Cellular and molecular events in early and mid gestation porcine implantation sites: a review

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Commercial, North American pork breeds (*Sus scrofa*) experience significant loss of genetically-normal conceptuses during the peri-implantation (attachment) period and at mid-gestation (day 50 to 90 of the 114 day porcine gestation interval). Although exact causes for these losses are not defined, asynchronous *in-utero* development and deficits in vascularization of the endometrium and placenta appear to be involved. Understanding of normal maternal-fetal dialogue is critical to develop breeding or therapeutic strategies that improve fetal health and overall litter size in commercial pigs. The non-invasive, epitheliochorial porcine placenta permits investigation of maternal or fetal compartments without cross contaminating cells. We developed and use protocols to capture single, homogenous populations of porcine cells (endometrial lymphocytes, dendritic or endothelial cells) from histological sections using laser capture microdissection (LCM), a powerful tool for study of gene expression that reflects the *in vivo* environment. These data are compared with gene expression in biopsies of endometrium and of trophoblast from the same, attachment sites. Here we review justifications for selection of the genes we have studied and our published and in progress work. These data provide new insights into the roles of the endometrial immune environment in the regulation of the success and failure of porcine conceptuses.

Introduction

Prenatal mortality is a major economic concern for commercial swine producers in. North America. In gilts, approximately, 20 to 30% of conceptuses die between gestation days (gd)12-30 and another 10-15% are lost by midgestation (114 day pregnancy, Pope *et al.* 1986, Pope 1988, Pope 1994). The ovulation rate in these animals is 16 to 18 with > 95% fertilization. This should result in 14 to 17 embryos at the beginning of pregnancy. By farrowing however, litters are reduced to ~10 (Pope 1994). The pig uterus is estimated to have the capacity to carry 12 to 14 fetuses to term (Freking *et al.* 2007).

Conceptus-derived growth factors supplemented by histiotrophic nutrition derived from the maternal uterine glands (Spencer & Bazer, 2004) support pre-elongation conceptuses. Growth and development of post-attachment (>gd15) conceptuses require endometrial-placental interactions that greatly expand local, through attachment site endometrial angiogenesis. Most

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porcine gestational losses occur as elongating conceptuses start secreting estrogen to promote endometrial attachment (Geisert *et al.* 1982). It is difficult to quantify losses of elongating conceptuses between gd13 to 20 due to their extreme fragility (Pope 1994). Trophoblast-derived estrogen is considered to signal maternal recognition of pregnancy and alters the endometrium to support attachment (Geisert & Yelich 1997). The estrogen secreted by the earliest elongating blastocysts creates a hostile endometrial *milieu* for less advanced conceptuses. Asynchronous development is a major factor influencing the first peri-attachment wave of conceptus loss (Pope 1994). A mixture of interferons (IFN) unique to porcine trophoblast is produced by early, peri-attachment embryos. Immune IFNG and IFND reach peak levels at gd15 (La Bonnardière *et al.* 1991, Cencic & La Bonnardière 2002). Porcine trophoblastic IFNs, unlike IFNT in cattle and sheep, do not act on corpora lutea but alter polarity of the uterine epithelium through the gain of basal tight junctions during conceptus attachment (Cencic *et al.* 2003).

In other species, roles have been identified for the immune system in promotion of implantation and conceptus survival as well as in conceptus demise (Raghupathy 2001, Croy *et al. 2006*, Seavey & Mosmann 2008). In humans, pregnancy success is associated with a switch from a type 1 dominant, pro-inflammatory cytokine profile (the normal non pregnant state) to a type 2 dominant, anti-inflammatory profile in blood and in endometrium between mid to late pregnancy (Raghupathy 2001, Borzychowski *et al.* 2005, Aris *et al.* 2008).). Type 1 cytokine dominance in later gestation is considered to compromise pregnancy success (Raghupathy & Kalinka 2008). As in humans and mice, early porcine pregnancy is dominated by pro-inflammatory type I cytokines such as IFN-γ and TNF-α (Croy *et al.* 2006, Tayade *et al.* 2007, Curry *et al.* 2008). In addition to creating the endometrial cytokine *milieu*, the endometrial immune systems of humans and mice are strongly angiogenic in early gestation. The studies reviewed and reported here establish that immune cells of early porcine gestational endometrium make important, exquisitely localized contributions to conceptus attachment sites (CAS) that regulate conceptus fates.

