Expression of chemokine decoy receptors and their ligands at the porcine maternal–fetal interface

Jocelyn M Wessels¹, Nicola F Linton¹, Marianne J van den Heuvel¹, Sonya A Cnossen¹, Andrew K Edwards¹, Barbara Anne Croy¹,² and Chandrakant Tayade¹,²

Successful pregnancy requires coordinated maternal–fetal cross-talk to establish vascular connections that support conceptus growth. In pigs, two waves of spontaneous fetal loss occur and 30–40% of conceptuses are lost before parturition. Previous studies associated these losses with decreased angiogenic and increased inflammatory cytokines. Chemokines, a sub-category of cytokines, and decoy receptors control leukocyte trafficking, angiogenesis and development. The availability of chemokines is regulated by three non-signalling decoy receptors: chemokine decoy receptor (D6), Duffy antigen receptor for chemokines (DARC) and Chemocentryx decoy receptor (CCX CKR). We hypothesized that the expression of these receptors and their chemokine ligands regulate the porcine pregnancy success or failure. Here, we describe for the first time the transcription and translation of all three decoy receptors and several chemokine ligands in endometrium and trophoblast associated with healthy and arresting conceptuses at gestation day (gd) 20 and gd50. Among decoy receptors, transcripts for DARC were significantly reduced in endometrium, whereas that for CCX CKR were significantly increased in endometrium and trophoblast at gd50 arresting compared with healthy sites. However, western blot analysis revealed no differences in decoy receptor expression between healthy and arresting tissues. Transcripts for decoy receptor ligands CCL2, CCL3, CCL4, CCL5, CCL11, CCL19, CCL21, CXCL2 and CXCL8 were stable between healthy and arresting littermates. Quantification by SearchLight chemiluminescent protein array confirmed ligand expression at the protein level. These data indicate that decoy receptors and ligands are expressed at the porcine maternal–fetal interface and dysregulation of decoy receptor (DARC and CCX CKR) transcripts occurs at sites of fetal arrest.

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INTRODUCTION

Decoy receptors are non-signalling, membrane-bound receptors that regulate cell trafficking, inflammatory responses, immune reactions, angiogenesis and apoptosis by binding, internalizing and degrading their chemokine ligands.¹⁻⁷ Recently, the expression of chemokines and their receptors by cell types such as endothelial cells, trophoblast cells and leukocytes has been shown to contribute to important physiological developments in the uterus before and during human and mouse pregnancy.⁸⁻⁹ The chemokine family consists of a number of 8–12 kDa proteins that mediate leukocyte trafficking to sites of inflammation and injury. Coordination of cell migration is also regulated by chemokines during routine tissue maintenance.³⁻¹⁰ Chemokines are divided into four families (CC, CXC, CX3C and C) on the basis of the arrangement of four conserved cysteine residues on the mature protein.⁶⁻¹⁰ They can also be classified as inducible or constitutive, with the former describing chemokines controlling the inflammatory response and the latter defining those responsible for homeostatic cell trafficking.³⁻¹¹ Chemokines elicit their effects by binding to specific G-protein-coupled receptors. Both chemokines and their receptors have redundancies, each receptor can bind several chemokines, and most chemokines can bind more than one receptor.¹²⁻¹³ Chemokines are key regulators of leukocyte migration, blood vessel development and are thought to have a role in trophoblast migration during human pregnancy.⁹⁻¹⁶

Aside from binding to signaling chemokine receptors, chemokine activity is regulated by decoy receptors. Three decoy receptors are known in humans and mice: the chemokine decoy receptor (D6), which binds at least 12 CC chemokines, the Duffy antigen receptor for chemokines (DARC), which binds at least 10 CC chemokines and 9 CXC chemokines, and the Chemocentryx chemokine receptor (CCX CKR), which binds at least 3 CC chemokines.²⁻¹⁷⁻²⁹ D6 and DARC bind inducible inflammatory chemokines whereas CCX CKR binds constitutive homeostatic chemokines.²⁷⁻²⁹ Previous studies have reported D6, DARC and CCX CKR expression by trophoblast cells in species with invasive hemochorial placentae.²⁻¹⁵⁻¹⁶⁻²⁹⁻³¹ Using D6 knockout mice, Martinez de la Torre et al.² showed the ability of D6 to

