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Interferon regulatory factor 6 (IRF6) is expressed in the ovine uterus and functions as a transcriptional activator

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Abstract

Interferon tau (IFNT), the maternal recognition of pregnancy signal in sheep and other ruminants, is secreted by the conceptus and regulates the expression of a number of genes in a cell-specific manner within the uterus. The response of different endometrial cell types to IFNT appears to be specified by IFN regulatory factors (IRFs). IRF2, a potent repressor of gene transcription, is expressed only by luminal (LE) and superficial glandular epithelia (sGE), whereas IRF1 and IRF9, activators of gene transcription, are expressed only in GE and stromal cells of the uterus during early pregnancy. In the present study, IRF6 was found to be expressed in LE/sGE and middle GE of the ovine uterine endometrium as well as conceptus trophoctoderm. IRF family members can regulate transcription via IFN-stimulated response elements (ISREs). Transient transfection analyses found that IRF6 enhanced basal activity of ISRE-containing promoters, but did not enhance IFNT stimulation of ISRE-containing promoters in variety of different cell types. Further, IRF6 did not cooperate with IRF1 or reduce IRF2 repression of ISRE-containing promoter activity. These results establish that IRF6 is a transcriptional activator that is preferentially expressed in the endometrial epithelia and conceptus trophoctoderm. IRF6 is hypothesized to play critical roles in endometrial gene expression as well as in conceptus trophoctoderm growth and differentiation.

1. Introduction

Biochemical signaling between the conceptus (embryo and associated extraembryonic membranes) and the mother must occur prior to and following conceptus attachment to the uterine luminal epithelium (LE) to prevent the mother from returning to cyclicity and to establish an altered physiological milieu favorable for implantation, placentation, and survival and development of the conceptus. In sheep, mononuclear trophoctoderm cells of the conceptus secrete copious amounts of interferon tau (IFNT), a Type I IFN, from Day 11 to Days 21 to 25 of pregnancy (Bazer et al., 1992). In sheep, IFNT silences *estrogen receptor alpha* (*ESR1*) gene transcription in endometrial LE and superficial glandular epithelia (sGE) (Fleming et al., 2001), which inhibits *ESR1* induction of *oxytocin receptor* gene transcription, thereby preventing oxytocin-induced release of luteolytic pulses of prostaglandin F2 alpha (PGF) and maintaining production of progesterone by the corpus luteum. Progesterone is essential for establishment and maintenance of pregnancy. In addition, IFNT stimulates a number of IFN-

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stimulated genes (ISGs) in a cell-specific manner within the uterus (Spencer et al., 2008b, Spencer et al., 2007). These ISGs are hypothesized to regulate endometrial receptivity to implantation and placentation, as well as survival, growth and development of the conceptus.

The classical pathway of Type I IFN action is via activation of the Janus kinase/signal transducers and activators of transcription (JAK-STAT) signaling pathway (Stark et al., 1998). Using cell lines deficient in various components of this pathway, IFNT was found to activate the JAK-STAT pathway similar to other Type I IFNs (Stewart et al., 2001a). STAT1, STAT2 and IFN regulatory factor 9 (IRF9), which comprise the IFN-stimulated gene factor 3 (ISGF3) transcription factor complex that transactivates ISG expression via IFN-stimulated response elements (ISREs) in their promoter regions, are up-regulated by IFNT only in GE and stromal cells of the ovine uterus during early pregnancy and are down-regulated or absent in LE/sGE of the endometrium between Days 11 and 19 of pregnancy (Choi et al., 2001). The lack of STAT1, STAT2, IRF9 and other classical ISGs, such as *ISG15* and *beta-2 microglobulin (B2M)*, in LE/sGE and cell-specific effects of IFNT on uterine gene expression is hypothesized to involve IFN regulatory factors (IRFs) (Choi et al., 2001, Choi et al., 2003, Spencer et al., 2008a, Spencer et al., 2007).

The IRFs are key regulators of IFN induction during antiviral responses (Stark et al., 1998, Mamane et al., 1999). The nine mammalian members of the *IRF* gene family share a conserved 115 amino acid N-terminal DNA binding domain (DBD) that bind ISREs and closely related IRF elements in the promoter region of target genes (Mamane et al., 1999). Most IRFs contain a transcriptional activation domain, although IRF2 contains a potent transcriptional repression domain. The transcriptional regulatory functions of most IRFs, with the exception of IRF6, have been determined (Mamane et al., 1999, Cheng et al., 2006, Takaoka et al., 2005). In the ovine uterus, the IRF2 repressor is expressed exclusively by endometrial LE/sGE during the estrous cycle and expression increases during early pregnancy (Choi et al., 2001). Available results support the hypothesis that increased expression of *IRF2* in endometrial LE/sGE during early pregnancy represses transcription of many classical ISGs, such as *STAT1*, *STAT2*, *IRF9*, *ISG15* and *B2M* (see (Choi et al., 2001, Spencer et al., 2008a, Spencer et al., 2007). Thus, the induction and stimulation of many classical ISGs by conceptus IFNT is restricted to endometrial stromal cells and GE, which are IRF2-negative although IFN receptors are abundant on LE/sGE (Rosenfeld et al., 2002). In addition, IRF2 is involved in IFNT inhibition of *ESR1* transcription in LE/sGE (Fleming et al., 2006, Fleming et al., 2001).

