Pig Conceptuses Secrete Estrogen and Interferons to Differentially Regulate Uterine STAT1 in a Temporal and Cell Type-Specific Manner

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Conceptus trophoderm and uterine luminal epithelial cells interact via endocrine, paracrine, and autocrine modulators to mediate pregnancy recognition and implantation. Pig conceptuses not only release estrogens for pregnancy recognition but also secrete interferons during implantation. Because interferon-stimulated genes are increased by interferons secreted for pregnancy recognition in ruminants, we asked whether the interferon-stimulated gene, STAT1, is up-regulated in pig endometrium by conceptus estrogens and/or interferons. STAT1 expression in response to day of pregnancy, estrogen injection, and intrauterine infusion of conceptus secretory proteins in pigs indicated 1) estrogen increases STAT1 in luminal epithelial cells, 2) conceptus secretory proteins that contain interferons increase STAT1 in stroma, 3) STAT1 increases in close proximity to the conceptus, and 4) early estrogen results in conceptus death and no STAT1 in stroma. The interactions of estrogen and interferons to regulate cell-type-specific expression of STAT1 highlight the complex interplay between endometrium and conceptus for pregnancy recognition and implantation. (Endocrinology 148:4420–4431, 2007)

Implantation is the process by which the blastocyst attaches to the uterus for juxtaposition of embryonic and maternal circulations leading to the establishment of a functional placenta and successful pregnancy. During the peri-implantation period of pregnancy, uterine luminal epithelial (LE) cells and conceptus trophoderm develop adhesion competence in synchrony to initiate an adhesion cascade within a restricted period of the uterine cycle termed the window of receptivity. These cells orchestrate bidirectional interactions between the blastocyst and uterine endoderm involving spatiotemporally regulated endocrine, paracrine, and autocrine modulators that mediate cell-cell and cell-matrix interactions (1–7). The trophoderm layer of the blastocyst produces a factor(s) that signals pregnancy recognition as well as forms the placental membranes that are ultimately responsible for ensuring pregnancy success.

Uterine endometrial responses to implantation are complex. In addition to remodeling of the uterine LE (8), both LE and glandular epithelia (GE) secrete histotroph to nourish and support development of the conceptus (9). Uterine stroma transforms (i.e. decidualization) to control movement of the conceptus through the uterine wall during implantation while generating a cytokine-rich environment that directly promotes angiogenesis to ensure sufficient blood flow to the placenta for hematotrophic nourishment of fetal development (10–12).

In pigs, pregnancy recognition is the result of conceptus secretion of estrogens on d 11 and 12 of pregnancy to redirect prostaglandin $F_2\alpha$ secretion from the uterine vasculature to the uterine lumen where it is sequestered away from the corpora lutea (13–15). In addition, conceptus estrogens modulate uterine gene expression responsible for endometrial remodeling from 13–25 of gestation required for implantation (16). Secreted phosphoprotein 1 (SPP1, or osteopontin) is an extracellular matrix protein induced by estrogen in LE where it is hypothesized to influence trophoderm and LE adhesion, signal transduction, and cell migration (17). Conceptus estrogen secretion also correlates with conceptus secretion of IL-1β, which may in turn modulate uterine response to this cytokine (18). The importance of estrogen to implantation of pig conceptuses is underscored by the fact that premature exposure of the pregnant uterus to estrogen on d 9 and 10 results in degeneration of all pig conceptuses by d 15 (19).

Peri-implantation pig conceptuses also secrete interferons (IFNs) during the peri-implantation period. Cultured conceptuses from d 11 of pregnancy were first shown to secrete proteins that cross-reacted with antiserum against IFNα (20), but peak antiviral activity was not measured until d 14 and -15 conceptuses were cultured (21). The major species (75% of antiviral activity in pig conceptus secretory proteins) is the type II IFNγ and the other (25%) is the type I IFNβ (22, 23).
However, in contrast to sheep conceptuses, in which a type I IFN (IFNγ) is the signal for maternal recognition of pregnancy (24), the IFNs produced by pig conceptuses do not appear to be antioestrogenic. Intruterine infusion of conceptus secretory proteins on d 12 and 15 of the estrous cycle had no effect on interestrus interval or temporal changes in plasma progesterone concentrations (25).

IFNγ increases expression of a number of IFN-stimulated genes (ISGs) in the stroma of the ruminant uterus, including MX1 and signal transducer and activator of transcription 1 (STAT1) (7, 26, 27). It is noteworthy that paracrine actions of pig conceptus IFNs are also suggested by localization of IFN receptors on endometrial epithelial cells (23) and expression of MX1 in the stroma of pigs on d 18 of pregnancy (28). Although effects of these IFNs on pig endometrium have not been determined, emerging evidence suggests that induction or increases in ISGs in the endometrium by conceptus IFNs is a phenomenon of early pregnancy in many mammals and may facilitate establishment of a uterine vascular supply to the conceptus (29–34).