Transcripts for genes regulating angiogenesis in porcine endometrium and CAS

VEGF and VEGFR-mediated angiogenesis at porcine implantation sites

Angiogenesis is the process of generation of new blood vessels from existing vasculature. It requires endothelial cell activation, tip cell differentiation, stalk cell proliferation and lumen formation. Maturation of new blood vessel is accompanied by recruitment of pericytes, smooth muscle cells and circulating bone-marrow derived cells (Takakura 2006, Grunewald *et al.* 2006). This is coupled with formation of an underlying basement membrane (Holderfield & Hughes 2008). Extensive, localized endometrial angiogenesis occurs during pregnancy to support each developing conceptus and its placental exchange system.

Angiogenesis is regulated by variety of growth factors but VEGF and the VEGF receptor (R) system appear to be the most important and have been targeted clinically in humans (Loges *et al.* 2009). VEGFs are a family of heparin binding growth factors. In humans, at least eight *VEGF* isoforms (*VEGF121, VEGF145, VEGF148, VEGF165, VEGF165b, VEGF183, VEGF189, and VEGF206*) are generated by alternative splicing of a single *VEGF* mRNA (Breen 2007). Similarly, eight *VEGF* splice variants have been identified in pigs. Of these, splice variants, *VEGF*120, 144, 164, 164b, 188 and 205 are exactly one amino acid shorter than the human variants while *VEGF* 147 and 182 are unique porcine isoforms (Ribeiro *et al.* 2007). VEGF signals through VEGFRI, VEGFRII and VEGFR III and binds additionally to soluble VEGFRI (sVEGFRI), a splice variant of VEGFRI and to neuropilin-1 (NRP1). VEGFRI acts primarily as a regulator of VEGF availability (Holderfield & Hughes 2008, Zygmunt *et al.* 2003). Soluble VEGFRI, which is elevated in human and mouse pregnancy complications (Levine *et al.* 2004),

prevents VEGF signaling by sequestering VEGF (Zygmunt *et al.* 2003, Breen 2007). VEGF promotes migration of vascular smooth muscle cells through its binding to VEGFRI and to NPR1 (Banerjee *et al.* 2008). It induces endothelial cell migration and proliferation primarily through binding to VEGFRII (Holderfield & Hughes 2008). VEGFRII is the dominant receptor promoting endothelial cell permeability. Placenta growth factor (PGF) is a high affinity ligand for VEGFR1 (Carmeliet 2001).

To understand the dynamic features of angiogenic gene expression at the porcine maternal-fetal interface, mRNA comparisons for *VEGF, PGF, VEGFRI* and *VEGFRII* were carried out in various endometrial tissue microdomains and in endometrial biopsies and trophoblasts at gd20 and 50 from healthy conceptus attachment sites (H-CAS) and arresting conceptus attachment sites (A-CAS). Details of sample processing and techniques are summarized in Fig. 1 and are previously published (Tayade *et al.* 2006, 2007, Linton *et al.* 2008). Angiogenic gene profiles were studied by quantitative real time PCR analyses relative to housekeeping gene, *ACTB* (β-*actin*). Transcripts for *VEGF, PGF, VEGFRI* and *VEGFRII* were detected in virgin uterus but their abundance differed between mesometrial and anti-mesometrial sides. In pregnancy, A-CAS at both gd20 and 50 had severely reduced numbers of *VEGF* transcripts in endometrial biopsies compared with time-matched endometrium from H-CAS. *VEGF* transcripts in trophoblast were relatively stable between H-CAS and A-CAS. These differences suggested the maternal compartment had sensed "danger" signals emitted from individual conceptuses and was adjusting to promote demise of that conceptus while the conceptus maintained *VEGF* transcription in its trophoblast for survival. Transcripts for *VEGFRI and II* were variable between H-CAS and A-CAS during both gd20 and 50 time points (Tayade *et al.* 2007).

