A microarray analysis for genes regulated by interferon- τ in ovine luminal epithelial cells

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Abstract

Interferon- τ (IFNT) is released by preimplantation conceptuses of ruminant species and prepares the mother for pregnancy. Although one important function is to protect the corpus luteum from the luteolytic activity of prostaglandin-F 2 α , IFNT most likely regulates a range of other physiological processes in endometrium. Here, an immortalized cell line from ovine uterine luminal epithelial cells was treated with IFNT for either 8 or 24 h. RNA was subjected to cDNA microarray analysis, with RNA from untreated cells as the reference standard. Of 15 634 genes, 1274 (8%) were IFNT responsive at $P < 0.01$ and 585 at $P < 0.001$ to at least one treatment. Of the latter, 356 were up-regulated and 229 down-regulated. Increasing IFNT concentrations from 10 ng/ml to 10 μ g/ml had minor effects, and most genes up- or down-regulated at 8 h were regulated similarly at 24 h. Although IFNT influences many genes implicated in antiviral activity and apoptosis, its action also likely regulates prostaglandin metabolism, growth factors and their receptors, apoptosis and the nuclear factor (NF)- κ B cascade, extracellular matrix accretion, angiogenesis, blood coagulation, and inflammation. In particular, it increased mRNA concentrations of genes related to the vascular endothelial growth factor R2 pathway of angiogenesis and down-regulated ones associated with hypoxia. Two genes implicated in the antiluteolytic actions of IFNT (encoding cyclooxygenase-2 and the oxytocin receptor respectively) were down-regulated in response to all treatments. IFNT targets a complex range of physiological processes during the establishment of pregnancy.

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Introduction

Interferon- τ (IFNT) is generally regarded as the primary signal for maternal recognition of pregnancy in the true ruminant species such as cattle and sheep and helps to maintain the structural and functional integrity of the corpus luteum (CL) at a time in a nonpregnant animal that this structure would be poised to regress (Roberts *et al.* 1992). By such intervention, IFNT allows progesterone production by the CL to continue and hence maintains the endometrium in a receptive state. The elongating conceptus secretes IFNT in large amounts during this period, which immediately precedes definitive attachment of trophoctoderm to the uterine epithelium, i.e. $\sim$ 12–21 days in sheep (Hansen *et al.* 1988, Farin *et al.* 1990) and $\sim$ 14–25 days in cattle (Helmer *et al.* 1987, Farin *et al.* 1990). In its luteoprotective role, IFNT prevents or diminishes oxytocin-induced 'pulses' of prostaglandin F2 α (PGF2 α ; PGF) from the uterus, most likely by

suppressing the expression of oxytocin receptors (OTR) on the luminal and upper glandular epithelial cells lining the uterus (Spencer *et al.* 1995a). Hence, the immediate target for IFNT action is on these epithelial cells, which are exposed to very high concentrations of this protein for an extended period of time.

IFNT is a member of the Type I IFN family (Roberts *et al.* 1997). These cytokines have broad pleiotropic actions on their target cells (Stark *et al.* 1998). Type I IFNs bind to a common receptor complex consisting of two subunit polypeptides (IFNAR1 and IFNAR2; Pestka *et al.* 2004), both of which are highly concentrated in uterine epithelial cells relative to other cells in ovine endometrium (Rosenfeld *et al.* 2002). Upon binding to the receptor, Type I IFNs activate the well-studied JAK–STAT signaling pathway (Stark *et al.* 1998), leading to the induction of several downstream effector genes, often named interferon-stimulated genes or ISGs. In addition, Type I IFNs activate additional signal transduction

pathways, including components of the MAPK pathway (Uddin *et al.* 2000).

