Interleukins, interferons, and establishment of pregnancy in pigs

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Abstract

Early pregnancy in mammals requires complex and highly orchestrated cellular and molecular interactions between specialized cells within the endometrium and the conceptus. Proinflammatory cytokines are small signaling proteins released by leukocytes that augment innate and adaptive immune responses. They are also released by the mammalian trophectoderm as the conceptus apposes the uterine surface for implantation. On approximately day 12 of development in pigs, the conceptus undergoes a rapid morphological transformation referred to as elongation while simultaneously releasing estrogens and a novel conceptus form of interleukin-1 beta (IL1β). Following elongation, pig conceptuses express interferon gamma (IFNγ) and, in lesser amounts, interferon delta (IFNδ). Significant IFN signaling takes place within the endometrium between day 14 and 18 of pregnancy as the conceptus intimately associates with the uterine epithelium. Based on studies carried out in pigs and other mammals, the combined spacio-temporal activities of conceptus estrogens, IL1β, and IFN set in motion a series of coordinated events that promote establishment of pregnancy. This is achieved through enhancement of conceptus development, uterine receptivity, maternal–fetal hemotropic exchange, and endometrial leukocyte function. These events require activation of specific signaling pathways within the uterine luminal epithelium, glandular epithelium, and stroma. Here, we review proinflammatory cytokine expression by pig conceptuses and the hypothesized actions of these molecules during establishment of pregnancy.

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Introduction

The first 3 weeks of development are the most critical for survival of the early mammalian embryo or conceptus (embryo and extraembryonic membranes). During this time, the blastocyst hatches from the zona pellucida exposing the conceptus to a lavish uterine environment that consists of a complex mixture of maternal secretory proteins and small molecular weight molecules. Attachment and implantation follows when the conceptus becomes intimately associated with the uterine luminal epithelium (LE), glandular epithelium (GE), stromal fibroblasts, and maternal immune cells. There is a defined “window” of uterine receptivity, which allows the trophoblast to attach and, in some species, invade beyond the epithelial barrier until pregnancy is established. Throughout the entire process, as many as 25–60% of conceptuses fail to develop and establish pregnancy, a phenomenon commonly referred to as early embryonic mortality (Wilmut et al. 1986).

Proinflammatory cytokines are small signaling proteins released by leukocytes that control innate and adaptive immune responses. They also mediate communication between the early conceptus and endometrium, promoting implantation and establishment of pregnancy (Simón et al. 1998, Ross et al. 2003a, Granot et al. 2012). Recent clinical observations made during human embryo transfer may reflect the importance of a proinflammatory environment at the site of embryo implantation. It has been shown that mechanically manipulating the endometrium before embryo transfer can be more than double the rate of implantation, pregnancy, and live birth in women who experienced repeated pregnancy failure (Granot et al. 2012). Proinflammatory cytokines, released by the damaged endometrium, are believed to promote implantation.

Interleukins (IL) and interferons (IFN) are some of the most well-studied proinflammatory cytokines in mammalian reproduction. Studies on primates and rodents indicate that the early embryo releases interleukin-1 beta (IL1β), increasing adhesion molecule expression within the endometrium and invasive characteristics of the cytotrophoblast (Librach et al. 1994, Simón et al. 1998). The pig is unique in that the IL1B gene is duplicated, resulting in a novel gene
and conceptus form of IL1β (interleukin-1 beta 2; IL1β2) (Vandenbroeck et al. 1993, Groenen et al. 2012, Mathew et al. 2015). Conceptus expression of IL1β2 is followed by abundant production of interferon gamma (IFNγ) by pig conceptuses and, in lesser concentrations, a pregnancy-specific type I IFN, interferon delta (IFNδ) (La Bonnardière et al. 1991). These cytokines are expressed during the synthesis of conceptus estrogens for maternal recognition of pregnancy (Bazer & Thatcher 1977). Other proinflammatory cytokines are present at the conceptus–maternal interface during establishment of pregnancy in pigs including interleukin-6 (IL6), interleukin-18 (IL18), leukemia inhibitory factor (LIF), and tumor necrosis factor alpha (TNFα) (Yu et al. 1998, Modrić et al. 2000, Ashworth et al. 2010, Blitk et al. 2012). Many of these cytokines stimulate production and release of prostaglandins (PG) that may contribute to the inflammatory environment within the endometrium (Waclawik 2011, Geisert et al. 2015). The combined spacio-temporal activities of these molecules on specific cell types within the endometrium set in motion a series of coordinated events that promote survival of the early pig conceptus (Bazer & Johnson 2014).

Development and implantation of the early pig conceptus

Within 5 days of estrus and breeding, more than 20 pig embryos can enter the uterine lumen. There, the blastocyst hatches from the zona pellucida (day 6–7) and is exposed to the uterine histotroph; a collection of ions and proteins including proteases, growth factors, cytokines, and nutrient transport molecules that are secreted by the uterine LE and GE cells. The histotroph is released in response to ovarian progesterone (P4) and promotes growth and development of the conceptus. The spherical pig blastocyst continues to grow and expand within the uterine lumen until approximately day 11 of gestation. At that time, the conceptus transforms morphologically and rapidly elongates at an extraordinary speed. During rapid elongation, cellular mitosis decreases and cellular hypertrophy and migration of the surrounding trophoblast cells results in extensive remodeling of the conceptus (day 11 or 12; Fig. 1A) (Geisert et al. 1982, Bazer & Johnson 2014). The pig conceptus becomes ovoid, tubular (15 mm by 50 mm), and then filamentous (1 mm by 200 mm) in less than 3 h, extending through a complex landscape of endometrial crypts and folds at a rate of 30–45 mm/h (Geisert et al. 1982, Bazer & Johnson 2014). Elongation is a critical stage of development for the early pig conceptus, defining the uterine surface area for its individual placental attachment. The conceptus will continue to grow and expand into the uterine lumen reaching over a meter in length while attaching to the uterine LE between day 13 and 18 of gestation (Bazer & Johnson 2014).

