Differential Gene Expression in Endometrium, Endometrial Lymphocytes, and Trophoblasts during Successful and Abortive Embryo Implantation

Chandrakant Tayade, Gordon P. Black, Yuan Fang and B. Anne Croy

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Differential Gene Expression in Endometrium, Endometrial Lymphocytes, and Trophoblasts during Successful and Abortive Embryo Implantation

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Prenatal mortality reaching 30% occurs during the first weeks of gestation in commercial swine. Mechanisms for this are unknown although poor uterine blood supply has been postulated. In other species, vascular endothelial growth factor, hypoxia-inducible factor 1-α, and IFN-γ regulate gestational endometrial angiogenesis. Vascular endothelial growth factor and hypoxia-inducible factor 1-α are also important for placental angiogenesis while trophoblastic expression of Fas ligand is thought to protect conceptuses against immune-mediated pregnancy loss. In this study, we document dynamic, peri-implantation differences in transcription of genes for angiogenesis, cytokine production, and apoptosis regulation in the endometrium, and laser capture microdissected endometrial lymphocytes and trophoblasts associated with healthy or viable but arresting porcine fetuses. In healthy implantation sites, endometrial gene expression levels differed between anatomic subregions and endometrial lymphocytes showed much greater transcription of angiogenic genes than trophoblasts. In arresting fetal sites, uterine lymphocytes had no angiogenic gene transcription and showed rapid elevation in transcription of proinflammatory cytokines Fas and Fas ligand while trophoblasts showed elevated transcription of IFN-γ and Fas. This model of experimentally accessible spontaneous fetal loss, involving blocked maternal angiogenesis, should prove valuable for further investigations of peri-implantation failure of normally conceived and surgically transferred embryos in many species, including the human. The Journal of Immunology, 2006, 176: 148–156.

The maternal-fetal interface is a dynamic site in which fetally derived trophoblast cells interact with maternal tissue that includes immune cells. During early pregnancy in many species, including pigs, maternal endometrium becomes enriched for cells of the innate immune system, particularly NK cells (1–4). Uterine NK (uNK) cells have been most extensively studied in humans, mice, and rats, species with invasive hemochorial placentation. In these species, uNK cell recruitment is associated with decidual induction and does not require a conceptus, although presence of a conceptus is required in humans, mice, and rats. Invasive hemochorial placentation is associated with healthy or viable but arresting porcine fetuses. In healthy implantation sites, endometrial gene expression levels differed between anatomic subregions and endometrial lymphocytes showed much greater transcription of angiogenic genes than trophoblasts. In arresting fetal sites, uterine lymphocytes had no angiogenic gene transcription and showed rapid elevation in transcription of proinflammatory cytokines Fas and Fas ligand while trophoblasts showed elevated transcription of IFN-γ and Fas. This model of experimentally accessible spontaneous fetal loss, involving blocked maternal angiogenesis, should prove valuable for further investigations of peri-implantation failure of normally conceived and surgically transferred embryos in many species, including the human. The Journal of Immunology, 2006, 176: 148–156.

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1 The porcine partial coding sequences presented in this article have been submitted to GenBank under the following accession numbers: AY616676 (VEGF), AY836553 (HIF-1α), AY562551 (IFN-γ), AY781397 (FasL), AY781398 (Fas), AY577818 (IL-1β), and AY577819 (IL-1R).
2 Address correspondence and reprint requests to Dr. Chandrakant Tayade, Department of Biomedical Sciences, University of Guelph, Guelph, Ontario, Canada N1G2W1. E-mail address: ctayade@uoguelph.ca
3 C.T. and G.P.B. contributed equally to this work.
4 Abbreviations used in this paper: uNK, uterine NK; gd, gestation day; HIF-1α, hypoxia-inducible factor 1-α; VEGF, vascular endothelial growth factor; LCM, laser capture microdissection; NP, nonpregnant; FasL, Fas ligand; aRNA, antisense RNA.