Several ISGs are regulated by IFNT in the endometrium of cattle and sheep (Austin *et al.* 1996, Chen *et al.* 2006, Gray *et al.* 2006). In sheep, at least, increased expression of some of these genes has been noted in stromal and immune cells resident in the uterus as well as in the surface and glandular epithelium (Johnson *et al.* 1999a, 2000, 2001a, 2002, Choi *et al.* 2001). In addition, different ISGs show complex and distinct patterns of expression within the endometrium, suggesting that regulation of gene expression is complex. Most of the endometrial ISGs so far studied are ones anticipated to be up-regulated as part of the classical antiviral response. It remains unclear whether the activation of these viral-response genes has any role in preparing the uterus for pregnancy. However, at least three genes, specifically those encoding estrogen receptor- α (*ESR1*; Spencer *et al.* 1995b, Spencer & Bazer 1996), the *OTR*; Spencer *et al.* 1995a, Spencer & Bazer 1996), and cyclooxygenase-2 (*PTGS2*; Xiao *et al.* 1998, 1999, Binelli *et al.* 2000, Pru *et al.* 2001) have been implicated in the antiluteolytic action of IFNT and reported to be down-regulated following IFNT exposure. Confusingly, others have been unable to confirm this result for *PTGS2*, which has variously been reported to be up-regulated (Asselin *et al.* 1997a, 1997b, Emond *et al.* 2004) or unaffected by IFNT (Kim *et al.* 2003b). We recently showed that *OTR* and *PTGS2* gene expression were down-regulated at day 14 of pregnancy in sheep and by treatment of ewes with IFNT and concluded that the mechanism whereby IFNT acts as an antiluteolysin still remains unclear, although a strong case can be made for the involvement of *OTR* (Chen *et al.* 2006).

The endometrium responds to IFNT secreted by the conceptus in a paracrine manner. The surface epithelium of the uterine lumen is probably the primary target for IFNT, although there is also evidence that this cytokine can reach cells in the underlying stroma (Johnson *et al.* 1999b, 2000, 2001b, Hicks *et al.* 2003) and even the myometrium (Ott *et al.* 1998, Johnson *et al.* 1999d, Hicks *et al.* 2003), suggesting that the epithelium does not act as a completely impermeable barrier to conceptus-derived proteins. Although cell lines from a number of sources have been used to explore the biological activities of IFNT, including ones from nonendometrial tissues, such as L929 (mouse fibroblast) cells, WISH (human amnion) cells, Daudi (human B-cell) cells, human 2fGH (human fibrosarcoma) cells, and U3A (STAT1-deficient 2fTGH) cells (Alexenko *et al.* 1997, 1999, Stewart *et al.* 2001b, Kim *et al.* 2003a), cells or tissue explants from endometrium clearly provide the best models for studying the physiological role of IFNT. In this paper, we have chosen to examine the effects on an immortalized luminal epithelial (LE) cell line initially isolated at day 5 of the estrous cycle (Johnson *et al.* 1999c). The LE line has been relatively well characterized and mimics at least some of

the responses of the ovine uterus to IFNT in terms of ISG expression (Johnson *et al.* 1999c, Stewart *et al.* 2001a). These cells express estrogen and progesterone receptors (Johnson *et al.* 1999c) and both Type I IFN receptor subunits, IFNAR1 and IFNAR2 (Rosenfeld *et al.* 2002). They provide a convenient means for studying the effects of IFNT on gene expression in a specific ovine uterine cell type, although it would be naïve to assume that these cells can exactly recapitulate responses *in vivo*. Nevertheless, data from such cells can provide insight into the range of IFNT-regulated genes and pathways likely to be modulated by IFNT during pregnancy, and can be compared with existing *in vivo* information on gene expression changes that occur in response to the presence of a conceptus (Ishiwata *et al.* 2003, Bauersachs *et al.* 2006, Klein *et al.* 2006) or to exposure of the uterine tract to IFNT (Chen *et al.* 2006, Gray *et al.* 2006).

We have examined two kinds of response of the LE cells to IFNT, namely the effects of concentration and time of exposure, since both variables have been reported to be important. For example, IFNT increased PGE2 production in a dose-dependent manner (Asselin *et al.* 1997b, 1997c), and while low concentrations (equivalent to 5×10^{-8} M or less) inhibited the production of PGF in primary bovine endometrial cell cultures (Xiao *et al.* 1998, 1999, Asselin & Fortier 2000), high concentrations ($\sim 5 \times 10^{-7}$ M) promoted such production (Asselin *et al.* 1997a, 1997c, Parent *et al.* 2003). In addition, IFNT reduced *PTGS2* mRNA at 6 h or earlier but not at later time points (Xiao *et al.* 1999). To limit the number of analyses, we chose two exposure times: 8 and 24 h. The former was anticipated to provide data on 'early' and 'intermediate' genes, the second on genes whose regulation was late and possibly secondary to the initial response to IFNT.