Figure 1 Models representing the proinflammatory cytokine microenvironment created during rapid elongating and implantation of the pig conceptus. Between day 11 and 12 (A and C) and 15 and 18 (B and D) of pregnancy, pig conceptuses rapidly elongate and implant, respectively, along the mesometrium. (C; location of small box in image A) During rapid elongation (near day 12 of pregnancy), pig conceptuses release interleukin-6 (IL6) and abundant concentrations of interleukin-1 beta 2 (IL1β2) that act on receptors within the conceptus and uterine surface epithelium. Conceptus estrogens and IL1β2 activate estrogen receptor (ESR) and nuclear factor-kappa B (NF-kB) transcription factors, respectively, along the maternal interface likely modulating the expression of prostaglandin (PG) synthases, endometrial interleukin 1 beta 1 (IL1β1), leukemia inhibitory factor (LIF), and endometrial IL6. (D; location of small box in image B) Near day 15 of pregnancy, pig conceptuses release peak concentrations of interferon gamma (IFNγ) and, in lesser amounts, interferon delta (IFNδ). Together, IFNγ and IFNδ trigger the activation of janus-associated kinases and signal transducers and activators of transcription (Jak-STAT) signaling pathways and expression of interferon regulatory factor 1 (IRF1) in stroma fibroblasts (F) and deep glandular epithelium (dGE) that stimulate the expression of classical ISGs. Within the surface epithelium, conceptus estrogens increase the expression of interferon regulatory factor 2 (IRF2), which, in conjunction with ubiquitin-specific protease (USP), may inhibit the expression of classical ISGs. Progestomedins and conceptus IFNs are proposed to induce and stimulate, respectively, the expression of non-classical ISGs within the uterine surface epithelium. Endometrial interleukin-18 (IL18) is released into the uterine lumen and is hypothesized to further stimulate IFNγ production by the pig conceptus. T, trophoblast; LE, luminal epithelium; sGE, surface glandular epithelium; BV, blood vessel; IL1R1, interleukin-1 receptor 1; IL6R, interleukin-6 receptor; LIFR, leukemia inhibitory factor receptor; IFNγR1, interferon gamma receptor; IFNδR, interferon alpha receptor.
Within attachment sites, surface area is increased by the presence of endometrial folds, surface epithelial folds, and microvilli between the trophoblast and dome-shaped LE cells, which are coated by a thick glycocalyx (Dantzer 1985, Keys & King 1990). The window of receptivity for conceptus attachment is programmed by ovarian P4 secretion. In mammals, P4 down-regulates nuclear progesterone receptors (PGR) within the uterine LE and surface GE (sGE) cells (Bazer & Johnson 2014). Loss of PGR in the pig uterine surface epithelium (Geisert et al. 1994) reduces the expression of mucin-1 (MUC1), a large glycoprotein regulated by P4 that inhibits attachment of the pig conceptus (Bowen et al. 1996). The loss of PGR also allows for the expression of estrogen receptor alpha (ESR1), which can be activated by conceptus estrogens secreted between day 10 and 18 of gestation (Geisert et al. 1993). It is hypothesized that binding of estrogens to surface epithelial ESR1 stimulates the secretion of ‘estromedins’ such as phosphoprotein 1 (SPP1; also known as osteopontin), fibroblast growth factor 7 (FGF7), and prostaglandin E2 (PGE2) that aid in adhesion, proliferation, and implantation of the pig conceptus (Fig. 1C and D) (Waclawik 2011, Bazer & Johnson 2014). Although PGR expression decreases in the uterine surface epithelium, expression is unchanged in the stromal fibroblast cells, deep glandular epithelium (dGE), and myometrium (Geisert et al. 1994). Within these tissues, P4 stimulates the release of “progestomedins” that act on the uterine surface epithelium and conceptus (Bazer & Johnson 2014). During rapid elongation, pig conceptuses also abundantly express a unique form of IL1β.

**Interleukin-1**

Proinflammatory cytokines IL1β and interleukin-1 alpha (IL1α) serve as central mediators of inflammation (Sims & Smith 2010). Hematopoietic cells such as monocytes, macrophages, and skin dendritic cells secrete IL1β during infection. IL1β increases the expression of endothelial cell adhesion molecule and blood vessel permeability, thus allowing peripheral blood leukocytes to extravasate and migrate into infected tissues. Furthermore, IL1β is an endogenous pyrogen, likely contributing to leukocyte proliferation and migration by inducing fever (Sims & Smith 2010).

The IL1 (IL1β and IL1α) are collectively referred to as IL1) and IL1 receptor family currently consists of 22 molecules that include proinflammatory cytokines IL1β and IL1α, an IL1 receptor antagonist (IL1RA), an IL1 receptor accessory protein (IL1RAP), a functional IL1 receptor (IL1R1), and a decoy receptor the IL1 receptor type 2 (IL1R2) (Sims & Smith 2010). Release of active IL1β occurs following formation of the inflammasome, a multi-protein complex associated with the caspase-1 (CASP1) protease, within the cell cytoplasm. Active CASP1 cleaves pro-IL1β and pro-IL18, another member of the IL1 family, resulting in the formation of mature, functional, proinflammatory cytokines.