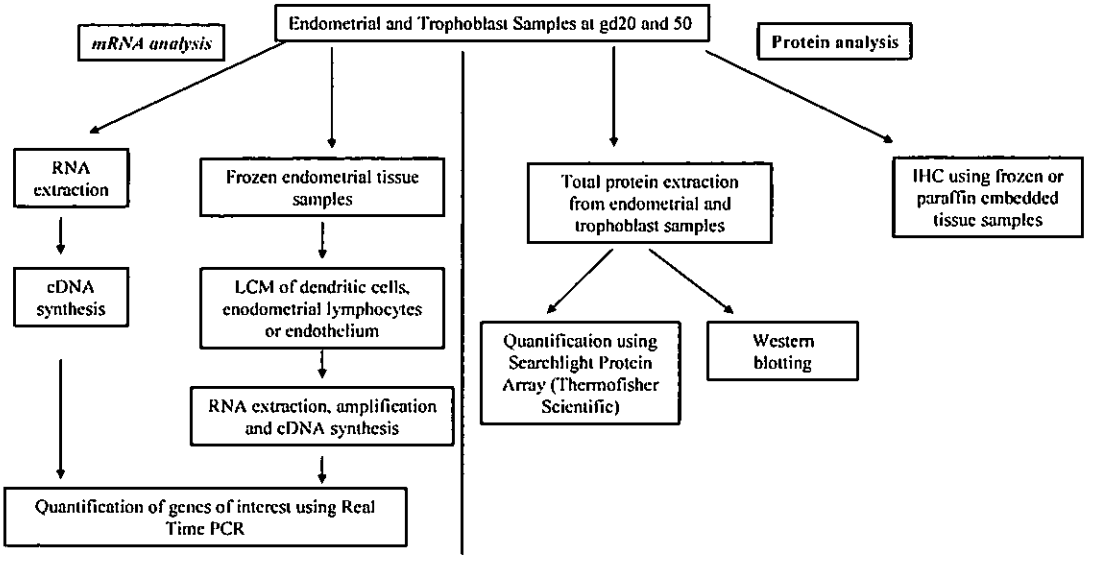

Fig. 1 Diagrammatic representation of sample utilization in various downstream applications. Each sample is processed either for mRNA or protein analysis as described in Tayade *et al.* 2006, 2007. All samples were collected from specific pathogen-free Yorkshire gilts housed at Arkell Swine Research Station, University of Guelph. Littermate H-CAS and A-CAS were identified based on gross fetal membrane vasculature at gd20 and length/weight parameters at gd50 (Tayade *et al.* 2006, 2007). The data were analyzed (unless otherwise stated) by PROC MIXED procedure of SAS using a mixed linear model that included the effects of gestation date, tissue, and health, as well as their interactions.

Neuropilins, semaphorins and plexins in angiogenesis

The numerous VEGF isoforms bind endothelial cell receptors that include the VEGFRI and VEGFRII signaling tyrosine kinase receptors and the non-signaling co-receptor NRP1. NRP1 and NRP2 were identified in 1995 in the nervous system as receptors for class 3 semaphorins (SEMA) which repel axonal outgrowth (Satoda *et al.* 1995, He & Tessier-Lavingne 1997, Kolodkin *et al.* 1997). They were subsequently found to act in endothelial cells as receptors for several pro-angiogenic factors, including $VEGF_{165}$ (Soker *et al.* 1998), PGF (Soker *et al.*1998) hepatocyte growth factor (Sulpice *et al.* 2008), and galectin1 (Hsieh *et al.* 2008). Interestingly, NPR1 as well as VEGF and VEGFRII are up-regulated on endothelial cells in hypoxic conditions (Ottino *et al.* 2004).

While NPR1 and NPR2 are only 45% homologous, they are structurally similar with five extracellular domains, each of which has specific binding partners and a short cytoplasmic tail incapable of signal transduction. The a1 and a2 domains bind SEMAs. The b1 and b2 domains bind VEGF isoforms and heparin and are implicated in cell-cell adhesion. The c domain is responsible for dimerization. As shown in Table I, NPRs also differ in their VEGF and SEMA binding partners. NPRs themselves are non-signaling co-receptors which require complexing with receptors with signaling capability to effect downstream functions. NPR1, which is expressed on endothelial cells, is associated with angiogenesis and arterial differentiation while NPR2, expressed in veins and lymphatic vessels, is involved in lymphangiogenesis (Karkkainen & Alitalo 2002, Yuan *et al.* 2002).