Although gene transcript profiling for IFN-responsive genes in human cells was first performed in 1998 (Der *et al.* 1998), there have been few other comprehensive studies since then in any species. Genes that respond to ovine IFNT have been described in human fibroblast cells (Stewart *et al.* 2001b, Kim *et al.* 2003a). In addition, our laboratory has examined the expression of 70 genes considered likely to be IFNT responsive in endometrium of ewes that were either pregnant or had been administered IFNT (Chen *et al.* 2006), while, in the same species, Gray *et al.* (2006) used a microarray representing ~ 5000 ovine genes to study genes regulated during pregnancy and in response to a combination of IFNT and progesterone. In the present study, we have made the use of a 15 634 bovine unigene set to analyze IFNT responses in LE cells. There were four goals. The first was to determine whether the changes we observed earlier (Chen *et al.* 2006) in endometrium of ewes, including effects on genes influencing prostaglandin metabolism, would be reproduced in the epithelial cell line. The second and third goals were to determine whether raising the concentration and time of exposure

to IFNT would influence gene expression profiles, recalling that the surface epithelium of the intact uterus must be exposed to unusually high amounts of the cytokine for several days. The final goal was to generate a comprehensive list of genes regulated by IFNT in ovine endometrium. Presumably, the responses of some but not necessarily all of these genes provide the basis for how IFNT mediates changes in the mother that provide for a successful establishment of pregnancy.

Materials and Methods

Cell culture

The LE cells were maintained in Dulbecco's modified Eagle's medium with F12 salts (DMEM/F12; Sigma–Aldrich Corp.), pH 7.4, containing 10% (v:v) fetal bovine serum (Gibco-BRL) and penicillin/streptomycin/amphotericin B solution (100 IU/ml, 0.1 mg/ml, 0.25 µg/ml; Gibco-BRL) as described previously (Johnson *et al.* 1999c).

Recombinant ovIFNT

Recombinant protein was produced as described elsewhere (Ealy *et al.* 1998a, 1998b). Antiviral assays to assess potency of the preparation were performed on MDBK cell (ATCC#CCL22, American Type Culture Collection; Ealy *et al.* 1998b). The antiviral activity of this preparation of IFNT was 2×10^8 international units/mg.

Treatment of cells with IFNT

For the microarray experiments, three stocks of LE cells, frozen on different occasions, were used. Each experiment, which was performed thrice, employed three replicate groups of cells representing the three different cell stocks. Both experiments had an identical experimental design. In each, cells were allowed to reach ~80% confluence in 75 cm tissue culture flasks. The medium was then replaced with 5.0 ml fresh DMEM/F12 containing either recombinant ovine IFNT at two different concentrations (10 ng/ml; $\sim 5 \times 10^{-10}$ M and 10 µg/ml; $\sim 5 \times 10^{-7}$ M) or with the IFNT vehicle (1×PBS) as a control (Parent *et al.* 2003). The lowest concentration of

IFNT was close to the K_D for the ovine receptor, while the highest was supersaturating, but probably closer to IFNT concentrations in the uterine lumen at the time of maximal production by the conceptus. Cells were exposed to IFNT for either 8 or 24 h before isolation of RNA. There were, therefore, 36 samples (9 per treatment) of RNA prepared (see below). Cultures of pooled, untreated (control) cells that were harvested at the 8-h time point provided the reference RNA for both the microarray and real-time PCR analyses (Table 1).

Microarray analysis

The DNA array slides were made by spotting cDNA PCR products in 3×SSC solutions onto the poly-L-lysine coated glass slides. Each array contained 17 692 cDNA probes from an available cDNA library of 16 156 probes (Smith *et al.* 2001) and a bovine ovary EST library with 1536 probes (Takasuga *et al.* 2001). In addition, the new library represented 15 634 unique sequences.