Binding of IL1β to IL1R1 in the target cell membrane results in juxtapostitioning of the toll interleukin-1 receptor (TIR) domains within the cytoplasmic region of IL1R1 and IL1RAP (Sims & Smith 2010). This effectively recruits myeloid differentiation primary response protein 88, IL-1R-associated kinase 4, tumor necrosis factor receptor-associated factor 6, and other downstream intermediates. These recruited molecules collectively signal activation of nuclear factor-kappa B (NF-κB) transcription factors and mitogen-activated protein kinases (MAPK) (Sims & Smith 2010). The abundance of cytokine receptor within a target tissue, including reproductive tissues, can greatly influence the activation of second messenger pathways and cytokine activity.

Nuclear factor-kappa B transcription factors are evolutionarily conserved modulators of gene expression that control cell activity during innate and adaptive immune processes (Hayden & Ghosh 2012). NF-κB influences the expression of cytokines, growth factors, adhesion molecules, immunoreceptors, antigen presentation molecules, and other transcription factors (Hayden & Ghosh 2012). For this reason, NF-κB can have a large effect on cell function and its activity is tightly regulated by feedback mechanisms. Uncontrolled, IL1 signaling, and/or NF-κB activation can lead to autoimmune, autoinflammatory, infectious, and degenerative diseases that include malignant cancer and type II diabetes (Dinarello 2011). For this reason, the IL1-NF-κB signaling pathway is a target in many disease or cancer therapies.

**IL1 and reproduction**

Interleukin-1 beta is believed to be an ancient mediator of vertebrate reproduction and placental viviparity. It has been detected within the reproductive tissues of animals using very different reproductive strategies including oviparous, ovuliparous, and aplacental viviparous species (Bird et al. 2002, Jantra et al. 2007). In addition, IL1β is expressed within the placenta of mammalian and non-mammalian vertebrates such as squamate reptiles (Paulesu et al. 2005). In some placental mammals, including primates, rodents, and pigs, considerable IL1 cross talk occurs between the conceptus and endometrium during implantation (Simón et al. 1998, Ross et al. 2003a, Mathew et al. 2015).

In primates and rodents, IL1β is released by the early blastocyst and has a direct effect on uterine receptivity, modulating alpha V (AV) and beta 3 (B3) integrin subunits within the uterine LE (Simón et al. 1997, 1998). In primates, IL1β may promote cytotrophoblast invasion and can be detected within the villous cytotrophoblast, extravillous intermediate trophoblast, syncytiotrophoblast, as well as maternal stromal decidua cells (Librach et al. 1994, Simón et al. 1994). The mouse blastocyst expresses Il1b and Il1r1 during early...
pregnancy and induction of decidualization increases IL1 mRNA within the murine endometrium (Choudhuri & Wood 1993, Krüssel et al. 1997). The importance of IL1β during early pregnancy in mice is unclear. Intrapерitoneal infusion of IL1RA between day 2.5 and 7 of pregnancy in mice results in implantation failure (Simön et al. 1998). Knockout mice lacking Il1r1 are fertile, however, having only slightly reduced litter sizes (Abbondanzo et al. 1996). Furthermore, mouse conceptuses lacking Il1b, Il1a, or Il1b/Il1a develop normally, suggesting that other factors may compensate for the lack of IL1 signaling within the endometrium (Horai et al. 1998).

Tuo et al. (1996) were the first to report that elongating pig conceptuses express IL1β, a phenomena that was further characterized by Ross et al. (2003a). In pigs, conceptus expression of IL1β is maximal at the height of rapid elongation (approximately day 12 of development) but then decreases 2000-fold and is nearly undetectable a few days later (Ross et al. 2003a). During peak expression, IL1β is one of the most abundant transcripts in the pig conceptus. Intrauterine IL1β protein concentrations approach 4000 ng per uterine horn (Ross et al. 2003b) and can be detected within the uterine lumen between day 12 and 15 of gestation (Ross et al. 2003a).

Novel pig interleukin-1 beta

The theory that pigs may have an alternate IL1β gene was proposed more than 20 years ago (Vandenbroeck et al. 1993). This was not confirmed, however, until recent assembly and analysis of the pig genome (Groenen et al. 2012). It is now accepted that two IL1β genes are present within pig chromosome 3: interleukin-1 beta 1 (IL1β1) and IL1β2. The IL1β1 gene transcribes the prototypical cytokine in pigs and is expressed by adult tissues including blood leukocytes and the endometrium (Mathew et al. 2015). The IL1β2 gene is transcribed by the early pig conceptus (Mathew et al. 2015). Conceptus expression of IL1β2 is greatest during elongation; however, transcripts have been detected in the day 6 pig blastocyst (D J Mathew, MC Lucy and RD Geisert 2015, unpublished observations). Transcripts for IL1β2 have not been detected in adult tissues, suggesting that expression of this cytokine may be unique to the conceptus (Mathew et al. 2015).

Interleukin-1 beta 2 apparently arose from a gene duplication of IL1β1. The genes are tandem within the chromosome and both have seven exons (Mathew et al. 2015). Compared with IL1β1, however, IL1β2 transcribes an alternate exon 1 that is further upstream of exon 2. As a result, IL1β1 spans approximately 7 kb and IL1β2, 16.5 kb (Mathew et al. 2015). In theory, reconfiguration of the IL1β2 adjacent promoter region could have changed its transcriptional regulation and may partially explain why IL1β2 is expressed by the conceptus but not by other pig tissues (Mathew et al. 2015).