A comprehensive search for all IRF family members in the ovine uterine endometrium found expression of only IRF1, IRF2, IRF6 and IRF9 (Choi et al., 2001). IRF6 is a member of the *IRF* gene family based on the presence of the N-terminal DBD characteristic of IRFs and has much greater sequence homology with IRF5 in both the DBD and the C-terminal domain than with the other paralogs (Mamane et al., 1999, Nguyen et al., 1997). The relative lack of information on the transcriptional function and target genes regulated by IRF6 is somewhat surprising in view of its strong genetic association with cleft lip and/or palate, one of the most common birth defects of humans (Cobourne, 2004). Mutation of *IRF6* has been strongly associated with human craniofacial developmental defects in van der Woude and popliteal pterygium syndromes as well as in nonsyndromic cleft lip and palate and tooth agenesis worldwide (Cobourne, 2004, Blanton et al., 2005, Zuccherro et al., 2004, Kondo et al., 2002, Gorlin et al., 1968). Besides the better known craniofacial defects, popliteal pterygium syndrome also includes genitourinary abnormalities and digital and limb anomalies (Gorlin et al., 1968). *IRF6* is expressed in epithelial cells of several tissue types in mouse, chicken, zebrafish and *Xenopus laevis* embryos (Ben et al., 2005, Knight et al., 2006, Washbourne and Cox, 2006, Hatada et al., 1997). *Ir6* is found in a variety of adult mouse tissues, including eyes, liver, lung, skin, spleen, placenta and testes (Kondo et al., 2002). In contrast to knockout mice for the other IRFs which have compromised immune responses, *Ir6* null mice have

morphological defects that include severely abnormal skin, limb and craniofacial development resulting in perinatal lethality, consistent with the defects seen to varying extents in humans with *IRF6* mutations (Ingraham et al., 2006, Richardson et al., 2006). Thus, *Ir6* is a primary regulator of keratinocyte proliferation and differentiation during embryogenesis in mice (Ingraham et al., 2006, Richardson et al., 2006).

These studies determined the temporal and spatial patterns of *IRF6* expression in the ovine uterus during early pregnancy and whether *IRF6* functions as an activator or repressor of ISRE-regulated promoter activity and effects of IFNT. The results indicate that *IRF6* is exclusively expressed in endometrial epithelia of the ovine uterus and in conceptus trophoctoderm. Further, *in vitro* transfection assays found that *IRF6* is a transcriptional activator, but it does not enhance IFNT actions on ISRE-containing promoters.

2. Materials and Methods

2.1. Cells and reagents

Human 2fTGH (parental), U3A (STAT1-deficient 2fTGH) fibrosarcoma cells (Pelligrini), immortalized ovine endometrial luminal epithelial cells (LE) (Johnson et al., 1999) and bovine endometrial cells (BEND) (Staggs et al., 1998) were maintained in DMEM-F12 medium (Sigma-Aldrich Corp., St. Louis, MO) supplemented with penicillin/streptomycin sulfate/ amphotericin B (PSA) solution (Invitrogen, Carlsbad, CA) and fetal bovine serum (Hyclone, Logan, UT) (5% FBS for 2fTGH and U3A and 10% FBS for LE and BENDs). Ovine mononuclear trophoctoderm cells (oTr1) (Farmer et al., 2008) were maintained in DMEM-F12 plus PSA, 1 mM pyruvate, 2 mM L-glutamine, non-essential amino acids, 700 nM insulin and 10% FBS (complete trophoblast medium). Recombinant ovine IFNT (IFNT) (10^8 antiviral units/mg) was prepared and assayed as described previously (Van Heeke et al., 1996). Restriction endonucleases, T4 DNA ligase, cell culture lysis reagent and luciferase substrate were purchased from Promega (Madison, WI). *Ex Taq*TM polymerase (Takara Bio USA, Madison, WI) was used. Plasmid DNAs were purified by the alkaline lysis method with kits from Qiagen (Qiagen, Valencia, CA). An affinity purified rabbit polyclonal antibody against *IRF6* was purchased from Active Motif (Carlsbad, CA).

2.2. Cloning of ovine *IRF6* and luciferase constructs

An ovine endometrial *IRF6* DNA was cloned as described previously for ovine *IRF1* and *IRF2* (Choi et al., 2001). Briefly, an ovine *IRF6* partial cDNA was amplified by reverse transcription-polymerase chain reaction (RT-PCR) of total RNA isolated from Day 15 pregnant ovine endometrium with primers designed from the human orthologue (forward: 5'-CCA GAA CG TGG ATC AGC-3' and reverse: 5'-GAC CTC CAG GAT CAG TCC-3'). The PCR reaction was performed using PCR Optimized Buffer F (Invitrogen, Carlsbad, CA) as follows: 1) 95 °C for 2 min; 2) 1.5 min at 95 °C, 2 min at 42 °C, 1 min at 72 °C for 4 cycles; 3) 30 sec at 95 °C, 30 sec at 53 °C, 1 min at 72 °C for 30 cycles; and 4) 72 °C for 7 min. The partial ovine endometrial *IRF6* cDNA was cloned into pCRII plasmid vector using a T/A cloning Kit (Invitrogen), verified by sequencing, and used to screen a Lambda Zap II cDNA library made from Day 15 pregnant ovine endometria (Stratagene, LaJolla, CA) using methods described previously (Choi et al., 2001). A full-length ovine endometrial *IRF6* cDNA (GenBank AF228446) was subcloned into pEF1-Myc/His LacZ mammalian expression vector (Invitrogen) by standard methods.