Table I. Neuropilin (NRP) Interactions

Gene	Pro-angiogenic Ligands	Pro-angiogenic Complex Partners	Anti-angiogenic Complex Partners	Anti-angiogenic Ligands
NRP1	VEGF165, VEGF-B, VEGF-E, PGF, HGF, PDGFBB, FGF2, TGF-β, galectin1	VEGFRI, VEGFRII,	PLXNA1, PLXNA2, PLXNA3, PLXNA4, PLXND1, integrin β1, LICAM	SEMA3A SEMA3B SEMA3C SEMA3D
NRP2	VEGF145, VEGF-C, PGF, HGF	VEGFRI, VEGFRII, VEGFRIII,	PLXNA1, PLXNA2, PLXNA3, PLXNA4, PLXND1, NRCAM	SEMA3C, SEMA3D SEMA3F, SEMA3G

HGF: hepatocyte growth factor; LICAM: neural cell adhesion molecule L1 (LICAM); NRCAM: neuronal cell adhesion molecule.

NPRs bind Class 3 SEMA, which are a large and diverse family with pivotal roles in axon guidance, organogenesis, neoplastic transformation, immune responses, vascularization and angiogenesis (Soker *et al.* 1998). SEMA functions through two receptor families; the NRPs and the plexins (PLXN). SEMA3s, a seven member family, are the only secreted semaphorins. Six SEMA3s bind to NRP1, NRP2 or both (see Table I).The seventh, SEMA3E, binds to the receptor plexin-D1, which in turn complexes with NRPs (Kolodkin *et al.* 1997). SEMA3A and SEMA3F bound by NRP and complexed with a plexin (PLXN) family member are anti-angiogenic because the complex suppresses endothelial cell migration (Guttmann-Raviv *et al.* 2007). Similarly, complexing NRPs and PLXN with SEMA3B and SEMA3F inhibited adhesion, migration and proliferation of lung cancer-derived tumor cells but the mechanism of these interactions is unknown. SEMA3A, SEMA3D, SEMA3E, and SEMA3G also have anti-tumorigenic properties and independently reduce the density of blood vessels in tumors (Neufeld & Kessler 2008).

The roles of NRPs in blood vessel development during embryogenesis and tumour progression are well-studied. However, their roles in the only normal angiogenesis found in adults, the cycling female reproductive tract, have not been thoroughly addressed. Pavelock *et al.* found that physiological variations in different sex hormones, particularly progesterone, increased

NPR1 mRNA in rat uterus (Pavelock *et al.* 2001). These data gave us the impetus to quantify expression of NPRs and their binding and complexing partners in the virgin and pregnant porcine uterus. Transcripts of *NPR1* and *NPR2*, *SEMA*-A3 *C3, E3, F3* and *G3* as well as *PLXN A1, A2, A3, A4* and *D1* were detected in the virgin pig uterus as well as in endometrial and trophoblast samples at gd20 and 50. Quantification of these genes is in progress (M van den Heuvel, unpublished observations).

Lymphocytes and dendritic cells in the promotion of endometrial angiogenesis during pregnancy

We found porcine endometrium of early pregnancy contained mildly elevated (~3x) numbers of uterine natural killer (uNK) cells, a unique lymphocyte subset, between gd15 to 28. During pregnancy, these cells were found scattered throughout the stroma, beneath luminal epithelium, around blood vessels and uterine glands (Engelhardt *et al.* 2002). This interval coincides with onset of angiogenesis at CAS. In contrast to the recruitment of uNK cells in humans and mice that is driven by decidual cells, porcine uNK cells were not recruited in pseudopregnancy but required conceptus-derived signals that are not yet identified (Engelhardt *et al.* 2002). Porcine uNK cells are agranular and their identification required dual antibody staining against CD16 and CD8 surface receptors to distinguish them from T cells. Recently, Dimova *et al.* (2007) examined changes in endometrial T cells (CD4 and CD8) during early porcine pregnancy. T-cells formed clusters in areas of conceptus attachment but no significant differences were found in their numbers at or between CAS. T lymphocytes increased in the sub-epithelium from 15% of leukocytes in the pre-attachment phase to 54% right after attachment at gd15. Maximal T lymphocyte numbers reached 85% at gd30 after the formation of placenta (Dimova *et al.* 2007). In our studies, we noticed scattered lymphocytes in the luminal epithelium but due to lack of commercially available porcine specific monoclonal antibodies, phenotypic characterization of the intra-epithelial lymphocytes could not be carried out. Thus, our studies of gene expression in endometrial lymphocytes have not yet been refined to address lineage subsets. We employ frozen endometrial tissue sections rapidly stained with haematoxylin and eosin and laser capture microdissection (LCM) to collect pure populations of either lymphocytes or dendritic cells (DC; Tayade *et al.* 2006, Linton *et al.* 2008) to address our hypothesis that immune cells contribute to and regulate endometrial angiogenesis during porcine pregnancy.