RNA was isolated from individual plates of cells using RNA STAT-60 (Tel-Test, Friendswood, TX, USA) according to manufacturer's recommendations. The quantity of RNA for each extraction was determined by measuring absorbance at 260 nm; purity was calculated from the ratios of absorbance at 260 and 280 nm; and quality was evaluated by agarose gel electrophoresis. Extracted RNA was stored at -80°C . For the microarray analysis, the triplicate samples for each of the four experimental treatments were pooled (RNA ratio 1:1:1) to provide four pools of RNA. Since the experiment was performed thrice, there were 12 RNA sample pools in total, which were each analyzed through a comparison with a single sample of control RNA from untreated cells. Thus, each RNA sample represents pooled material from three different isolates of LE cells, thereby minimizing any confounding effect of variation among different batches of cells.

The microarray analysis utilized also-called Reference Design approach in which we compared RNA from the various treatment groups with the control RNA, following the microarray protocol from TIGR (The Institute for Genomic Research; Hegde *et al.* 2000). RNA (15 µg) was reverse transcribed using

Table 1 Real-time PCR analysis of mRNA expression in ovine luminal epithelial cells treated with interferon- τ (IFNT).

Gene	None	IFNT (0.001 mg/ml)		IFNT (10 mg/ml)	
		8 h	24 h	8 h	24 h
<i>ISG15</i>	1.0±0.5	8.7±0.9* (23.9)	6.1±0.8* (30.7)	6.6±0.8* (13.8)	7.3±0.9* (30.7)
<i>OTR</i>	1.0±0.2	0.30±0.1* (0.40)	0.34±0.1* (0.77)	0.45±0.1* (0.73)	0.39±0.1* (0.51)
<i>IGF2</i>	1.2±0.4	0.43±0.1* (0.88)	0.31±0.2* (0.69)	0.28±0.1* (0.81)	0.35±0.1* (0.79)
<i>PTGS2</i>	1.0±0.1	0.32±0.1* (0.65)	0.36±0.1* (0.77)	0.40±0.1* (0.61)	0.42±0.2* (0.76)
<i>HIF1A</i>	1.1±0.4	0.26±0.1* (0.80)	0.22±0.08* (0.77)	0.37±0.1* (0.79)	0.29±0.2* (0.83)

*Effect of treatment ($P<0.01$). Real-time PCR was used to measure the fold decrease or increase in target gene mRNA in IFNT-treated cells. The values presented are from independent experiments, each conducted in triplicate. Values in parentheses were the changes in expression as assessed on the microarrays. OTR was not represented on the microarray.

SuperScript III reverse transcriptase (Invitrogen) to provide incorporation of the reactive amine derivative of dUTP, 5-(3-aminoallyl)-2'-deoxyuridine 5'-triphosphate (Sigma) into the cDNA. After removing free nucleotides, the aminoallyl-labeled samples were coupled to dye. The cDNAs from treated and control were labeled separately with either cyanine 3 (Cy3) or Cy5 dyes by standard procedures (Amersham Biosciences). The resulting labeled probes were purified, mixed together, and then redissolved in 25 µl hybridization solution (50% formamide, 5×SSC, 0.1% SDS, 10 µg ovine Cot-1 DNA, and 10 µg poly-A in water). The samples were denatured and then applied to the microarrays. After incubation at 42 °C for 16 h, the arrays were sequentially washed with 1×SSC/0.2% SDS, 0.1×SSC/0.2% SDS, and 0.1×SSC solutions. The arrays were then dried and scanned using a Genepix 4000B scanner (Axon Instruments, Union City, CA, USA) and analyzed by the Genepix Pro software. As a control, the dye labeling was reversed in one of the hybridizations (dye-swap control).

Data analysis

The intensity value of each spot for Cy3 and Cy5 fluorescence (media foreground intensity minus media background intensity) was analyzed by R/MAANOVA (MicroArray ANOVA; Wu *et al.* 2002), which runs in the statistical software program 'R' (Beckers *et al.* 1988) from www.r-project.org. Briefly, data were first normalized by means of the regional Lowess algorithm to account for systematic intensity dependence and spatial variation in microarray log ratios, which is to correct the bias caused largely by differences of dye incorporation (Berger *et al.* 2004). Then, a two-stage ANOVA model was applied to the data. In the first stage, the model was

$$Y_{ijkgr} = \mu + A_i + D_j + AD_{ij} + r_{ijkgr}$$

where Y is the log intensity reading for a particular gene on a certain array, e.g. array i , dye j of RNA variety k ; μ is the overall mean; A_i is the effect of array i ($i=1, \dots, 12$), D_j is the effect of dye j ($j=1, 2$); and AD is the effect of array and dye interactions. This is the cross-gene stage of the calculation, which removes the overall effects of array, dye effects, and the effects of array and dye interactions.