IL1β1 and IL1β2 mRNAs are translated into 267 amino acid pro-proteins that are 85% similar (Mathew et al. 2015). In mammals, CASP1 proteolytically cleaves pro-IL1β in two sequentially conserved locations, resulting in the formation of mature or active IL1β with a molecular weight of approximately 17 kDa (Hailey et al. 2009). Compared with pro-IL1β1, the full-length IL1β2 sequence has an amino acid deletion and insertion, yet retains both CASP1 sites (Mathew et al. 2015).

CASP1 processing of pro-IL1β2, however, may not be the same as for pro-IL1β1. When Degrelle et al. (2009) conducted a proteomic analysis of elongating pig conceptuses, they detected multiple IL1β proteins with varying molecular weight and/or isoelectric points. The detected proteins are likely IL1β2 variants arising from alternate CASP1 activity or pro-IL1β2 processing by other proteases. In support of this, Katebi et al. (2010) predicted IL1β1 and IL1β2 (pro and mature) protein structures using an iterative threading assembly refinement (I-TASSER) program. They suggest that an amino acid substitution near CASP1 site 2 in pro-IL1β2 could reduce the protease’s activity. In addition, we predicted and compared IL1β1 and IL1β2 solvent-accessible surface areas using DNASTAR’s latest protein structure assembly program, NovaFold. Visual comparison of the NovaFold-predicted pro-structures revealed that pro-IL1β2 might have a large steric hindrance partially concealing the first CASP1 site (Fig. 2). In the study by Degrelle et al. (2009), a fully processed IL1β lacking the pro-domain and with a molecular weight of approximately 18 kDa was detected. At least some pro-IL1β2, therefore, may

![Figure 2 Solvent-accessible surface areas of pro-IL1β1 and pro-IL1β2 protein structures predicted using DNASTAR NovaFold computer program. Four amino acids N-terminal and C-terminal to the CASP1 sites are depicted in green and blue color respectively. Compared with pro-IL1β1, pro-IL1β2 was predicted to have a large steric hindrance, resembling a Y, covering CASP1 site 1. The opposite was true for CASP1 site 2. Alternatively, a large molecular structure covers CASP1 site 2 in pro-IL1β1 but not pro-IL1β2. The bottom structures are the top structures rotated 180° around a vertical axis and tilted to the right 90°.](image-url)
be proteolytically modified by CASP1 in a manner that is similar to pro-IL1β1.

Fully processed IL1β1 and IL1β2 are 92% similar within the mature region (Mathew et al. 2015). The high rate of amino acid conservation within this region suggests that mat-IL1β2 also interacts with the IL1R1. Compared with mat-IL1β1, however, mat-IL1β2 was predicted to have three less receptor-binding sites and an inserted proline and four non-conserved amino acid substitutions in locations predicted to interact with the IL1R1 (Fig. 3) (Mathew et al. 2015). Three of these substitutions in mat-IL1β2 result in a complete change of amino acid side-chain charge and solvent-accessible surface area when compared with mat-IL1β1 (Fig. 3). The second receptor-binding substitution in mat-IL1β2 includes the addition of a glutamic acid (glutamate; Glu16) in place of a histidine (His15; mat-IL1B1) with negative- and positive-charged side chains respectively (Fig. 3) (Mathew et al. 2015). Human IL1β has an uncharged glutamine in this location and its mutation to a glycine results in complete loss of receptor-binding activity (Vigers et al. 1997). Based on this information, one might conclude that non-conserved amino acid substitutions in mat-IL1β2 could affect its interaction with the IL1R1 resulting in functional differences between mat-IL1β1 and mat-IL1β2. In further support of this, when the activities of recombinant IL1β1 and IL1β2 were tested during pig endometrial explant experiments, mat-IL1β2 had a lesser capacity to activate NF-κB in uterine LE cells and increase NF-κB-responsive gene expression within total endometrium (Mathew et al. 2015).

**IL1β2 and the conceptus**

The autocrine effects of IL1β2 on the pig conceptus are unknown. Pig conceptuses abundantly and temporally express IL1β2, IL1R1, and IL1RAP during rapid elongation and estrogen synthesis for maternal recognition of pregnancy (Fig. 1A and C). Therefore, it has been suggested that IL1β2 is involved in these processes (Ross et al. 2003a, Degrelle et al. 2009, Mathew et al. 2015). This theory is supported by the observation that IL1β increases aromatase expression and estrogen synthesis in human cytotrophoblast cells (Nester 1993). In addition, after a global proteomic analysis of early pig conceptuses, IL1β was regarded as a focal protein between three primary protein networks that are expressed during rapid elongation (Degrelle et al. 2009). These networks include proteins involved in cellular assembly and organization, embryonic development, as well as cell growth and proliferation. Interleukin-1 beta 2 may promote trophoblast cell reorganization and motility by increasing cell membrane fluidity and/or by modifying actin molecules during rapid elongation (Geisert et al. 1982, 2015). Interleukin-1 beta has been shown to increase the motility of airway epithelial cells, neutrophils, and tumor cells through modifications made to cell integrins and actin filaments (Ma et al. 2014).