The sequence of PRE2-TATA-LUC (a gift from Dr. Ming-er Tsai, Baylor College of Medicine, Houston, TX) was determined by standard methods. Oligonucleotides corresponding to the sense (5'-CTA GCCAGCTGA GAG GGT ATA TAA TGG ATC TGC GAT CTA AGT AAG CTT GGC ATT CCG GTA CTG TTG GTA AAA-3') and antisense

(5'-GAT CTT TTA CCA ACA GTA CCG GAA TGC CAA GCT TAC TTA GAT CGC AGA TCC ATT ATA TAC CCT CTC AGC TGG-3') strands of the core promoter were synthesized (Integrated DNA Technologies, Inc., Coralville, IA) and annealed. The double-stranded oligonucleotide was cloned into the luciferase vector pGL3-Basic (Promega, Madison, WI) that had been digested with *Bgl* II and *Nhe* I to create pTATA-LUC. Five copies of an ISRE was inserted by ligating a double-stranded oligonucleotide (sense strand 5'-CGA TAT CCT GCA GGA AAC TGA AAC TGA AAC TGA AAC TGA AAC TGA AAC TGA AAC TGA AAC TGA AAC TGA AAC TG-3'; antisense 5'-CTA GCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCC TGC AGG ATA TCG AGC T-3') into pTATA-LUC that had been digested with *Sac* I and *Nhe* I to create 5xISRE-TATA-LUC. The reporter construct containing the IFNT-responsive bovine *ISG17* promoter (*ISG17*-LUC) (Perry et al., 1999) was kindly provided by Dr. Thomas R. Hansen (Colorado State University, Fort Collins).

2.3. Collection of Ovine Uterine Tissues

Crossbred Suffolk ewes (*Ovis aries*) were observed daily for estrus in the presence of vasectomized rams and used in the experiments after they exhibited at least two estrous cycles of normal duration (16–18 days). All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Teaching and Research and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

At estrus (Day 0), ewes were mated to either an intact or a vasectomized ram and then hysterectomized (n = 5 ewes/day) on either Day 11, 13 or 15 of the estrous cycle, Days 11, 13, 15, 17 or 19 of pregnancy, Days 10, 12, 14 or 16 of the estrous cycle or Days 10, 12, 14, 16, 18 or 20 of pregnancy. Pregnancy was confirmed on Days 10 to 16 post-mating by the presence of a morphologically normal conceptus(es) in the uterus. At hysterectomy, several sections (~0.5 cm) from the mid-portion of each uterine horn ipsilateral to the corpus luteum (CL) 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). 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. The uterine lumen was flushed with saline on Days 10 to 16 of estrous cycle or Days 10 to 16 of pregnancy. The presence of a morphologically normal conceptus was evidence for ewes being pregnant. It was not possible to obtain uterine flushings on either Day 18 or Day 20 of pregnancy because the conceptus is firmly adhered to the endometrial luminal epithelium (LE) and basal lamina on those days of pregnancy.

2.4. In Situ Hybridization Analyses

Location of *IRF6* mRNA in sections (5 µm) of ovine uteri from Days 11, 13, and 15 of the estrous cycle and Days 11, 13, 15, 17 and 19 of early pregnancy was determined by radioactive *in situ* hybridization analysis as described previously (Choi et al., 2001). Briefly, deparaffinized, rehydrated and deproteinized uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized ovine *IRF6* partial cDNA using *in vitro* transcription with [α -³⁵S]-UTP. After hybridization, washing and ribonuclease A digestion, slides were dipped in NTB-2 liquid photographic emulsion (Kodak, Rochester, NY), and exposed at 4°C for two weeks. Slides were developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Fairlawn, NJ), 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 and darkfield illumination using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX) fitted with a Nikon DXM1200 digital camera.

2.5. Immunohistochemical Analyses

Immunocytochemical localization of IRF6 protein in ovine uteri was performed as described previously (Song et al., 2005) in sections from Days 10, 12, 14 and 16 of the estrous cycle and Days 10, 12, 14, 16, 18 and 20 of pregnancy with affinity purified rabbit anti-human IRF6 polyclonal antibody at 0.5 µg per ml final dilution. Antigen retrieval was performed by using a boiling citrate buffer, and negative controls included substitution of purified rabbit IgG for the primary antibody at the same final concentration. Images of representative fields were recorded using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX) fitted with a Nikon DXM1200 digital camera.