The residual of the first stage is used as the input for the second stage, which is the gene-specific model.

$$r_{ijkgr} = G + AG_i + DG_j + VG_k + \varepsilon_{ijkgr}$$

Here, G is the average effect of the gene, AG is the array-by-gene variation, and DG is the dye-by-gene variation. VG is the effects of the treatment on the expression of the gene of interest. The error ε is the residual. A permutation F -test (Cui & Churchill 2003) was applied by MAANOVA, and the adjusted P values were calculated for every gene to indicate if expression was changed by

treatment. Finally, a two-dimensional hierarchical clustering analysis was performed using GeneCluster (www.broad.mit.edu/cancer/software/genecluster2/gc2.html) and TreeView software (Eisen *et al.* 1998; <http://rana.lbl.gov/EisenSoftware.htm>; Fig. 1). We combined use of the DAVID Bioinformatics Resources Data Base (2006; <http://david.abcc.ncifcrf.gov/>; Dennis *et al.* 2003, Brunner *et al.* 2004) with manual examination of regulated genes to provide functional annotation clustering.

Real-time PCR analysis

The relative expression levels of five selected ovine genes were determined by real-time PCR in part to confirm the reproducibility of the microarray and also to provide additional quantitative data for genes of particular interest. Primers specific for the genes for ISG-15/17, originally known as ubiquitin cross-reactive protein (*ISG15*), *PTGS2*, *OTR*, insulin-like growth factor-2 (*IGF2*), and hypoxia-inducible factor 1 subunit α (*HIF1A*) were designed with Primer Express software (Applied Biosystems, Foster City, CA, USA). The primer pairs are listed in Supplementary Table 1 which can be viewed online at www.reproduction-online.org/supplemental/.

Real-time PCR was performed and analyzed by using an ABI prism 7500 sequence detection system (Applied Biosystems). The protocol for real-time RT-PCR analysis has been described earlier (Chen *et al.* 2006). Relative expression in comparison with ribosomal protein L19 (*RPL19*) was calculated by the comparative C_T method (threshold cycle). The relative quantitation of candidate gene expression in each treatment groups was determined by using the comparative C_T method ($2^{-\Delta\Delta C_T}$) as described in user bulletin 2 ABI Prism 7500 Sequence Detection System.

Each of the five transcripts was amplified in triplicate in a single PCR run. The entire experiment was then repeated with RNA from two additional experiments from the same treatment to provide a total of three replicates. Real-time PCR quantification of gene expression level in each treatment was the mean of three real-time RT-PCR experiments. Differences in relative mRNA expression between experimental groups were assessed by one-way ANOVA, followed by pairwise comparison and by using a least significant difference test (Brunner *et al.* 2004). All experimental data are shown as the mean $\pm$ S.E.M. Values were considered significantly different at $P < 0.05$.

Results

Identification of IFN- τ responsive genes

Using the linear mixed model described in the Material and Methods, we identified 585 genes that had altered expression level at an adjusted P value < 0.001 following IFNT treatments (Supplementary Table 2 which can

Figure 1 Two-way hierarchical cluster analysis of gene expression in control- and IFNT-treated LE cells. Here we show only the 585 genes that are differentially regulated ($P < 0.001$). The gene set depicts two major groups (up- or down-regulated), which can be further divided into five subgroups (subgroups 1–5). The data clustered according to treatment time (8 and 24 h) and dose of IFNT used (L, low; H, high).