Endometrial lymphocytes obtained from healthy and arresting conceptus attachment sites at gd20 and gd50 were screened for the expression of *VEGF, PGF, VEGFRI and VEGFRII* by quantitative real time PCR. Endometrial lymphocytes had a much greater abundance of *VEGF* transcripts than endometrial endothelial cells or trophoblasts. Attachment sites associated with arresting conceptuses had a severe reduction in *VEGF* and gain in *PGF* transcripts in lymphocytes. Lymphocytes preferentially expressed transcripts for *VEGFRI* whereas trophoblasts had abundant *VEGFRII* transcripts showing there are differences in the mechanisms that regulate angiogenesis in the maternal and fetal compartments (Tayade *et al.* 2007).

In humans, a population of immature dendritic cells was identified in decidua via their surface expression of DC-SIGN (Dendritic cell specific ICAM grabbing non-integrin, DC-SIGN) (Kammerer *et al.* 2003, 2008). These antigen presenting cells were in contact with uNK cells in the vicinity of spiral arteries, an interaction requiring DC-SIGN on the DCs and ICAM-3 on the uNK cells (Kammerer *et al.* 2003). Because uNK cells secrete abundant VEGF but do not express VEGFRI or II (Li *et al.* 2001), we hypothesize that endometrial DC-SIGN$^+$ DCs and uNK cells may additionally communicate via VEGF and its receptors. This would also predict perivascular co-localization of these cell types to sites of active endothelial cell proliferation (Grunewald *et al.* 2006). Using a cross-reactive anti-human DC-SIGN antibody, we identified DC-SIGN+ DCs

in porcine endometrium. In contrast to human endometrium where the cells are only present in pregnancy, both virgin and gestational porcine endometrium contained DC-SIGN+ cells (Linton *et al.* 2008). In pregnancy, DC-SIGN+ cells were frequently associated with lymphocytes near blood vessels. The numbers of DC-SIGN+ cells appeared to be stable between early and mid pregnancy. Using a modified immunohistochemistry protocol for rapid staining, we immunostained DC-SIGN+ DCs in frozen porcine endometrial sections, isolated them using LCM and recovered RNA for analysis. We report for the first time that porcine DC-SIGN+ DCs transcribe angiogenic factors (*VEGF, VEGFRI and II*) and thus contribute to the regulation of angiogenesis at CAS (Linton *et al.* 2008 and N F Linton, unpublished observations).

Roles of chemokines and chemokine decoy receptors at the porcine maternal fetal interface

Chemokines are families of small cytokines characterized by the presence of four conserved cysteine molecules. They range from 8 to 11 kDa and are active over a 1 to 100 ng/ml range in concentration. The major function of chemokines is to direct immune cell movement towards chemotactic stimuli (Charo & Ransohoff 2006). Chemokines are classified as CC, CXC, CX3C or XC (Charo & Ransohoff 2006). Almost all somatic cell types synthesize pro-inflammatory as well as homeostatic chemokines. Chemokines can be induced by variety of stimuli including viruses, bacteria, pro-inflammatory cytokines, anaphylatoxin C5a, leukotriene B_4, and IFNs (Drake *et al.* 2002, Huang *et al.* 2006). Although their main function is chemo-attraction, they also participate in angiogenesis, haematopoiesis, and regulate activation, proliferation, differentiation, and apoptosis in the cells they attract (Drake *et al.* 2002, Hannan *et al.* 2006).

