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Pregnancy and interferon tau regulate *RSAD2* and *IFIH1* expression in the ovine uterus

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Abstract

Radical S-adenosyl methionine domain containing 2 (*RSAD2*) encodes a cytoplasmic antiviral protein induced by interferons (IFN). Interferon-induced with helicase C domain 1 (*IFIH1*) is a RNA helicase involved in innate immune defense against viruses, growth suppression, and apoptosis. Interferon tau (IFNT), a Type I IFN produced by the peri-implantation ruminant conceptus, acts on the uterine endometrium to signal pregnancy recognition and promote receptivity to implantation. Transcriptional profiling identified *RSAD2* and *IFIH1* as IFNT regulated genes in the ovine uterine endometrium. This study tested the hypothesis that *RSAD2* and *IFIH1* were induced in the endometrium in a cell type-specific manner by IFNT from the conceptus during early pregnancy. Endometrial *RSAD2* and *IFIH1* mRNA increased between days 12 and 16 of pregnancy, but not of the estrous cycle. In pregnant ewes, *RSAD2* and *IFIH1* mRNAs increased in endometrial glands, and stroma and immune cells, but not in the luminal epithelium. Neither gene was expressed in the trophectoderm of day 18 or 20 conceptuses. Progesterone (P4) treatment of ovariectomized ewes did not induce expression *RSAD2* or *IFIH1* mRNA in the endometrium; however, intrauterine injections of IFNT induced expression of *RSAD2* and *IFIH1* mRNA in endometria of ewes treated with P4, as well as in ewes treated with P4 and the progesterone receptor antagonist, ZK 136,317. These results indicate that conceptus IFNT induces both *RSAD2* and *IFIH1* in a P4-independent manner in the ovine uterine endometrium. These two IFNT-stimulated genes are proposed to have biological roles in the establishment of uterine receptivity to the conceptus during implantation through induction of an antiviral state and modulation of local immune cells in the endometrium.

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Introduction

Interferon tau (IFNT), the maternal recognition of pregnancy signal in ruminants (sheep, cattle, goats), is secreted by the elongating peri-implantation conceptus (embryo–fetus and associated membranes) and inhibits development of the endometrial luteolytic mechanism (Spencer & Bazer 2002, 2004). IFNT is produced by sheep conceptuses between days 10 and 21 of gestation with maximal production on days 14 to 16 (Farin *et al.* 1989, Guillomot *et al.* 1990). During pregnancy recognition, IFNT acts in a paracrine fashion on endometrial luminal epithelium (LE) and superficial ductal glandular epithelium (sGE) of the ovine uterus to repress transcription of the estrogen receptor alpha gene (Spencer *et al.* 1996, Fleming *et al.* 2001), thereby preventing estrogen induction of expression of the oxytocin receptor gene (Fleming *et al.* 2006) which precludes oxytocin-induced endometrial release of luteolytic pulses of prostaglandin F2 alpha (Spencer & Bazer 2004). The antiluteolytic actions of IFNT allow maintenance of a functional corpus luteum and secretion of progesterone (P4), which is the hormone of pregnancy necessary for successful implantation and development of the conceptus to term (Spencer & Bazer 2004). In addition, to antiluteolytic effects on the endometrium, IFNT induces a number of IFN-stimulated genes (ISGs) in a cell-specific manner within the endometrium, and ISGs are hypothesized to play important roles in uterine receptivity and conceptus implantation during establishment of pregnancy (Hansen *et al.* 1999*a*, Spencer & Bazer 2004, Gray *et al.* 2006, Klein *et al.* 2006). Several ISGs are first induced by P4 and stimulated by IFNT, whereas other genes are stimulated by IFNT from the conceptus in a P4-independent manner (Gray *et al.* 2006).

Recent transcriptional profiling experiments identified *RSAD2* and *IFIH1* as genes induced by IFNT from the conceptus in ovine and bovine endometria during early pregnancy (Gray *et al.* 2006, Klein *et al.* 2006). Radical S-adenosyl methionine domain containing

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2 (*RSAD2*; alias viperin) is a cytoplasmic antiviral protein induced by Type I IFNs that can inhibit infection of cells with human cytomegalovirus (Chin & Cresswell 2001). Interferon-induced with helicase C domain 1 (*IFIH1*; alias MDA5) is a RNA helicase that through its ATP-dependent unwinding of RNA, promotes mRNA degradation by specific RNases and is involved in innate immune defense against viruses as well as cellular growth suppression (Kang *et al.* 2002, 2004). IFIH1 senses intracellular viral infection and triggers innate antiviral responses including the production of Type I IFNs (Yoneyama *et al.* 2005). Both *RSAD2* and *IFIH1* are produced during a viral infection in response to IFNs to limit viral replication and modulate subsequent adaptive immunity (Katze *et al.* 2002, Helbig *et al.* 2005). Similar to other Type I IFNs, IFNT elicits antiviral, antiproliferative, and immunomodulatory activities in homologous and heterologous cells (Pontzer *et al.* 1991, Alexenko *et al.* 1997, Khan *et al.* 1998, Johnson *et al.* 1999*a*, 1999*b*, 1999*c*, 1999*d*, 1999*e*). Induction of an antiviral state in the endometrium during early pregnancy may be beneficial by inhibiting sexually transmitted viruses as well as modulating local immune cells to promote tolerance of the allogeneic conceptus and stimulating production of cytokines beneficial for conceptus survival and growth (Hansen 1995, Tekin & Hansen 2002, Croy *et al.* 2003*b*).

Although *RSAD2* and *IFIH1* have been identified as pregnancy- and IFNT-stimulated genes in the ovine uterine endometrium, the temporal and spatial alterations in their expression in the endometrium during early pregnancy and in response to P4 and IFNT have not been investigated. Our working hypothesis that RSAD2 and IFIH1 are induced in the endometrium in a cell-type specific manner by IFNT from the conceptus during early pregnancy and have biological roles in establishing uterine receptivity to implantation by the conceptus. As first step in testing this hypothesis, studies were conducted to determine effects of: (1) stage of the estrous cycle and early pregnancy on *RSAD2* and *IFIH1* expression in the ovine uterus; (2) P4 and IFNT on *RSAD2* and *IFIH1* expression in the ovine uterus; and (3) IFNT on *RSAD2* and *IFIH1* expression in ruminant endometrial cell lines.