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uteri of commercial pigs using quantitative real-time PCR. Endometrium, laser capture microdissected (LCM) endometrial lymphocytes and trophoblasts were compared. By gd20, arresting fetuses (viable but destined to die (Fig. 2)) could be identified. Relative gene expression from gd20 was therefore compared between samples from littermates who would have different gestational outcomes. Our results suggest porcine uterine lymphocytes are more actively involved in angiogenesis and oxygen sensing than trophoblasts and that the maternal endometrium and its lymphocytes acutely withdraw all vascular support from arresting conceptuses. This occurs simultaneously with a highly localized, cytokine-based immune attack.

Materials and Methods

Animals and tissue collection

Allogeneic-specific, pathogen-free, line-bred Yorkshire gilts (n = 18; Arkell Swine Research Station, University of Guelph) were either not mated or bred twice, 24 h apart, naturally or by artificial insemination at their first estrus. Reproductive tracts were recovered immediately after abattoir slaughter at the University of Guelph using protocols approved by the institutional Animal Care Committee. The gd was estimated from the first mating day.

Uteri were transported on ice to an RNase decontaminated dissection area and opened longitudinally along the antimesometrial side. For non-pregnant (NP) uteri, endometrial biopsies were collected from random antimesometrial and mesometrial (side of uterine artery entry) sites. At gd15, attachment sites were identified under dissecting microscope magnification; later stages were visualized without aid. Within litters, gd21 and 23 conceptuses were not similar and they were grouped as healthy or arresting by fetal length, weight, and vascularity of the placental membranes (Fig. 2). For each pregnant uterus, three or four healthy implantation sites were studied as individual samples. When arresting fetal sites were recognized, each one was collected separately, resulting in at least three additional sites being studied per dam in each later pregnancy. Conceptuses enclosed in membranes were peeled from each pregnant uterus and analyzed separately. Gestational sacs were opened to identify fetus and amnion. The trophoblast was then dissected free from these tissues and placed in RNA...
lysis buffer. After removing conceptuses, endometrial biopsies (~30 mg) were collected immediately under the attachment sites (mesometrial, antimesometrial to the attachment or between two healthy attachment sites. Endometrial biopsies were either placed in 600 μl of lysis buffer or embedded in OCT (Thermo Shandon) for cryosectioning.

**RNA isolation from embryo attachment sites and trophoblasts**

Tissues in RLT buffer (RNase mini kit; Qiagen) were disrupted using Kontes pestles (Fisher Scientific). Total RNA was extracted following manufacturer’s instructions. Briefly, tissue lysate was centrifuged (15,000 × g, 3 min) and the cleared lysate was mixed with 700 μl of 70% ethanol. The reaction mixture was washed over an RNasey mini column. The RNA was eluted using 50 μl of nuclease-free water and quantified by a RNA/DNA calculator (Genequant Pro; Promega) and stored at −80°C.