**IL1β2 and the endometrium**

Pig conceptus IL1β2 may create a proinflammatory microenvironment within the endometrium that promotes implantation and establishment of pregnancy (Fig. 1A and C). Studies suggest that this environment could provide additional histotroph proteins for nourishment of the pre-implanting conceptus, endometrial estrogen, and prostaglandin (PG) synthesis during maternal recognition of pregnancy and endometrial architecture changes for implantation (Table 1) (Ross et al. 2003a, Degrelle et al. 2009, Franczak et al. 2010).
Table 1 Proinflammatory cytokine, source tissue, location of receptor, proposed actions during establishment of pregnancy, and corresponding references for cytokines detected within the pig conceptus or endometrium during rapid elongation (days 11–12 of pregnancy).  

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Source</th>
<th>Receptor location</th>
<th>Proposed actions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1β1</td>
<td>Endometrium</td>
<td>IL1R1</td>
<td>Increase endometrial estrogen and PG synthesis during MRP</td>
<td>Franczak et al. (2010, 2013)</td>
</tr>
<tr>
<td></td>
<td>Endometrium and conceptus</td>
<td>IL1R1</td>
<td>Uterine epithelial cell proliferation</td>
<td>Jeong et al. (2015)</td>
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<tr>
<td>IL1β2</td>
<td>Conceptus</td>
<td>IL1R1</td>
<td>Alterations to uterine surface architecture for implantation</td>
<td>Ross et al. (2003a), Mathew et al. (2015)</td>
</tr>
<tr>
<td></td>
<td>Endometrium and conceptus</td>
<td>IL1R1</td>
<td>Modulation of maternal immune responses to conceptus tissues</td>
<td>Geisert et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>Endometrium and conceptus</td>
<td>IL1R1</td>
<td>Direct actions on conceptus development (elongation)</td>
<td>Tuo et al. (1996), Ross et al. (2003a,b), Degrelle et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>Endometrium and conceptus</td>
<td>IL1R1</td>
<td>Increase conceptus and endometrial estrogen and PG synthesis during MRP</td>
<td>Ross et al. (2003a), Franczak et al. (2010, 2013)</td>
</tr>
<tr>
<td></td>
<td>Endometrium and conceptus</td>
<td>IL1R1</td>
<td>Increase expression of endometrial IL1-stimulated genes that contribute to conceptus development and attachment</td>
<td>Tuo et al. (1996), Ross et al. (2003a), Seo et al. (2011, 2012), Mathew et al. (2015)</td>
</tr>
<tr>
<td>LIF</td>
<td>Endometrium</td>
<td>LIFR</td>
<td>Direct actions on conceptus development and attachment</td>
<td>Modrić et al. (2000), Blítek et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Endometrium and conceptus</td>
<td>LIFR</td>
<td>Endometrial estrogen and PG synthesis during MRP</td>
<td>Modrić et al. (2000), Blítek et al. (2012)</td>
</tr>
<tr>
<td>IL6</td>
<td>Endometrium and conceptus</td>
<td>LIFR</td>
<td>Direct actions on conceptus development and attachment</td>
<td>Franczak et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>Endometrium and conceptus</td>
<td>LIFR</td>
<td>Endometrial estrogen and PG synthesis during MRP</td>
<td>Yu et al. (1998), Waclawik et al. (2010), Franczak et al. (2013)</td>
</tr>
</tbody>
</table>

*IL1, interleukin-1; IL1β1, interleukin-1 beta 1; IL1β2, interleukin-1 beta 2; IL1R1, interleukin-1 receptor type 1; LIF, leukemia inhibitory factor; LIFR, leukemia inhibitory factor receptor; IL6, interleukin-6; IL6R, interleukin-6 receptor; TNFα, tumor necrosis factor alpha; TNFR, tumor necrosis factor receptor. *Receptor presence suspected but not verified.

2013, Seo et al. 2011, Mathew et al. 2015). These events are likely triggered, in part, by IL1β1 and IL1β2 activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPK, and NF-κB within the endometrium (Jeong et al. 2015, Mathew et al. 2015). Studies of immortalized porcine LE cells suggest that IL1β activates p38 MAPK and ERK1/2 signaling pathways, the latter promoting epithelial cell proliferation (Jeong et al. 2015). In addition, elongating day 12 pig conceptuses or recombinant IL1β2 can activate the p65 subunit of NF-κB in pig uterine LE cells (Fig. 4) (Mathew et al. 2011, 2015).

The proinflammatory microenvironment may include an IL1-positive feedback loop involving conceptus IL1β2 and endometrial IL1β1. Elongating pig conceptuses that abundantly express IL1β2 increase the expression of IL1R1 and IL1RAP in the adjacent uterine epithelium (Mathew et al. 2011, Seo et al. 2012). In addition, recombinant IL1β2 and recombinant human
IL1β increase the expression of IL1β1 and IL1 signaling factors (IL1R1 and IL1RAP) in pig endometrial explants respectively (Seo et al. 2012, Mathew et al. 2015). It is well known that IL1 increases blood vessel permeability and leukocyte extravasation in peripheral tissues; therefore, it is possible that IL1β1 and IL1β2 synergistically modulate these processes during implantation in pigs. An increase in endometrial blood vessel permeability is temporally associated with conceptus release of IL1β2 (Keys & King 1988).

The IL1β1 and IL1β2 feedback loop may be partially controlled by NF-κB transcription factors activated by IL1 (Ross et al. 2010, Mathew et al. 2015). It is well known that IL1 and NFκB subunits are regulated by NF-κB activity. Considering that an over-amplification of the proinflammatory state could have consequences on the establishment of pregnancy, it is possible that reduced activity of IL1β2 helps to minimize the magnitude of the feedback loop.

