A Review of Gene Expression in Porcine Endometrial Lymphocytes, Endothelium and Trophoblast During Pregnancy Success and Failure

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Abstract. Meat pig breeds used commercially in North America lose significant numbers of genetically-normal fetuses in the peri-implantation (attachment) period and at mid-gestation (day 50 of the 114 day gestation interval). Fetal demand that is in excess to the placental blood supply is thought to underlie these waves of fetal loss. In many species, the endometrium of early normal pregnancy is enriched in innate immune cells, particularly uterine natural killer (uNK) cells. In pigs, a species with epitheliochorial placentation, conceptuses mediate about a three-fold enrichment in uNK cells at attachment sites but the functions of these cells are unknown. In species with hemochorial placentation, uNK cells are highly enriched during the process of decidualization and promote endometrial angiogenesis. We have conducted molecular analyses using pure samples of endometrial lymphocytes or endothelium and trophoblast from healthy and arresting conceptus attachment sites in Yorkshire gilts immediately post-attachment [gestation day (GD) 20] and at mid pregnancy (GD50). In healthy sites, angiogenesis was more robustly promoted by lymphocytes than by trophoblasts. An early sign of impending fetal arrest was loss of vascular endothelial growth factor (VEGF) transcription from the lymphocytes and elevation in transcription of the pro-inflammatory gene Interferon (IFN)-γ. We have postulated that newly differentiated endometrial endothelial cells, not fetal trophoblasts, are damaged by the maternal withdrawal of vascular support and onset of inflammation and that this endometrial damage contributes significantly to peri-implantation fetal death.

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as blastocysts transform into long (20 cm), thin, filamentous shapes and start secreting estrogen. Endometrial attachment is completed by GD15 [6] and invokes the recruitment of uterine leukocytes enriched in a natural killer (uNK) cell subset. There is no subsequent invasion or implantation of fetal cells into maternal tissue.

Development in the peri-attachment interval depends upon conceptus-derived growth factors. During early trophoblast elongation, this becomes supplemented by histotrophic nutrition derived from maternal uterine glands. Histotrophic factors appear to regulate the placental surface area available to each conceptus [9]. Post-attachment conceptus growth and development subsequently come to depend upon endometrial-placental interactions. Factors hypothesized to play important roles in pre- and peri-implantation porcine conceptus development are summarized in Fig. 1.

By late pregnancy, a major increase in maternal blood supply to the uterus sustains fetal nutrition. Indeed, porcine endometrial tissue is estimated to increase 15 fold during pregnancy and the uterus 100 fold when conceptus weights are included [10]. To service this gain in tissue, the size and number of uterine blood vessels is greatly enlarged during gestation. For many years, efforts have been made to improve litter sizes in North American meat pigs through identification of and selective breeding for genes responsible for uterine capacity and placental efficiency (i.e. the ratio of fetal weight to placental weight). Significant improvement has not occurred [11, 12]. Nutritional studies (improved diet balance or vitamin supplementation) also failed to show consistent beneficial effects [13, 14]. Thus, the critical steps controlling successful interactions between porcine conceptuses and their endometrial environment have remained elusive.

In published experiments, we addressed the question of whether the lymphocytes recruited to porcine attachment sites between GD15–23 transcribed angiogenic molecules. During this study, we observed heterogeneity between the fetuses in every litter (Fig. 2). Although the fetuses were all alive, they could be readily categorized as healthy or arresting based on disparity in

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**Fig. 1.** Summarizes key factors that influence successful conceptus growth during the pre-implantation and peri-implantation periods. Genetically normal, North American commercial meat pigs experience unexplained fetal losses. One wave occurs immediately after attachment of the blastocyst to the uterus and another at mid pregnancy (day 50 of the 114 day gestation interval).
Angiogenesis During Porcine Pregnancy