**LCM and RNA amplification**

Sections from frozen endometrial biopsies were cut (7 μm) and stained with a modified rapid H&E protocol to identify uterine lymphocytes. All solutions, including the stains, were supplemented with 0.5 μg/μl RNase inhibitor (Promega). Slides were air-dried (5 min), placed into a slide box over desiccant and moved to the LCM (Pix Cell IIe; Arcturus). Endometrial lymphocytes were transferred individually to high-sensitivity LCM caps using laser pulse settings of 55 mW for 0.7 ms at 7.5 μm. LCM was performed rapidly at room temperature. Five hundred lymphocytes were captured for each sample. RNA was extracted using a Picopure RNA isolation kit and ExtracSure assembly in an alignment tray (Arcturus) as per manufacturer’s instructions. Briefly, the cap containing laser-captured cells was aligned in the ExtracSure assembly and filled with 10 μl of extraction buffer. After centrifugation to collect cell extract in a microcentrifuge tube, 10 μl of 70% ethanol were added to the cell extract and loaded onto a preconditioned RNA purification column. The cell extract was purified by the RNA purification column. The RNA was eluted in 11 μl of nuclease-free water and stored at −80°C. RNA amplification was conducted using the MessageAmp II antisense RNA (aRNA) kit (Ambion) as per the manufacturer’s instructions. The procedure consisted of reverse transcription with oligo(dT) primer bearing a T7 promoter and in vitro transcription of DNA using T7 RNA polymerase to produce copies of aRNA for each mRNA in the sample. Briefly, to 11 μl of aRNA (2 μg), 1 μl of T7 oligo(dT) primer was added, and the mixture was incubated (10 min, 70°C). Eight microliters of reverse transcription master mix were added to each sample and incubation continued (2 h, 42°C). The resulting cDNA was stored at −20°C. PCR-amplified products of VEGF, HIF-1α, IFN-γ, TNF-α, IL-1, IL-1R, Fas ligand (FasL), and Fas were cloned using the TOPO-TA cloning kit (Invitrogen Life Technologies) as per manufacturer’s instructions. Plasmid DNA was purified by the Genelute plasmid DNA purification kit (Sigma-Aldrich). Sequencing was done at the Molecular Biology SuperCentre, University of Guelph. Sequences were analyzed by the BLASTN program of the National Center for Biotechnology Information portal and deposited to GenBank.

### Quantitative real-time PCR

Real-time PCR (LightCycler; Roche Diagnostics) was used to quantify expression of target genes relative to β-actin in the endometrial biopsies and in lymphocytes. Each sample was analyzed at least twice and averaged. Primers, designed using the Primer 3 software program ([http://frodo.wi.mit.edu/cgi-bin/primer3_web.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3_web.cgi)), are given in Table I. The Quantitect SYBR green I PCR mix kit (Qiagen) was used for the quantification of gene expression. LightCycler reactions were performed in 20 μl of total reaction volume as per manufacturer’s instructions. PCR products were gel-purified using the Wizard DNA purification system (Promega) and/or plasmid DNA with specific inserts were quantified and diluted serially to generate standard curves for each gene. The LightCycler program for each gene was denaturation (94°C, 15 min); PCR amplification and quantification (95°C, 10 s; 58°C, 5 s; 72°C, 20 s) with the fluorescence measurement at specific acquisition temperatures for 5 s, repeated for 45 cycles. The melting program was 70–95°C at the rate of 0.1°C/s with continuous fluorescence measurement, with the final cooling step at 40°C. Data were quantified using ReQuant LightCycler analysis software; the normalized ratio was calculated by the software using the following formula: ((median [target]/median [reference])/median [targetcal]/median [referencecal] × correction factor) × multiplication factor. ReQuant software was used to calculate the normalized ratio using the coefficient file. The correction and multiplication factors were used to correct the PCR efficiency and variations between the different real-time PCR runs. Second-derivative maximum analysis, arithmetic baseline adjustment, and polynomial calculation methods were used for quantification. Baseline curve, melting curve, melting point, crossing point, slope error (0.1–0.5), and correlation (r-1) were critically monitored for each round of analysis. The ratio between the target gene and β-actin was used as a level of mRNA expression.

### Statistical analyses

Statistical analyses were performed by the nonparametric Friedman test using SAS software (SAS 8.2; SAS Institute) for comparison among the groups. Post hoc analysis for planned comparisons between different paired groups was done using the Wilcoxon signed rank test. A value of p < 0.05