Involvement of the IL1-NF-κB system during early pregnancy is not unique in pigs. Primate and rodent conceptus IL1β acts on the uterine surface epithelium to promote implantation and the effect is likely mediated by NF-κB transcription factors (Laird et al. 2000, Page et al. 2002, Nakamura et al. 2004, King et al. 2010). NF-κB increases transcripts for prostaglandin synthases and inflammatory cytokines such as prostaglandin-endoperoxide synthase 2 (PTGS2), IL6, and LIF that are essential for the establishment of pregnancy (Stewart et al. 1992, Laird et al. 2000, Nakamura et al. 2004). Prostaglandin-endoperoxide synthase 2 is a rate-limiting enzyme during PG synthesis and is essential for the implantation and decidualization in mice (Lim et al. 1997). This may also be true for pigs. Blocking prostaglandin synthesis in pigs results in implantation failure (Kraeling et al. 1985). Pig conceptus IL1β2 may increase endometrial PTGS2 activity by activating NF-κB. Transcripts for PTGS2 increase in uterine LE cells on day 12 of pregnancy and recombinant IL1β2 increased the expression of PTGS2 in endometrial explants that had activated NF-κB (Ashworth et al. 2006, Mathew et al. 2015). Prostaglandins, released by the conceptus and endometrium, have proinflammatory properties and essential functions during implantation (Kennedy et al. 2007, Waclawik 2011, Seo et al. 2014).

IL6 and LIF

Inflammatory cytokines IL6 and LIF have important functions during implantation in mammals. A decrease in endometrial IL6 and LIF mRNAs during early pregnancy in humans is correlated with infertility (Laird et al. 2000) and implantation does not occur in Lif-knockout mice (Stewart et al. 1992). Little is known about the effects of these cytokines during the establishment of pregnancy in pigs. Receptors for IL6 and LIF are expressed by both the conceptus and uterine surface epithelium between day 10 and 14 of pregnancy in pigs, indicating that these cytokines have important functions during implantation (Modrić et al. 2000, Blitek et al. 2012).

Endometrial expression of LIF and IL6 are under control of IL1 and estrogen activation of NF-κB and ESR transcription factors respectively. Inhibition of NF-κB activity blocks IL1-induced IL6 and LIF expression within cultured human endometrial epithelial cells (Laird et al. 2000) and delays implantation in mice (Nakamura et al. 2004). In the latter study, implantation was partially rescued after uterine viral transfection of Lif cDNA (Nakamura et al. 2004). In mice, ovarian estrogen also increases endometrial Lif expression during implantation, and LIF protein can replace nidatory estrogen by inducing implantation and decidualization (Chen et al. 2000).

Pig conceptus IL1β2 and estrogens may control IL6 and LIF expression within the endometrium. Transcripts for IL6 and LIF increase within the pig endometrium between day 10 and 12 of gestation and in response to conceptus-conditioned culture media (Blitek et al. 2012). Peak concentrations of LIF proteins were detected in pig uterine flushings on day 12 of pregnancy and are temporally associated with conceptus expression of IL1β2 and estrogen synthesis during maternal recognition of pregnancy (Blitek et al. 2012). IL1β2 could theoretically increase the expression of IL6 in conceptus tissues via IL1R1. Transcripts for IL6 were found to transiently increase in pig conceptuses during elongation, a time when IL1R1 and IL1β2 are abundant in conceptus tissues (Blitek et al. 2012). Within the endometrium, interactions between transcriptional regulators activated by IL1β, such as NF-κB, in combination with those activated by estrogen and progesterone, may be complex yet optimize the adjacent uterine environment for implantation of the pig conceptus (Quaedackers et al. 2007, King et al. 2010).

Interferons

IFNs are expressed by the peri-implantation primate, rodent, and ungulate trophoblast and coordinate essential interactions within the uterus during the establishment of pregnancy (Bazer et al. 2009). Conceptus IFNs are the maternal recognition of pregnancy signal in ruminants (cattle, sheep, and goats), acting on the endometrium to indirectly maintain P4 synthesis by the CL (Imakawa et al. 1987, Spencer et al. 2008). Conceptus IFNs also stimulate the expression of classical (antiviral) and non-classical IFN-stimulated genes (ISGs) that are under spacio-temporal regulation within the mammalian endometrium (Spencer et al. 2008). Although the functions of endometrial ISGs remain unclear, studies investigating IFN signaling within the uterus of livestock species suggest that they...
modulate maternal immune tolerance of the implanting conceptus, endometrial architecture changes for uterine receptivity, and vascular remodeling for maternal–fetal nutrient and waste exchange (Table 2).

Mammalian IFNs are classified under two main types: type I and type II. Type I IFNs include interferon alpha (α), beta (IFNβ), kappa (IFNκ), omega (IFNω), epsilon (IFNε), tau (IFNτ), and IFNδ. Some type I IFNs consist of more than one subtype; however, all bind the same receptor complex (the interferon alpha receptor (IFNαR)) to signal a biological response. The type II interferon consists of one member, IFNγ, which does not share structural similarity with type I IFNs. IFNγ has its own receptor complex, the interferon gamma receptor (IFNγR) (Platianias 2005).