Materials and Methods

Animals

Mature crossbred Suffolk sheep (*Ovis aries*) were observed daily for estrus in the presence of vasectomized rams and used in the experiment after they exhibited at least two estrous cycles of normal duration (16–18 days). At estrus, ewes were assigned randomly to cyclic or pregnant status. All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Teaching and Research, and were approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Experimental design

Study 1

At estrus (day 0), ewes were mated to either an intact or vasectomized ram as described previously (Spencer *et al.* 1999*a*) and then hysterectomized ($n=5$ ewes/day) on day 10, 12, 14, or 16 of the estrous cycle or day 10, 12, 14, 16, 18, or 20 of pregnancy. To confirm the pregnancy status, the uterine lumen was flushed with saline on days 10–16 of pregnancy and examined for the presence of a morphologically normal conceptus(es). At hysterectomy, several sections (~0.5 cm) from the mid-portion of each uterine horn ipsilateral to the corpus luteum were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO, USA). Several sections (1–1.5 cm) from the middle of each uterine horn were embedded in Tissue-Tek OCT compound (Miles, Oneonta, NY, USA), frozen in liquid nitrogen vapor, and stored at −80 °C. The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA extraction. In monovulatory pregnant ewes, uterine tissue samples were marked as either contralateral or ipsilateral to the ovary bearing the corpus luteum; no tissues from the contralateral uterine horn were used for this study.

Study 2

Sixteen cyclic ewes were ovariectomized and fitted with intrauterine (IU) catheters on day 5 post-estrus as described previously (Gray *et al.* 2006) and injected daily i.m. with 75 mg P4 between days 5 and 16. Ewes were then assigned randomly ($n=5$ ewes/treatment) to receive one of the following treatment regimens between days 11 and 16: (1) P4 and daily IU infusions of control serum proteins (P4+CX); (2) P4 and 75 mg of ZK136,317 (Schering, Berlin, Germany), a progesterone receptor (PGR) antagonist and CX proteins (P4+ZK+CX); (3) P4 and IU IFNT (2×10^7 antiviral units) (P4+IFN); or (4) P4 and ZK and IU IFNT (P4+ZK+IFN). The P4 and ZK were administered daily in corn oil vehicle. Both uterine horns of each ewe received twice daily injections of either CX proteins (50 μg/horn per injection) or recombinant ovine IFNT (5×10^6 antiviral units/horn per injection with CX proteins). Recombinant ovine IFNT was produced in *Pichia pastoris* and purified as described previously (Van Heeke *et al.* 1996). Proteins were prepared for IU injection as described previously (Spencer *et al.* 1999*b*). This regimen of P4 and IFNT mimics the effects of P4 and the conceptus on endometrial expression of hormone receptors and IFNT-stimulated genes during early pregnancy in ewes (Spencer *et al.* 1995, Johnson *et al.* 2001, Kim *et al.* 2003). All ewes were hysterectomized on day 17. The uterus was processed for histology and the endometrium obtained for RNA extraction as described in Study 1.

Cell culture

Immortalized ovine uterine endometrial LE cells were cultured as described previously (Johnson *et al.* 1999*c*). Bovine endometrial (BEND) cells (Johnson *et al.* 1999*a*) were kindly provided by Dr Thomas R Hansen (Colorado State University, Fort Collins, CO, USA). Ovine LE and BEND cells were maintained in 150 mm culture dishes containing DMEM (Dulbecco's modified essential medium) with F-12 salts (DMEM-F12; Sigma-Aldrich Corp.) supplemented with 5% serum and antibiotics. When cells reached 70–80% confluency, they were treated with either IFNT (2×10^7 antiviral unit (AVU)/ml) or left untreated as a control for 24 h in serum-free medium. The experiment was independently repeated three times in each cell type.

RNA isolation

Total cellular RNA was isolated from frozen endometrium or cultured cells using the Trizol reagent (Gibco-BRL) according to manufacturer's recommendations. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis respectively.

Cloning of partial cDNAs for ovine RSAD2 *and* IFIH1

Partial cDNAs for ovine *RSAD2* and *IFIH1* mRNAs were amplified by RT-PCR using total RNA endometrial tissues from day 18 of pregnancy using specific primers based on human *RSAD2* mRNA (Genbank NM_080657; forward, 5′-GAG GCC AAG AAA GGT CTG C-3′; reverse, 5′-CCA AGA ACG CTT CAA ACT CC-3′) and human *IFIH1* mRNA (Genbank AF095844; forward, 5′-TTC CGC AAA GAG TTC AAA CC-3′; reverse, 5′-AAT GTG TTC TTC GGG TTT GG-3′). The RT of cellular total RNA into cDNA was performed, as described previously (Stewart *et al.* 2000). The PCR amplification was conducted as follows for *RSAD2* and *IFIH1*: (1) 95 °C for 5 min; (2) 95 °C for 30 s, 56.5 °C for 40 s (for *RSAD2*), 57 °C for 40 s (for *IFIH1*), and 72 °C for 1 min for 35 cycles; and (3) 72 °C for 10 min. The partial cDNAs for ovine *RSAD2* and *IFIH1* PCR products were cloned into pCRII using a T/A Cloning Kit (Invitrogen) and their sequences verified using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

Slot blot hybridization analyses

Steady-state levels of mRNA in ovine endometrium were assessed by slot blot hybridization as described previously (Spencer *et al.* 1999*c*, Choi *et al.* 2001*a*). For *RSAD2* and *IFIH1* antisense cRNA probes, the plasmids were linearized with XbaI and *in vitro* transcription was conducted with SP6 RNA polymerase. Sense cRNA probes were generated using BamHI and T7 RNA polymerase. Radiolabeled antisense and sense cRNA probes were then generated by *in vitro* transcription with [α-^{32}P]-UTP. Denatured total endometrial RNA (20 μg) from each ewe was hybridized with radiolabeled cRNA probes. To correct for variation in total RNA loading, a duplicate RNA slot membrane was hybridized with radiolabeled antisense 18S cRNA (pT718S; Ambion, Austin, TX, USA). Following washing, the blots were digested with RNase A and radioactivity associated with slots quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ, USA).

