Pregnancy and interferon τ regulate DDX58 and PLSCR1 in the ovine uterus during the peri-implantation period

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Abstract

Interferon τ (IFNT), the pregnancy recognition signal in ruminants, abrogates the luteolytic mechanism for maintenance of the corpus luteum for production of progesterone (P4). This study examined the expression of DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (DDX58) and phospholipid scramblase 1 (PLSCR1) mRNAs in the ovine uterus as these genes were increased most in 2fTGH (STAT1 positive) cells by IFNT. The results of this study indicated that IFNT induces expression of DDX58 and PLSCR1 mRNAs in the ovine uterus, which confirmed the results of the in vitro transcriptional profiling experiment with the 2fTGH (parental STAT1 positive) and U3A (STAT1 null) cell lines. Steady-state levels of DDX58 and PLSCR1 mRNAs increased in cells of the ovine uterus between days 12 and 20 of pregnancy, but not between days 10 and 16 of the estrous cycle. The expression of DDX58 and PLSCR1 mRNAs was greatest in endometrial stromal cells, but there was transient expression in uterine luminal and superficial glandular epithelial cells. P4 alone did not induce expression of DDX58 and PLSCR1 mRNAs; however, intrauterine injections of IFNT did induce expression of DDX58 and PLSCR1 mRNAs in the endometria of nonpregnant ewes independent of effects of P4. These results indicate that IFNT induces expression of DDX58 and PLSCR1 in ovine endometrial cells via the classical STAT1-mediated cell signaling pathway. Based on their known biological effects, DDX58 and PLSCR1 are IFN-stimulated genes, which may increase the antiviral status of cells of the pregnant uterus to protect against viral infection and/or enhance secretion of type I IFNs that inhibit viral replication.


Introduction

Interferon τ (IFNT), the maternal recognition signal in ruminants (sheep, cattle, goats), is produced by mononuclear trophoectoderm cells of peri-implantation conceptuses (embryo/fetus and associated membranes) to prevent the development of the endometrial luteolytic mechanism (Bazer et al. 2010). IFNT is secreted by ovine trophoectoderm cells between days 10 and 25 of pregnancy (Ashworth & Bazer 1989) with maximal production between days 14 and 16 (Farin et al. 1989, Roberts et al. 1999). As a novel member of the type I IFN family, IFNT also possesses antiviral, antiproliferative, and immunomodulatory activities (Ponzter et al. 1991, Alexenko et al. 1997, Khan et al. 1998, Johnson et al. 1999c). In the ovine uterus, IFNT acts in a paracrine manner on endometrial luminal (LE) and superficial glandular epithelia (sGE) to suppress transcription of estrogen receptor α and oxytocin (OXT) receptor (Spencer & Bazer 1996, Fleming et al. 2001), thereby preventing endometrial release of OXT-induced luteolytic pulses of prostaglandin F₂α (Bazer 1992, Spencer et al. 2004). The antiluteolytic action of IFNT allows maintenance of a functional corpus luteum and its secretion of progesterone (P₄), the hormone of pregnancy, which is required for successful establishment and maintenance of pregnancy (Spencer et al. 2004, Bazer et al. 2010). In addition to its antiluteolytic actions, IFNT increases expression of several IFN-stimulated genes (ISGs) that are hypothesized to be important for endometrial differentiation and implantation of the conceptus (Hansen et al. 1999, Bazer et al. 2010). These ISGs include STAT1 and STAT2 (Johnson et al. 1999a, 1999d, Stewart et al. 2001a), IFN regulatory factor-1 (IRF1; Spencer et al. 1998, Johnson et al. 1999a, 1999d, Stewart et al. 2001a), IRF9 (Stewart et al. 2002), ISG15 (Johnson et al. 1999a, 1999b, 1999d, 2000, Stewart et al. 2001a), MX (Ott et al. 1998), OAS (Mirando et al. 1991, Johnson et al. 2001), MIC (Choi et al. 2003), B2M (Vallet et al. 1991, Choi et al. 2003), LGALS15 (Gray et al. 2004), WNT7A (Kim et al. 2003),
CTSL1 (Song et al. 2005), CST3 (Song et al. 2006b), RSAD2, and IFIHI (Song et al. 2007).