Chemokine decoy receptors are non-signaling, membrane-bound receptors. They are responsible for regulation of cell trafficking, inflammatory responses, immune reactions, angiogenesis, and apoptosis by internalizing and degrading chemokines (Mantovani *et al.* 2001, Mantovani *et al.* 2003, Borroni *et al.* 2008). In humans and mice, three chemokine decoy receptors have been characterized. D6 and DARC (duffy antigen receptor for chemokines) bind inflammatory chemokines while CCX CKR binds homeostatic chemokines (Gosling *et al.* 2000, Townson & Nibbs 2002). In humans and mice, D6 is expressed by invasive trophoblast cells (Montavani *et al.* 2003, Martinez de la Torre *et al.* 2007). D6 knockout mice are fertile but have compromised pregnancy outcome if inoculated with lipopolysaccharide or auto-immune human anti-phospholipid antibodies during pregnancy (Martinez de la Torre *et al.* 2007). DARC and CCX CKR are also found in human and mouse placentas (Girard *et al.* 1999, Townson & Nibbs 2002, Martinez de la Torre *et al.* 2007). However, roles for decoy receptors and their binding chemokines have not been characterized in spontaneous fetal loss or in pregnancies with non invasive trophoblast. We have investigated selected members of these molecular families that are expected in porcine pregnancy.

Transcripts for chemokines that expected to bind to porcine D6 (*CCL2, CCL3L1, CCL4, CCL5* and *CCL11*), DARC (*CCL2, CCL5, CCL11* and *CXCL2*) or CCX CKR (*CCL21*) were assessed in paired gd20 and 50 endometrial and trophoblast samples from H-CAS and A-CAS. Transcripts were found for all of the genes in gd20 and gd50 endometrium and trophoblast. No significant differences were found in the expression of these chemokines between healthy and arresting conceptus attachment sites (Wessesl *et al.* 2007 and J M Wessels, unpublished observations).

Endometrial transcripts for *D6* were more abundant than those in trophoblast at gd20 and 50. Transcript numbers were stable at healthy versus arresting sites. In contrast, transcripts for the homeostatic decoy receptor *CCX CKR* were elevated in gd50 trophoblasts and endometrium

from A-CAS (J M Wessels, unpublished observations). Both molecules were localized by immunohistochemistry to uterine epithelium at the CAS. Thus, the importance of inflammation in gestational failure in pigs appears to be much less than in mice while dysregulation of porcine homeostasis may have a greater role in both maternal and fetal tissues. The role of homeostatic chemokines decoy receptors in pregnancy failure or success in other species has not been studied yet. Thus, the relevance of these findings for species with non epitheliochorial placentation is currently unknown and needs to be defined.

Role of pro-inflammatory cytokines at the porcine maternal fetal interface

The adverse effects of elevated pro-inflammatory cytokines during human and mouse pregnancy are extensively studied (Polgar & Hill 2002, Dent 2002, Patrick & Smith 2002). Several reports linked TNFA, combined with IFNG and IL-1B in promoting pathology and pregnancy failure. In women with recurrent spontaneous abortions, synergistic effects of TNFA and IFNG lead to endothelial cell injury, reduced blood supply and subsequent embryonic death (Stemmer 2000). In addition to the deficits in endometrial angiogenesis described above at porcine A-CAS, we have documented elevations in proinflammatory cytokine gene transcripts (*IFNG*, *TNFA* and *IL-1B*). Endometrial lymphocytes appeared to switch transcription abruptly from angiogenic to pro-inflammatory cytokine genes.

Based on these findings we proposed paradigm shifting functions for endometrial lymphocytes (Leonard *et al.* 2006). We feel the elevated pro-inflammatory cytokines attack maternal endothelial cells ultimately restricting blood supply to an already stressed conceptus rather than envisioning trophoblast as the target of immune attack. This raises the possibility that elevations in pro-inflammatory cytokines are an aftermath of impending conceptus death. In pigs, this immune signaling is unlikely to be an immune scavenging signal because we did not observe signs of inflammation (infiltration of neutrophils/lymphocytes) at A-CAS at gd20 or 50. Definition of this cause and effect relationship will require further study. We found significantly elevated expression of *IFNG* in endometrial lymphocytes and trophoblasts collected from gd20 A-CAS but not in gd50 A-CAS. Rather, *TNFA* was elevated in lymphocytes obtained from gd50 A-CAS. The dominance of *IFNG* during early loss (gd20) and *TNFA* during mid gestational loss (gd50) suggests distinct immune mechanisms effect conceptus health at different stages of gestation (Tayade *et al.* 2007).