Our working hypothesis is that pig conceptus IFNs increase uterine endometrial expression of the ISG STAT1 during pregnancy and that STAT1 has biological roles in uterine receptivity and conceptus implantation and development. Indeed, STAT1 activation generally results in transcription of genes that are antiproliferative, proapoptotic, and proinflammatory that could profoundly influence endometrial remodelling for implantation and placentation (35). However, in ruminants, endometrial ISG expression is a result of the signal for pregnancy recognition from the conceptus. Pigs use estrogens, not IFNs, for pregnancy recognition. Therefore, the objectives of the present studies were to determine whether STAT1 is expressed in the pig endometrium during pregnancy and, if so, whether STAT1 expression is regulated by conceptus estrogen and/or conceptus secretory proteins that contain IFNγ and IFNβ. Results provide compelling evidence that pig conceptus trophectoderm cells orchestrate precise temporal and cell-type-specific changes in uterine STAT1 expression through initial secretions of estrogen, followed by IFNβ and IFNγ.

Materials and Methods

Animals and tissue collection

Experimental and surgical procedures complied with the Guide for Care and Use of Laboratory Animals and were approved by the Texas A&M University Laboratory Animal Care or the Oklahoma State Institutional Care and Use Committees. Pigs were observed daily for estrus (d 0) and exhibited at least two estrous cycles of normal duration before use in studies.

Study 1. To evaluate the effect of pregnancy on gene expression, pigs were assigned randomly to either cyclic or pregnant status. Those in the pregnant group were bred when detected in estrus and 12 and 24 h later. Pigs were hysterectomized on either d 5, 9, 12, or 15 of the estrous cycle or d 9, 10, 12, 13, 14, 15, 20, 25, 30, 35, 40, 60, or 85 of pregnancy (n = 3 pigs/d status).

Study 2. To evaluate the effect of estrogen on uterine gene expression, pigs were assigned randomly to receive daily injections (im) of either 5 mg 17β-estradiol benzoate (Sigma; 5 mg in 5 ml corn oil) or 5 mg in 5 ml corn oil (Sigma) on d 11, 12, 13, 14, and 15 post estrus. On d 12 post estrus (coincident with secretion of IFNs by pig conceptuses) (20–22), each pig was surgically implanted with two indwelling Alzet osmotic pumps (Durect Corp., Cupertino, CA) with a constant delivery rate of 10 µl/h. Each uterine horn was isolated via midline celiotomy, clamped, and severed from the uterine body at approximately 5 in. from the uterotubal junction while preserving the mesometrium and vascular supply to the uterine horn. The transected ends of each uterine horn and uterine body were sutured closed and the serosa of the antimesometrial borders of the horn and body sutured together to prevent twisting of the horn. For each pump, a catheter was attached and inserted approximately 2 cm into the lumen of one uterine horn. Before surgery, pumps were filled and equilibrated per manufacturer’s instructions. For each pig, one uterine horn was infused by a pump filled with porcine serum albumin (35 mg; Sigma), whereas the other uterine horn was infused by a pump filled with porcine conceptus secretory proteins (CSPs) (35 mg). All pigs were hysterectomized on d 16 post estrus (coincident with maximal antifl ocal activity in pig uterine flushings) (21).

Study 3. To evaluate the effect of pig conceptus secretory proteins on uterine gene expression, pigs (n = 3) were injected (im) with 5 mg 17β-estradiol benzoate (Sigma; 5 mg in 5 ml corn oil) on d 11, 12, 13, 14, and 15 post estrus. On d 12 post estrus (coincident with secretion of IFNs by pig conceptuses) (20–22), each pig was surgically implanted with two indwelling Alzet osmotic pumps (Durect Corp., Cupertino, CA) with a constant delivery rate of 10 µl/h. Each uterine horn was isolated via midline celiotomy, clamped, and severed from the uterine body at approximately 5 in. from the uterotubal junction while preserving the mesometrium and vascular supply to the uterine horn. The transected ends of each uterine horn and uterine body were sutured closed and the serosa of the antimesometrial borders of the horn and body sutured together to prevent twisting of the horn. For each pump, a catheter was attached and inserted approximately 2 cm into the lumen of one uterine horn. Before surgery, pumps were filled and equilibrated per manufacturer’s instructions. For each pig, one uterine horn was infused by a pump filled with porcine serum albumin (35 mg; Sigma), whereas the other uterine horn was infused by a pump filled with porcine conceptus secretory proteins (CSPs) (35 mg). All pigs were hysterectomized on d 16 post estrus (coincident with maximal antifl ocal activity in pig uterine flushings) (21).

Preparation of porcine CSPs. As previously described (36, 37), conceptuses from d 15–17 pregnant pigs (coincident with maximal production of IFNs by conceptuses) (20–22) were recovered by flushing uterine horns, cultured for 30 h, dialyzed (MWCO 3500; Spectrum Laboratories, Inc., Rancho Dominguez, CA), concentrated (MWCO 5000; Millipore Corp., Bedford, MA), filter sterilized, assayed for protein concentration, and stored at 4 C.