Angiogenesis is a process in which new capillaries develop from the pre-existing vessels. Physiological angiogenesis occurs at the maternal fetal interface and is crucial for growth and development of conceptuses. To meet the requirements of rapidly growing pig fetuses, placentae either increase in attachment surface area over the endometrium to become large (the strategy in North American meat breeds such as Yorkshire) or increase in vascular density (the

vasculature and color of the fetal membranes and in fetal length and weight. We compared transcripts in biopsies of trophoblast and endometrium with those in two endometrial cell types, lymphocytes and endothelial cells, for the two categories of attachment sites. The lymphocytes and endothelial cells were recovered as pure populations of ~500 cells from cryostat sections using laser capture microdissection. Quantitative, realtime PCR was used to assess relative gene expression in each cell type. Beta actin was selected as the most stable of three housekeeping genes examined across the interval of study and was used for calculation of relative gene expression. Our comparisons of the levels of transcription of angiogenic factors in endometrium, in endometrial cell types and in trophoblasts from healthy and arresting porcine littermate attachment sites has linked loss of tissue oxygen sensing and angiogenic molecules from endometrial lymphocytes with fetal growth arrest [15, 16]. These studies, their background and extension are summarized below.

Fig. 2. Illustrates a dissected pig uterus that exposes the lumen and its contents at GD30. Healthy conceptuses (sites A and B) occupy a greater endometrial surface than the arresting conceptus that lies between them. Health status of the conceptuses was characterized on the basis of disparity in the fetal membrane vasculature, in fetal length and in fetal weight. Five hundred endometrial lymphocytes or endothelial cells were captured using laser capture microdissection (LCM) from each attachment site after conceptus removal. Gene expression was compared between endometrial lymphocytes, endometrial endothelium, mixed cell endometrial biopsies and trophoblasts collected from the same sites.
strategy in Chinese Meishan pigs, a highly prolific breed that lacks meat qualities desired in North America [17]). Chinese Meishan pigs have similar ovulation rates and uterine sizes to Yorkshire pigs but farrow 4–5 more piglets per litter. This biological difference is attributed to the Chinese Meishan’s physiological strategy of markedly increasing the vascular density of each small placenta to overcome potential limits in uterine capacity [18]. In contrast, fetuses of North American meat breeds enter a competition for access to maternal nutrients by covering more and more of the endometrial surface [11, 19]. Recently, a positive correlation was reported in pigs genetically selected for high placental efficiency and expression of vascular endothelial growth factor family related genes (VEGF, Flt-1 and KDR) [20]. The opposite outcome has been reported by others [21].

VEGF is a secreted, glycoprotein homodimer, when bound to one of its two specific membrane receptors, VEGF-RI (Flt-1) and VEGF-RII (KDR), is responsible for endothelial cell proliferation, migration and capillary permeability [22, 23]. VEGF and its receptors are present at the pig maternal fetal interface from early to late gestation [24, 25]. Understanding the roles of VEGF-RI and VEGF-RII in VEGF-promoted angiogenesis is complicated because a soluble (s)VEGF-RI (sFlt-1) exists. This soluble receptor was first cloned from human umbilical vein endothelial cells [26] and is generated by alternative splicing of VEGF-R1 pre-mRNA. sVEGF-RI binds VEGF at high affinity but signaling is blocked because there is no tyrosine kinase domain on this receptor [27]. As an antagonist of VEGF, sVEGF-RI regulates the levels of bioavailable VEGF during pregnancy [28]. sVEGF-RI has been implicated, along with soluble endoglin, in the pathogenesis of pre-eclampsia, a common but critical hypertensive disorder in pregnant women [29–31]. sVEGF-RI has not been reported in pigs. Placenta growth factor (PlGF), a splice variant belonging to VEGF family also binds to VEGF-RI and has direct angiogenic function as well as decoy activity [32, 33].

Recruitment of Uterine Natural Killer Cells During Porcine Pregnancy

Early pregnancy-associated porcine endometrium (GD15–28) is enriched two to three fold in uterine natural killer (uNK) cells [34]. Most of the hypotheses regarding potential functions of these cells derive from more detailed investigations that have been conducted on murine and human uNK cells. There is some weakness in this logic because mice and humans are species in which endometrial decidualization accompanies pregnancy and this event, not trophoblast, recruits uNK cells. The level of uNK cell enrichment is also more minor than in mice and humans and porcine uNK cells are usually agranular, contrasting with the numerous cytoplasmic granules found in mouse uNK cells. Porcine uNK cells are spherical in shape and have a relatively small diameter (5 µm) compared with mouse uNK cells that range in diameter between 49–80 µm [34, 35]. Using monoclonal antibodies against specific surface markers, Bischof and his colleagues showed that porcine uterine lymphocytes were packed beneath the luminal epithelium, around glands and blood vessels and scattered throughout the stroma [36, 37].

