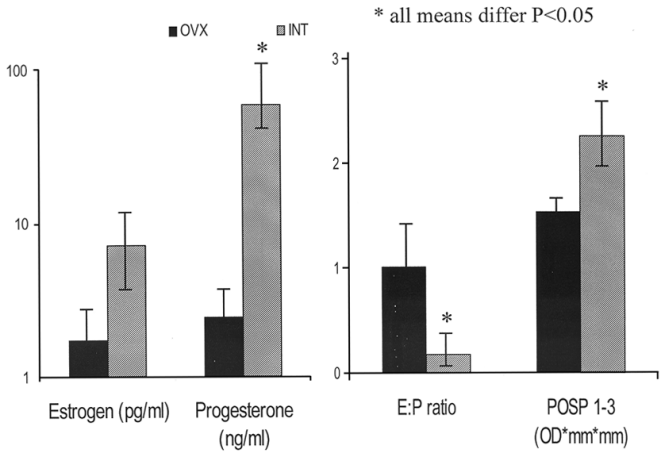

Figure 3. Plasma concentrations of estrogen (E) and progesterone (P), and the E:P ratio, in samples obtained from the oviductal vein under general anesthesia, between 12 and 24 h after ovulation in unilaterally ovariectomized gilts. Quantification of porcine oviductal secretory proteins (POSP 1-3) in oviductal flushings also collected at the time of surgery was carried out using Western blot analysis. The data indicate that high steroid concentrations perfusing the oviductal vasculature ipsilateral to the remaining ovary were associated with higher concentrations of oviductal proteins. (S. Novak, W.T. Dixon, and G.R. Foxcroft, unpublished observations).

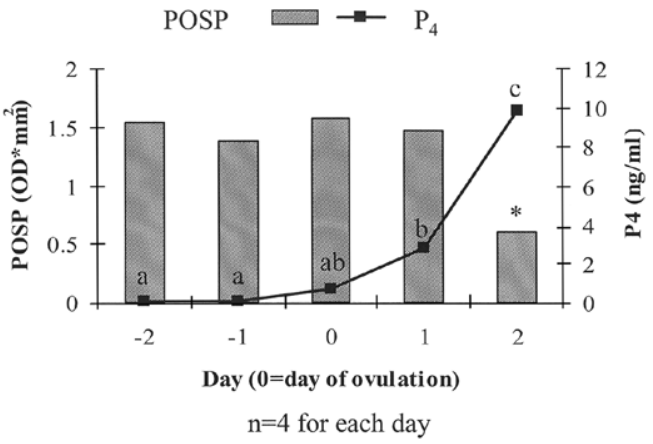

Figure 4. Quantification of porcine oviduct secretory protein (POSP) over the peri-ovulatory period in gilts. Lower concentrations of POSP on d 2 were associated with an increase in peripheral plasma progesterone (P4) concentrations at this time. (S. Novak, W. T. Dixon, and G. R. Foxcroft, unpublished observations).