Microarray analysis

For study 4, microarray analysis was conducted using a spotted cDNA array representing mRNA transcripts from pig brain, uterus endometrium, oocytes, early embryos, peri-implantation conceptuses, and fetal and ovarian tissues (developed at the University of Missouri using procedures previously described) (38).

Total endometrial RNA (20 µg) was reverse transcribed and labeled using the 3DNA Array 50 Expression Array Detection Kit (GeneChip Inc., Hatfield, PA). Four hybridizations were conducted per the manufacturer’s recommendations. For each replication, the total cDNA synthesis reaction volume for both treatments for each day was combined, cDNA volume concentrated to 3–10 µl (Microcon YM-30; Millipore), nuclelease-free water added to a final volume of 10 µl, and slides hybridized (10 µl concentrated cDNA, 25 µl 2× formamide hybridization buffer, 2 µl LNA dT blocker, and 13 µl nuclease-free water) at 53 C for 16 h in a humidified hybridization cassette using a 22 × 40 mm LifterSlip (Scientific Co., Portsmouth, N.H.). After hybridization, slides were washed (2× SSC/0.2% SDS at 65 C for 15 min; 2× SSC, at room temperature for 15 min; and 0.2× SSC at room temperature for 15 min), rinsed in 95% ethanol for 2 min, and dried on a slide centrifuge. Secondary hybridizations were conducted at 50 C for 3 h, washed, and dried as before. Each microarray slide was scanned with the Cy3 and Cy5 channels using the ScanArray Express (PerkinElmer Life Sciences Inc., Wellesley, MA). Laser power and photomultiplier tube gain were adjusted for each slide to minimize variation between wavelengths. GenePix Auto Processor 3.0 software was used for data preprocessing, background correction, Local Loess pin-by-pin intensity normalization,

RNA isolation and analyses

RNA isolation. Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations.

RT-PCR analysis. Partial cDNAs for porcine IFNα and porcine IFNγ were amplified by RT-PCR as previously described (39). For IFNα, conceptus total RNA from d 14 of pregnancy was reverse transcribed, and then gene-specific primers (GenBank accession no. Z22706; forward 5′-ATGGATTGTCCCCATGTAGG-3′ and reverse 5′-CTGAGCTAC-CAGGGTTACC-3′) were used. For IFNγ, porcine uterine endometrial RNA from d 15 of pregnancy was reverse transcribed, and then gene-specific primers (GenBank accession no. AY188890; forward 5′-CACGCTTGGCTGACCTTG-3′ and reverse 5′-GAAATGCCCTGT-TATCCTTG-3′) were used. Both PCR products were cloned into a pCRII cloning vector using the TA cloning kit (Invitrogen) and confirmed by sequence analysis. A BLAST search for each was conducted to ensure that only target genes were evaluated.

Northern blot analysis. As previously described (41), 8 μg total RNA per lane was hybridized with a radiolabeled antisense human STAT1 cRNA probe (42) generated by in vitro transcription with [α-35S]uridine 5′-triphosphate (PerkinElmer) and a MAXIscript kit (Ambion, Austin, TX). Hybridization signals were detected by exposure to a PhosphoImager screen and visualized using a Typhoon 8600 variable mode imager (Molecular Dynamics, Piscataway, NJ).

Slot blot analysis. As previously described (41), 20 μg total RNA per slot was hybridized with a radiolabeled antisense human STAT1 cRNA generated by in vitro transcription with [α-35S]uridine 5′-triphosphate (PerkinElmer). After hybridization, washes, and RNase A digestion, autoradiography was performed using NTB liquid photographic emulsion (Eastman Kodak, Rochester, NY). Slides were exposed at 4°C, developed in Kodak D-19 developer, counterstained with Harris’ modified hematoxylin (Fisher Scientific, Fairlaw, NJ), dehydrated, and protected with coverslips.

Immunofluorescence analysis

As previously described (44), frozen pig uterine cross-section (8–10 μm) were fixed in −20°C methanol, washed in PBS containing 0.3% vol/vol Tween 20, blocked in 10% normal goat serum, incubated overnight at 4°C with 30 μg/ml mouse antihuman STAT1 (610185; BD Biosciences Pharmingen, San Jose, CA) or 25 μg/ml mouse antiporcine IFNγ (I7662-18P; U.S. Biological, Swampscott, MA) or mouse IgG (negative control; Sigma), and detected with fluorescein-conjugated goat antimouse IgG (Chemicon International, Temecula, CA). Slides were overlaid with Prolong antifade mounting reagent (Molecular Probes, Eugene, OR) and a coverslip.

Photomicrography

Digital photomicrographs of in situ hybridization (autoradiographic film overview as well as representative bright-field and dark-field images of liquid emulsion autoradiography) and immunofluorescence staining were evaluated using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axioplan HR digital camera and Axiovision 4.3 software. Photographic plates were assembled using Adobe Photoshop (version 6.0; Adobe Systems Inc., San Jose, CA).