Role of Endometrial (Maternal) Lymphocytes in Promotion of Angiogenesis

We have now examined the transcription of VEGF, PI GF, VEGF-RI and VEGF-RII in lymphocytes and endometrium and in endometrial and trophoblast biopsies from healthy and arresting littermate attachment sites at GD20 and GD50. In comparison with lymphocytes from non-pregnant uteri, transcripts for VEGF (Fig. 3) and PlGF (data not shown) were progressively elevated in lymphocytes from healthy attachment sites between GD15–50 [15, 16, 38]. These findings were supported by immunohistochemistry, which demonstrated translation of these genes by lymphocytes as well as by other cell types. Thus, porcine endometrial lymphocytes share the ability to promote and regulate angiogenesis with uNK cells found in the decidualized endometria of species with hemochorial placentation (humans [39–41] and mice [42]).

Lymphocyte transcription of VEGF was at almost undetectable levels when the cells were recovered from sites showing the earliest visible signs of arrest in fetal development (GD20) [15]. Fewer (P<0.001) VEGF transcripts were also found at GD50 in lymphocytes associated with arresting
versus healthy conceptuses. Interestingly, the pattern of PI GF transcription in lymphocytes from arresting sites differed from that of VEGF. PI GF transcription became elevated during both early and mid gestation failure (manuscript in preparation). These patterns of change were masked when transcripts from mixed cell endometrial biopsies were analyzed [Fig. 3; 15, 38]. Because PI GF is a competitive antagonist for VEGF and has direct effects on cell proliferation and migration, it is unclear whether elevated PI GF in lymphocytes from failing sites is a process to release more bioavailable VEGF or has a direct effect on the lymphocytes. In mice, PI GF plays a role in the terminal maturation of uNK cells. Thus, it is possible that elevated PI GF may be a mechanism to enhance uNK cell maturation and production of IFN-γ in compensation for reduced VEGF. Endometrial lymphocytes not only produced angiogenic factors but showed gestation time dependent changes in expression of VEGF-RI and VEGF-RII. VEGF-RI was more abundantly transcribed by lymphocytes in healthy implantation sites than VEGF-RII [38]. Fetal arrest had little effect on the level of transcription of either receptor.

Endometrial Endothelial Cells and Implantation Site Angiogenesis

Endothelial cells are very flat (1–2 µm thick), have a central nucleus, and are 10–20 µm in diameter. They form pavement-like patterns along
the lumen surfaces of vessels [43]. Endothelial cells
cover the intimal surfaces of blood vessels to form
the interface between blood and tissue. Endothelial
cells form new capillaries from preexisting blood
vessels, largely in response to VEGF-mediated
signaling. Paracrine signals provided by
endothelial cells to surrounding cells can also
promote stem cell development and organ
formation during fetal development and in adult
endothelial niches [44]. Activated endothelial cells
synthesize degradative enzymes such as matrix
metalloproteinases and secrete them into
surrounding tissue. The extracellular matrix is then
degraded allowing endothelial cell migration into
the surrounding area. Activated endothelial cells
also initiate proliferation and form tubes that
construct the new blood vessel meshwork [45].

Endothelial cells are major components of uterine
stroma. In endothelial cells from healthy
attachment sites, more transcriptions of VEGF (Fig.
3) and PlGF (data not shown) was present than in
endothelium from non-pregnant uteri. Fetal arrest
was associated with declining endothelial cell
transcription of VEGF but stable expression of
PlGF. Endothelium transcribed both VEGF-RI and
VEGF-RII with VEGF-RII being more abundant.
Thus, lymphocytes and endothelium in
mesometrial endometrium are expected to have
differing responses to VEGF in their shared
environment due to differential expression of the
VEGF receptors. VEGF-RI and VEGF-RII
expression by endothelial cells fluctuated in sites of
fetal arrest [38].