Insulin like growth factors 1 and 2 in porcine fetal loss

Insulin like growth factors (IGF-1 and IGF-2) are small polypeptides of approximately 7kDa implicated in regulation of fetal and placental growth. They promote cellular differentiation, proliferation and migration and inhibit apoptosis. These processes are involved in the extensive remodeling that occurs during development of the placenta and its endometrial attachment site. The IGFs bind with high affinity to IGF receptors, namely IGF1R and IGF2R. IGF1R is a member of the tyrosine kinase family and is structurally related to the insulin receptor (Jones & Clemmons 1995, Butler & LeRoith 2001). IGF1R binds with equal affinity to both IGF-1 and IGF-2, where as IGF2R binds only IGF-2 with high affinity (Pollak 2008). The bio-availability and biological actions of IGFs are regulated by at least six insulin-like growth factor binding proteins (IGFBP1 to 6, Clemmons 1997). IGF ligation to binding proteins may augment or inhibit IGF action. Several proteases cleave IGFBPs, reducing or eliminating their ability to bind IGFs. From a series of knockout studies, it is clear that IGF2 rather than IGF1 plays important roles in mouse placental and fetal development (Baker *et al.* 1993).

The IGF system has been extensively studied during the porcine estrous cycle and in early pregnancy (Simmen *et al.* 1992, Ashworth *et al.* 2005). *IGF-1* mRNA decreased while *IGF-2* mRNA increased as pregnancy advanced. Highest levels were found in the placenta followed by endometrium and myometrium (Simmen *et al.* 1992). There is a spatiotemporal relationship between increased uterine IGF-1 and IGF-2 and estrogen synthesis by the elongating conceptuses at gd10 to 13 (Letcher *et al.* 1989, Geisert *et al.* 2001). Increased uterine IGF is associated with aromatase production for trophoblast estrogen synthesis (Green *et al.* 1995). Ashworth *et al.* (2005), documented premature loss of IGFs during the period of conceptus elongation by early exposure of pregnant gilts to estrogen. This loss of IGFs was attributed to proteolysis of IGFBPs due to endocrine disruption caused by exogenous administration of estrogen at the time of conceptus elongation (Ashworth *et al.* 2005).

We addressed whether IGF-1 and 2 are directly linked with porcine conceptus arrest at gd20 and 50. Transcripts for *IGF-1* and *IGF-2* were quantified in trophoblast and endometrial biopsies at gd20 and gd50 by real time PCR and expressed as a ratio of target gene (*IGF*) to *ACTB* (Fig. 2A and B). At gd20, trophoblast from A-CAS had fewer *IGF-1* transcripts than trophoblast from H-CAS. At gd50, trophoblasts had more transcripts than gd50 endometrial samples ($P < 0.05$). In gd20 endometrial samples (Fig. 2A), *IGF-1* was more abundant than in gd50 endometrium ($P < 0.05$). These data are consistent with other reports that *IGF-1* declines as pregnancy advances (Simmen *et al.* 1992, Ashworth *et al.* 2005). *IGF-2* transcripts were 10-fold higher in both endometrial and trophoblast samples than *IGF-1* transcripts. No significant differences were found in either tissue at gd20 or gd50 related to conceptus health status. Our results are in agreement with previous reports that *IGF-2* is predominantly expressed over *IGF-1* during porcine pregnancy (Simmen *et al.* 1992). Our studies did not provide any evidence that IGFs are directly linked with porcine fetal arrest. More comprehensive studies that includes IGF receptors and IGFBPs are in progress.