Statistical analysis

Data were subjected to least-squares ANOVA using the general linear models procedures of the Statistical Analysis System (SAS, Cary, NC). All slot blot hybridization data were analyzed using the 18S rRNA as a covariate to correct for differences in RNA loading. Data from study 1 were analyzed for effects of day and status and their interaction where appropriate. For all other studies, effects of treatment were determined by preplanned orthogonal contrasts. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. Data are presented as least-squares means with se.

Results

STAT1 increases in pregnant uterine endometrium in a cell-type-specific manner

The human cDNA for STAT1 detected an approximately 4.2-kb mRNA by Northern blot analysis of pig total endometrial mRNA from d 15 of pregnancy (data not shown). This mRNA was similar in size to that detected using the same cDNA in sheep endometrial total mRNA. Pregnancy-specific up-regulation of endometrial STAT1 mRNA was evaluated using slot blot hybridization (Fig. 1A). Steady-state levels of STAT1 mRNA in pig endometrium decreased between d 9 and 12 of the estrous cycle (P < 0.07). However, during pregnancy, STAT1 mRNA levels increased between d 10 and 20 (P < 0.01), decreased between d 20 and 25 (P < 0.07), decreased again between d 35 and 40 (P < 0.07), and remained low thereafter (Fig. 1A).

Temporal and spatial changes in STAT1 mRNA (Fig. 1B) and protein (Fig. 1C) in the endometrium of cyclic and pregnant pigs were assessed by in situ hybridization and immunofluorescence analysis, respectively. STAT1 mRNA was low in all endometrial cell types on d 9–15 of the estrous cycle. In contrast, STAT1 mRNA increased in LE cells between d 9 and 12 of pregnancy. A second increase in STAT1 mRNA was observed in endometrial stromal and GE cells between d 12 and 15 of pregnancy. STAT1 mRNA remained high in LE, stroma, and GE cells through d 20 of pregnancy, after which levels decreased to those observed on d 9 (Fig. 1B). Consistent with in situ hybridization results, immunoreactive STAT1 protein was low in endometrium during the estrous cycle but was present in LE cells on d 12 of pregnancy and prominent in endometrial LE and stromal cells on d 15 (Fig. 1C).

Collectively, these data document two STAT1 expression events in endometria during the peri-implantation period of pigs: 1) STAT1 increases in LE cells on d 12 when elongated pig conceptuses secrete estrogen for pregnancy recognition (13–15), and 2) a second STAT1 increase in endometrial stroma and GE cells between d 12 and 15 temporally correlates with increased antiviral activity measured in uterine flushes exposed to conceptus secretion of IFNγ and IFNα (20–22). Interestingly, expression of STAT1 in pig stroma and GE is similar to that observed in sheep endometrium in response to conceptus IFNγ, but up-regulation of STAT1 in LE is unique to the pig, which uses estrogen for pregnancy recognition.

IFNα and IFNγ are synthesized by pig conceptuses

Two major species of pig conceptus IFNs had previously been identified, the type II IFNγ and the type I IFNα (22, 23).
FIG. 1. Study 1: STAT1 increases in pregnant uterine endometrium in a cell-type-specific manner. A, Steady-state levels of mRNA for STAT1 in pig endometrium during the estrous cycle and pregnancy. The mRNA levels are expressed as least-square means of relative units of counts per minute with overall SEM, are normalized for differences in sample loading using 18S rRNA, and represent 20 μg total endometrial mRNA per sample. B, In situ hybridization analysis of STAT1 mRNA in cross-sections of pig uterus. Corresponding bright-field and dark-field images from different days (D) of the estrous cycle (C) and pregnancy (P) are shown. A representative section from D15P hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. Width of each field is 940 μm. C, Immunofluorescence localization of STAT1 protein in frozen cross-sections of pig endometrium from d 12 and 15 of the estrous cycle (C) and pregnancy (P). A representative section from D12P immunolocalized with nonrelevant mouse IgG serves as a negative control. Width of each field is 540 μm. PL, Placenta; ST, stratum compactum stroma; Tr, trophectoderm.
Although these proteins were shown to be coexpressed in d-16 pig trophoderm, no rigorous temporal and spatial localization of these genes was performed. Therefore in the present studies, pig-specific cRNA probes to IFNγ and IFNδ and antiserum to IFNγ were used to determine temporal and spatial localization of these IFNs in pig conceptus and uterine tissues (Fig. 2). RT-PCR analysis for IFNδ in d-14 conceptuses detected an approximately 296-kb mRNA (Fig. 2A). Sequence analysis identified that the PCR product was pig IFNδ; however, levels of IFNδ mRNA were not sufficiently high to be detectable in pig trophoderm by our in situ hybridization procedure (data not shown). In contrast, robust hybridization was detected for IFNγ using in situ hybridization (Fig. 2B). IFNγ was not evident in endometrium during the estrous cycle but was expressed in a pregnancy-specific manner by a population of cells scattered within the endometrial stroma on d 9 and 12. It is likely these are endometrial lymphocytes that have been reported to express IFNγ.