**Trophoblasts in Implantation Site Angiogenesis**

Trophoblasts produce angiogenic, anti-
angiogenic and vasoactive that regulate blood
vessel development and patterning in the fetal
membranes. In species with invasive trophoblasts,
trophoblasts also effect structural changes on
endometrial vessels. For example, trophoblast cells
are found in the walls and lumens of human and
murine spiral arteries [39, 46] and endothelium
may be replaced by intravascular trophoblasts that
acquire endothelial cell-like molecular expression.
It is also possible that distant maternal arterial
function is modified by “conducted responses”
originating from more proximal interactions
between trophoblasts and endothelial or vascular
smooth muscle cells and moving rapidly along the
vessel wall [47]. Murine trophoblasts express
VEGF and Proliferin, angiogenic factors critical for
maternal vascular development and anti-
angiogenic factors such as s-VEGF-R1 and
Proliferin-related protein. The latter antagonizes
the angiogenic effects of Proliferin [48] and is
expressed by the spongiotrophoblast layer, the
layer immediately under VEGF-expressing
trophoblast giant cells [49]. Anti-angiogenic factors
found just beneath the trophoblast giant cell layer
could prevent the growth of maternal endothelium
into spongiotrophoblast.

There is limited information regarding the
regulatory role of trophoblasts in angiogenesis
during porcine pregnancy. Porcine trophoblast
cells synthesize and secrete the steroid hormones
and cytokines required to establish and maintain
pregnancy [50]. IFN-γ and IFN-δ are secreted in
large amounts between GD12 and 20 with peak
synthesis at GD15 and 16 [51]. It is postulated that
IFN-γ plays important roles in early embryo
attachment and development [52]. Since pig
trophoblast lacks the IFN-γ receptor at the time of
IFN-γ and IFN-δ secretion, maternal epithelium is
considered the most probable and perhaps the only
target of trophoblastic IFNs [51, 53]. We postulate
that porcine trophoblastic IFNs activate
recruitment and function of endometrial
lymphocytes and thereby play a central role in
regulation of endometrial angiogenesis. Winther
et al. (1999) demonstrated by immunostaining that
VEGF, VEGF-R1 and VEGF-R1I expression by endothelial cells fluctuated in sites of
fetal arrest [38].

We have quantified the transcription of VEGF,
PlGF, VEGF-R1 and VEGF-R11 in porcine
trophoblasts. At healthy sites, trophoblast
transcription of VEGF was high and exceeded that
of PlGF at both GD20 and GD50 [15, 38]. VEGF-R11
was more abundantly expressed by trophoblasts
than VEGF-R1. At both GD20 and GD50,
trophoblasts from arresting sites had lower
expression of VEGF, PlGF and VEGF-R11 than
found in healthy sites. VEGF-R11 expression
differed by gestation time not by the health status
of the attachment site.

Trophoblasts had many fewer transcripts for
VEGF than lymphocytes from healthy conceptus
attachment sites (Fig. 3), suggesting that maternal
immune cells are major promoters of endometrial
angiogenesis from arresting GD20 sites showed a significant deficit in VEGF transcripts while failing GD50 trophoblasts did not. This suggests maternal lymphocytes sensed warnings, perhaps of conceptus origin, and responded by shutting down the mother’s angiogenesis support system for that specific placenta. Trophoblast-directed angiogenesis appeared to decline synchronously with that in endometrium at GD20 but remained elevated at GD50. This latter finding suggests that, in some instances of fetal loss, maternal endometrium may be the prime regulator of fetal demise through inadequate support for angiogenesis.

Conclusions

Maternal and fetal angiogenesis are both required for successful gestation. Commercial North American meat pigs experience high rates of fetal loss during attachment to the uterus and at mid gestation. These losses appear to be associated with interconceptus competition for endometrial space and inadequate vascular development. Using the techniques of laser capture microdissection and quantitative realtime PCR, we found that porcine uterine lymphocytes and endothelium from healthy attachment sites transcribe VEGF and PI GF more abundantly than trophoblast from the same attachment site. This was confirmed by immunohistochemistry. Further, we found that the lymphocytes preferentially transcribed VEGF-RI while endothelium and trophoblasts preferentially transcribed VEGF-RII. GD50 fetal arrest sites showed VEGF transcription at normal levels in trophoblast but at greatly reduced levels in endometrial lymphocytes from the same site. These data suggest that immune cells detect trophoblast-derived signals of stress and locally withdraw maternal vascular support to ensure a specific conceptus will not develop further. Strategies to elevate endometrial angiogenesis in commercial meat pigs during the peri-attachment to mid gestational interval would be predicted to enhance the number of live-born/litter.

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