Type I and II IFNs trigger the expression of classical ISGs through activation of the janus-associated kinases and signal transducers and activators of transcription (Jak-STAT) signaling pathways. Interferon signaling involves binding of type I and II IFNs to IFNαR and IFNγR, respectively, resulting in phosphorylation and activation of STAT (González-Navajas et al. 2012, Bazer 2013). Type I IFN signaling can activate STAT1 and STAT2, resulting in the formation of the interferon-stimulated gene factor 3 (ISGF3) complex. The ISGF3 can bind IFN-stimulated response elements (ISRE) within promoter regions of DNA and up-regulate ISGs (González-Navajas et al. 2012, Bazer 2013). Formation of STAT1 dimers during IFN signaling leads to the expression of interferon regulatory factor 1 (IRF1), a protein that can also bind and activate ISREs. There are additional signaling cascades activated by type I and type II IFNs other than Jak-STAT and perhaps explain the pleiotropic effects of these cytokines within the endometrium (González-Navajas et al. 2012, Bazer 2013).

### IFNγ/IFNδ and reproduction

The discovery that IFNγ serves as the maternal recognition of pregnancy signal in ruminants prompted investigations of IFN signaling within the reproductive tissues of other mammals, including pigs (Imakawa et al. 1987). By 1989, it had been confirmed that antiviral activity could be detected in pig uterine flushings and conceptus-conditioned culture medium between day 12 and 17 of gestation (Cross & Roberts 1989). It was determined later that pig conceptuses express the type II IFN, IFNγ, and a newly discovered type I IFN, IFNδ (Lefèvre et al. 1990, La Bonnardière et al. 1991).

Interferon gamma is the dominate pig conceptus IFN, accounting for 75% of antiviral activity in uterine flushings compared with IFNδ (25% of activity). Interferon gamma transcripts are found in conceptus RNAs between day 13 and 20 of development (Joyce et al. 2007a), and peak concentrations of IFNγ protein (250 μg per uterine horn) are detected in uterine flushings near day 15 of pregnancy (Fig. 1B and D) (Cencić & La Bonnardière 2002). Interferon gamma protein is released by the trophoblast and has been localized within cytoplasmic vesicles at the cell apical surface (Lefèvre et al. 1990, Joyce et al. 2007a). The proinflammatory cytokine IL18, released by the pig endometrium between day 15 and 18 of gestation, may stimulate conceptus IFNγ release (Table 2) (Ashworth et al. 2010). Only transcripts for IFNδ, expressed by the conceptus between day 14 and 20 of development, have been detected (Joyce et al. 2007a).

Interferon gamma receptors are expressed in the day 10 pig conceptus; however, the endometrium is considered to be the primary target (D’Andréa & La Bonnardière 1998). Endometrial IFNγR is abundant on day 15 of pregnancy and is temporally associated with peak production of IFNγ by conceptuses (D’Andréa &

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### Table 2 Proinflammatory cytokine, source tissue, location of receptor, proposed actions during establishment of pregnancy, and corresponding references for cytokines detected within the pig conceptus or endometrium during implantation (days 15–18 of pregnancy).^6^

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Source</th>
<th>Receptor location</th>
<th>Proposed actions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ and IFNδ</td>
<td>Endometrium (IFNγ)</td>
<td>IFNγR and IFNαR^b</td>
<td>Increase endometrial ISGs that contribute to conceptus development and attachment; Endometrial blood vessel modifications for maternal–fetal hematropic transport; Direct actions on conceptus development</td>
<td>Joyce et al. (2007a,b, 2008), Kim et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Conceptus (IFNγ and IFNδ)</td>
<td>Endometrium and conceptus</td>
<td>Alterations to uterine surface epithelial architecture during implantation; Influence of endometrial leukocyte function and immune tolerance to conceptus tissues</td>
<td>Joyce et al. (2008), Kim et al. (2012)</td>
</tr>
<tr>
<td>IL18</td>
<td>Endometrium</td>
<td>IL18R^b</td>
<td>Conceptus IFNγ release</td>
<td>Ashworth et al. (2010)</td>
</tr>
</tbody>
</table>

^6^IFNγ, interferon gamma; IFNδ, interferon delta; IFNγR, interferon gamma receptor; IFNαR, interferon alpha receptor; IL18, interleukin-18; IL18R, interleukin-18 receptor; ISGs, interferon-stimulated genes. ^b^Receptor presence suspected but not verified.
La Bonnardière 1998). Interferon gamma is considered to promote endometrial architectural changes during implantation by modulating the expression of zonula occluden 1 (ZO1), a tight junction protein, along the uterine surface epithelium (Table 2) (Cenci et al. 2003). In addition, conceptus IFNs are transferred across the uterine epithelial barrier and modulate the expression of ISGs within the stroma (Cenci et al. 2003, Joyce et al. 2007a,b, 2008).

Classical and non-classical endometrial ISGs

Estrogens, released by the conceptus between day 10 and 18 of pregnancy, are the initial maternal recognition of pregnancy signal in pigs (Bazer & Thatcher 1977, Bazer 2013). Unlike sheep and cattle, IFNs are not antiluteolytic in pigs, and infusion of IFNγ and IFNα into the pig uterus does not extend the interestrous interval or the functional life span of CL (Lefèvre et al. 1998).