FIG. 2. Study 1: IFNγ and IFNδ are synthesized by pig conceptuses. A, RT-PCR analysis of IFNγ mRNA in two d-14 pig conceptus total RNA preparations. B, In situ hybridization analysis of IFNγ mRNA in cross-sections of the interface between pig uterus and conceptus. Corresponding bright-field and dark-field images from different days (D) of the estrous cycle (C) and pregnancy (P) are shown. A representative section from D15P hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. On D9P and D12P, arrows indicate a population of cells within the endometrial stroma that express IFNγ mRNA. Width of each field is 940 μm. ST, Stratum compactum stroma; Tr, trophoderm.
IFNγ in the pig (45). Pig conceptus trophectoderm cells expressed high amounts of IFNγ mRNA by d 13 of pregnancy, and IFNγ remained readily detectable through d 20 (Fig. 2B). Similar to a previous report (23), immunoreactive IFNγ was localized to perinuclear membranes typically occupied by endoplasmic reticulum and Golgi apparatus as well as cytoplasmic vesicles within trophectoderm cells, suggesting trafficking and secretion of IFNγ into the uterine lumen for access to endometrium (data not shown).

**Estrogen injections given im increase STAT1 in uterine luminal epithelium**

The timing of STAT1 expression in uterine LE coincides with the secretion of estrogen by pig conceptuses to signal pregnancy recognition. The potential involvement of estrogen in endometrial LE STAT1 expression was evaluated by exogenous estrogen administration in postestrus cyclic pigs.

The im injection of estradiol benzoate did not alter steady-state levels of STAT1 mRNA compared with corn oil injection in total pig endometrium, of which LE cells compose a small proportion (P > 0.1; Fig. 3A). However, in situ hybridization and immunofluorescence staining for STAT1 revealed that estradiol benzoate increased STAT1 mRNA and protein in the endometrial LE (Fig. 3, B and C). No increase in STAT1 gene expression was observed in the LE of corn-oil-treated pigs. STAT1 is therefore among the first genes shown to be regulated by estrogen in the pig uterine LE where it is temporally available to participate in pregnancy recognition and/or the adhesion cascade for implantation.

**Intrauterine infusion of pig conceptus secretory proteins that contain IFNα and IFNγ increases STAT1 in uterine stroma**

To investigate protein paracrine signals, including IFNα and IFNγ, directed from the conceptus, CSPs were infused into the uterine lumen of estrogen-treated postestrus cyclic pigs. CSPs increased steady-state levels of endometrial STAT1 mRNA expression over intrauterine infusion of control serum proteins (P = 0.08; Fig. 4A). Consistent with slot blot hybridization results, in situ hybridization and immunofluorescence analyses for STAT1 demonstrated increased expression in the endometrial stratum compactum stroma of d-16 cyclic pigs intrauterine infused with CSP (Fig. 4, B and C). No increase in STAT1 was observed when control serum proteins were infused into the uteri of d-16 cyclic pigs. Therefore, similar to sheep, STAT1 increases in the sub-LE uterine wall, coincident with exposure to IFNs, where it is temporally available to facilitate remodeling within the stromal compartment of the uterus for implantation and placentation.

**Uterine STAT1 increases in close proximity to paracrine release of IFNγ by implanting conceptuses**

Figure 5 illustrates the spatial distribution of STAT1 mRNA in relation to location of the conceptus within the uterine lumen using autoradiographs of serial uterine cross-sections probed with IFNγ and STAT1 cRNAs, respectively. Significantly, STAT1 increases in uterine endometrial LE,
FIG. 4. Study 3: intrauterine infusion of pig CSPs that contain IFN\(\gamma\) and IFN\(\beta\) increase STAT1 in uterine stroma. A, Steady-state levels of STAT1 mRNA in endometrium of pigs intrauterine infused with control serum proteins or with CSPs. The mRNA levels are expressed as least-square means of relative units of counts per minute with overall SEM, normalized for differences in sample loading using 18S rRNA, and represent 20\(\mu\)g total endometrial mRNA per sample. * Statistically different from control at \(P < 0.08\). B, In situ hybridization analysis of STAT1 mRNA in uterine cross-sections from control serum protein- and CSP-infused pigs. Corresponding bright-field and dark-field images of uterine endometrium are shown. A representative section from d 15 of pregnancy hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. Width of each field is 940\(\mu\)m. ST, Stratum compactum stroma. A representative section from d 15 of pregnancy immunolocalized with nonrelevant mouse IgG serves as a negative control. Width of each field is 540\(\mu\)m. ST, Stratum compactum stroma.