Available evidence indicates that IFNT induces dimerization of type I IFN receptors in cells of the ovine uterus (Han et al. 1997), and hence, phosphorylation of receptor-activated STATs (Johnson et al. 1999a, 1999b, Stewart et al. 2001a, 2001b) leading to formation of two transcription factor complexes: ISG factor 3 (ISGF3; STAT1:STAT2:IRF9 complex) and γ-activation factor (GAF; STAT1 homodimer; Stewart et al. 2001a, 2001b). Those transcription factor complexes translocate to the nucleus and bind to specific DNA sequences to activate transcription of target genes (Decker et al. 1991, Shuai et al. 1992, Pine et al. 1994). For example, GAF (Decker et al. 1991, Shuai et al. 1992) regulates transcriptional activities of genes containing a γ-activation sequence, such as IRF1 (Pine et al. 1994). In contrast, ISGF3 (Schindler et al. 1992) regulates transcription of genes containing IFN-stimulated response elements (ISREs), such as STAT1, STAT2, IRF9, and OAS (Reich et al. 1987, Levy et al. 1988). In the ovine uterus, IFIRF1 is a transcriptional activator that binds to both ISREs and IRF elements (Fujita et al. 1988, Harada et al. 1989, 1990, 1994, Karin 1991, Stark & Kerr 1992, Nguyen et al. 1997, Mamane et al. 1999). In contrast, IRF2 is a potent transcriptional repressor (Senger et al. 2000). IRF2 is constitutively expressed in the ovine endometrial LE (oLE) and sGE, but increases during early pregnancy to prevent induction or increased expression of classical (STAT1 dependent) IFNT-stimulated genes (Choi et al. 2001). Therefore, classical IFNT-stimulated genes are expressed by endometrial stromal cells and middle to deep uterine GE cells of the ovine uterus (Johnson et al. 2000, 2001, Choi et al. 2001, 2003). However, P4 and/or IFNT act to induce and regulate expression of a novel set of genes in uterine LE and sGE via an unidentified novel cell signaling pathway(s) as these cells lack both P4 receptors (PGR) and STAT1 (Bazer et al. 2010).

It has been reported that the 2fTGH (parental) cells derived from a human fibrosarcoma and STAT1-null U3A cells derived from the 2fTGH parental cells are models for assessing the effects of IFNT on STAT1-dependent and STAT1-independent gene expressions respectively (Shuai et al. 1992, Stewart et al. 2002, Kim et al. 2003). These cells recapitulate cell-type-specific responses of the ovine uterus to IFNT in terms of IFNT signaling and ISG expression (Johnson et al. 1999b, 2000, 2001, 2002, Choi et al. 2001, 2003). Therefore, these cells were used to determine the effects of IFNT on expression of DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (DDX58). DDX58 is a member of DEXH box family that consists of two caspase recruitment domains (CARDs) at the N-terminus and an RNA helicase domain at the C-terminus (Sun 1997, Wu et al. 2000) as they were the two genes most highly upregulated in 2fTGH cells by IFNT. It also contains the conserved DEAD motif as a putative RNA helicase induced by retinoic acid in differentiating promyelocytic leukemia cells (Sun 1997). Full-length DDX58 interacts with intracellular double-stranded RNA (dsRNA) to augment type I IFN production in response to viral infection in an ATP-dependent manner and CARD of DDX58 activates IRF3 and nuclear factor κ-B, subunit 1 (NFκB1; Yoneyama et al. 2004). Furthermore, DDX58 is induced by IFNG and regulates chemokine, CXC motif, ligand 11 (CXCL11) production in human cervical cancer cell lines and normal human endometrium (Yuzawa et al. 2008). Phospholipid scramblase 1 (PLSCR1) is an endofacial plasma membrane protein that mediates calcium-dependent transbilayer movement of membrane phospholipids. It was identified as an ISG in oligonucleotide array analyses and is required for maximal antiviral activity of type I IFNs (Basse et al. 1996, Zhou et al. 1997, Der et al. 1998, Dong et al. 2004). In the uteri of pregnant rats, there are two isoforms of PLSCRs, but this is not the case for PLSCR1 (Phillippe et al. 2006).

The expression of DDX58 and PLSCR1 genes in the ovine uterus with respect to temporal and cell-specific changes during the estrous cycle and early pregnancy, and in response to P4 and IFNT is not known. However, they are ISGs that can increase the antiviral state of cells to protect them from viral infection. Therefore, our working hypothesis was that DDX58 and PLSCR1 are induced in the ovine uterus in a cell-type-specific manner by IFNT from the conceptus during early pregnancy to mediate biological effects relative to the establishment of uterine receptivity to implantation by the ovine conceptus. Accordingly, this study was conducted to 1) identify ISGs induced by recombinant ovine IFNT (roIFNT) in cells expressing STAT1 (human 2fTGH cells) or not expressing STAT1 (U3A STAT1-null 2fTGH cells) and 2) determine the effects of the estrous cycle, pregnancy, and roIFNT on expression of DDX58 and PLSCR1 in the ovine uterus.