In sheep and cattle, conceptus IFNα stimulates classical and non-classical ISGs within the endometrial stroma/dGE and LE/sGE respectively. Within the stroma/dGE, IRF1 and ISGF3 respond to IFNγ by modulating the expression of classical ISGs that are believed to be essential for the establishment of pregnancy (Bazer et al. 2009). Within the LE/sGE, IFNα stimulates IRF2, a potent inhibitor of IRF1 and ISGF3 activity. The IRF2 blocks the expression of classical ISGs including major histocompatibility complex class 1 polypeptide (MIC), beta 2 microglobulin (B2M), ESR1, and ISGF3 assembly factors (Choi et al. 2001, Spencer et al. 2008). Down-regulation of ESR1 prevents luteolysis in sheep (Spencer et al. 2008). IFNα and progesterone (via progestomedins) are hypothesized to stimulate and induce, respectively, non-classical ISG expression within the uterine LE/sGE. This may be achieved through a non-canonical second messenger pathway (independent of the STAT) that includes activation of PI3K and MAPK signaling cascades. Non-classical ISGs that increase in the uterus LE/sGE may affect conceptus development, glucose, and amino acid transport into the lumen and trophoblast attachment during implantation (Bazer et al. 2009).

Classical and non-classical ISGs are differentially regulated within the pig uterus during early pregnancy; however, the mechanism by which this is achieved involves the combined actions of P4, conceptus estrogens, and IFNs. Pig conceptus IFNγ and IFNα likely act synergistically to activate IRF1 and stimulate the expression of classical ISGs within the uterine stroma/dGE (Joyce et al. 2007a, Johnson et al. 2009). Stroma expression of IRF1 increases during early pregnancy in pigs and is temporally associated with an increase in classical ISGs localized within the stroma and GE including STAT1, STAT2, myxovirus resistance 1 (Mx1), and major histocompatibility complex (MHC) class I and II molecules (Joyce et al. 2007a,b, 2008, Kim et al. 2012). In addition, osmotic pump release of conceptus secretory proteins (CSP) into the pig uterine lumen will increase IRF1 in the stroma (Joyce et al. 2007b). Unlike sheep, estrogens (rather than IFNs) from the porcine conceptus increase the expression of IRF2 within the uterine LE.

In sheep, activation of IRF2 in the uterine LE/sGE blocks the expression of classical ISG STAT1. This does not appear to be true for pigs as conceptus estrogens also increase the expression of STAT1 in this tissue (Joyce et al. 2007a). Later in pregnancy, STAT1 increases within the stroma/dGE in response to pig conceptus IFNs (Joyce et al. 2007a). Activation of STAT1 during early pregnancy may be necessary for endometrial remodeling in the preparation of implantation (Johnson et al. 2009).

Endometrial MHC

The MHC is responsible for the majority of immune responses during tissue graft rejection. Pig trophoblast cells do not express MHC class I (classical or non-classical) or class II molecules during implantation (Joyce et al. 2008). This is considered to camouflage the conceptus from maternal immune responses that would be detrimental to conceptus survival. MHC molecules are expressed within the porcine endometrium, however, and are regulated by conceptus IFNs (Joyce et al. 2008).

Joyce et al. (2008) characterized endometrial β2m and classical (SLA1, SLA2, and SLA3) and non-classical (SLA6, SLA7, and SLA8) MHC class I molecules during the estrous cycle and early pregnancy in pigs. They concluded that pig conceptus IFNs increase MHC class I molecules within the stroma during implantation (day 10–25); however, conceptus estrogens decrease their expression within the LE by up-regulating IRF2 after initial attachment of the trophectoderm. In addition, the decrease in MHC class I molecules in the uterine LE coincides with an increase in the expression of an ubiquitin-specific protease (USP). The USP protein also represses type I IFN signaling and may therefore work with IFR2 in decreasing classical ISGs in the pig uterine surface epithelium (Joyce et al. 2008). Joyce et al. (2008) suggested that loss of β2m and MHC class I molecules along the conceptus–maternal interface could minimize negative immune responses against the conceptus; however, their increase within the stroma may facilitate endometrial vascular changes that support pregnancy. These conclusions are based on the studies in primates and rodents, in which it was found that uterine NK cells release IFNγ-promoting uterine vascular modification (Ashkar et al. 2000). In addition, the non-classical MHC class I molecule, HLA-G, regulates angiogenesis during trophoblast invasion in humans (Le Bouteiller et al. 2007).
MHC class II molecules can present exogenous-derived peptides on antigen-presenting cells to CD4+ helper T cells. Pig MHC class II molecules, SLA-DQ (A and B), increase in the subepithelial stroma and blood vessels on day 15 of pregnancy and in pig endometrial explants treated with recombinant IFNγ (Kim et al. 2012). An increase in endometrial MHC class II molecules in response to IFNγ may serve as a means of protecting the mother and conceptus from uterine pathogens. In addition, Kim et al. (2012) speculated that conceptus IFNs increase their expression in the adjacent stroma, facilitating the activation of CD4+ T lymphocytes to promote immune tolerance of the implanting pig conceptus.

Summary and conclusion

The cellular and molecular interactions that control early pregnancy in mammals are multifaceted and complex; however, common features do exist across species. Ongoing investigations of early pregnancy in primates, rodents, and agricultural animals help to delineate biological pathways that contribute to the establishment of pregnancy or alternatively reproductive failure. During early pregnancy in mammals, proinflammatory cytokines are released by the conceptus and endometrium to promote embryonic survival. In pigs, conceptus IL1 and IFNs likely create a balanced proinflammatory microenvironment within the endometrium by spacio-temporally activating MAPK, NF-κB, and Jak-STAT signaling pathways within the uterine epithelium and stroma. Interleukin-1 beta 2, a newly discovered IL1, may trigger similar pathways in the conceptus, promoting embryonic growth and rapid elongation. In addition, other inflammatory mediators such as LIF, IL6, IL18, and PG may compliment this environment, contributing to embryonic survival and establishment of pregnancy in pigs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Cytokines and embryo implantation in pigs


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