Inappropriate early estrogen results in demise of embryos and loss of IFN-induced STAT1

Early uterine exposure to estrogen on d 9 and 10 of pregnancy results in total embryo loss by d 15–17 of pregnancy (46). Analysis of uterine STAT1 mRNA expression using this experimental model system provides an opportunity to investigate the interrelationship between paracrine release of estrogens and IFNs by pig conceptuses.

Total endometrial STAT1 mRNA was 2.1-fold lower on d 15 of early estrogen-treated than control pregnant pigs (\(P = 0.06\); Fig. 6A). Although STAT1 mRNA increased in LE of all animals in the study, stromal expression of STAT1 was observed only in control pregnant pigs. No stromal expression of STAT1 was detected in pigs exposed to early estrogen (Fig. 6B). These results indicate that inappropriate estrogen, leading to progressive conceptus degeneration, compromises paracrine release of IFN\(\beta\) and IFN\(\gamma\), which are required for stromal expression of STAT1.

Discussion

Our results demonstrate that cell-type-specific induction of STAT1 in the pig uterus is differentially regulated by conceptus signals. Estrogen secretion by the conceptus on d 12, which is the signal for maternal recognition of pregnancy, temporally correlates with STAT1 expression in the LE, and treatment of cyclic pigs with exogenous estrogen increased STAT1 in the LE. Stromal induction of STAT1 correlates with IFN\(\beta\) and IFN\(\gamma\) secretion by the conceptus, and intrauterine infusion of CSPs, which contain IFN\(\beta\) and IFN\(\gamma\), into cyclic pigs treated with exogenous estrogen increased STAT1 compared with intrauterine infusion of control proteins, similar to that observed on d 15 of pregnancy.

Up-regulation of STAT1 within uterine LE, stroma, and GE in close proximity to the implanting conceptus implies paracrine regulation of STAT1 by conceptus estrogens and IFNs. A similar conceptus-associated pattern of LE gene expression has previously been observed for SPP1, a gene that increases in the pig uterine LE in response to conceptus estrogen (17). It is likely that estrogen effects on the endometrium are restricted to regions near the conceptus due to sulfatase activity of trophectoderm. During pregnancy, pig endometrium rapidly converts estradiol to the biologically inactive estrone sulfate, and concentrations of estrone sulfate are high within the uterine lumen of pregnant pigs (47). Trophectoderm has sulfatase activity that restores the biological activity of estrogen, allowing for a localized effect of estrogen to up-regulate STAT1 and SPP1 in LE.

In contrast, it is somewhat surprising that initial increases in stromal STAT1 are restricted to sites of intimate association between the conceptus and uterus, given that IFN\(\gamma\) synthesis and secretion by pig conceptuses appears to be similar in stroma, and GE cells in close proximity to the implanting conceptus that expresses estrogens, IFN\(\beta\), and IFN\(\gamma\), with the amount of STAT1 decreasing as distance from the point of contact between conceptus and uterus increases. These data strongly suggest that STAT1 expression is regulated by paracrine secretions, i.e. estrogens and/or IFN\(\beta\) and IFN\(\gamma\), from the implanting pig conceptus.
magnitude to IFNγ production by sheep conceptuses (Fig. 2, B and C) (25). Indeed, STAT1 increases universally in the stroma and GE of pregnant sheep without regard to conceptus location within the lumen, presumably due to the high levels of secretion of IFNγ by conceptuses (39, 48). One explanation for the spatial pattern of STAT1 expression observed in the pig uterus is that IFNα and IFNγ act synergistically to up-regulate ISGs. Interaction between type I and type II IFNs has been previously demonstrated (49). It is plausible that high levels of IFNγ act on uterine stromal and GE cells to increase intracellular stores of ISGF3 so that the much lower levels of IFNα can maximally up-regulate STAT1 in close proximity to the implanting pig conceptus.

To the best of our knowledge, this is the first report demonstrating estrogen regulation of STAT1 gene expression. Induction of STAT1 in LE may be the result of direct transcriptional activation. The protein kinase regulated by RNA (PKR) is an ISG, yet in the absence of IFN, induction can be mediated by Sp1 (50). Interestingly, estrogen regulates oxytocin receptor expression in the uterine LE of sheep through GC-rich Sp1 promoter elements (51). It is plausible that in a similar manner, estrogen up-regulates STAT1 gene expression in pig LE through binding of Sp1 sites. Alternatively, induction of STAT1 in LE may be indirect through the induction of a putative estromedin. This estromedin would be released from uterine cells, because exogenous estrogen, in the absence of a conceptus, induced STAT1 in uterine LE. Stromal estromedins that regulate gene expression in adjacent epithelia have been reported in the murine uterus (52).

However, estrogen receptor α (ESR1) is nearly undetectable in pig endometrial stroma from d 5–15 of pregnancy (53), and only low levels of estrogen receptor β (ESR2) have been detected in total pig endometrium (54, 55). Because ESR1 is prominent in pig LE through d 12 of pregnancy (53), it is possible that estrogen binds ESR1 in LE to induce release of an unknown autocrine factor that up-regulates STAT1 in LE.