be viewed online at www.reproduction-online.org/supplemental/). At least another ~ 700 genes (a total of 1274 or 8% of all genes) were IFNT responsive if the threshold of significance was raised to an adjusted P value < 0.01 (data available upon request). Among the 585 genes in Supplementary Table 2, 567 have annotations that are based on sequence similarity with known genes. We applied a two-way (genes and treatments) hierarchical clustering method (Eisen *et al.* 1998) on the 585 genes regulated by IFNT relative to the expression of the same genes in untreated cells that had been isolated 8 h after the addition of IFNT to the treatment groups (Fig. 1). The treatments clustered into two main branches, which corresponded to the 8 and 24 h treatments. The genes regulated by the low (10 ng/ml) and high (10 μ g/ml) treatment groups formed two groups within these main branches of the tree, i.e. according to the duration of the treatments. Two major groups of genes can be easily

recognized, one that consists of genes that were up-regulated in one or more of the treatments and a second that consists of down-regulated genes. A few genes were up-regulated at one time point and down-regulated at the other. In general, a concentration of 10 ng/ml IFNT was about as effective as 10 μ g/ml. Based on visual inspection of the expression patterns, the genes illustrated in Fig. 1 could be divided arbitrarily into five subgroups, although there were distinct expression patterns visible within each grouping.

Members of subgroup 1 (Supplementary Table 3 which can be viewed online at www.reproduction-online.org/supplemental/), which contains 68 genes, were generally expressed more strongly at 8 h than at 24 h (Fig. 1), although there were several exceptions. The genes in subgroup 1 encode products with a broad range of biochemical roles. Among them are the genes for transcription factors associated with downstream action

of Type 1 IFN, including *RNF31* (interferon-stimulated transcription factor 3- γ 48 kDa) and interferon regulatory factor 7 (IRF7), three guanylate-binding proteins (*GBP4*, *GBP5*, and *GEM*), and a few genes encoding inflammatory cytokines, e.g. *TNFSF10* and *IL6ST*. The extent of up-regulation of the subgroup 1 genes by IFNT at both the low and high concentrations was relatively modest when compared with many of the genes in subgroup 2 discussed below.

Subgroup 2 comprises 252 genes (Supplementary Table 4 which can be viewed online at www.reproduction-online.org/supplemental/) whose expression was, in general, sustained over 24 h (Fig. 1). Subgroup 2 contained the majority of the genes that were up-regulated fivefold or more by IFNT. Many of these highly up-regulated genes have long been recognized as responsive to Type I IFN, and many are included in the original list of genes identified by microarrays in human cells (Der *et al.* 1998, Kim *et al.* 2003a). They include *Mx1*, *ISG15*, interferon-induced protein 6-16 (*G1P3*), interferon-induced protein with tetratricopeptide repeats (*IFIT2*), 2'-5'-oligoadenylate synthetase (*OAS1*), IFN-induced protein-35 (*IFI35*), multiple (*MHC*) genes from classes I and II, and β -2-microglobulin (*PDLIM3*). Many of the proteins encoded by these subgroup 2 genes contribute to the antiviral response of cells targeted by Type 1 IFN and are 'classical' ISGs (Stark *et al.* 1998).

Subgroup 3 consists of 31 genes that were modestly, but significantly up-regulated by IFNT at 8 h but provided inconsistent temporal patterns of expression thereafter. The genes did not appear to fall into any particular functional class (Supplementary Table 5 which can be viewed online at www.reproduction-online.org/supplemental/).

Subgroup 4 is comprised 87 genes that were down-regulated by all IFNT treatments (Fig. 1; Supplementary Table 6 which can be viewed online at www.reproduction-online.org/supplemental/). IFN responses were, in general, sustained over the 24-h exposure to IFNT. This cohort contains several genes of potential interest in endometrial responses to pregnancy, including ones encoding

metalloproteinases, the OTR, prostaglandin synthase, and IGF-2, which are discussed later.

The final subgroup contains 123 genes that were generally more strongly down-regulated at 24 h than at 8 h (Supplementary Table 7 which can be viewed online at www.reproduction-online.org/supplemental/). Again the responses to the high IFNT concentration were only marginally different from those at the low concentration.

Validation of gene expression by real-time PCR

To validate our results from the multiple microarray analyses, we performed real-time PCR experiments on five selected genes. *ISG15* was chosen as a positive control for validating the experimental procedure because it has been previously shown to be up-regulated by IFNT in ovine LE cells (Johnson *et al.* 1999c). We selected *PTGS2*, *IGF2*, and hypoxia-inducible factor-1 α subunit (*HIF1A*), because we had previously demonstrated these genes to be down-regulated in endometrium from ewes that were either pregnant or, alternatively, treated with IFNT (Chen *et al.* 2006). *OTR* was selected because of its importance in controlling endometrial responses to oxytocin. In general, the results from real-time PCR experiments showed the same trends as the microarray experiments (Table 2), although the magnitude of fold-changes was sometimes quite different. One possible explanation is that the real-time PCR experiments were performed on un-pooled samples of RNA obtained from individual plates of cells, while the microarrays employed pooled RNA from replicate samples within an experiment.