Results

Upregulated genes in 2fTGH cells by roIFNT

In this study, we have used 2fTGH (parental) cells derived from a human fibrosarcoma and STAT1-null U3A cells derived from the 2fTGH parental cells to assess the effects of IFNT on STAT1-dependent and STAT1-independent gene expressions respectively (Shuai et al. 1992, Stewart et al. 2002, Kim et al. 2003). These cells recapitulate cell-type-specific responses of the ovine uterus to IFNT in terms of IFNT signaling and ISG expression (Johnson et al. 1999b, 2000, 2001, 2002, Choi et al. 2001, 2003). Therefore, these cells were used to determine the effects of IFNT on expression of DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (DDX58). DDX58 is a member of DEXH box family that consists of two caspase recruitment domains (CARDs) at the N-terminus and an RNA helicase domain at the C-terminus (Sun 1997, Wu et al. 2000) as they were the two genes most highly upregulated in 2fTGH cells by IFNT. It also contains the conserved DEAD motif as
Table 1 Real-time RT-PCR analysis of mRNA expression in 2fTGH and U3A cells treated with recombinant ovine interferon-τ (roIFNT).

<table>
<thead>
<tr>
<th>Genes</th>
<th>2fTGH (none versus IFNT)</th>
<th>U3A (none versus IFNT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDX58</td>
<td>+26.2*</td>
<td>+3.34†</td>
</tr>
<tr>
<td>PLSCR1</td>
<td>+5.5*</td>
<td>+2.0†</td>
</tr>
<tr>
<td>GALBP3</td>
<td>+2.4†</td>
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</tr>
<tr>
<td>MYCL1</td>
<td>+1.5†</td>
<td>+1.9</td>
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<td>TGFBR1</td>
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<tr>
<td>ANKFY1</td>
<td>+1.8‡</td>
<td>+1.4</td>
</tr>
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<td>HES4</td>
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</tr>
<tr>
<td>MMP23</td>
<td>+3.1†</td>
<td>+1.2</td>
</tr>
<tr>
<td>HONXD3</td>
<td>+1.6</td>
<td>+1.6</td>
</tr>
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</table>

*P<0.001; †P<0.01; ‡P<0.05. Real-time quantitative RT-PCR was used to determine fold increases in target gene mRNAs in 2fTGH and U3A control cells and in cells treated with roIFNT. The values presented are from independent experiments, each conducted in triplicate.

expression of DDX58 (26.2-fold), PLSCR1 (5.5-fold), HES4 (4.2-fold), and PKD2 (3.2-fold) (P<0.001); MMP23 (3.1-fold), GALBP3 (2.4-fold), ANKFY1 (1.8-fold), FGFI1A (1.8-fold), and MYCL1 (1.5-fold) (P<0.01); and CREF1 (1.8-fold) (P<0.05) mRNAs in 2fTGH cells. However, RT-PCR analyses for these genes in U3A (STAT1 deficient) cells indicated that roIFNT did not increase expression of DDX58 and PLSCR1 mRNAs.

Western blot analyses for DDX58 and PLSCR1 proteins in 2fTGH and U3A cells treated with roIFNT in a time-dependent manner

Parental 2fTGH and U3A cells were treated with roIFNT for 0, 6, 12, or 24 h. Western blot analyses of whole cell extracts detected immunoreactive DDX58 (~110 kDa) and PLSCR1 (about 35.1 kDa) in both the cell lines, but the abundance of these proteins increased (P<0.01) in response to IFNT only in 2fTGH cells in a time-dependent manner (Fig. 1). This indicates that the roIFNT induced increase in DDX58 and PLSCR1 mRNAs and proteins was STAT1 dependent. Also of interest were reports that DDX58 and PLSCR1 are expressed in HeLa cells and in rat uteri (Phillipe et al. 2006, Yuzawa et al. 2008). Therefore, we hypothesize that DDX58 and PLSCR1 genes are induced in the ovine uterus in a cell-type-specific and STAT1-dependent manner by IFNT from the conceptus during early pregnancy to affect uterine receptivity to implantation by the ovine conceptus.