Although estrogen regulation of STAT1 gene expression has not previously been reported, estrogen has been shown to induce transcriptional activation of STATs (56). This activation required cytoplasmic localization of ESR1 and ESR2 (55). Additionally, MAPK, Src-kinase, and phosphatidylinositol-3-kinase activity were involved in this activation (56). Because ESR1 is present in the pig uterine LE at the time of estrogen release by conceptuses (53), and ESR2 is barely detectable in the pig uterus (55), it is likely that STAT1 regulation would be mediated via ESR1. However, estrogen receptor involvement may not be essential. Kennedy and co-workers (57) used estrogen receptor-negative osteoblast and breast cancer cells to demonstrate estrogen-dependent STAT1 activation.

Although estrogens, secreted by pig conceptuses on d 12 of gestation, function to establish pregnancy (13–15), secretion of estrogen also overlaps with initiation of attachment of the conceptus to the uterine luminal surface for implantation on d 13 of pregnancy (58). Indeed, both pregnancy recognition and implantation require rapid morphological elongation of trophectoderm that coincides with elevated conceptus estrogen synthesis and release (16). The timing and extent of estrogen exposure can have dramatic effects on conceptus development and survival. Insufficient distribution of estrogen, as seen in litters with fewer than two piglets per uterine horn at the time of trophectoderm elongation, results in failure to prevent luteolysis and subsequent termination of pregnancy (59). On the contrary, adverse timing of estrogen exposure in the form of naturally occurring alfatoxins in moldy corn on d 9 and 10 of gestation results in conceptus degeneration during the period of placental attachment to the uterine surface (46, 60). Indeed, conceptus estrogens are believed to regulate implantation success by altering gene expression, including SPP1 (17) and IL-1β (18), in uterine LE to initiate a cascade of molecular events that modifies the luminal glycocalyx for conceptus implantation (58, 61). It is reasonable to propose that elongating pig conceptuses secrete estrogen to increase expression of STAT1 in LE and that this gene has a role in pregnancy recognition and/or the adhesion cascade for implantation.

Pig conceptuses also secrete IFNs during the peri-implantation period (22), a phenomenon shared by humans,
rodents, and ruminants (24, 29, 33). However the secretion of both type I and type II IFNs, IFN\(\delta\) and IFN\(\gamma\), respectively, is unique to the pig. In general, both type I and II IFNs can induce STAT1 through the classical Janus kinase-STAT signaling pathway leading to γ-activation factor binding of γ-activated sequence (GAS) elements and induction of gene transcription (62). In addition, IFN\(\delta\) signals through a similar, yet distinct, pathway leading to ISGF3 complex binding of IFN-stimulated response elements in the promoters of several ISGs to initiate transcription (63).

Although the type I IFNo and type II IFN\(\gamma\) each induce expression of largely nonoverlapping sets of genes, they can also act in concert to produce synergistic interactions leading to mutual reinforcement of physiological responses (64). This synergy has been demonstrated for cooperative induction of ISGs such as STAT1. Normally relatively nonresponsive to IFN\(\gamma\), combined treatment of cells with IFN\(\gamma\) followed by IFN\(\alpha\) results in higher-magnitude ISG induction (64). In addition, cotreatment with IFN\(\gamma\) and IFN\(\alpha\) extends the period of ISG expression over IFN\(\alpha\) alone (49). For typical ISGs, type I IFNs induce rapid expression, independent of protein synthesis, followed by a protein synthesis-dependent suppression of transcription within 6 h. IFN\(\gamma\) overrides IFN\(\alpha\)-induced ISG repression, allowing continuous expression of ISGs for greater than 24 h (49). Clearly, IFN\(\delta\) and IFN\(\gamma\) may profoundly influence uterine physiology through cooperative induction of cytokine-specific transcription factors, such as STAT1, that allow reinforcement of effects of distinct cell-surface ligands while maintaining the specificities of the individual inducing IFNs.

It is estimated that the IFNs control the transcription of several hundred genes to influence cell functions. STAT1 activation mediates transcriptional responses to many cytokines and growth factors that are generally antiproliferative, proapoptotic, and proinflammatory (35). Interestingly, recent work by Hartman et al. (65) has shown that gene transcription that results from STAT1 activation depends on the type of interferon that activates STAT1. Because STAT1 homodimers form after both IFN\(\gamma\) and IFN\(\alpha\) stimulation, it was expected that IFN\(\gamma\)-induced STAT1 binding sites would predominately overlap with those of the IFN\(\alpha\)-induced STAT1 sites. However, IFN\(\gamma\) and IFN\(\alpha\) treatments result in dramatic differences in target sites for STAT1 binding. Sixty-six percent of the STAT1 binding sites observed with IFN\(\gamma\) treatment were absent in IFN\(\alpha\)-treated cells, and 75% of IFN\(\alpha\)-induced STAT1 binding sites were not present in IFN\(\gamma\)-treated cells (65). Indeed, IFN\(\gamma\)-induced STAT1 homodimers bind sites not occupied by STAT1 upon IFN\(\alpha\) induction, and vice versa, indicating that many STAT1 IFN\(\gamma\) sites are not used in the IFN\(\alpha\) response, and many STAT1 IFN\(\alpha\) sites are not used in the IFN\(\gamma\) response (65). Therefore, pig conceptuses possess the potential for highly complex and differential cell-type-specific gene expression in the mesenchymal component of the pregnant uterus through conceptus cosecretion of IFN\(\delta\) and IFN\(\gamma\) to induce STAT1.