2.6. Transient transfections and luciferase assays

Transient transfections of 2fTGH and U3A cells were conducted as described previously (Fleming et al., 2006). The oTr1 cells were seeded to about 90% confluency before transfection of each well with 1 µg luciferase construct and 1 µg expression plasmid or pEF1-Myc/His/LacZ vector lacking an insert or containing full-length ovine IRF1, ovine IRF2 or ovine IRF6 cDNAs in the presence of ExGen 500 *in vitro* transfection reagent (Fermentas, Inc., Glen Burnie, MD) according to the manufacturer's instructions. Cells were maintained in complete trophoblast medium containing 10% FBS during transfection and an overnight incubation (approximately 14–16 h). The oTr1 cells then were cultured in serum-free, antibiotic supplemented DMEM-F12 medium and treated with IFNT (10^4 antiviral units/ml) in the same medium for 24 h before collecting cell lysates. BEND cells were cultured similarly to oTr1 cells except that antibiotic supplemented DMEM-F12 plus 10% FBS was used during the transfection and the overnight recovery period. The ovine LE cells were seeded to 67% confluency and transferred to serum-free antibiotic containing DMEM-F12 before transfection with DNAs and ExGen 500 *in vitro* transfection reagent as described above. In order to maintain good cell condition throughout the transfection and to avoid cytopathic effects resulting from overgrowth and crowding of this rapidly proliferating immortalized cell line, the overnight recovery period was omitted and IFNT treatments were added to LE cells in serum-free medium approximately 2 to 4 h after addition of the DNA complexes. The LE cells continued to proliferate under these conditions and typically achieved 100% confluency by the time cells were lysed 24 h after the addition of DNA complexes to the monolayers. Cell lysates were collected and luciferase assays were conducted as described previously (Fleming et al., 2006). In experiments with all cell types, the amount of reporter plasmid per well and the total amount of DNA per well was kept constant. The pEF1-Myc/His/LacZ empty vector was used, as necessary, to maintain constant amounts of DNA when expression plasmids were used at varying ratios. Each DNA and treatment combination was tested in four replicate wells in each experiment, and all experiments were repeated independently a minimum of three times.

2.7. Statistical analyses

The effect of the IRFs or IFNT on activities of reporter constructs in transient transfection assays were analyzed by least squares ANOVA using the General Linear Models procedure of the Statistical Analysis System (Cary, NC). A *P* value of 0.05 or less was considered statistically significant effect of treatment.

3. Results

3.1. IRF6 expression in the ovine uterus and conceptus

An ovine *IRF6* cDNA (GenBank Accession AF228446) of 1404 nucleotides was isolated as described from a Lambda Zap II cDNA library of Day 15 pregnant ovine endometrium. The ovine endometrial *IRF6* cDNA had high sequence identity with *IRF6* cDNAs of human (93%) and other mammalian species. *In situ* hybridization analyses detected *IRF6* mRNA in the LE/

sGE and GE of endometria from cyclic and early pregnant ewes. The abundance of *IRF6* mRNA in the LE/sGE, and to some extent in GE, increased between Days 11 and 17 of pregnancy, but not between Days 11 and 15 of the estrous cycle, and *IRF6* mRNA was detected in the conceptus trophoctoderm on Day 17 of pregnancy (Fig. 1). Immunohistochemical analyses of IRF6 protein in cyclic and pregnant ewes indicated that IRF6 protein was similar between Days 10 and 16 of the estrous cycle, increased in abundance between Days 10 and 20 of pregnancy in LE/sGE and GE, and was detected in trophoctoderm and extra-embryonic endoderm of conceptuses (Fig. 2). IRF6 protein increased in LE/sGE and, to some extent, in GE in pregnant ewes. In addition, IRF6 protein was detected in the trophoctoderm of the conceptus as was particularly abundant in the nuclei of trophoblast giant binucleate cells. The IRF6 protein was present in both the cytoplasm and nucleus of LE/sGE, GE and trophoctoderm cells.

3.2. Biological activity of IRF6 in human 2fTGH and U3A cells

In the early pregnant ovine uterus, the transcriptional activators IRF1 and IRF9 are expressed in stromal cells and GE, whereas the transcriptional repressor IRF2 is expressed in LE/sGE that lack detectable STAT1, STAT2, IRF9 and IRF1 (Choi et al., 2001). Transient transfection analyses were conducted in human 2fTGH and U3A STAT1 null cells to determine if ovine endometrial IRF6 was an activator or repressor of transcription and if it was involved in responses of cells to IFNT. In human 2fTGH cells, IFNT treatment alone increased ($P < 0.05$) activity of the 5xISRE-TATA-LUC reporter about 1.5-fold. Co-transfection with IRF6 or IRF1 stimulated ($P < 0.05$) basal activity of the reporter only slightly (1.3-fold) compared to the empty vector (EF1-LacZ), but co-transfection with both IRF6 and IRF1 did not have ($P > 0.10$) additive effects on 5xISRE-TATA-LUC activity (Fig. 3). Further, IRF6 and IRF1 alone or together failed ($P > 0.10$) to increase IFNT stimulation of 5xISRE reporter activity in 2fTGH cells. In contrast, co-transfection of ovine IRF2 repressed ($P < 0.01$) both basal and IFNT-stimulated reporter activity.