Semiquantitative RT-PCR analysis

RSAD2 and *IFIH1* mRNA levels in immortalized ovine endometrial LE and BEND cells were assessed using semi-quantitative RT-PCR as described previously (Stewart *et al.* 2000). Briefly, isolated total cellular RNA was treated with RQ1 RNase Free-DNase1 (Promega) and then ethanol-precipitated. The cDNA was synthesized from total cellular RNA (5 μg) isolated from both cell-lines using random and oligo (dT) primers and SuperScript II Reverse Transcriptase (Life Technologies). Newly synthesized cDNA was acid-ethanol precipitated, resuspended in 20 μl sterile water, and stored at −20 °C. The cDNAs were diluted (1:10) in sterile water before use in PCR. The primers, PCR amplification and verification of their sequences were conducted as described in the section on cloning partial cDNAs. Housekeeping β-actin (ACTB) primers were forward (5′-ATGAAGATCCTCACGGAACG-3′) and reverse (5′-GAAGGTGGTCTCGTGAATGC-3′), which amplified a 270-bp product. PCR amplification was conducted as follows for ACTB: (1) 95 °C for 5 min; (2) 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min for 25 cycles; and (3) 72 °C for 10 min. After PCR, equal amounts of reaction product were analyzed using a 1.5% agarose gel, and PCR products were visualized using ethidium bromide staining. The amount of DNA present was quantified by measuring the intensity of light emitted from correctly sized bands under u.v. light using a ChemiDoc EQ system and Quantity One software (Bio-Rad).

In situ *hybridization analyses*

Location of mRNA expression in uterine sections (5 μm) was determined by radioactive *in situ* hybridization analysis as described previously (Spencer *et al.* 1999*c*, Choi *et al.* 2001*a*). Briefly, deparaffinized, rehydrated and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized *RSAD2* and *IFIH1* partial cDNAs using *in vitro* transcription with [α-^{35}S]-UTP. After hybridization, washing, and RNase A digestion slides were dipped in NTB-2 liquid photographic

emulsion (Kodak), and exposed at 4 °C for 1–2 weeks. Slides were developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Fairlawn, NJ, USA), and then dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed with Permount (Fisher). Images of representative fields were recorded under brightfield or darkfield illumination using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX, USA) fitted with a Nikon DXM1200 digital camera.

Statistical analyses

All quantitative data were subjected to least-squares analyses of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Slot blot hybridization data were corrected for differences in sample loading using the 18S rRNA data as a covariate. Data from Study 1 were analyzed for effects of day, pregnancy status (cyclic or pregnant), and their interaction. Data from Study 2 were analyzed using orthogonal contrasts (P4+CX vs P4+IFN; P4+ZK+CX vs P4+ZK+IFN; and P4+CX vs P4+ZK+CX) to elucidate effects of treatment. Semi-quantitative RT-PCR data was analyzed using the ACTB data as a covariate. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. A *P*-value of 0.05 or less was considered significant. Data are presented as least-square means (LSM) with standard errors (S.E.).

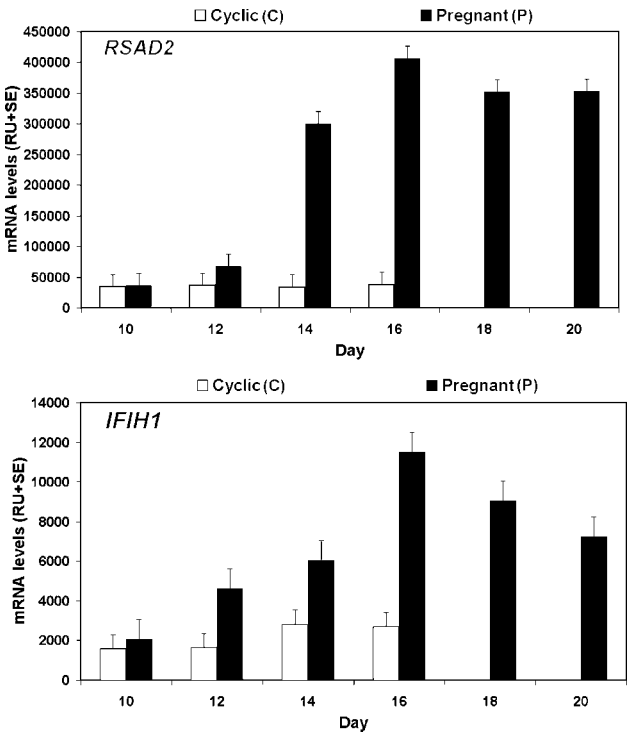

Figure 1 Steady-state levels of *RSAD2* and *IFIH1* mRNAs in endometria from cyclic and early pregnant ewes as determined by slot blot hybridization analysis. In cyclic ewes, *RSAD2* mRNA level was low between days 10 and 16. In contrast, *RSAD2* mRNA increased ($P<0.01$) sixfold between days 12 and 16 and was maintained to day 20. Similarly, *IFIH1* mRNA was very low in the endometria of cyclic ewes and increased ($P<0.01$) about 2.5-fold between days 12 and 16 of pregnancy. Data are expressed as LSM relative units (RU) with standard error (S.E.).