Although it is well established that conceptus IFN\(\tau\) induces expression of numerous ISGs in the stroma and GE of ruminants (66), the pregnancy-specific roles of uterine ISGs in general remain conjectural. Recent analysis of human endometrium by Hess et al. (34) may shed light on ISG function(s) within the endometrium. In these studies, culture medium from human trophoblasts was incubated with decidualizing endometrial stromal cells, and global gene expression was assessed in the decidualized cells. IFN-induced or related genes constituted a significant percentage of those that were up-regulated in decidualizing cells in response to paracrine signals from the trophoblast (34). Human trophoblasts have previously been reported to produce IFN (29), and clearly, results from Hess et al.
(34) and others (30–33) indicate decidualizing stroma of mice and humans undergoes an IFN-like response similar to that observed in the endometrial stroma of ruminants, and now pigs. Interestingly, a decidual-like transformation has previously been reported in the pregnant endometrial stroma of sheep, suggesting that the endometrium of noninvasive implanting species undergoes remodeling somewhat similar to that observed in the decidua of invasive implanting species (67). Therefore, it is likely that ISGs facilitate remodeling within the stromal compartment of the uterus for implantation and placentation across disparate mammalian species. As players in decidual/stromal remodeling, individual ISGs could be involved in protecting the fetal semi-allograft from immune rejection, in limiting conceptus invasion through the uterine wall, and/or in establishing a vascular supply to the conceptus. Because IFNy, a protein secreted by pig conceptuses, is believed to initiate endometrial vascular development (68), it is reasonable to hypothesize that conceptus-derived IFNs up-regulate ISGs such as STAT1 to facilitate vascular changes necessary to provide hema-totropic support to the developing conceptus. Whether this is the case or not, it is becoming increasingly clear that IFN induction of genes within the uterine stroma of mam-mals is a universal response to, or component of, a pro-gressing pregnancy.

It is particularly intriguing that IRF1, a prototypic IFNy/STAT1-responsive gene (69), is not detectable in the uterine LE of peri-implantation sheep and pigs (27, 70). However, the concomitant expression of interferon regulatory factor 2 (IRF2), a potent transcriptional repressor of ISGs (71, 72), in LE has led to the hypothesis that interferon regulatory factor 2 prevents ISG expression in LE, whereas ISG expression in the underlying stroma continues unabated (27, 70). Indeed, most ISGs, including STAT1 are not expressed in the LE of sheep (7). Results of the present study indicate that conceptus estrogen secretion on d 12 alters this physiology by inducing STAT1 in pig LE without the subsequent induction of IRF1. Given that the human IRF1 promoter contains seven Sp1 sites (73) and that estrogen is capable of regulating gene transcription through ESR1/Sp1 interactions (53), estrogen may also regulate IRF1 expression. Indeed, 17β-estradiol can down-regulate IRF1 expression (74, 75) without affecting STAT1 expression (74), and this effect appears to be mediated by ESR1 (75, 76). The temporal/spatial pattern of uterine ESR1 correlates with this idea (53). ESR1 is detectable in pig LE on d 15 where STAT1, but not IRF1, is expressed (70). ESR1 is not present in d-15 stroma where both STAT1 and IRF1 are prominently expressed (70).

Collectively, in vivo results support the conclusion that conceptus estrogen and IFNs regulate uterine ISGs in a complex cell-type-specific manner. In the case of STAT1, conceptus estrogen increases STAT1 in LE as part of the pregnancy recognition signal that may also serve to remodel the apical surface of LE to allow attachment of the conceptus, whereas conceptus IFNγ and IFNγ increase STAT1 in the underlying uterine stroma that may play a role in the remodeling of the uterine wall for maximal blood flow to the developing fetus. It is likely that many other uterine genes considered critical for pregnancy success are regulated by similar interplay between conceptus steroid and protein secretion. Because the trophoblasts of ruminants, rodents, and primates share with pigs the secretion of multiple paracrine factors that profoundly affect uterine gene expression and uterine remodeling, insights from the present studies impact our understanding of early pregnancy across mammalian species. Although the key players at the uterine-placental interface require further definition, the interactions of estrogen, IFNs, and STAT1 described here highlight the complex, precisely orchestrated interplay between endometrium and conceptus that influences conceptus survival, implantation, and development.

Acknowledgments

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