In U3A STAT1 null cells (Fig. 4), IFNT did not ($P > 0.10$) stimulate 5xISRE-TATA-LUC reporter activity due to the lack of STAT1 and the inability to form the ISGF3 transcription factor complex (Choi et al., 2001, Stewart et al., 2002). Transfection of U3A cells with either IRF6 or IRF1 did not ($P > 0.10$) affect basal activity of the reporter, whereas IRF2 alone or when co-transfected with either IRF6 or IRF1 repressed ($P < 0.01$) basal activity of the reporter alone.

Next, 2fTGH cells were transfected with a constant amount of IRF6 or IRF1 and increasing amounts of the IRF2 repressor (Fig. 5). In the absence of IRF2, IRF6 and IRF1 stimulated ($P < 0.05$) basal activity of the reporter by 1.5- and 1.3-fold, respectively, but neither potentiated IFNT stimulation of 5xISRE-TATA-LUC reporter activity. IRF2 decreased ($P < 0.01$) both basal and IFNT-stimulated reporter activity in a dose-dependent manner in cells co-transfected with either IRF6 or IRF1. Ratios of IRF2 to IRF6 (or IRF1) as low 1:40 (10 ng IRF2 DNA/well) had pronounced effects on reporter activity, although on a fold-change basis IFN still stimulated reporter activity to approximately the same extent as in cells transfected with lower amounts of IRF2 DNA. Higher relative amounts of IRF2 compared to IRF6 or IRF1 (1:4, or 100 ng IRF2 DNA/well) further reduced ($P < 0.01$) reporter activity and completely abolished IFNT stimulation.

3.3. Biological activity of IRF6 in ruminant endometrial cells

The effect of IRF6 on 5xISRE-TATA-LUC reporter activity was conducted next in an immortalized ovine LE cell line (Johnson et al., 1999). In untreated LE cells, IRF6 stimulated ($P < 0.05$) basal activity of the 5xISRE-TATA-LUC reporter by almost 4-fold compared to the empty vector control, whereas IRF1 had no effect ($P > 0.10$) on reporter activity (Fig. 6). IFNT

stimulated ($P<0.01$) reporter activity about 7-fold in cells transfected with the empty vector, 4-fold in cells transfected with IRF1, and 1.7-fold in cells transfected with IRF6.

In a bovine endometrial cell line (BEND), IRF6 stimulated ($P<0.05$) basal activity of the 5xISRE-TATA-LUC reporter approximately 3-fold in cells transfected with the empty vector (Fig. 7). Transfection of IRF1 did not ($P>0.10$) increase basal activity of the reporter. IFNT induced ($P<0.01$) a 19.3-fold increase in 5xISRE-TATA-LUC reporter activity in cells transfected with the empty vector, but neither IRF1 nor IRF6 enhanced ($P>0.10$) IFNT stimulation of reporter activity. On a fold-change basis, IFNT stimulated ($P<0.01$) reporter activity 19-fold and 14-fold for empty vector and IRF1-transfected cells, respectively, and 4.6-fold for IRF6-transfected cells, reflecting the higher basal reporter activity in the IRF6-transfected cells. Transfection of ovine IRF2 decreased ($P<0.01$) basal reporter activities in a dose-dependent manner regardless of whether IRF6 or IRF1 was co-transfected into the cells.

A similar experiment was conducted in BEND cells using the promoter region of the bovine *ISG15* gene that contains four ISREs (Perry et al., 1999). IRF6, but not IRF1, stimulated ($P<0.05$) basal activity of the bovine *ISG15*-LUC reporter approximately 1.5-fold (Fig. 8). Treatment with IFNT induced a 4-fold increase ($P<0.05$) in reporter activity in cells transfected with the empty vector, but neither IRF6 nor IRF1 enhanced ($P>0.10$) the response of the reporter to IFNT. Co-transfection of IRF2 decreased ($P<0.01$) both basal and IFNT-stimulated reporter activity in BEND cells transfected with the empty vector, IRF6 or IRF1.

3.4. Biological activity of IRF6 in ovine trophectoderm cells

Since IRF6 is expressed in trophectoderm cells of the ovine conceptus, IRF6 activity in the oTr1 trophectoderm cell line was determined. Transfection of IRF6, but not IRF1, increased ($P<0.05$) basal activity of the 5xISRE-TATA-LUC reporter 5-fold compared to the empty vector (Fig. 9). IFNT increased ($P<0.05$) activity of the reporter by 4.8-fold in cells transfected with the empty vector and 4.1-fold in IRF1-transfected cells. In IRF6 transfected cells, IFNT stimulated ($P<0.05$) reporter activity only 1.7-fold. Both basal and IFNT-stimulated luciferase activity depended on the presence of the concatamerized ISRE in front of TATA in the luciferase reporter (Fig. 9).