Results

RSAD2 *and* IFIH1 *expression increases in the endometrium by a cell type-specific manner*

Expression levels of *RSAD2* and *IFIH1* mRNAs in the endometrium of cyclic ewes were low and not affected ($P>0.10$) by day (Fig. 1). In contrast, *RSAD2* mRNA increased ($P<0.01$, quadratic) about sixfold between days 12 and 16 and was maintained through day 20 in pregnant ewes. Similarly, *IFIH1* mRNA increased ($P<0.01$, quadratic) about 2.5-fold between days 12 and 16. The presence of a conceptus increased endometrial *RSAD2* and *IFIH1* mRNA between days 10 and 16 ($P<0.01$, day x status; Fig. 1).

In situ hybridization analyses determined the location of *RSAD2* (Fig. 2) and *IFIH1* (Fig. 3) mRNAs in uteri of cyclic and pregnant ewes. *RSAD2* mRNA was low and not different between uteri from day 10 cyclic and pregnant ewes (Fig. 2). Between days 10 and 12 of pregnancy, *RSAD2* mRNA increased in the middle glands and to a lower extent in the stratum compactum stroma. Between days 14 and 20 of pregnancy, *RSAD2* mRNA was present predominantly in the endometrial glands, and stroma and immune cells, but not in LE, sGE, myometrium, or conceptus trophectoderm. Interestingly, *RSAD2* mRNA declined in the endometrial glands after day 16 of pregnancy. Similar to *RSAD2*, *IFIH1* mRNA was low and not different between uteri from day 10 cyclic and pregnant ewes (Fig. 3). Between days 10 and 12 of pregnancy, *IFIH1* mRNA increased slightly in the middle glands and stratum compactum stroma. Between days 14 and 18 of pregnancy, *IFIH1* mRNA was present predominantly in the stratum compactum stroma of the endometrium, middle glands, and immune cells, and not observed in the endometrial LE, sGE, myometrium, or conceptus trophectoderm. The melanocytes underneath the LE do not have *IFIH1* mRNA, but appear white in darkfield photomicrographs. Between days 18 and 20, *IFIH1* mRNA abundance declined in the endometrial stroma. The presence of *RSAD2* and *IFIH1* mRNAs in immune cells within the endometrium was based on visual observations of cell morphology.

Intrauterine administration of recombinant ovine IFNT induces RSAD2 *and* IFIH1 *mRNA in the ovine endometrium*

In order to determine if differences in expression of the selected genes in endometrium of pregnant compared

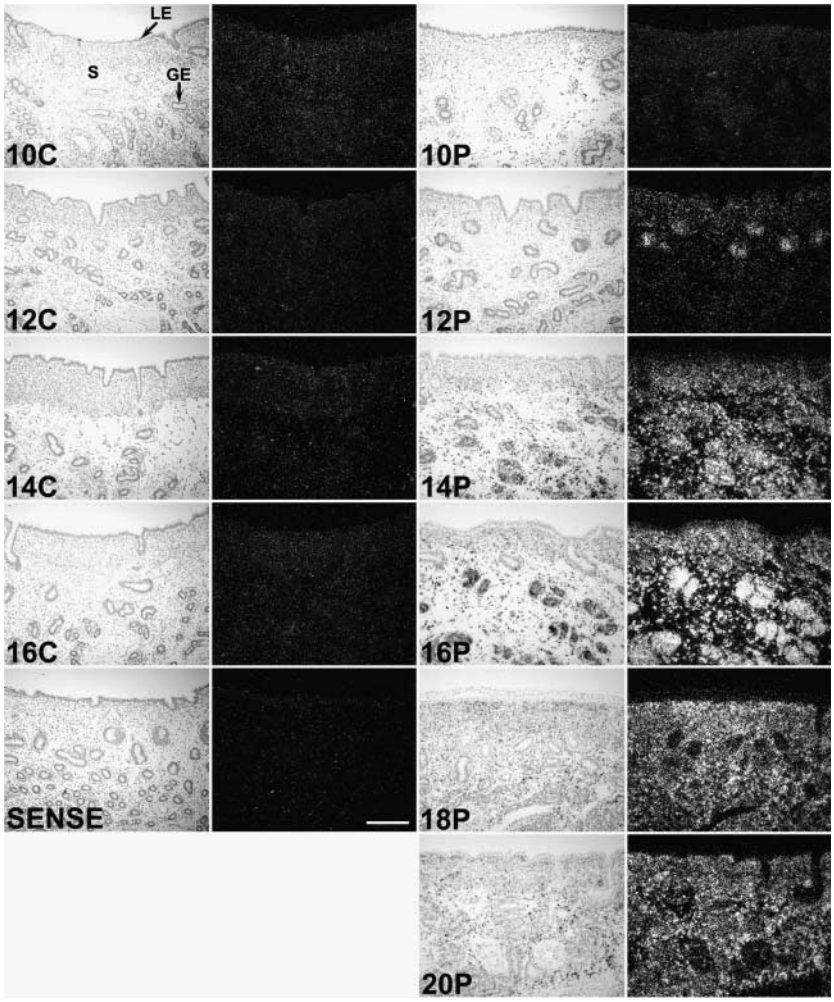

Figure 2 *In situ* hybridization analyses of *RSAD2* mRNA in uteri of cyclic and pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radio-labeled antisense or sense ovine *RSAD2* cRNA probes. *RSAD2* mRNA is expressed detected in endometrial stroma, glands, and resident immune cells. Legend: LE, luminal epithelium; GE, glandular epithelium; M, myometrium; S, stroma; Tr, trophectoderm. Scale bar represents 10 μm.

with cyclic ewes was due to IFNT from the conceptus, cyclic ewes were ovariectomized and fitted with IU catheters on day 5 and hysterectomized on day 17 (see Fig. 4A). Treatment of ewes with the ZK 136,317 PGR antagonist did not affect ($P>0.10$, P4+CX vs P4+ZK+CX) endometrial *RSAD2* or *IFIH1* mRNA abundance (Fig. 4B). For ewes receiving P4 alone, IU recombinant ovine IFNT increased ($P<0.001$) steady-state levels of *RSAD2* and *IFIH1* mRNAs 10-fold and 8.3-fold respectively, in the endometria ($P<0.001$, P4+CX vs P4+IFN; Figs 4B and 5A). Similarly, for ewes receiving P4+ZK treatment, IU recombinant ovine IFNT increased *RSAD2* and *IFIH1* mRNAs in the endometrium about 9.3-fold and 5.6-fold respectively ($P<0.001$, P4+ZK+CX vs P4+ZK+IFN).