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Decoy receptor mRNA quantification at the porcine maternal–fetal interface

Transcripts for the chemokine decoy receptors D6, DARC and CCX CKR were isolated from non-pregnant (dystrous phase of the estrous cycle) porcine endometrium (n=3–5 uteri) and at gd20 (n=3–9 attachment sites) and gd50 (n=3–9 attachment sites; Figure 1a), and in gd20 (n=3–9 attachment sites) and gd50 (n=3–9 attachment sites) trophoblast samples (Figure 1b). DARC transcripts were found to be significantly increased (P<0.05) in endometrium during pregnancy when compared with non-pregnant endometrium. No differences were found between D6 or CCX CKR transcript numbers in the non-pregnant versus pregnant endometrium. Significantly fewer transcripts for DARC (P<0.05) were present in endometrium from arresting versus healthy conceptus attachment sites at gd50. In contrast, CCX CKR transcripts were significantly elevated (P<0.05) at gd50 in both maternal (endometrium) and fetal (trophoblast) tissue harvested from arresting sites when compared with that harvested from healthy sites. No detectable differences were found between healthy and arresting sites for the decoy receptor D6 in either tissue (Figure 1). All of the decoy receptors studied have a stable transcript expression between gd20 and gd50, in both tissues.

Figure 1  Relative mRNA levels of chemokine decoy receptors D6, DARC and CCX CKR in NP (gray filled bars, n=3–5 uteri) and in paired tissue samples (n=3–9 attachment site pairs) from healthy (white filled bars) and arresting (black filled bars) attachment sites in endometrium (a) and trophoblast (b) at gd20 and gd50. Transcripts for DARC were significantly decreased (*P<0.05) in endometrium associated with arresting conceptus attachment sites, at gd50. In addition, CCX CKR transcripts were significantly elevated (*P<0.05) at gd50 in both maternal and fetal tissues associated with arresting conceptuses. DARC transcripts (*P<0.05) were found to be significantly elevated in the endometrium during pregnancy. Transcript abundance was quantified by real-time PCR and normalized as a ratio to β-actin on a logarithmic scale. Histogram bars represent group mean plus s.e.m. Gd, gestation day; NP, non-pregnant.

RESULTS

Decoy receptor mRNA quantification at the porcine maternal–fetal interface

Transcripts for the chemokine decoy receptors D6, DARC and CCX CKR were isolated from non-pregnant (dystrous phase of the estrous cycle) porcine endometrium (n=3–5 uteri) and at gd20 (n=3–9 attachment sites) and gd50 (n=3–9 attachment sites; Figure 1a), and in gd20 (n=3–9 attachment sites) and gd50 (n=3–9 attachment sites) trophoblast samples (Figure 1b). DARC transcripts were found to be significantly increased (P<0.05) in endometrium during pregnancy when compared with non-pregnant endometrium. No differences were found between D6 or CCX CKR transcript numbers in the non-pregnant versus pregnant endometrium. Significantly fewer transcripts for DARC (P<0.05) were present in endometrium from arresting versus healthy conceptus attachment sites at gd50. In contrast, CCX CKR transcripts were significantly elevated (P<0.05) at gd50 in both maternal (endometrium) and fetal (trophoblast) tissue harvested from arresting sites when compared with that harvested from healthy sites. No detectable differences were found between healthy and arresting sites for the decoy receptor D6 in either tissue (Figure 1). All of the decoy receptors studied have a stable transcript expression between gd20 and gd50, in both tissues.

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Decoy receptor protein quantification at the porcine maternal–fetal interface

Western blot analysis of decoy receptors in matched samples (endometrium and trophoblast) of healthy and arresting attachment sites at gd20 \((n=3 \text{ uteri})\) and gd50 \((n=3 \text{ uteri})\) are summarized in Figure 2. A specific band of the expected size confirmed that the antibody was crossreactive (Figure 2a). Protein levels were quantified by scanning densitometry. Results are summarized in Figure 2b. There was no difference found in the level of expression of D6, DARC or CCX CKR at healthy versus arresting attachment sites, in either tissue examined.