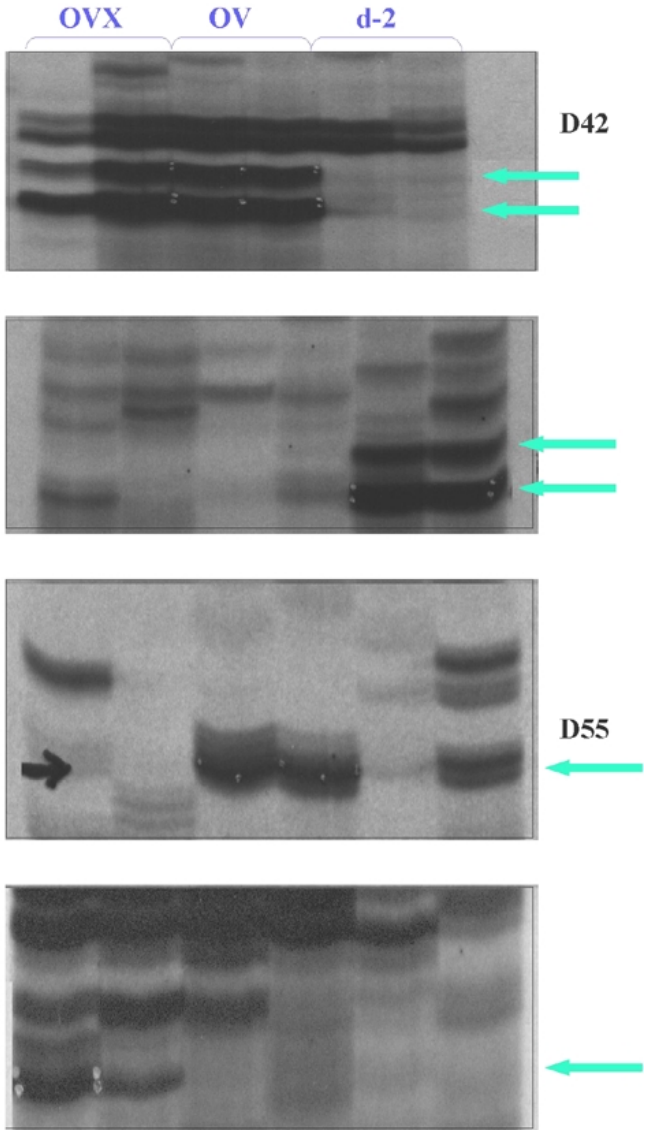

Figure 5. A DD-RT-PCR analysis of oviductal tissue obtained from an intact, cyclic gilt 2 d before ovulation (d –2), and from a unilaterally ovariectomized gilt in the 12- to 24-h period after ovulation, on the side bearing the ovary (OV) or without an ovary (OVX). Duplicate samples of the same mRNA sample were processed to confirm the repeatability of the results. Arrows indicate amplified gene fragments that appear to be differentially expressed either temporarily (d –2 vs postovulation) or in response to a different hormonal environment (OV vs OVX). (B. R. Treacy, W. T. Dixon, and G.R. Foxcroft, unpublished observations).

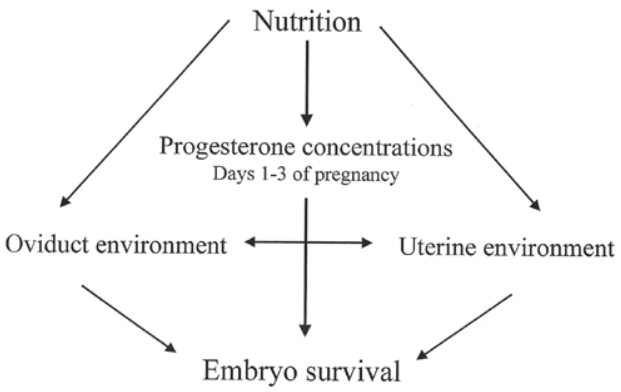

Figure 6. Schematic representation of possible direct and indirect effects of nutrition on the oviductal and uterine environment with possible consequences for embryonic survival in pigs. Progesterone mediated effects in a critical period of early pregnancy may be an important component of the interaction between nutrition and early embryonic development.

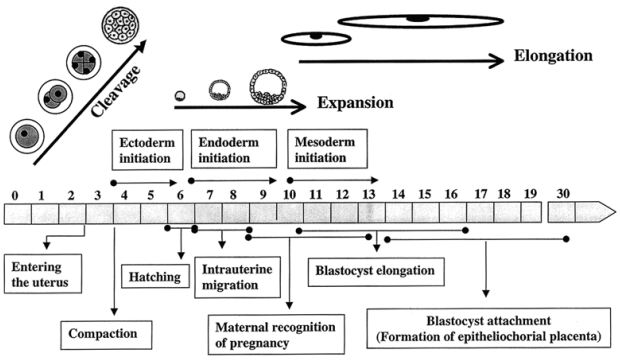

Figure 7. A schematic representation of early embryonic development during the first 30 d of gestation in pigs.