Discussion

These experiments, employing microarrays based on bovine EST sequences, allowed us to collect data on over 15 000 genes, the majority of which were at least partially annotated by comparison with human data. Our study identified a large number of genes that have altered expression patterns when LE cells were treated with IFNT. We did not employ a cutoff based on a particular value of

Table 2 Regulated genes in prostaglandin metabolic pathway.

Unigene cluster ID	Gene name	Gene symbol	8 h (10 ng/ml)	8 h (10 μ g/ml)	24 h (10 ng/ml)	24 h (10 μ g/ml)
Bt.23094	Prostaglandin-F synthase 1 (PGF synthase 1)	<i>AKR1C1</i>	0.5	0.65	0.6	0.7
	Prostaglandin G/H synthase 2 precursor					
Bt.15758	Cyclooxygenase-2 (COX-2)	<i>PTGS2</i>	0.65	0.77	0.61	0.76
Bt.8953	Prostaglandin E synthase (PGES)	<i>PTGES</i>	1.41	1.4	1.37	1.93
Bt.9662	Prostaglandin E receptor 4 (PGE receptor, EP4)	<i>PTGER4</i>	1.84	1.75	1.85	1.81
Bt.22672	NAD ⁺ -dependent 15-hydroxyprosta glandin dehydrogenase (PGDH)	<i>PGDH</i>	2.09	1.47	1.86	1.45
Bt.2990	Prostaglandin transporter (PGT)	<i>SLC21A2</i>	4.33	1.25	2.51	1.6

fold-change, e.g. twofold, since there is no reason to assume that values of less than this arbitrary value are of minor significance. Also in absence of a detailed time-course, it is not possible to infer whether a low fold-change at 8 h, for example, does not reflect a more major change at an earlier or later time point. We chose to concentrate on the 'top' 585 genes (of which 567 were at least partially annotated) where the changes were highly significant (adjusted $P < 0.001$). Of these genes, 356 were up-regulated, while the remainder showed a reduction in expression. Although some of these genes were late responders, most were fully up- or down-regulated within 8 h. In addition, the lowest concentration of IFNT, which was deliberately chosen to be close to the K_D for the ovine receptor (Knickerbocker & Niswender 1989, Li & Roberts 1994), was generally about as effective as the highest concentration ($\sim 100 \times K_D$) in causing changes in gene expression, although there were some exceptions (Supplementary Table 2). This result is consistent with the assumption that Type I IFNs are effective at low receptor occupancy (Alexenko *et al.* 1997).

Other experiments employing microarrays to study the effects of IFNA and IFNB on human cells also indicated that large numbers of genes are responsive to Type I IFN (Der *et al.* 1998). Kim *et al.* (2003a) identified 101 IFNT-responsive genes in human U3A (STAT1-deficient 2fTGH) cells. Most recently, Gray *et al.* (2006) identified 180 genes that were regulated by IFNT independently of progesterone in day 14 ovine endometrium. Yan *et al.* (2004) in a proteomics analysis performed on human liver cells reported that as many as 1364 polypeptides were regulated by IFNA. In addition, these data illustrate the many downstream effects of Type I IFN on their targets. As in all previous studies, the majority of the IFN-responsive genes shown in Supplementary Table 2 were difficult to categorize, possibly reflecting the broad, pleiotropic effects of Type I IFN on their target cells. Many of these same IFNT-responsive genes, particularly ones implicated in an antiviral response, have been observed to be regulated during pregnancy in cattle and sheep, although it is clear that early maternal responses to the presence of a conceptus are likely to be more than simply a result of exposure of LE cells to IFNT. However, a number of interesting patterns of gene expression change that might relate to the effects of IFNT during pregnancy could be discerned.