Immunolocalization of decoy receptors D6, DARC and CCX CKR

The site of expression of the decoy receptors D6, DARC and CCX CKR in endometrial tissue was addressed using immunohistochemistry (gd20: \(n=3 \text{ uteri}, \text{gd50}: n=3 \text{ uteri}\)). At gd20 and gd50, anti-D6 immunoreactivity was observed on endometrial and glandular epithelia, as well as on dispersed stromal cells (Figures 3a–d). D6 expression was generally uniform in epithelium and stroma in both healthy and arresting attachment sites. DARC had peri-vascular and epithelial expression at gd20 and gd50 (Figures 3e–h). Its expression seemed uniform between healthy and arresting tissues, as well as between gestation days. Anti-CCX CKR immunoreactivity was present at both gd20 and gd50 on luminal and glandular epithelia and on perivascular stroma (Figures 3i–l). At gd50, CCX CKR expression seemed to be more intense and more abundant, especially surrounding the vasculature, in endometrium from arresting sites compared with healthy sites.

Chemokine mRNA quantification at the porcine maternal–fetal interface

Transcripts for selected chemokines known to bind to D6 only (CCL3 and CCL4), to both D6 and DARC (CCL2, CCL5 and CCL11), to DARC only (CXCL2 and CXCL8) or to CCX CKR only (CCL19 and CCL21) were found in endometrium of five non-pregnant animals (Figure 4a). CCL5 transcripts were significantly reduced \((P<0.05)\) in the pregnant versus non-pregnant endometrium. Pregnancy did not induce changes in any other chemokine transcripts (non-pregnant versus gd20 and gd50 combined). Transcripts for CCL2, CCL3, CCL4, CCL5, CCL11, CCL19, CCL21, CXCL2 and CXCL8 were also identified in endometrium (Figure 4a) and in trophoblast (Figure 4b) from five
Lymphocytes make important contributions to endometrial angiogenesis during early mammalian pregnancy. The positioning of lymphocytes and endothelial progenitor cells is regulated by decoy receptors and chemokines. Decoy receptors and spontaneous fetal loss. The first aim of this study was to determine whether decoy receptors and their chemokine ligands are expressed at the maternal–fetal interface of pigs, a species with non-invasive epitheliochorial placentation. The second aim was to determine if these factors are differentially expressed between healthy and spontaneously arresting porcine conceptus attachment sites at the peri-attachment stage of pregnancy (gd20) and at mid-gestation (gd50). Table 1 summarizes the significant findings.

In diestrous (non-pregnant) porcine endometrium, DARC transcript numbers were lower than that in endometrium from pregnant animals. As DARC controls local inflammation and cell trafficking, its strict regulation during pregnancy may be necessary to attract appropriate, pregnancy-protective cells to the maternal–fetal interface. Non-pregnant porcine endometrium also contained an abundance of transcripts for the D6/DARC-binding chemokine CCL5, which agrees with observations in cycling human endometrium wherein the inflammatory chemokines CCL2 and CCL5 predominate. In general, mid-cycle porcine endometrium had more pro-inflammatory than homeostatic chemokine transcripts, indicating that D6, DARC and their ligands are the most likely chemokine-based interactions to participate in the recruitment of T cells, macrophages and neutrophils during the estrous cycle. With the exception of fewer CCL5 transcripts, the state of pregnancy did not significantly alter the abundance of chemokine mRNA present in the maternal endometrium.

Porcine endometrium and trophoblast express all three chemokine decoy receptors (D6, DARC and CCX CKR) and several of their ligands as transcripts and protein. This is the first report of decoy receptor and chemokine ligand expression in pigs or in any species showing non-invasive epitheliochorial placentation. It is also the first to quantify decoy receptor expression independently in maternal and fetal tissue. Decoy receptor transcription and translation occurred in both endometrium and trophoblast. D6 transcripts were more abundant than CCX CKR transcripts, but both were lower than the number of transcripts in gd20 endometrium. Anti-D6 immunoreactivity was observed on endometrial epithelium in arresting sites as compared with healthy sites. There were no other detectable differences in chemokine transcript numbers between healthy and arresting sites for the rest of the chemokines investigated in endometrium or trophoblast. Transcripts of CCL2 and CCL4 decreased significantly (P < 0.05 for each) in number between gd20 and gd50 in both endometrium and trophoblast.