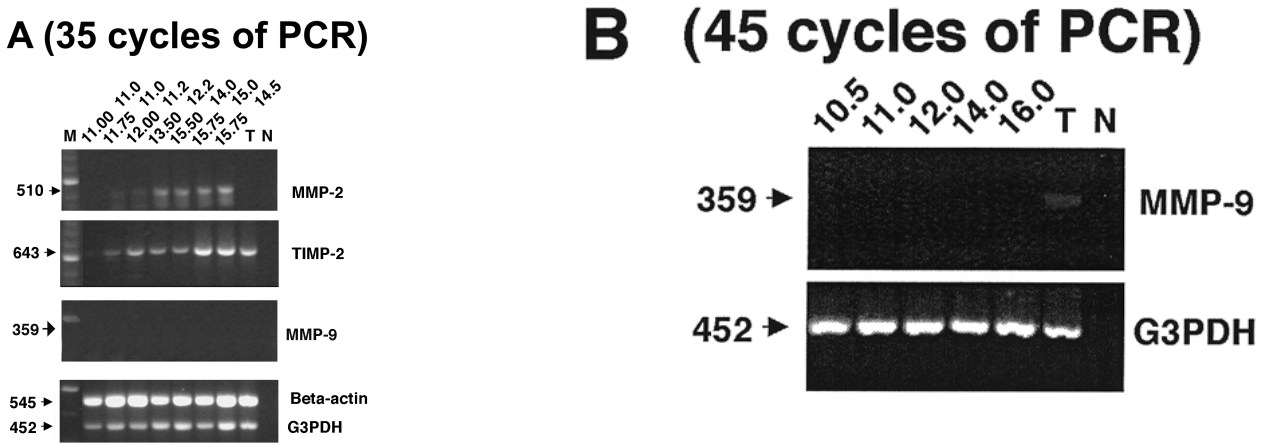

Figure 8. (A) Results of RT-PCR amplification of MMP-2, MMP-9, TIMP-2 , β-actin, and glucose-3 phosphate dehydrogenase (G3PDH) transcripts in blastocysts during the peri-implantation period. Two series of pregnant gilts were used in this experiment, and their developmental stages are indicated above the gels. Days of pregnancy in the left panel were determined on the basis of the time of the peak of the preovulatory LH surge, and those in the right panel were determined relative to the onset of estrus. T, Jag-1 cells; N, negative control. PCR was carried out with 35 cycles for each target gene in this experiment. RT-PCR products were analyzed in 1.2 % agarose gel stained with ethidium bromide. The sizes of PCR products for target genes are indicated on the left side of gels. B-Actin and(or) G3PDH were amplified as internal controls for gene expression. (B) Results after amplification of MMP-9 where 45 cycles were applied (L. Jiang, G. R. Foxcroft, and W. T. Dixon, unpublished observations).

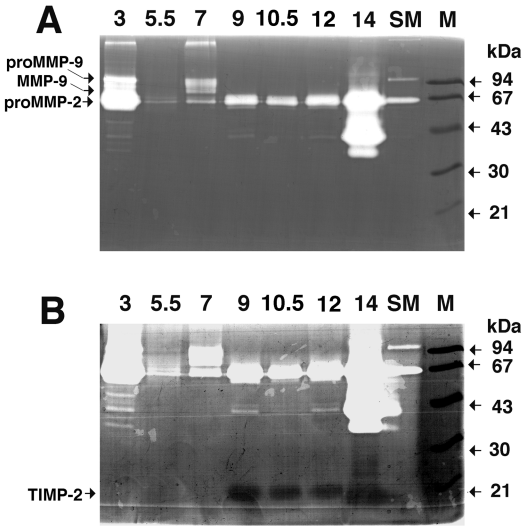

Figure 9. Gelatin zymographic (top) and reverse zymographic (below) analysis of MMP and TIMP in uterine flushings of gilts obtained on d 3, 5.5, 7, 9, 10, 1,1 and 14 of pregnancy relative to onset of estrus. Ten micrograms of total protein was loaded on each lane. SM, 20 µg of total proteins from bovine skeletal muscle homogenate as positive controls for proMMP-2 and proMMP-9; M, molecular weight marker (L. Jiang, G. R. Foxcroft and W. T. Dixon, unpublished observations).