DDX58 and PLSCR1 expression increases in ovine endometria in a cell-type-specific manner

Partial cDNAs for ovine DDX58 and PLSCR1 mRNAs were amplified by RT-PCR using total RNA from endometria collected on days 16 and 18 of pregnancy and specific primers (Table 2). The cDNAs were cloned and sequences were verified (data not shown). Steady-state levels of DDX58 and PLSCR1 mRNAs in uterine endometria from cyclic and pregnant ewes, determined by slot blot hybridization analyses (Fig. 2), were affected by day (P<0.01), pregnancy status (P<0.01), and day by pregnancy status interaction (P<0.01). The expression of DDX58 and PLSCR1 mRNAs in the endometria of cyclic ewes was low and not affected (P>0.10) by day of the estrous cycle. However, expression of DDX58 mRNA increased (P<0.01) 3.54-fold between days 10 and 16, and then declined slightly to day 20 of pregnancy. The expression of PLSCR1 mRNA also increased (P<0.01) about 4.92-fold between days 10 and 18 of pregnancy. In situ hybridization analyses revealed cell-specific expression of DDX58 and PLSCR1 mRNAs in uteri of cyclic and pregnant ewes (Figs 3 and 4). The expression of DDX58 mRNA in uterine LE was not different between day 10 cyclic and day 10 pregnant ewes, but between days 12 and 16 of pregnancy, DDX58 mRNA increased in stromal cells, but was undetectable in LE and sGE (Fig. 3). In cyclic ewes, PLSCR1 mRNA was detected in endometrial LE and sGE on days 10 and 12 and at very low abundance between days 14 and 16 of the estrous cycle (Fig. 4). Similarly, in pregnant ewes, PLSCR1 mRNA was detected mainly in LE and sGE on days 10 and 12 and then increased in abundance in stratum compactum stroma and sGE between days 14 and 20 of pregnancy.

Intrauterine administration of roIFNT increases DDX58 and PLSCR1 mRNAs in the endometrium

In order to determine whether the differences in the expression of DDX58 and PLSCR1 genes in the uteri of pregnant compared with cyclic ewes were due to IFNT from the conceptus, cyclic ewes were ovariolectomized and fitted with intrauterine (i.u.) catheters on day 5 after the onset of estrus. The ewes received daily i.m. injections of 25 mg P4 from day 5 until they were

**Figure 1** Detection of immunoreactive DDX58 and PLSCR1 proteins in human 2fTGH (parental) and U3A (STAT1-null) 2fTGH cell lines. Cells were treated with roIFNT (10^4 AVU/ml) for 0, 6, 12, or 24 h in each of three independent experiments. These results indicate that induction of DDX58 and PLSCR1 by roIFNT is STAT1 dependent. Immunoreactive proteins were detected using rabbit anti-DDX58 and anti-PLSCR1 antibodies and compared with the levels of mouse α-tubulin (TUBA) protein as a loading control.
hysterectomized on day 16. The treatment groups were as follows 1) P4 from days 5 to 16 and i.u. injections of control serum proteins (CX, 200 μg) at 0700 and 1700 h on days 11–16 (P4CX); 2) i.m. P4, i.u. CX proteins, and 75 mg ZK 136 317 (ZK, P4 receptor antagonist) from days 11 to 16 (P4CXZK); 3) i.m. P4 and i.u. roIFNT (1 × 108 antiviral units (AVU)/day) from days 11 to 16 (P4CIFNT); and 4) i.m. P4, i.u. roIFNT, and 75 mg ZK 136 317 (P4CIFNTZK).

Intrauterine administration of roIFNT increased expression of DDX58 and PLSCR1 mRNAs by six- and tenfold respectively in the endometrium of ewes (P4CX versus P4CIFNT, P < 0.001; Fig. 5A and B). Similarly, i.u. injections of roIFNT increased DDX58 and PLSCR1 mRNAs by four- and fourfold respectively in ewes treated with ZK (P4CXZK versus P4CX + ZK + IFN, P < 0.001) indicating an effect of roIFNT, which was independent of functional PGR. In situ hybridization analyses verified that roIFNT increased DDX58 and PLSCR1 mRNA expression in a cell-type-specific manner consistent with that observed in uteri from ewes on days 16 and 18 of pregnancy. The i.u. roIFNT increased both DDX58 and PLSCR1 mRNAs in stromal cells (Fig. 5C and D), but not in LE, GE, blood vessels, or myometrium (P < 0.05).

**Effects of IFNT on DDX58 and PLSCR1 in endometrial cells**

As compared with untreated ovine stromal cells maintained in serum-free medium, treatment with roIFNT for 0, 6, 12, or 24 h increased (P < 0.001) or induced expression of DDX58 and PLSCR1 mRNAs (Fig. 6A–C). In addition, as compared with untreated oLE and bovine endometrial (BEND) cells maintained in serum-free medium, treatment of both oLE and BEND cells with roIFNT increased (P < 0.001) or induced expression of DDX58 and PLSCR1 mRNAs (Fig. 7A–C).