4. Discussion

In the present study, *IRF6* mRNA was present only in the endometrial epithelia and conceptus trophectoderm during early pregnancy in sheep. The expression of *IRF6* in uterine epithelia is consistent with its expression in epithelia of tissues in other species (Washbourne and Cox, 2006, Hatada et al., 1997, Ben et al., 2005, Knight et al., 2006, Bailey et al., 2005). IRF6 levels in human mammary epithelial cells are highest in normal tissues, but diminished in mammary carcinomas (Bailey et al., 2005). The abundance of IRF6 was inversely correlated with the invasiveness of several breast cancer cell lines or tumor grade, which suggested that IRF6 declines as epithelial-mesenchymal transition progresses and cancer cells acquire more mesenchymal characteristics (Bailey et al., 2005). Alternatively, these observations may reflect higher rate of proliferation of breast cancer cells versus their fully differentiated normal counterparts since IRF6 was higher in quiescent cells than in dividing cells and decreased mitotic activity was associated with decreasing levels of IRF6 (Bailey et al., 2008). Similar to findings from studies of sheep placenta, *Irf6* is expressed in placenta of mice (Kondo et al., 2002). The role of *Irf6* in placental differentiation is not known, because homozygous *Irf6* null mice die shortly after birth (Richardson et al., 2006). Thus, IRF6 likely has a biological role in both ovine uterine endometrial epithelia and conceptus trophectoderm.

IFN plays a critical role in the induction and activation of several IRFs (Mamane et al., 1999, Nguyen et al., 1997, Stark et al., 1998, Platanias, 2005), but *IRF6* expression is expressed in

endometrial epithelia of ewes during the estrous cycle independent of IFNs. However, *IRF6* mRNA does increase in abundance in LE/GE between Days 11 and 17 of pregnancy in concert with onset of conceptus elongation and production of IFNT that is maximal between Days 14 and 16 of pregnancy (Ashworth and Bazer, 1989). These correlative results suggest that IFNT regulates *IRF6* and perhaps *IRF2* expression in LE/sGE, because *IRF2* mRNA and protein abundance also increase in uterine LE/sGE of early pregnant, but not cyclic ewes (Choi et al., 2001). Treatment of human MCF-10A mammary cancer cells with the classic IFN inducer poly dI-C resulted in a rapid phosphorylation and translocation of activated *IRF6* to the nucleus and later increased *IRF6* levels in the cytosol (Bailey and Hendrix, 2008). Future studies are required to dissect out the mechanism underlying pregnancy-specific increases in *IRF6* and *IRF2* expression in endometrial LE/sGE.

With the exception of human U3A cells, *IRF6* is a transcriptional activator of ISRE-containing promoter reporter constructs in transfected cells. The ability of *IRF6* to activate the ISRE-containing reporter constructs depended on the presence of an ISRE upstream of TATA in the TATA-based constructs. Constructs that lacked ISREs were not activated by either *IRF6* or *IRF1* in the absence or presence of IFNT. The dependence on the presence of an upstream ISRE suggests that the DBD of *IRF6* binds to ISRE and increases transcription via an intrinsic activation domain similar to most other IRFs, except *IRF2*. A high percentage of missense and nonsense mutations in *IRF6* in individuals with van der Woude's or popliteal pterygium syndromes occur in the DBD region, underscoring the importance of the interaction of *IRF6* with an ISRE or ISRE-like element in target genes (Kondo et al., 2002). Compared to the TATA-LUC reporter, the 5xISRE-TATA-LUC reporter had significantly greater basal activity in the absence of IFNT regardless of whether *IRF6* or *IRF1* was co-transfected. These findings suggest that an endogenous factor(s) interacts with the ISREs to increase basal levels of transcription. Several IRFs are likely to be expressed in cells used for transfection analyses. Indeed, *IRF5*, but not *IRF1*, *IRF3* or *IRF7*, stimulated basal activities of a reporter in 2fTGH cells (Barnes et al., 2001). Moreover, the C-terminal regions of IRFs contain domains termed IRF-association domains (IADs) required for interaction of IRFs with other IRFs or other transcription factors (Mamane et al., 1999, Taniguchi et al., 2001). Transactivation or repression is effected by these higher order complexes, and complexes of an IRF such as *IRF8* with different interacting partners can determine whether the complex functions as an activator or repressor (Levi et al., 2002). For instance, *IRF2* is normally a strong transcriptional repressor of many genes, but a PU.1-*IRF2*-*IRF8* complex activates the *neurofibromin 1* gene in myeloid cells, emphasizing the importance of heterocomplexes in regulatory activity of genes (Mamane et al., 1999, Nguyen et al., 1997, Huang et al., 2007). Indeed, a high proportion of the *IRF6* variants associated with van de Woude syndrome occur in the C-terminal protein interaction domain, suggesting that the *IRF6* IAD is important to its function in gene regulation (Kondo et al., 2002). Although *IRF6* is co-expressed with *IRF1* in ovine endometrial LE/sGE (Choi et al., 2001), co-transfection of *IRF6* and *IRF1* did not enhance transactivation of ISRE-containing reporter constructs in any cell type tested. Conversely, titrations of *IRF2* suggest that very low amounts of *IRF2* are sufficient to repress both the 5xISRE-TATA-LUC and *ISG17*-LUC reporter activities *in vitro*, and that neither *IRF6* nor *IRF1* is sufficient to prevent *IRF2*-mediated repression. The DBD of *IRF1* and *IRF2* have a high degree of sequence similarity (76%) and function through the same *cis* element (Nguyen et al., 1997, Harada et al., 1989). The DBD of *IRF6* has limited similarity to those of *IRF1* and *IRF2* (36% and 40%, respectively) and is most similar to the DBD of *IRF5* (63%) (Nguyen et al., 1997). Repression of basal reporter activity by *IRF2* suggests that it binds ISREs in the absence of IFNT and displaces *IRF6* and *IRF1* in co-transfected cells.