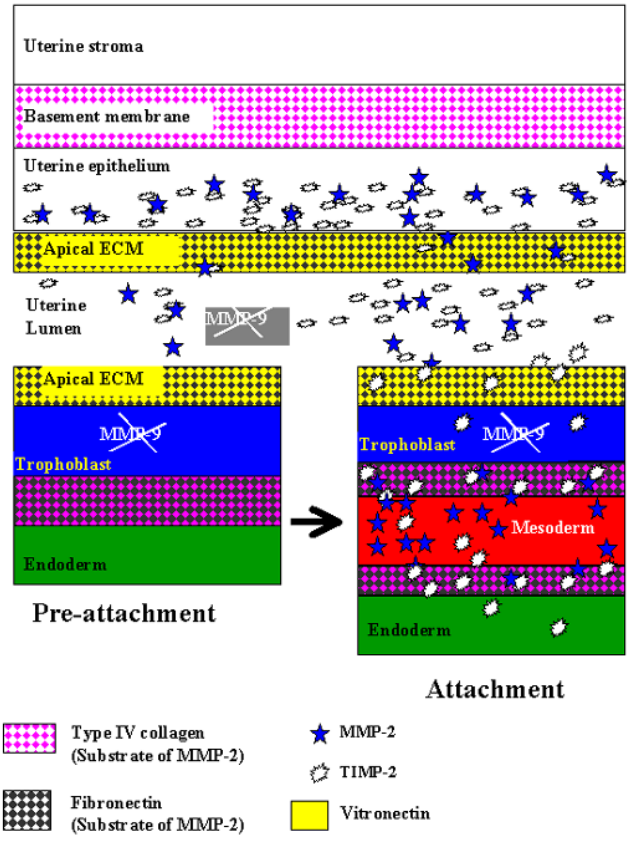

Figure 10. Schematic representation of the role of the matrix metalloproteinases (MMP) and their regulators in the peri-implantation period in pigs. The lack of expression of MMP-9 in the trophoblast and the colocalization of both TIMP-2 and MMP-2 in the uterine lumen are consistent with the noninvasive placentation in the pig. The balance between MMP-2 and TIMP-2 in the extraembryonic mesoderm will favor cell migration during mesodermal development.

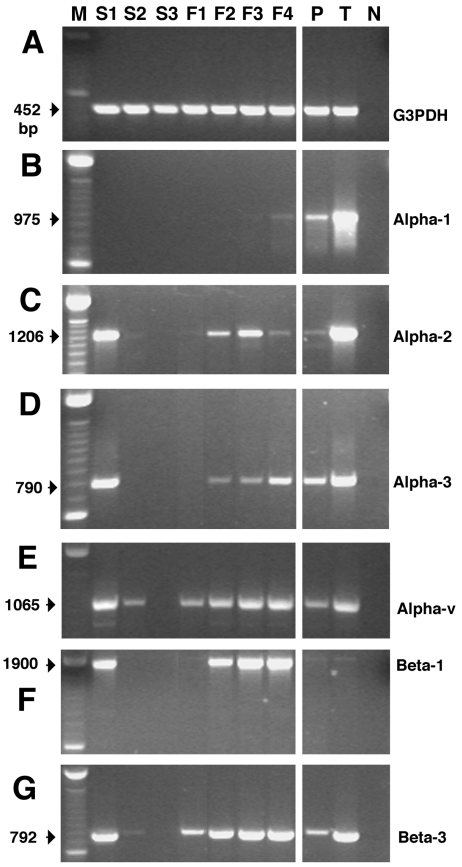

Figure 11. RT-PCR amplification of G3PDH (A) and integrin subunits α_1 (B), α_2 (C), α_3 (D), α_v (E), β_1 (F), and β_3 (G) in blastocysts, placental tissues, and Jag-1 cells. Total RNA was extracted from blastocysts from gilts representing d 9 (S1), 10 (S2), 10.5 (S3), 11 (F1), 12 (F2), 14 (F3), and 16 (F4) of pregnancy, respectively, from placental tissues on d 28 (P) and from trophoblast (Jag-1) cells (T). M, 100-bp DNA ladders; N, negative control. Names and predicted sizes of target genes are indicated to the right and left sides of the figures, respectively. PCR products were run in 1.2% (wt/vol) agarose gel, and gels were stained with ethidium bromide. (L. Jiang, G. R. Foxcroft and W. T. Dixon, unpublished observations).

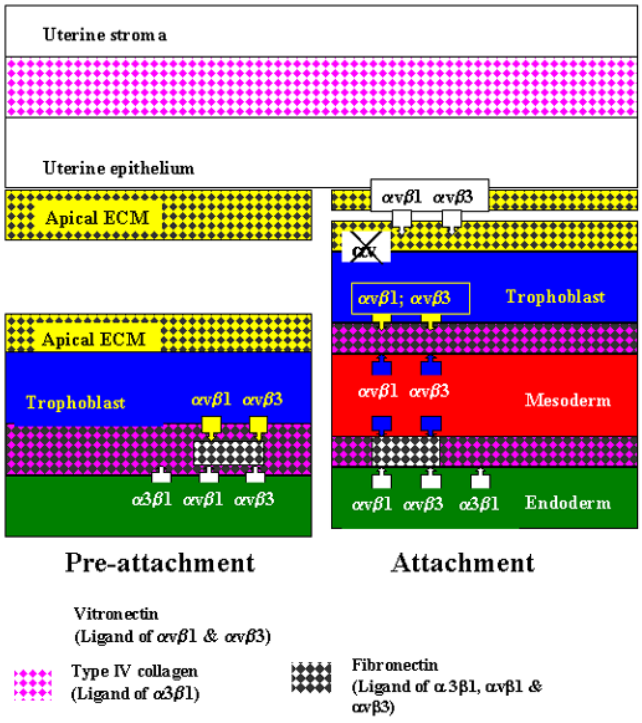

Figure 12. Schematic representation of the role of the integrin family in the peri-implantation period in pigs.