#### Table I. Primers used for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Porcine Gene</th>
<th>Primer Position</th>
<th>Primer Sequence</th>
<th>Product Size</th>
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<tr>
<td>VEGF</td>
<td>Forward</td>
<td>ACAGCGAAGGTCGAGGTCT</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAATGCTTCCTCCTGCTCTGA</td>
<td></td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Forward</td>
<td>TTGTAAGTTGGAACATGTAC</td>
<td>228</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>AGGCGTCTGATGTTTCCGA</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Forward</td>
<td>GTGAACCTGCTGAACTGATG</td>
<td>166</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCGCTGCTGATGTTTCCCTGA</td>
<td></td>
</tr>
<tr>
<td>FasL</td>
<td>Forward</td>
<td>GGGCTGCTGATGTTTCCCTGA</td>
<td>246</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTTCCAAGGAGGTATCTTGG</td>
<td></td>
</tr>
<tr>
<td>Fas</td>
<td>Forward</td>
<td>CCACCTGTGACATGTAAGTC</td>
<td>229</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATCATGTCGCCCTCTCTGAC</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward</td>
<td>ACTCACCCCTCCCTCTCTGT</td>
<td>224</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTCGATGTTGGAATGAGG</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward</td>
<td>CAACGTGGAAGCACATGAC</td>
<td>216</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCAGCAGACACACACCACCT</td>
<td></td>
</tr>
<tr>
<td>IL-1R</td>
<td>Forward</td>
<td>CTTCTGCTTCCGATTAACA</td>
<td>213</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGTGGGTGTTGATCACAAGGC</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward</td>
<td>ACATCTGCTGAAAGGTGAC</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACATCTGCTGAAAGGTGAC</td>
<td></td>
</tr>
</tbody>
</table>
was considered significant. Data are presented as box plots showing median and quartiles which were prepared using Statgraphics Centurion software.

Results

Cloning and analysis of partial coding sequences

VEGF, HIF-1α, IFN-γ, FasL, and Fas detected in porcine uterine lymphocytes and TNF-α, IL-1β, IL-1R detected in porcine endometrium were cloned in TOPO-TA cloning vectors. Basic local alignment search tool analysis revealed 98–100% sequence homology with the published nucleotide sequences.

Gene expression in endometrial biopsies

Virgin vs gd19. To determine whether genes promoting angiogenesis are expressed uniformly in pregnant uterus, endometrial biopsies of NP (n = 3) and gd19 (n = 3) gilts were studied for expression of VEGF, HIF-1α, IFN-γ, and TNF-α. All four genes were detected in NP endometrium, and their transcription was altered by pregnancy (Fig. 3). In pregnant uteri, the change in gene expression varied by anatomic location. For VEGF, IFN-γ, and TNF-α, transcription was significantly higher (p < 0.05, p < 0.01) in the mesometrial endometrium compared with antimesometrial and interconceputus attachment sites. For these genes, transcription in the mesometrial interconceputus endometrium was higher than in the antimesometrial endometrium. HIF-1α was most abundantly expressed in antimesometrial tissue (p < 0.05) at gd19, compared with mesometrial and interconceputus tissues.

Time-course analysis with living and dying fetuses. To define more precisely the control of mesometrial angiogenesis immediately postattachment, a time-course study was undertaken from gd15–23 with three or more pregnancies per time point. From gd20, arresting fetuses could be identified in each litter and tissues associated with each of these fetuses were processed separately from tissues associated with apparently healthy, viable conceptuses. In endometrium from attachment sites with healthy conceptuses, both VEGF (Fig. 4A) and HIF-1α (Fig. 4B) were expressed and expression progressively increased to gd23. No VEGF and minimal HIF-1α were found at attachment sites of arrested fetuses. In healthy attachment sites, endometrial transcription of IFN-γ was highest at gd15. For the other gd studied, levels were only slightly above those in virgin uteri (Fig. 4C) and no significant differences were found. Endometrium associated with arresting fetuses had much higher transcription of IFN-γ than endometrium associated

![FIGURE 3](http://www.jimmunol.org/)