**FIGURE 3** Anti-D6 (a–d), anti-DARC (e–h) and anti-CCX CKR (i–l) immunoreactivity in gd20 and gd50 endometrial sections from healthy and arresting conceptus attachment sites. D6 was localized in paraffin-embedded sections (5 μm) while DARC and CCX CKR were localized in frozen sections (5 μm) using standard fluorescent wide-field microscopy. Anti-D6 immunoreactivity was observed on endometrial epithelium (a, d), endometrial glands (b, c) and dispersed stromal cells (d). DARC expression was seen surrounding endometrial blood vessels (e, f, h) and on the endometrial epithelium (g). Endometrial CCX CKR immunoreactivity was localized at gd20 and gd50 to luminal (i) and glandular epithelium (j), dispersed stromal cells (k) and to peri-vascular cells (l). Anti-CCX CKR reactivity appeared greater in gd50 arresting compared with healthy endometrium. N=3 uteri. Bv, blood vessel; epi, epithelium; gd, gestation day; gl, gland; st, stroma. 200×.
Figure 4  Relative mRNA levels of decoy receptor-binding chemokines in NP(gray filled bars) and in paired tissue samples from healthy (white filled bars) and arresting (black filled bars) attachment sites in endometrium (a) and trophoblast (b) at gd20 and gd50. In both endometrium and trophoblast, relative transcript abundance for CCL2, CCL3, CCL4, CCL5, CCL11, CCL19, CCL21 and CXCL2 did not differ between healthy and arresting attachment sites at gd20 or gd50. CXCL8 transcripts were less abundant (*P<0.05) in gd50 endometrium isolated from arresting sites, and they were more abundant (*P<0.05) in gd50 arresting trophoblast when compared with gd50 tissues from healthy attachment sites. Transcripts of CCL2 (*P<0.05) and CCL4 (*P<0.05) were found to significantly decrease between gd20 and gd50 in both the endometrium and trophoblast. CCL5 (*P<0.05) transcripts were found to be significantly decreased in the endometrium during pregnancy. Transcript abundance was quantified by real-time PCR and normalized as a ratio to β-actin on a logarithmic scale. Histogram bars represent group mean plus s.e.m. N=5–12 attachment sites. Gd, gestation day.
Figure 5 A comparison of chemokine expression in porcine endometrium (a) and trophoblast (b). Proteins CCL2, CCL3, CCL4, CCL5, CCL11, CCL21 and CXCL8 were quantified by SearchLight chemiluminescent protein array in healthy (white filled bars) and arresting (black filled bars) gd20 and gd50 endometrium (a) and trophoblast (b). In both endometrium and trophoblast, the amount of protein for all of the aforementioned chemokines did not differ significantly between healthy and arresting attachment sites at gd20 or gd50. The amount of CXCL8 was found to decrease (*P<0.05) between gd20 and gd50 in both endometrium and trophoblast. Data are presented in pg/ml on a logarithmic scale. Bars represent group mean plus s.e.m. N=6 uteri. Gd, gestation day.
of DARC transcripts. Our mRNA data suggest that DARC is the predominant decoy receptor at the porcine maternal–fetal interface. In humans and mice, DARC is a potential candidate to control leukocyte recruitment at the maternal–fetal interface because it is expressed by endothelial cells, participates in chemokine transcytosis and releases chemokines when they are required. DARC also binds and sequesters CXCL8, a chemokine shown to induce trophoblast migration and invasion in vitro, and a variety of other pro-angiogenic chemokines. A deviation in DARC at the maternal–fetal border has the potential to contribute to aberrant angiogenesis and/or dysregulated trophoblast migration at arresting porcine fetal attachment sites.