### Table 2 Sequences of primers used for real-time RT-PCR and cloning.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequence (5′–3′): forward and reverse</th>
<th>GenBank accession</th>
<th>Product size (bp)</th>
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<td>CYC. B</td>
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<td>NM_000942</td>
<td>69</td>
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**For RT-PCR**

| DDX58      | GGAAGACCTGGACCTACCT                | NM_014314         | 450              |
| PLSCR1     | GAAATGCTTCACCCGGGAAATCTTGCTCTGCTCACTGCA | NM_021105         | 484              |

**DDX58** (=RIG-1), retinoic acid-inducible gene 1; **PLSCR1**, phospholipid scramblase 1; **PKD2**, protein kinase D2; **GALBP3**, galactoside-binding, soluble, 3 binding protein; **ANKFY1**, ANKHZN protein (ankyrin repeat and FYVE domain containing 1); **HES4**, bHLH factor Hes4; **FGF1A**, FGF-1 acidic-like protein; **MYCL1**, v-myc myelocytomatosis viral oncogene homolog 1; **CGREF1**, cell growth regulator with EF hand domain 1; **BRDT**, a testis-specific bromodomain-containing protein; **TGFBR1**, TGFβ receptor 1; **DHX33**, DEAH (Asp-Glu-Ala-His) box polypeptide 33; **MMP3**, matrix metalloproteinase 23A; **HoxD3**, homeobox D3.
by IFNT via an unidentified cell signaling pathway(s) (Bazer et al. 2010) The STATs are required for classical signal transduction elicited by IFNT in ovine uterine stromal cells and GE because they interact with specific IFN-responsive elements and/or STAT-interacting transcription factors in the nucleus to modulate expression of defined target genes.

This study first identified DDX58 and PLSCR1 as genes induced by ovine IFNT in human 2fTGH (parental), but not STAT1-null U3A cells by transcriptional profiling. Accordingly, we then determined the effects of the estrous cycle, pregnancy, and i.u. injections of rolIFNT on expression of those genes. The 2fTGH and U3A cells recapitulated endometrial cell-type-specific responses to IFNT in terms of type I IFN-receptor-induced cell signaling and STAT1-dependent expression of several classical ISGs (Shuai et al. 1992, Johnson et al. 1999b, 2000, 2001, 2002, Choi et al. 2001, 2003, Stewart et al. 2002). When 2fTGH and U3A cells were treated with IFNT, expression of PLSCR1 and DDX58 mRNAs and proteins was detected only in 2fTGH cells that expressed STAT1 (Fig. 1). These results indicate that genes responsive to IFNT in an STAT1-dependent manner can be identified in 2fTGH human fibroblast cells by transcriptional profiling and that the use of those cells for identification of genes potentially regulated by IFNT in the ovine endometrium during the peri-implantation period is valid as previously reported (Kim et al. 2003, Song et al. 2007).

This is the first known report of temporal and spatial alterations in expression of DDX58 and PLSCR1 genes in the uteri of cyclic and pregnant ewes. On the basis of the results of this study, we tested the hypotheses to understand the biological roles of DDX48 and PLSCR1 in the establishment and maintenance of pregnancy with particular emphasis on uterine receptivity to implantation by the ovine conceptus. Of interest is the increase in expression of these genes in the ovine endometrium that coordinates with maximum production of IFNT by the rapidly elongating ovine conceptus between days 13 and 16 of pregnancy (Ashworth & Bazer 1989).

Most IFNT-stimulated genes are expressed by endometrial stromal cells and middle to deep GE in the ovine uterus (Vallet et al. 1989, 1991, Shuai et al. 1992, Johnson et al. 1999b, Stewart et al. 2002, Bazer et al. 2008). This is due to the fact that IRF2, a potent transcriptional repressor, is expressed in LE and sGE that lack STAT1 (Senger et al. 2000, Choi et al. 2001). In this study, the PLSCR1 and DDX58 genes were upregulated in stromal cells, but not in uterine LE or sGE during pregnancy. Full-length DDX58 interacts with intracellular dsRNA to augment type I IFN production in response to viral infection in an ATP-dependent manner, and CARD of DDX58 activates IRF3 and NFkB1 (Yoneyama et al. 2004). There is also evidence for an important role for DDX58 cell signaling in innate

**Figure 2** Steady-state levels of DDX58 and PLSCR1 mRNAs in endometria from cyclic (C) and pregnant (P) ewes (study one). Significant \( P<0.01 \) changes in expression of DDX58 and PLSCR1 were due to day, pregnancy status, and day by pregnancy status interaction. Expression of DDX58 mRNA increased about 3.5-fold between days 10 and 16, and then declined slightly to day 20. In cyclic ewes, there was no effect \( P>0.10 \) of day of the estrous cycle. PLSCR1 mRNA increased about 4.9-fold between days 10 and 18 of pregnancy, but there was no effect \( P>0.10 \) of day of the estrous cycle. Data are expressed as least square means (LSM) relative units (RU) with S.E.M.