In situ hybridization analyses verified that IFNT increased *RSAD2* and *IFIH1* mRNA abundance in the endometrium (Figs 4C and 5B). Similar to day 16–18 pregnant ewes, *RSAD2* and *IFIH1* mRNA were increased by IFNT primarily in the endometrial stroma and immune cells and, to a lower extent, in the endometrial glands of uteri from ewes receiving P4+IFNT treatment. In P4+ZK ewes, IFNT increased *RSAD2* and *IFIH1* mRNA in the endometrial stroma and immune cells. Further, IFNT increased *IFIH1* mRNA in endometrial LE of ewes receiving P4+ZK treatment (Fig. 5B).

Effects of IFNT on RSAD2 and IFIH1 in endometrial cells

In untreated ovine endometrial LE (oLE) and BEND cells maintained in serum-free medium, *IFIH1* but not *RSAD2* mRNA was detected (Fig. 6). Treatment of both oLE and BEND cells with recombinant ovine IFNT increased ($P<0.0001$) *RSAD2* and *IFIH1* mRNA levels.

Discussion

During the peri-implantation period of pregnancy, gene expression in endometria of ruminants is programmed primarily by P4 from the ovarian corpus luteum and IFNT from the conceptus (Spencer & Bazer 2002, Spencer *et al.* 2004*c*). In the present study, we identified two antiviral-related genes, *RSAD2* and *IFIH1*, as being induced in the ovine endometrium in response to IFNT

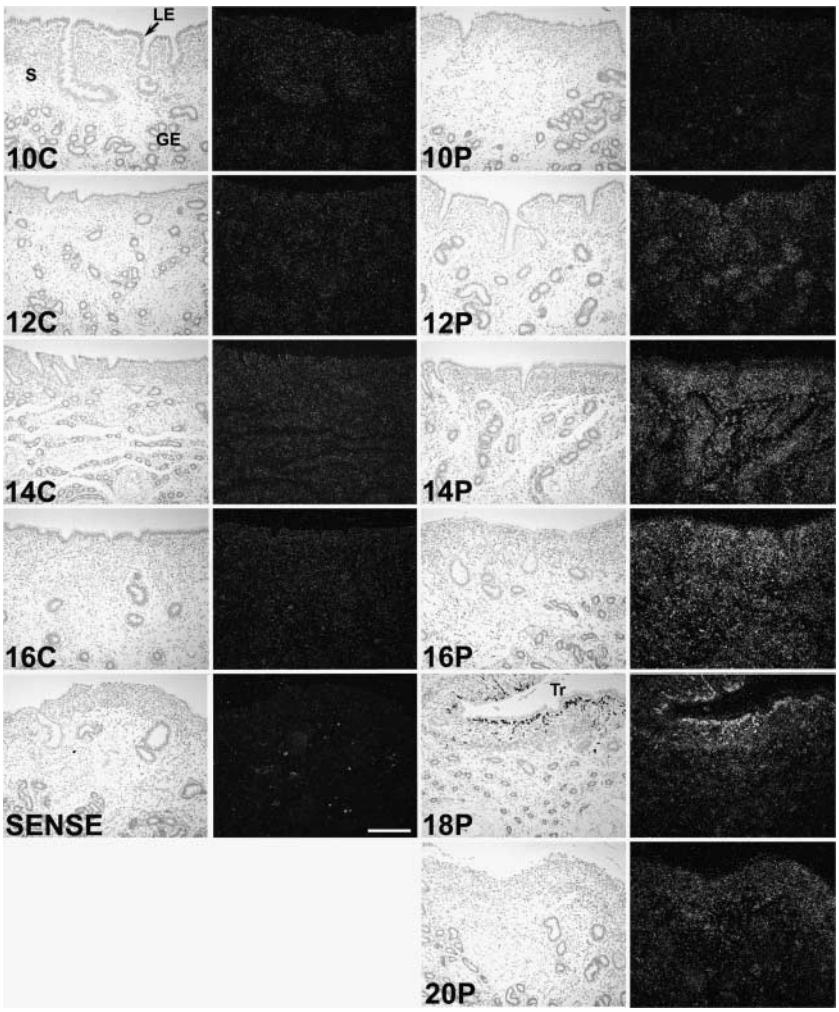

Figure 3 *In situ* hybridization analyses of *IFIH1* mRNAs in uteri of cyclic and pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radio-labeled antisense or sense ovine *IFIH1* cRNA probes. *IFIH1* mRNA was detected only in endometrial stroma and glands. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma; Tr, trophectoderm. Scale bar represents 10 μm.