In addition to regulating gene expression via classical ISREs found in many IFN-stimulated genes, comparisons of IADs with C-terminal domains of SMAD2 and SMAD4 predicted some conserved structural features in the SMADs and seven IRFs, including *IRF6*, but not *IRF1* or

IRF2 (Levi et al., 2002, Eroshkin and Mushegian, 1999, Qin et al., 2003). The C-terminal protein-binding domain of IRFs is sometimes termed SMIR (SMAD and IRF) (Eroshkin and Mushegian, 1999). The conserved predicted folding patterns suggest that SMADs and some IRFs may interact similarly with other proteins. X-ray crystallographic analyses confirmed that the C-terminus of IRF3 and the Mad homology domain 2 (MH2 domain) of SMAD 2 or SMAD3 have considerable similarities in structural and surface electrostatic potential (Qin et al., 2003, Takahashi et al., 2003). Although SMADs can bind DNA directly, the receptor-activated SMADs can physically interact with several sequence-specific transcription factors that are regulated by other signaling pathways as well as co-activators and co-repressors to form complexes with different transcriptional activities (Derynck and Zhang, 2003). This combinatorial approach affects the specificity of gene regulation by SMAD complexes and provides a mechanism for cross-talk among different signaling pathways (Zhang and Derynck, 1999). Thus, IRF6 interactions with other transcription factors may be important for its biological roles in endometrial epithelial cell functions as well as conceptus trophectoderm growth and differentiation. Indeed, IRF6 is involved in the development of multiple tissues and organs in the embryo (Knight et al., 2006, Richardson et al., 2006, Vieira et al., 2007, Xu et al., 2006).

In the present study, IRF6 did not affect 5xISRE reporter activity in U3A cells, a STAT1 null derivative of 2FTGH cells, used as a model for studies of gene regulation in LE/sGE of early pregnancy in sheep (Stewart et al., 2001b) which also lack STAT1 (Choi et al., 2001). During early pregnancy, IRF6 and IRF2 are both expressed in endometrial LE/sGE and increase in abundance in LE (Choi et al., 2001). The increase in *IRF2* expression in uterine LE/sGE of pregnancy is hypothesized to repress expression of most classical ISGs that contains multiple ISREs and are induced by Type I IFNs (Choi et al., 2001, Spencer et al., 2007, Spencer et al., 2004, Spencer et al., 2008a). Indeed, the LE/sGE of pregnancy lack detectable STAT1, STAT2, IRF9 and IRF1 (Choi et al., 2001), as well as ISG15, B2M and many other ISGs (Spencer et al., 2007, Spencer et al., 2004, Spencer et al., 2008a). Results of the present study support that hypothesis, because IRF2 dose-dependently inhibited IRF6 effects on basal activity of the ISRE-containing reporter constructs in all cells tested. Therefore, it is unlikely that IRF6 transactivates ISRE-containing genes in uterine LE/sGE, whereas IRF6 may regulate ISRE-containing genes in uterine GE and trophectoderm that are IRF2-negative. However, IRF6 may regulate expression of specific genes in endometrial LE/sGE as well as GE and trophectoderm through interactions with SMADs and other transcription factors.

In summary, IRF6 is co-expressed with IRF1 and IRF9, as well as IRF2 in ovine uterine GE and LE/sGE, respectively, and IRF6 is also expressed in trophectoderm of the conceptus. Moreover, IRF6 is a transactivator of ISRE-containing promoters that acts independent of IFNT. Based on differences in both their DBD and IADs, IRF6 and IRF1 and/or IRF2 likely regulate different, but possibly overlapping, expression of subsets of genes, depending on sequences of the *cis* elements in the promoters of the target genes and the different interacting proteins that may lead to the formation of different transcriptional regulatory heterocomplexes. Of particular interest are potential effects of IRF6 proliferation and differentiated functions of trophectoderm cells, as well as interactions between conceptus trophectoderm and extra-embryonic mesoderm during formation of the choriovitelline (yolk sac) and then chorioallantoic placenta during pregnancy in sheep (see (Kondo et al., 2002, Bailey et al., 2008, Bailey et al., 2005)). There is also the need to explore interactions between IRF6 and TGFB (Xu et al., 2006, Knight et al., 2006). For example, TGFB1 signaling involving SMADs can induce WNT-signaling by inhibiting DKK1 (Kane et al., 2008) and has been implicated in peri-implantation conceptus development in sheep (Dore et al., 1996, Dore et al., 1995). Future investigations should focus on determining the biological functions of IRF6 and target genes regulated by IRF6 in ovine uterine epithelia and conceptus trophectoderm during pregnancy.