**Discussion**

In this study, expression of DDX58 and PLSCR1 genes in the ovine endometrium were regulated by IFNT in an STAT1-dependent manner that was independent of functional PGR and P4. Implantation and establishment of pregnancy require that the peri-implantation ruminant conceptuses enter a receptive uterus and secrete IFNT (Bazer & Roberts 1983, Spencer et al. 1996, Bazer et al. 1998, 2010). Ovine IFNT prevents development of the luteolytic mechanism in the endometrium and acts as a pregnancy recognition signal, which communicates between the conceptus and maternal endometrium to allow for the establishment and maintenance of pregnancy, including implantation and synepitheliochorial placentation (Bazer et al. 1998, 2010). Furthermore, IFNT regulates the expression of ISGs that are considered essential for endometrial differentiation and implantation (Hansen et al. 1999, Bazer et al. 2010). Most classical IFNT-stimulated genes are expressed by endometrial stromal cells and middle to deep GE in the ovine uterus via STAT1-dependent cell signaling (Vallet et al. 1991, Shuai et al. 1992, Johnson et al. 1999b, Stewart et al. 2002). In ewes, IRF2 is a transcriptional repressor of STAT1 gene expression (Senger et al. 2000, Choi et al. 2001) that appears to be responsible for expression of a limited number of nonclassical ISGs (e.g. LGALS11, WNT7A, CTSL1, and CST3) by ovine uterine LE and sGE that are induced by P4 and further stimulated

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In pregnant ewes, PLSCR1 mRNA was detected in LE and sGE on days 10 and 12, but primarily in the stratum compactum stroma and sGE between days 14 and 20 of pregnancy. PLSCR1 is an endofacial plasma membrane protein that mediates calcium-dependent transbilayer movement of membrane phospholipids (Basse et al. 1996, Zhou et al. 1997). It was identified as an ISG in oligonucleotide array analyses (Der et al. 1998) and found to be required for maximal antiviral activity of type I IFNs (Dong et al. 2004).

In conclusion, the results of this study are the first, to our knowledge, to indicate that pregnancy and IFNT increase expression of DDX58 and PLSCR1 in an STAT1-dependent manner in ovine endometrial stromal cells, but not in uterine LE or GE during the peri-implantation period of pregnancy in ewes. The possibilities exist that DDX58 and PLSCR1 increase the antiviral state of the pregnant uterus to protect against viral infection and/or to enhance expression of type I IFNs that inhibit viral replication. These biological effects may be important in optimizing conditions for uterine receptivity to implantation and the establishment and maintenance of pregnancy in sheep.

Materials and Methods

Animals

Mature crossbred Suffolk ewes (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 pregnancy status. All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals and approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University.
Cell lines

The 2fTGH (parental) and U3A (STAT1-null 2fTGH) cell lines were derived from a human fibrosarcoma and responded to both type I and type II IFNs (Pellegrini et al. 1989). The 2fTGH cells were maintained in basal medium containing DMEM with F-12 salts (DMEM-F12; Sigma–Aldrich Corp.) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin/amphotericin solution (Invitrogen) as described previously (Stewart et al. 2001a, 2001b). The U3A cells were maintained in basal medium with hygromycin B (250 μg/ml; Invitrogen). rOIFNT was prepared and assayed for biological activity as described previously (Van Heeke et al. 1996). The biological activity of rOIFNT, expressed as AVU, was $1 \times 10^7$ AVU/mg protein. Monolayer cultures of 2fTGH and U3A cells were grown in culture medium to 80–90% confluency in 100 mm tissue culture plates ($n=3$ per treatment). Cells were either untreated to serve as a control or treated with rOIFNT ($10^4$ AVU/ml) for 0, 6, 12, or 24 h in each of three independent replicates. The dose of rOIFNT used was known to induce or increase expression of several ISGs in ovine uterine endometrial cells and human 2fTGH and U3A cell lines (Stewart et al. 2001a, 2001b, Kim et al. 2003). Total RNA was isolated and used for transcriptional profiling or RT-PCR. This design was used in three independent replicates.