Transcription of VEGF (A), HIF-1α (B), IFN-γ (C), and TNF-α (D) in endometrial subregions at gd19. For each region, samples were collected relative to multiple implant sites in three gilts. Three or more NP gilts were also studied. Samples were dissected from the mesometrial (entry site of maternal blood vessels), antimesometrial (opposite to mesometrial side), and mesometrial interconceputus endometrium. Gene expression is relative to β-actin and is expressed as a normalized ratio that differs between anatomical subregion. Significantly higher expression for VEGF, TNF-α, and IFN-γ was found mesometrially (*, p < 0.05, **, 0.01), compared with antimesometrially or in interconceputus endometrium. Gene expression is relative to β-actin and is expressed as a normalized ratio that differs between anatomical subregion. Significantly higher expression for VEGF, TNF-α, and IFN-γ was found mesometrially (*, p < 0.05, **, 0.01), compared with antimesometrially or in interconceputus endometrium. HIF-1α expression was significantly higher antimesometrially, compared with mesometrially and between conceptus attachment sites (p < 0.05). M, Mesometrial side; AM, antimesometrial side; IC, interconceputus. Data are presented by box plot showing median and quartiles.
with viable littersates ($p < 0.05$) at both gd21 and 23 and IFN-γ transcription increased ($p < 0.01$) in these sites between gd21 and gd23.

To determine whether changes in proinflammatory cytokines other than IFN-γ correlated with fetal arrest, TNF-α, IL-1β, and IL-1R were quantified in endometrium at gd21 and 23. All three genes were highly up-regulated in endometrium from arresting conceptus attachment sites (gd21, data not shown; gd23 (Fig. 4F)). IL-1β was the most elevated transcript followed by TNF-α, IFN-γ, and IL-1R. In the attachment sites of healthy conceptuses, endometrial transcription levels for these genes were significantly lower ($p < 0.01, p < 0.05$).

FasL, a molecule associated with apoptosis, was induced in the endometrium by pregnancy. Endometria associated with arresting fetuses had significantly higher levels ($p < 0.05$) of FasL expression than endometria associated with healthy fetuses (Fig. 4D). Expression of Fas in endometria from healthy implantation sites was variable but always lower than that in endometria from sites with arresting fetuses ($p < 0.05$; Fig. 4E). At gd23, Fas expression in arresting attachment sites was significantly higher than in healthy littermate sites ($p < 0.01$).

Endometrial lymphocytes

To determine the contribution of endometrial lymphocytes to angiogenesis during early pregnancy, a time-course analysis of transcription of VEGF, HIF-1α, and IFN-γ by pools of 500 LCM-captured mesometrial lymphocytes was conducted. VEGF expression progressively increased from NP to gd23, compared with gd15 and 19 ($*, p < 0.05$) with significantly higher VEGF expression in healthy gd21 attachment sites ($**, p < 0.01$). Unexpectedly, no VEGF and significantly lower HIF-1α expression were detected in attachment sites of arresting conceptuses at gd21, compared with healthy sites ($p < 0.05$). Both FasL and Fas were expressed in NP uterus but at lower levels. Elevated levels of FasL/Fas were detected at the attachment sites of arrested conceptuses ($*, p < 0.05; **, p < 0.01$) at both gd21 and 23, compared with healthy attachment sites. At gd15, significantly elevated Fas was found, compared with gd19 and gd21 healthy attachment sites ($p < 0.05$). Gene expression was normalized and expressed as a ratio of target to the housekeeping gene, β-actin. F. Expression of proinflammatory cytokines at the endometrial attachment sites of gd23-arresting fetuses. Significantly elevated IFN-γ, TNF-α, IL-1β, and IL-1R were observed at arresting sites, compared with healthy sites ($*, p < 0.05; **, p < 0.01$). ND, Not detected; H, healthy; A, arresting.
containing arresting fetuses. IFN-γ transcription was similar in lymphocytes from NP uteri and from healthy implantation sites but significantly higher in lymphocytes from attachment sites with arresting fetuses at gd21 (Fig. 5C; \( p < 0.05 \)), then declined at gd23 but remained significantly higher (\( p < 0.05 \)) than in healthy attachment site lymphocytes. In continuing studies at gd 30, we have found that the arresting embryos have, for the most part, been successfully eliminated. We therefore speculate that the implant site crisis started before we could see gross differences at gd21 and that it was resolving by gd23 when lower IFN-γ transcription was found in endometrial lymphocytes.