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Fig. 1. *In situ* hybridization analysis of *IRF6* mRNA in the ovine uterus

Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radiolabeled antisense or sense ovine *IRF6* cRNAs. *IRF6* mRNA was detected in the uterine LE/sGE and GE as well as trophoctoderm of the conceptus on Day 17 of pregnancy. Note the apparent increase in *IRF6* mRNA in LE/sGE during pregnancy. Legend: GE, glandular epithelium; LE, luminal epithelium; S, stroma; Tr, trophoctoderm. Scale bar represents 10 μ m.

Fig. 2. Immunohistochemical localization of IRF6 protein in uteri from cyclic and pregnant ewes
 In the IgG control, normal rabbit IgG was substituted for rabbit IgG specific for human IRF6. Sections were not counterstained. Note the immunoreactive IRF6 protein in uterine LE/sGE and GE, as well as conceptus trophoblast. IRF6 protein increased in uterine LE/sGE and GE during pregnancy. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma; Tr, trophoblast; En, endoderm. Scale bar represents 10 μ m in [A] and 100 μ m in [B].

Fig. 3. Effect of IRFs and IFNT on 5xISRE-TATA-LUC reporter activity in 2fTGH cells
Cells were co-transfected with indicated constructs (amount indicated in ng/well) and treated with nothing or IFNT (10^4 antiviral units) for 24 h. Transfection assays and luciferase activities were determined as described in Materials and Methods. The data are presented as mean relative light units (RLU) with standard deviation (SD). Four replicate determinations for each treatment group were conducted in each of three independent experiments. The asterisk (*) denotes a significant effect of treatment with IFNT on reporter activity ($P < 0.05$).

Fig. 4. Effect of IRFs and IFNT on 5xISRE-TATA-LUC reporter activity in U3A STAT1 null cells
Cells were co-transfected with indicated constructs (amount indicated in ng/well) and treated with nothing or IFNT (10^4 antiviral units) for 24 h. Transfection assays and luciferase activities were determined as described in Materials and Methods. The data are presented as mean relative light units (RLU) with SD. Four replicate determinations for each treatment group were conducted in each of three independent experiments. Note the lack of IFNT stimulation of reporter activity ($P>0.10$) in the transfected U3A cells.

Fig. 5. Effect of IRFs and IFNT on 5xISRE-TATA-LUC reporter activity in 2fTGH cells
Cells were co-transfected with indicated constructs (amount indicated in ng/well) and treated with nothing or IFNT (10^4 antiviral units) for 24 h. Transfection assays and luciferase activities were determined as described in Materials and Methods. The data are presented as mean relative light units (RLU) with SD. Four replicate determinations for each treatment group were conducted in each of three independent experiments. The asterisk (*) denotes a significant effect of treatment with IFNT on reporter activity ($P<0.05$).

Fig. 6. Effect of IRFs and IFNT on 5xISRE-TATA-LUC reporter activity in immortalized ovine endometrial luminal epithelia (LE) cells

Cells were co-transfected with indicated constructs (amount indicated in ng/well) and treated with nothing or IFNT (10^4 antiviral units) for 24 h. Transfection assays and luciferase activities were determined as described in Materials and Methods. The data are presented as mean relative light units (RLU) with SD. Four replicate determinations for each treatment group were conducted in each of three independent experiments. The asterisk (*) denotes a significant effect of treatment with IFNT on reporter activity ($P < 0.05$).

Fig. 7. Effect of IRFs and IFNT on 5xISRE-TATA-LUC reporter activity in a bovine endometrial cell line (BEND)

Cells were co-transfected with indicated constructs (amount indicated in ng/well) and treated with nothing or IFNT (10^4 antiviral units) for 24 h. Transfection assays and luciferase activities were determined as described in Materials and Methods. The data are presented as mean relative light units (RLU) with SD. Four replicate determinations for each treatment group were conducted in each of three independent experiments. The asterisk (*) denotes a significant effect of treatment with IFNT on reporter activity ($P < 0.05$).

Fig. 8. Effect of IRFs and IFNT on 5xISRE-TATA-LUC reporter activity in a bovine endometrial cell line (BEND)

Cells were co-transfected with indicated constructs (amount indicated in ng/well) and treated with nothing or IFNT (10^4 antiviral units) for 24 h. Transfection assays and luciferase activities were determined as described in Materials and Methods. The data are presented as mean relative light units (RLU) with SD. Four replicate determinations for each treatment group were conducted in each of three independent experiments. The asterisk (*) denotes a significant effect of treatment with IFNT on reporter activity ($P<0.05$).

Fig. 9. Effect of IRFs and IFNT on 5xISRE-TATA-LUC and TAT-LUC reporter activity in ovine trophoctoderm (oTr1) cells

Cells were co-transfected with indicated constructs (amount indicated in ng/well) and treated with nothing or IFNT (10^4 antiviral units) for 24 h. Transfection assays and luciferase activities were determined as described in Materials and Methods. The data are presented as mean relative light units (RLU) with SD. Four replicate determinations for each treatment group were conducted in each of three independent experiments. The asterisk (*) denotes a significant effect of treatment with IFNT on reporter activity ($P < 0.05$).