Both DARC and CCX CKR transcripts were significantly different between healthy and arresting porcine conceptus attachment sites at gd50 in endometrium (DARC and CCX CKR) and trophoblast (CCX CKR only). At gd50, DARC transcripts were significantly reduced in arresting endometrium, whereas CCX CKR transcripts were elevated in arresting endometrium and trophoblast. A recent study by Madigan et al. has implicated the decoy receptor D6 in fetal survival of allogeneic embryo transfers in mice, wherein D6 null embryos were less likely to survive after blastocyst transplant. Another study by Martinez de la Torre et al. showed the involvement of D6 in two models of induced fetal loss in mice. Although the mRNA results of the present study could not be confirmed at the protein level, the technical limitations of working with porcine tissues and many cross-species assays and reagents, most of which had limited sensitivity, must be considered. Interestingly, immunohistochemistry for CCX CKR did support the mRNA result of increased expression in gd50 endometrium at sites of fetal loss.

No differences in protein expression were observed for the decoy receptors’ chemokine ligands between tissues isolated from healthy versus arresting attachment sites. CXCL8 was found to significantly decrease in both tissues over gd20 to gd50, indicating that it may participate in attachment of the conceptus to the uterine wall in species with epitheliochorial placentation. The chemiluminescent array performed was a human array not specifically designed for porcine use. It was determined to be crossreactive with porcine samples, however, many of the chemokines measured were near the limit of detection for the assay when human samples are used. Further studies will be needed as porcine-specific reagents become available to quantify chemokines.

As Martinez de la Torre et al. and Madigan et al. showed, the dysregulation of decoy receptors at the maternal–fetal interface has the potential to participate in fetal loss. However, their specific cause or effect role of the decoy receptors and ligands in fetal loss is not elucidated and will require specific gene inactivation studies. It would be interesting to determine in future studies whether decoy receptors and chemokines have the ability to cause spontaneous loss, or whether they contribute to the removal of a fetus at the site of a failed pregnancy. Nevertheless, decoy receptors and chemokines seem to have functions at the maternal–fetal interface that remain to be fully understood.

This study reports for the first time the transcription and translation of decoy receptors and their chemokine ligands in endometrium and trophoblast from the same porcine conceptus attachment site, and permitted their quantitative comparisons during healthy and arresting, early and mid-gestation porcine pregnancies. Even though technical limitations precluded strong quantitative confirmation of the mRNA data at protein level, this study shows that non-invasive trophoblast expresses decoy receptors. These findings indicate that these decoy receptors have roles, other than to regulate trophoblast invasion, during pregnancy. This study also localizes expressed decoy receptors to positions that implicate them in the recruitment of cells to the maternal–fetal interface in pigs, a species with epitheliochorial placentation. Finally, we suggest from the specific Q-PCR studies that a dysregulation of chemokine decoy receptors may exist at arresting conceptus attachment sites and that this could have pivotal roles in determining conceptus fate by altering the ratios of recruited cell types at the sensitive maternal–fetal interface.
METHODS
Porcine tissue samples
Specific pathogen-free Yorkshire pigs were obtained from Arkell Swine Research Station (University of Guelph, Guelph, ON, Canada). First estrous cycle pigs (gilts) were bred twice, either artificially or naturally. Gestational age was determined from the first date of mating. Tissues were collected from six pregnant animals at gd20 (peri-attachment loss), and six pregnant animals at gd50 (post-attachment loss). Non-pregnant endometrium was obtained from five gilts during diestrous. All animal procedures were performed in accordance with protocols approved by the Animal Care Committee, University of Guelph.

Reproductive tracts were collected at the University of Guelph abattoir post-slaughter, transported to the laboratory on ice and immediately examined for gross abnormalities. Uteri were opened longitudinally along the anti-mesometrial side, exposing all conceptuses which were then classified as healthy or arresting on the basis of disparities in length, weight and vasculature as previously described.33,34,45 Any conceptuses of questionable gestational fate or advanced resorption were omitted from the study. For every pregnant reproductive tract, matching biopsies of mesometrial endometrium and trophoblast were collected from healthy and arresting sites after teasing the trophoblast away from the endometrium. Samples were individually collected from three healthy conceptuses and for all arresting conceptuses identified. Tissues were studied as independent attachment sites and were not pooled within a litter. For non-pregnant uteri, endometrial biopsies were collected from random mesometrial sites. Each tissue sample collected (endometrium or trophoblast) was divided and used for three analyses: RNA isolation, protein isolation and histology (paraffin-embedded and were flash frozen in liquid nitrogen-cooled isopentane. (Cryomatrix, ThermoShandon, Pittsburg, PA, USA) in a plastic biopsy mold.