FasL and Fas (Fig. 5, D and E) were transcribed by endometrial lymphocytes from NP animals and the transcripts were significantly elevated after gd15 (\( p < 0.01, p < 0.05 \)). In healthy attachment sites, lymphocyte transcription of both genes peaked at gd21 and then declined. Both genes were more abundantly transcribed in lymphocytes associated with implantation sites containing arresting fetuses and, again, levels at gd21 exceeded those at gd23.

Trophoblasts

Trophoblasts were dissected from normal and arresting fetuses and examined for gene expression at gd21 and gd23. VEGF transcription increased (\( p < 0.01 \)) between gd21 and 23 in trophoblasts from healthy fetuses but, at both time points, trophoblasts had fewer transcripts than did maternal lymphocytes from the same implantation site (Fig. 6A). VEGF transcription was significantly lower in trophoblasts from arresting compared with healthy fetuses (\( p < 0.05 \)) and declined from gd21 to 23. HIF-1α transcription decreased in trophoblasts between gd21 and 23 whether or not the fetus was arresting (\( p < 0.05 \); Fig. 6B). Trophoblastic IFNs are produced abundantly in pigs from gd12. At gd21, there was no noticeable difference in IFN-γ expression by trophoblasts from the two types of littermates (Fig. 6C), but by gd23, IFN-γ expression had increased ~10-fold in trophoblasts of arresting fetuses (\( p < 0.05 \)). Transcription of FasL (Fig. 6D) and Fas (Fig. 6E) changed dynamically in the trophoblasts of healthy gd21 and 23 fetuses with the decline of FasL and elevation of Fas (\( p < 0.05 \)). Low FasL transcription and high Fas transcription were found in trophoblasts from arresting fetuses. The elevated expression of both IFN-γ and Fas in gd23 trophoblasts indicates that the decline of expression of other genes in trophoblasts from arresting fetuses was not yet due to loss of trophoblast cell viability.

**Discussion**

Angiogenesis is a carefully regulated process that occurs in both maternal and fetal implantation site tissues. The pig is an excellent...
animal for study of implantation site angiogenesis and its maternal-fetal cross-regulation because maternal and fetal tissues are clearly distinguishable and can be studied independently as pure cell types. Pig placentation is simply apposed epitheliochorial; the uterine epithelium is not breached by trophoblasts (20). The endometrium was not uniform in its expression of VEGF, the gene we selected as a prototypic marker for angiogenesis. The highest VEGF expression occurred mesometrially, where major branches of the uterine and ovarian arteries supply the uterus. Species with epitheliochorial placentation use growth of subendothelial capillary networks to expand maternal blood supply to the placenta rather than spiral arterial modification. Nonetheless, porcine endometrial lymphocytes share angiogenic potential with uNK cells from hemochorial implantation sites of women and mice (7, 21). When compared with entire endometrium, transcription of VEGF was much higher in lymphocytes than in the complex tissue, suggesting lymphocytes may be major stimuli for endometrial angiogenesis during gestation. VEGF transcription in lymphocytes also exceeded that in trophoblasts, the interface tissue more commonly studied.

Lymphocytes in normal porcine implantation sites transcribe IFN-γ, as reported for uNK cells in species with hemochorial placentation (5–7). Previously, IFN-γ in porcine implantation sites was solely attributed to trophoblasts. Our relative quantification indicated that lymphocytes, not trophoblasts, are likely the major producers of this cytokine in early porcine implantation sites. TNF-α transcription was not found in the lymphocytes from normal implantation sites but low, differentially distributed levels occurred in healthy implantation site endometrium (Fig. 3D).

uNK cells are enriched 2- to 3-fold in pig mesometrial endometrium early after trophoblast attachment (1), but because we dissected all lymphocytes, IFN-γ production cannot be attributed exclusively to uNK cells. Similarly, contributions of lymphocyte subsets other than uNK cells to angiogenesis and to oxygen sensing cannot be established until reagents become available for rapid discrimination of porcine uterine lymphocyte subsets and permit reassessment of our data using lymphocyte subset analysis. We also cannot assume that the lymphocyte subset mixtures we studied on different gd or between living and arresting littersmates were similar. The transcriptional changes we documented may reflect either activation of cells already at attachment sites, transcripts in newly recruited cells or a mixture of both. It is anticipated that many genes, in addition to those examined, are altered in healthy...
implant sites and that novel as well as classical mechanisms could contribute to implantation site angiogenesis.