from the conceptus in a P4-independent manner. These genes were selected for analysis based on transcriptional profiling studies of ruminant endometria (Gray *et al.* 2006, Klein *et al.* 2006) as well as knowledge that both *RSAD2* and *IFIH1* are produced during a viral infection in response to IFNs to limit viral replication and modulate subsequent adaptive immunity (Katze *et al.* 2002, Helbig *et al.* 2005). In the present study, the ontogeny of *RSAD2* and *IFIH1* in the ovine endometrium correlates directly with increasing amounts of IFNT produced by the rapidly elongating conceptus, which is maximum between days 14 and 16 and declines thereafter, as the trophectoderm begins implantation and trophoblast giant binucleate cells begin to differentiate (Guillomot *et al.* 1990). Clearly, P4 and IFNT have complex, independent, and complementary effects on expression of a number of genes in the ovine endometrium during early pregnancy (see Gray *et al.* 2006, Klein *et al.* 2006). In the present study, P4 was found to be not required for IFNT induction of *RSAD2* and *IFIH1* in the endometrium, which is similar to findings for other IFNT-stimulated genes in the ovine endometrium including CXCL10 (chemokine (C-X-C motif) ligand 10), IFITM3 (interferon induced transmembrane protein 3 (1-8U)), B2M (beta-2-microglobin), MIC (MHC class I polypeptide-related alpha chain), and STAT1 (Gray *et al.* 2006). Further, treatment of ovine endometrial LE and BEND cells with recombinant ovine IFNT induced *RSAD2* and *IFIH1* expression without a requirement for serum or P4 in the medium. In contrast, IFNT induction of several non-classical IFN-stimulated genes, such as LGALS15 (galectin 15), CTSL (cathespin L), and CST3 (cystatin C), in endometrial LE and sGE is dependent on P4 (Gray *et al.* 2004, Song *et al.* 2005, 2006), which is hypothesized to involve P4 down-regulation of the PGR in those epithelia (Spencer *et al.* 2004*c*, Gray *et al.* 2006).

In the ovine uterus, induction of *RSAD2* and *IFIH1* mRNA by the presence of the conceptus during pregnancy and by IFNT was limited to endometrial stroma and middle to deep glands, as well as resident immune cells based on visual observations of cell morphology. The majority of ISGs induced by IFNT without a requirement for P4 in the ovine uterus are restricted to endometrial stroma and middle to deep glands as well as immune cells (see (Spencer *et al.*

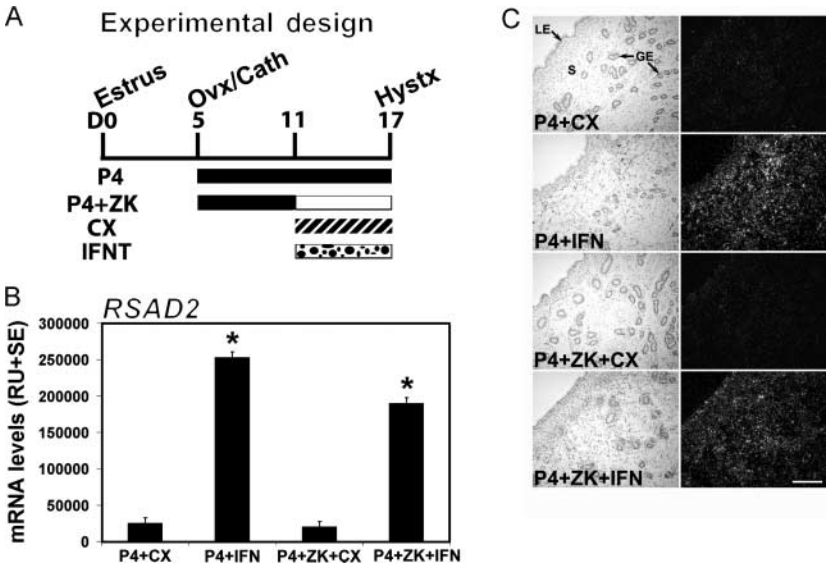

Figure 4 Effects of progesterone and IFNT on *RSAD2* mRNA in the ovine uterus. (A) Experimental design. See Materials and Methods for complete description. Legend: CX, control serum proteins; Hystx, hysterectomy; Ovx/Cath, ovariectomy and uterine catheterization; P4, progesterone; IFNT, recombinant ovine interferon tau; ZK, ZK137,316 anti-progestin. (B) Steady-state levels of *RSAD2* mRNA in endometria as determined by slot blot hybridization analysis. Intrauterine infusion of IFNT increased *RSAD2* mRNA by 10-fold in the endometrium (P4+CX vs P4+IFN, $P<0.001$), but not in ewes receiving the ZK anti-progestin (P4+IFN vs P4+ZK+IFN, $P>0.10$). Similarly, IFNT increased *RSAD2* mRNA 9.3-fold in ewes receiving ZK anti-progestin (P4+ZK+CX vs P4+ZK+IFN, $P<0.001$), but not in ewes receiving the ZK anti-progestin (P4+IFN vs P4+ZK+IFN, $P>0.10$). The asterisk (*) denotes an effect of treatment. (C) *In situ* hybridization analysis of *RSAD2* mRNA expression. *In situ* hybridization analyses verified that roIFNT increased *RSAD2* mRNA expression in a cell-type specific manner in P4-treated ewes consistent with increased expression in uteri from day 16 and 18 pregnant ewes. Intrauterine injections of IFNT increased *RSAD2* and *IFIH1* mRNAs in endometrial stroma and glands, but not LE, blood vessels or myometrium. Further, IFNT increased *RSAD2* mRNA in endometria of P4+ZK-treated ewes. Scale bar represents 10 μm.