Tissues for cryostat sectioning were placed into OCT embedding medium (Tissue-Tek, Miles Scientific, Elkhart, IN, USA), routinely processed, cut and mounted as 5-μm sections. Frozen tissue sections were dewaxed in xylene, rehydrated in graded ethanol and placed on charged glass slides.

Quantitative real-time PCR
Total RNA was extracted from samples using RNeasy mini kits (Qiagen, Mississauga, ON, Canada) according to the manufacturer’s instructions. RNA concentration and purity were determined using the GeneQuant pro RNA/DNA calculator (Pharmacia, Baie d’Urfe, QC, Canada), along with MagicMark protein ladder (Invitrogen), and run for 85 s at 100V at 4°C. Reagents were for each sample. RNA concentrations were determined using a modified Bradford assay (Coomassie Protein Assay Reagent, Thermo-Scientific, Fisher, Whitby, ON, Canada) and read at 595 nm using the GeneQuant pro RNA/DNA calculator. Samples were either used for western blot or SearchLight chemiluminescent protein array analysis. Samples for western blotting were diluted to 5 μg ml⁻¹, dispersed in 10 μl aliquots into clean tubes and stored at −80°C. Western blots were performed for reduced β-actin, D6, DARC and CCX CKR (Abcam Inc., Cambridge, MA, USA for all antibodies), as no SearchLight chemiluminescent protein array was available for these receptors. Protein aliquots were reduced by adding 2% β-mercaptoethanol into 10 μl of 2× protein loading buffer (0.1 M Tris HCl, pH 6.8, 16% glycerol, 2% SDS and a few grains of bromophenol blue) and heating to 85°C for 4 min before loading onto an acrylamide gel. Each sample was pipetted into individual lanes of a 4–20% gradient gel (Invitrogen, Burlington, ON, Canada), along with MagicMark protein ladder (Invitrogen), and run for 90 min at 125 V. The proteins were then transferred to PVDF membrane (Roche Diagnostics) overnight at 20V at 4°C. The membranes were rinsed in Tris-buffered saline with tween-20 (TBST), then blocked in 1% milk in TBST, then covered with primary antibody and rotated overnight at 4°C. The antibodies used and their concentrations are detailed in Table 3. After three washes in TBST, the membranes were covered with primary antibody and rotated overnight at 4°C. The antibodies used and their concentrations are detailed in Table 3. After three washes in TBST, the appropriate secondary antibody was applied and incubated at room temperature for 1 h. Membranes were washed three times in TBST before

Table 2 A list of PCR primers and partial coding sequence GenBank accession numbers

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Table 3 A list of antibodies, suppliers, supplied concentration and volume, isotype and amount of antibody used in this study for immunohistochemistry and western blotting

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<td>0.2 µg µl⁻¹</td>
<td>500 µl</td>
<td>Mixed donkey</td>
<td>1:200</td>
<td>NA</td>
</tr>
<tr>
<td>Alexa Fluor 588 goat anti-rabbit IgG</td>
<td>Invitrogen</td>
<td>0.2 µg µl⁻¹</td>
<td>500 µl</td>
<td>Mixed goat</td>
<td>1:200</td>
<td>NA</td>
</tr>
<tr>
<td>Goat anti-mouse</td>
<td>Thermo Scientific, Ottawa, ON, Canada</td>
<td>0.01 µg µl⁻¹</td>
<td>2 ml</td>
<td>Mixed goat</td>
<td>NA</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-goat IgG</td>
<td>Sigma Aldrich, St Louis, MO, USA</td>
<td>5 mg ml⁻¹</td>
<td>1 ml</td>
<td>Mixed rabbit</td>
<td>NA</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-rabbit IgG</td>
<td>Thermo Scientific</td>
<td>10 µg µl⁻¹</td>
<td>2 ml</td>
<td>Mixed goat</td>
<td>NA</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

Abbreviations: BSA, bovine serum albumin; Ig, immunoglobulin; TBST, Tris-buffered saline with tween-20.