Immortalized ovine uterine endometrial LE cells were cultured as described previously (Johnson et al. 1999a, 1999d). BEND cells (Johnson et al. 1999b) 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 with F-12 salts (DMEM-F12; Sigma–Aldrich Corp.) supplemented with 5% FBS and antibiotics. When cells reached 70–80% confluency, they were treated with either rOIFNT ($2 \times 10^7$ AVU/ml) or left untreated as a control for 24 h in serum-free medium. The experiment was independently repeated in three replicates for each cell type.

Experimental design

Study one

At estrus (day 0), ewes were mated to either intact or vasectomized rams and then hysterectomized ($n=5$ ewes/day) on either day 10, 12, 14, or 16 of the estrous cycle or day 10, 12, 14, 16, 18, or 20 of pregnancy as described previously (Spencer et al. 1999a). Pregnancy was confirmed on days 10–16 post mating by the presence of a morphologically normal conceptus(es) in the uterus. At hysterectomy, several sections ($\sim 0.5$ 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 \(-80^\circ C\). The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at \(-80^\circ C\) for RNA extraction. In monovulatory pregnant ewes, uterine tissue samples were marked as either contralateral or ipsilateral to the ovary bearing the CL. No tissues from the contralateral uterine horn were used for this study.

**Study two**

In order to determine whether the differences in expression of *DDX58* and *PLSCR1* genes in the uteri of pregnant compared with cyclic ewes were due to IFNT from the conceptus, 16 cyclic ewes were ovariectomized and fitted with i.u. catheters on day 5 post estrus. Ewes were then assigned randomly \((n=4\) ewes/treatment) to receive daily i.m. injections of \(P_4\) between days 5 and 16, and either a PGR antagonist \((ZK 136 317; Schering, Berlin-Wedding, Germany)\) or no PGR antagonist \((ZK 136 317)\) and daily i.u. infusions of either control serum proteins or roIFNT protein between days 11 and 16 were as follows: 1) \(25 \text{ mg } P_4\) and \(200 \mu g\) control \((\text{CX})\) serum proteins \((P_4 + \text{CX}); 2) P_4, 75 mg \(ZK 136 317\), and CX proteins \((P_4 + ZK + \text{CX}); 3) P_4\) and roIFNT \((1 \times 10^6 \text{ AVU})\) \((P_4 + \text{IFN});\) or 4) \(P_4\), \(ZK 136 317\), and roIFNT \((P_4 + ZK + \text{IFN}).\) All ewes were hysterectomized on day 16. At hysterectomy, several sections \((\approx 0.5 \text{ cm})\) from the midportion of each uterine horn 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). Several sections \((1–1.5 \text{ cm})\) from the middle of each uterine horn were embedded in Tissue-Tek OCT compound (Miles), frozen in liquid nitrogen vapor, and stored at \(-80^\circ C\). The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at \(-80^\circ C\) for RNA extraction.

**RNA isolation**

Total cellular RNA was isolated from frozen ipsilateral endometrium (studies one and two) using Trizol reagent (Gibco-BRL) according to the manufacturer’s recommendations. The quantity and quality of total RNA were determined by spectrometry and denaturing agarose gel electrophoresis respectively.

**Real-time quantitative RT-PCR analysis**

The cDNA was synthesized from total cellular RNA \((5 \mu g)\) isolated from cells using random primers (Invitrogen), oligo(dT) primers, and SuperScript II Reverse Transcriptase (Invitrogen) as
Effect of IFNT treatment. Cells were treated with roIFNT (10^4 AVU/ml) for 0, 6, 12, or 24 h in each of three independent experiments. (A) All PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide. Total cellular RNA from the endometria of a day-16 pregnant ewe and sterile water (no template) were taken as positive and negative controls respectively. (B and C) A graph illustrating the effect of IFNT on relative mRNA levels for DDX58 and PLSCR1 is presented below each gel, and the asterisk (*) denotes a significant (P<0.001) effect of IFNT treatment.

Figure 6: Semiquantitative RT-PCR analyses of DDX58 and PLSCR1 mRNAs in total cellular RNA isolated from immortalized ovine endometrial stromal cells. Cells were treated with roIFNT (10^4 AVU/ml) for 0, 6, 12, or 24 h in each of three independent experiments. (A) All PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide. Total cellular RNA from the endometria of a day-16 pregnant ewe and sterile water (no template) were taken as positive and negative controls respectively. (B and C) A graph illustrating the effect of IFNT on relative mRNA levels for DDX58 and PLSCR1 is presented below each gel, and the asterisk (*) denotes a significant (P<0.001) effect of IFNT treatment.