Hypoxia is a potent stimulus for VEGF production and is thought to be essential for development of both embryonic and placental vasculature in early human pregnancy (22, 23). HIF-1α, a prime regulator of oxygen homeostasis, binds to the hypoxia response element in the VEGF promoter (24) and is reported to regulate peri-implantation angiogenesis in humans (25), mice (26), and sheep (27). Our finding of HIF-1α expression in a species with epitheliolarchorial placentation suggests that transcription is promoted by attachment and growth of conceptuses (Fig. 4C). Induction was, however, variable (Fig. 3B), and there was significant transcription of HIF-1α in the antimesometrial endometrium.

The pig provides a strong experimental model for investigations of peri-implantation spontaneous loss of apparently normal conceptuses. The fetuses we classified as arresting were not dead. Indeed, their trophoblasts showed elevated transcription of some genes, indicative of a response to stress or environmental change. A highly localized regulatory step effectively blocked maternal transcription of VEGF and HIF-1α. The most probable source for a highly localized regulator, not affecting the entire litter, is trophoblasts within the individual implantation site. A number of danger signals have been described that are recognized by immune cells (28). Most of these evoke dendritic or phagocytic cell recognition of dying cells (29, 30). Danger signals include natural or endogenous adjuvants such as stress and heat shock proteins, fragments of fibronectin, hyaluronic acid, free DNA, CpG oligonucleotides, and uric acid (31, 32). Elevated circulating fetal DNA and trophoblast membrane fragments are associated with human fetal stress (33, 34). TLRs bind some danger molecules and may directly activate lymphocytes. Endometrial immunity could also become activated via the complement cascade (35, 36), changes in soluble forms of trophoblast transplantation Ags (37), or induction by trophoblast of adjuvant cytokines such as IL-12 (38). The finding of elevated IFN-γ gene expression in trophoblasts from arresting fetuses strongly supports the last mechanism. A trophoblast-derived natural adjuvant signal could induce the endometrial cytokine storm documented with gd21 and 23 fetal retardation. A key question is what cell types and tissues are targets of this cytokine aggression? We suggest the target is neither trophoblast nor fetus. Rather, we hypothesize that the immune storm is directed toward the maternal vasculature and its purpose is to eliminate/destroy maternal support for a dangerous, stressed, or about-to-fail conceptus by initiation of death in active endothelial tip cells and in those uNK cells providing the strongest support for angiogenesis.

Support for endometrial targets also comes from our examination of FasL/Fas expression. Trophoblasts dynamically express both molecules differentially between viable and arresting conceptuses. Thus, there is no absolute requirement for death signals of maternal origin. FasL and Fas are also elevated in the endometrium and in endometrial lymphocytes associated with arresting, compared with healthy, conceptuses. This combined elevation would be expected to protect some but not all lymphocytes and some but not all endometrial cells from death signals.

Further studies will be needed to resolve when heterogeneity for survival becomes established between porcine littersmates and whether mechanisms defined in this model of peri-implantation pregnancy failure are applicable to species with other placental types, particularly humans. We have documented for the first time the major and relative contributions of endometrial lymphocytes to both angiogenesis and oxygen sensing in normal implantation sites and the complete cessation of maternal angiogenesis in sites with arresting conceptuses. This report has also defined an experimental pregnancy model that can be interrogated in a precise and detailed way not only to promote agriculture but also to give insight into potential pathways for human embryo loss following conception or assisted reproductive techniques.

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Disclosures

The authors have no financial conflict of interest.

References