Immunohistochemistry
Antibodies to D6 (R&D Systems Inc., Minneapolis, MA, USA), DARC (Abcam Inc.) and CCX CKR (Abcam Inc.) were used to analyze tissue sections of endometrium from healthy and arresting sites. The size of protein isolated by each antibody was confirmed by western blot. As a negative control, parallel tissue sections were stained with a comparable isotype control. Localization of D6 was performed in parafin-embedded tissue. Sections (5 µm) were deparaffinized, heated for 8 min in citrate buffer for antigen retrieval, placed in 3% H₂O₂ to block endogenous peroxidases for 5 min, rinsed three times in affinized, heated for 8 min in citrate buffer for antigen retrieval, placed in 3% H₂O₂ to block endogenous peroxidases for 5 min, rinsed three times in phosphate-buffered saline, blocked in 1% bovine serum albumin (Fisher Scientific, Ottawa, ON, Canada) for 30 min and incubated overnight at 4 °C with 50 µl of primary antibody. The antibody concentrations used are detailed in Table 3. Negative control sections on the same slide were incubated under identical conditions, with 50 µl of an isotype control antibody. Slides were rinsed in phosphate-buffered saline and fluorescently tagged secondary antibodies were applied to the appropriate sections for 30 min. Coverslips were added using Aqua Poly/Mount mounting media (Polysciences, Inc., Warrington, PA, USA). Localization of DARC and CCX CKR used 5-µm-thick frozen sections. Slides were warmed to room temperature, fixed with pre-chilled methanol (Fisher Scientific) and allowed to sit in three changes of sterile 1% phosphate-buffered saline for 3 minutes each. Sections were blocked and stained as above. Tissue sections were visualized and photographed using a Leica DMLB wide-field fluorescent microscope and QCapture Pro software (QImaging Corporation, Surrey, BC, Canada). Images for each decy receptor were captured using identical camera conditions to allow for visual comparison between gd and health status.

Statistical analyses
A statistical consultant used the PROC MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA) to create a mixed linear model that included the effects of gestation day, tissue and health, as well as their interactions. The model also accounted for any clustering effects of litter. Data were logarithmically transformed to meet the assumption of normalcy required by the PROC MIXED procedure. This model was used on all mRNA and SearchLight protein quantification data. Results are presented as mean plus the s.e.m.. A P-value of <0.05 was considered significant. Although some data seemed to be statistically significant, they were not, unless marked by an asterisk (*). This occurred when there was an effect of gestation day, tissue and health in the model that had to be accounted for.

Scanning densitometry of western blots was analyzed by one way ANOVA. Individual densitometry value ratios were calculated for the genes of interest (D6, DARC, CCX CKR) versus the housekeeping gene (β-actin). Data were logarithmically transformed to achieve a normal distribution. Values for the quantity of each gene of interest relative to the quantity of β-actin were grouped according to health status and gestation day for maternal tissue (endometrium) and for fetal tissue (trophoblast). If significant differences (P<0.05) were noted, a Dunn's post hoc test was performed to determine where the difference lay. Results are presented as the mean plus s.e.m.

ACKNOWLEDGEMENTS
We thank the staff at Arkell Swine Research Station and the Meat’s Wing of the Department of Animal and Poultry Sciences, University of Guelph for their help with our experiments, and biological statistician Ms Anne Valliant for her help in designing our statistical model. This research was supported by funding from the Ontario Pork, NSERC, Agriculture and Agri-Food Canada, OMAFRA and Bioniche Life Sciences Inc., Belleville, ON, Canada.

NCBI GenBank Accession Numbers: Sus scrofa CCL2 mRNA, partial cds (EF107669.1); Sus scrofa CCL3L1 mRNA, partial cds (EF107671.1); Sus scrofa CCL4 mRNA, partial cds (EF107677.1); Sus scrofa CCL11 mRNA, partial cds (EF107670.1); Sus scrofa CCL11 mRNA, partial cds (EF179159.1); Sus scrofa CCL21-like mRNA, partial sequence (EU155482.1); Sus scrofa CXCL2-like mRNA, partial cds (EU280325.1); Sus scrofa CXCL8-like mRNA, partial cds (HM029251); Sus scrofa chemokine receptor D6 mRNA, partial cds (DQ437508.1); Sus scrofa CCX CKR-like mRNA, partial sequence (EU168334.1).


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