Steady-state levels of mRNA in ovine endometria were assessed by slot blot hybridization as described previously (Spencer et al. 1999b, Song et al. 2006a). For DDX58 and PLSCR1, antisense cRNA probes were generated by linearizing the pCRII-TOPO plasmid with XbaI and in vitro transcription with SP6 RNA polymerase and 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-32P]-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 Multimager (Molecular Dynamics, Piscataway, NJ, USA).

Figure 7: Semiquantitative RT-PCR analyses of DDX58 and PLSCR1 mRNAs in total cellular RNA isolated from immortalized ovine endometrial LE (oLE) and bovine endometrial (BEND) cells. Cells were treated with roIFNT (10^4 AVU/ml) for 0 or 24 h in each of three independent experiments. (A) All PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide. Total cellular RNA from the endometria of a day-16 pregnant ewe and sterile water (no template) were taken as positive and negative controls respectively. (B and C) A graph illustrating the effect of IFNT on relative mRNA levels for DDX58 and PLSCR1 is presented below each gel. The asterisk (*) denotes a significant (P<0.001) effect of IFNT treatment.

Cloning of partial cDNAs for ovine DDX58 and PLSCR1

Partial cDNAs for ovine DDX58 and PLSCR1 mRNAs were amplified by RT-PCR using total RNA endometrial tissues from days 18 of pregnancy using specific primers (Table 2). RT of cellular total RNA into cDNA was performed as described previously (Song et al. 2006a). PCR amplification was conducted as follows for DDX58 and PLSCR1: 1) 95°C for 5 min; 2) 95°C for 30 s, 64.5°C for 40 s (for DDX58), 64.5°C for 40 s (for PLSCR1), and 72°C for 1 min for 35 cycles; and 3) 72°C for 10 min. The partial cDNAs for ovine DDX58 and PLSCR1 PCR products were cloned into pCRII using a T/A Cloning kit (Invitrogen) and their sequences were verified using an ABI PRISM Dye Terminator Cycle Sequencing kit and ABI PRISM automated DNA sequencer (Perkin-Elmer Applied Biosystems, Norwalk, CT, USA).

slot blot hybridization analyses

Steady-state levels of mRNA in ovine endometria were assessed by slot blot hybridization as described previously (Spencer et al. 1999b, Song et al. 2006a). For DDX58 and PLSCR1, antisense cRNA probes were generated by linearizing the pCRII-TOPO plasmid with XbaI and in vitro transcription with SP6 RNA polymerase and 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-32P]-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 Multimager (Molecular Dynamics, Piscataway, NJ, USA).

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In situ hybridization analyses

Cell-specific mRNA expression in sections (5 μm) of the ovine uterus was determined by radioactive in situ hybridization analysis as described previously (Spencer et al. 1999B, Song et al. 2006a). Briefly, deparaffinized, rehydrated, and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized DDX58 and PLSCR1 partial cDNAs using in vitro transcription with [α-35S]-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 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.

Western blot analyses

Monolayer cultures of the 2fTGH (parental) and U3A (STAT1-deficient 2fTGH) cell lines were grown in culture medium to 80% confluence in 100 mm diameter dishes. Cells were then left untreated as a control or were treated with roIFNT (10^4 AVU/ml) for 0, 6, 12, or 24 h in each of three independent experiments. Protein concentrations were determined using the Bradford protein assay (Bio-Rad) with BSA as the standard. Whole cell extracts (20 μg/sample) were separated by 15% SDS–PAGE, transferred to nitrocellulose, and probed with rabbit anti-human DDX58 antibody (Abcam Inc., Cambridge, MA, USA) and rabbit anti-human PLSCR1 antibody (kindly provided by Dr Peter J Sims; Dong et al. 2004) or normal nonimmune rabbit IgG (Sigma) according to the manufacturer’s recommendations. Western blot analyses were conducted as described previously (Stewart et al. 2001a) using ECL (SuperSignal West Pico, Pierce, Rockford, IL, USA) and X-OMAT AR X-ray film (Kodak) according to the manufacturer’s recommendations.

Statistical analysis

All quantitative data were subjected to least squares regression analyses (ANOVA) using the general linear model procedures of 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 studies one and two were analyzed for the effects of day, pregnancy status (cyclic or pregnant), treatment, and their interactions. 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 0.10 was taken to indicate a trend and a P value of 0.05 or less was considered significant. Data are presented as least square means with S.E.M.S.

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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