2004*a*, 2004*c*) for review). A variety of ruminant and human cell lines have been used to determine that IFNT activates the classical JAK-STAT-IFN regulatory factor (IRF) signaling pathway utilized by other Type I IFNs that involves ISGF3 (STAT1, STAT2, ISGF3G complex), GAF (gamma activated unit) (STAT1 dimer), and IRF one (IRF1; see Stark *et al.* 1998, Spencer *et al.* 2004*c*). ISGF3 transactivates genes through binding an IFN-stimulated response element (ISRE), whereas GAF binds to a gamma activation sequence element in genes such as *IRF1*. Further, IRF1 transactivates genes through an IRF element (IRFE). Similar to findings for *RSAD2* and *IFIH1* in the present study, results of *in vivo* studies indicate that many classical IFN-stimulated genes (*STAT1*, *STAT2*, *IRF1*, *ISGF3G*, *GBP2*, *IFI6*, *IFI56*, *ISG15*, *MIC*, *B2M*, *OAS*) are not induced or increased by IFNT in LE and sGE of the sheep uterus (Johnson *et al.* 1999*d*, 2001, Choi *et al.* 2001*b*, 2003, Kim *et al.* 2003). This finding was initially surprising because all ovine endometrial cell types express IFNAR1 and IFNAR2 subunits of the common Type I IFN receptor (Rosenfeld *et al.* 2002). However, available results also indicate that IRF2, a potent transcriptional repressor of IFN-stimulated genes (Mamane *et al.* 1999), is expressed specifically in endometrial LE and sGE and represses transcriptional activity of promoters containing ISRE or IRFE (Choi *et al.* 2001*b*). Thus, IRF2 in LE and sGE is proposed to restrict IFNT induction of many IFN-stimulated genes to endometrial stroma and glandular epithelium. In fact, all components of ISGF3 (*STAT1*, *STAT2*, *ISGF3G*) and other studied IFN-stimulated genes (*B2M*, *GBP2*, *G1P2*, *ISG15*, *G1P3*, *IFI56*, *MIC*, *OAS*) contain ISREs in their promoters. Further, the promoter regions of the human and fish *RSAD2* genes contain multiple IRFEs (Chin & Cresswell 2001, Sun & Nie 2004). Similarly, the promoter region of the human *IFIH1* gene has predicted ISRE and IRFE (unpublished results). Thus, the constitutive presence and pregnancy-specific increase in IRF2 in ovine endometrial LE/sGE *in vivo* is proposed to prevent IFNT induction of *RSAD2* and *IFIH1* in those epithelia. P4 appears to be involved in this cell-type specification of IFNT actions, because IFNT induced *IFIH1* mRNA in the LE of the endometrium in P+ZK-treated ewes in the present study. Immortalized ovine endometrial LE and BEND cells lack or have very low levels of *IRF2* mRNA (Song & Spencer, unpublished results); thus, they are fully responsive to IFNT *in vitro* (Johnson *et al.* 1999*b*, Perry *et al.* 1999, Stewart *et al.* 2001). In human 2fTGH cells, Type I IFNB (interferon beta) can induce *IFIH1* expression, but this is not the case for STAT1 null U3A cells derived from 2fTGH cells (Kang *et al.* 2004). Thus, the classical JAK-STAT-IRF signaling pathway active in endometrial stroma and glands, and perhaps resident immune cells, is likely responsible for IFNT induction of

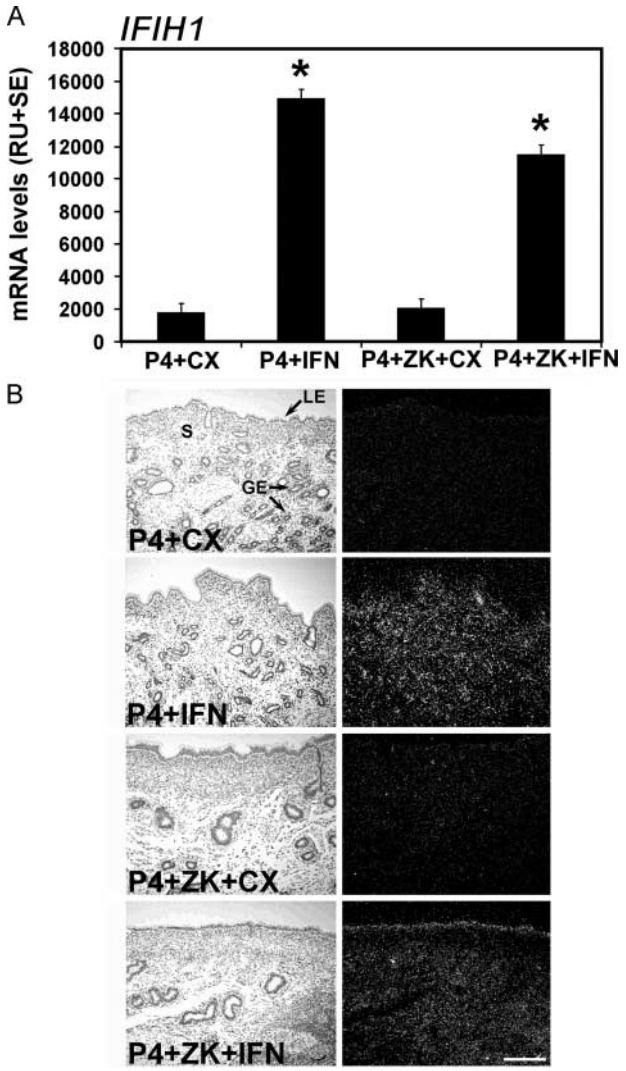

Figure 5 Effects of progesterone and IFNT on *IFIH1* mRNA in the ovine uterus. (A) Steady-state levels of *IFIH1* mRNA in endometria were determined by slot blot hybridization analysis. Intrauterine infusion of IFNT increased *IFIH1* mRNA about 8.3-fold in endometria (P4+CX vs P4+IFN, $P<0.001$), but not in ewes receiving the ZK anti-progestin (P4+IFN vs P4+ZK+IFN, $P>0.10$). The asterisk (*) denotes an effect of treatment. (B) *In situ* hybridization analysis of *IFIH1* mRNA expression. Intrauterine injections of IFNT increased *IFIH1* mRNA expression in a cell-type specific manner in P4-treated ewes consistent with the increased in expression in uteri from day 16 and day 18 pregnant ewes. Infusion of roIFNT increased expression of *IFIH1* mRNA in endometrial stroma and GE, but not LE, blood vessels or myometrium. Further, IFNT increased *IFIH1* mRNA in endometria from ewes treated with P4+ZK. Cross-sections of the uterine wall from treated-ewes were hybridized with radiolabeled antisense or sense ovine *IFIH1* cRNA probes. Scale bar represents 10 μm.

IFIH1 via activation and formation of the ISGF3 complex (Kang *et al.* 2004). One interesting finding of the present studies was the loss of *RSAD2* mRNA in the middle to deep endometrial glands between days 16 and 18 of pregnancy. This loss correlates with a reduction in IFNT production by the conceptus as well as a decline in IRF1 abundance in those glands (Choi *et al.* 2001*b*). Available evidence supports the concept that distinct cell-type specific differences exist in the ruminant endometrium with respect to responses to IFNT from the conceptus between the endometrial glands, stroma and resident immune cells.