Final remarks

Our molecular interrogation of endometrial attachment sites of live fetal littermates whose gestational fates differ, has identified clear roles for immune cells, both lymphoid and dendritic, in promotion of endometrial angiogenesis within CAS. Coincident with conceptus arrest, changes occur in the immune system that could have primary and/or secondary roles in the death of specific fetuses. The immune system appears to respond to fetal distress by removing vascular support for that CAS and locally elevating pro-inflammatory cytokines. These changes may be induced in resident cells because no significant alterations were found in chemokines ligands binding to pro-inflammatory decoy receptors or in the decoy receptor D6 between H-CAS and A-CAS. Alterations in expression of the homeostatic decoy receptor CCX-CKR between H-CAS and A-CAS suggest a new regulatory pathway that may contribute to porcine pregnancy success.

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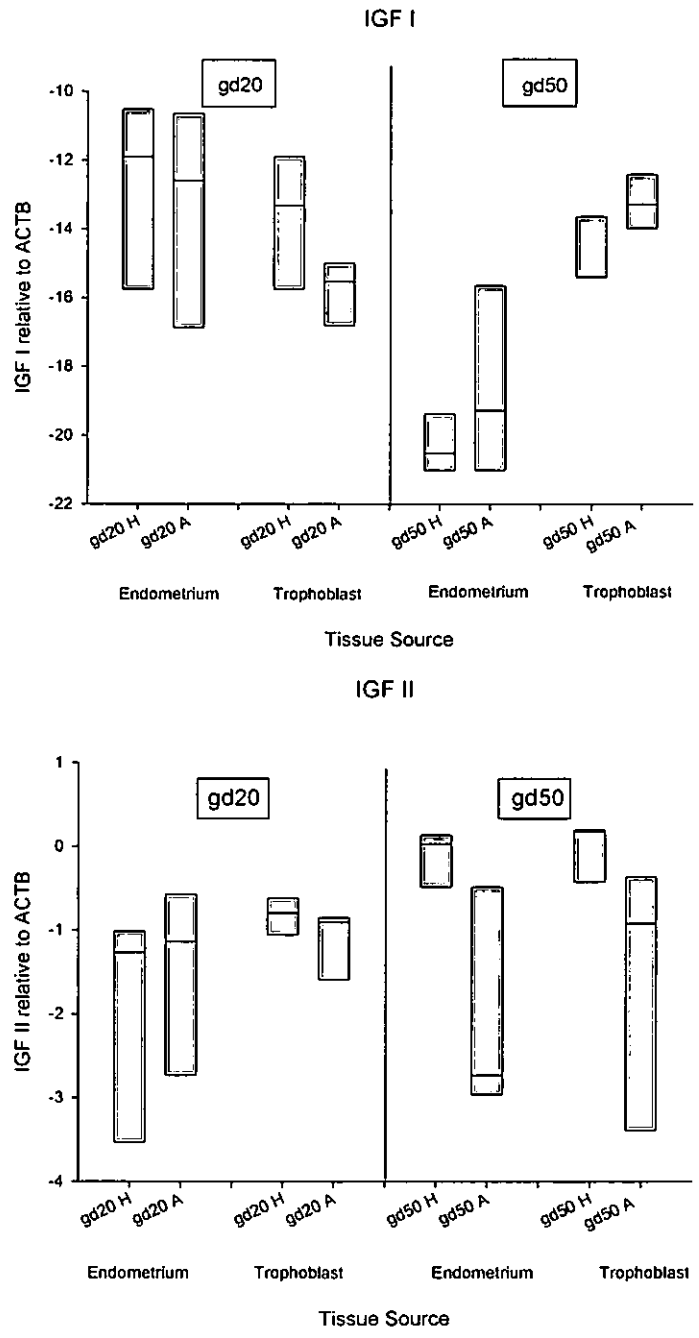

Fig. 2 Expression of *IGF1* (A) and *IGF2* (B) in porcine endometrium and trophoblasts at gd20 and 50. Expression was quantified by real time PCR using RelQuant software (Roche) and is relative to the housekeeping gene, *ACTB*. The LightCycler program was denaturation at 94 °C, 15 min; PCR amplification 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. Statistical analysis was performed using one way ANOVA and pair-wise multiple comparison procedure as per Holm-Sidak method. The bar in each box represents the data median with 6 samples per group. $P < 0.05$ was considered significant. gd: gestation days, H: healthy, A: arresting.

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