The IFNT-stimulated genes in endometria of ruminants are hypothesized to be important for conceptus implantation (Hansen *et al.* 1999*b*, Spencer *et al.* 2004*b*, Klein *et al.* 2006). RSAD2 contains a radical S-adenosylmethionine (SAM) domain that catalyzes diverse reactions, including unusual methylations, isomerization, sulfur insertion, ring formation, anaerobic oxidation, and protein radical formation. Radical SAM proteins function in DNA precursor, vitamin, cofactor, antibiotic and herbicide biosynthesis, and biodegradation pathways (Sofia *et al.* 2001) which could be important in endometrial cells during the peri-implantation period to support conceptus development and implantation. IFIH1 (alias melanoma differentiation associated gene 5) is a RNA helicase induced during differentiation, cancer reversion, and programmed cell death (Kang *et al.* 2002, 2004). IFIH1 acts to sense intracellular viral infection and mediate a signal for innate antiviral responses including production of Type I IFNs (Kang *et al.* 2002, Yoneyama *et al.* 2005). Other Type I IFNs (IFNA and IFNB) are not induced in the endometrium in response to IFNT (Spencer & Bazer, unpublished results).

One biological role of RSAD2 and IFIH1 could be to prevent viral infection of the uterus during the critical peri-implantation period of pregnancy, particularly when the conceptus does not have a developed immune system or antiviral defenses. *RSAD2* and *IFIH1* are implicated in establishing an antiviral state by modulation of innate immune responses. For example, stable expression of *RSAD2* in fibroblasts inhibits human cytomegalovirus infection (Chin & Cresswell 2001). Given that IFIH1 also has growth suppressive properties, IFNT induction may suppress the activation of cells within the endometrium, which could be beneficial for pregnancy. In other species such as rodents and humans, resident and recruited immune cells within the endometrium play important roles in placentation and the success of pregnancy (Croy *et al.* 2003*a*, 2003*b*). Unfortunately, knowledge of which immune cells are present in the ovine uterus during pregnancy and their biological functions is sparse. During the estrous cycle, the density of macrophages and T lymphocytes in the ovine and bovine uteri do not change (Hansen 1998). However, during early pregnancy, the number of $CD45R^+$ lymphocytes increases in both endometrium (Segerson *et al.* 1991) and uterine and jugular venous blood (Lee *et al.* 1988, Alders & Shelton 1990). It has been postulated that these are NK (natural killer) cells that produce factors to enhance establishment of pregnancy. In the present study, the number of *IFIH1*- and, in particular, *RSAD2*-positive immune cells

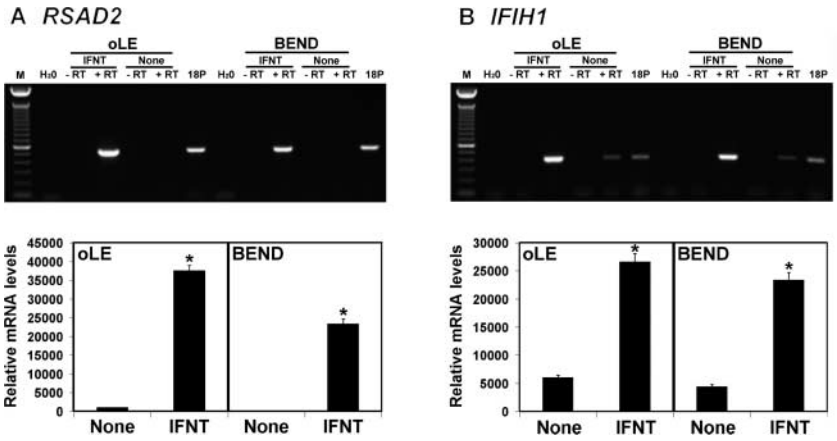

Figure 6 Semi-quantitative RT-PCR analyses of *RSAD2* and *IFIH1* mRNAs in total cellular RNA isolated from immortalized ovine endometrial LE (oLE) and bovine endometrial (BEND) cells. All PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide. Positions of the 100-bp (bp) DNA marker (M) ladder are shown. Total cellular RNA from endometria of a day 18 pregnant ewes and sterile water (no template) were positive and negative controls respectively. A graph illustrating the effect of IFNT on relative mRNA levels for RSAD2 and IFIH1 is presented below each gel, and the asterisk (*) denotes a significant ($P<0.001$) effect of IFNT treatment.

markedly increased in the endometria during pregnancy and in response to IFNT, but it is not clear whether these cells were recruited in response to IFNT or already present and stimulated by IFNT. The IFNT stimulated resident immune cells in the endometrium may migrate from the uterus, because IFNT-stimulated genes are higher in the peripheral blood leukocytes isolated from pregnant as compared with non-pregnant ewes and cows (Yankey *et al.* 2001). Eosinophils are also present in the endometrium of early pregnant ewes, and their numbers increase between days 11 and 19 of early pregnancy, perhaps due to actions of both P4 and perhaps IFNT (Asselin *et al.* 2001). In fact, IFNT possesses immunoregulatory activity and can inhibit mitogen-induced lymphocyte proliferation (Newton *et al.* 1989, Tekin *et al.* 2000) as well as modulate activity of NK cells (Tuo *et al.* 1993, Tekin & Hansen 2002). These effects of IFNT may prevent immune cell-mediated destruction of the conceptus (Hansen 1995). Finally, some IFNT-stimulated genes, such as CXCL10, from immune cells may have direct effects on conceptus implantation (Nagaoka *et al.* 2003, Imakawa *et al.* 2006). Collectively, available evidence supports the hypothesis that RSAD2 and IFIH1 modulate uterine receptivity to conceptus implantation by induction of an antiviral state and modulation of immune cell functions.

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