Although our results agree closely with an *in vivo* IFNT study performed on a limited number of selected genes (~ 70 vs 15 000 in the present study; Chen *et al.* 2006), they are difficult to compare with another, recent, *in vivo* study that utilized microarray technology (Gray *et al.* 2006). The latter utilized ~ 5000 ovine ESTs and a relatively complex experimental design, in which IFNT effects were distinguished from those of progesterone by exposing endometrium of some ewes to an antiprogesterin. Gray *et al.* (2006) described 180 genes regulated by IFNT, but many of these were not

represented in our gene list (Supplementary Table 2) and vice versa. Some of the more interesting differences are remarked upon later in the discussion that follows.

IFNT regulates the prostaglandin synthesis pathway

The production and release of uterine PGF is crucial to controlling the regression of the CL during early pregnancy in cattle and sheep (McCracken *et al.* 1999). Conversely, PGE₂ is luteotrophic (Magness *et al.* 1981, Thibodeaux *et al.* 1992). As shown in Fig. 2, the production of these prostaglandins is controlled by several enzymes, including PTGS2, PGE synthase (PGES), PGF synthase (AKR1C1), and PG 15-dehydrogenase (PTGES), and their metabolism by several other gene products, including 15-hydroxy prostaglandin dehydrogenase (PGDH) and the prostaglandin transporter (SLC21A2). Of these, PTGS2, which is responsible for the conversion of arachidonic acid into PGH₂, is considered to catalyze the rate-limiting step in the biosynthesis of prostaglandins in endometrial tissue (Simmons *et al.* 2004). In addition, an increase in the PGE₂:PGF ratio in favor of PGE₂ occurs during pregnancy in sheep and cattle (Silvia *et al.* 1984, Payne & Lamming 1994). The regulation of PTGS2 expression by IFNT has been exceedingly controversial, with some reporting an increase and others a decrease in expression of either the enzyme or the mRNA (see Introduction). In the present study, it is clear that PTGS2 and PGF synthase (AKR1C1) gene expression are down-regulated in LE cells in response to IFNT, while transcripts for PGE₂ synthase (PGES) increase in concentration. We obtained essentially identical results when comparing endometrium from day 14 pregnant and nonpregnant ewes and after ewes at day 14 of their estrous cycles had been treated with IFNT (Chen *et al.* 2006). These data contrast with other data (Gray *et al.* 2006) in which PTGS2 gene expression was up-regulated in response to IFNT

Figure 2 Prostaglandin metabolism in mammalian cells. The diagram is based on one downloaded from <http://www.genome.jp/kegg/>. Up or down arrows indicate whether the gene is positively or negatively regulated by IFNT in the LE cells.

treatment. We have no explanation for these differences in outcome. Changes in expression of other genes with a role in prostaglandin metabolism and action were also evident. Transcripts for *PGDH*, the PGE receptor (*PTGER4*), and the prostaglandin transporter (*SLC21A2*), for example, increased. Overall, our data are generally consistent with the view that IFNT treatment favors PGE production over PGF (Fig. 2).

None of the genes in Fig. 2 thought to be involved in PGF metabolism appeared to be differentially responsive as IFNT concentrations were raised from 10 ng/ml to 10 µg/ml. Thus, we were unable to provide any supporting evidence for the view (Asselin & Fortier 2000) that prostaglandin metabolism, and specifically the production of PGF, might differ according to the concentrations of IFNT employed, with low concentrations favoring lowered PGF production and high concentrations favoring an increase. On the other hand, transcript profiling can be misleading since it provides only information relating to mRNA concentrations and nothing about how well these messages are translated or the concentration of relevant proteins in the cells.

IFNT up-regulate components of signal transduction pathways

Type I IFN signaling has been studied extensively. Since all Type I IFN share the same receptor complex, IFNT is likely to activate some or all of the signal pathways employed by IFNA and IFNB, including the JAK–STAT, and MAPK pathways (de Veer *et al.* 2001). Our data indicate that IFNT, as expected, up-regulated genes encoding key components of these two signaling pathways, STAT1 and RNF31 in the JAK–STAT pathway and MAP3K14 (Supplementary Tables 2 and 3) from the MAPK pathway. Components of the two pathways have also been identified as IFNA-responsive genes in human cell lines (Der *et al.* 1998, de Veer *et al.* 2001). At least 16 transcription factors,

presumably downstream of IFNT, increased in expression, and at least three were down-regulated (Supplementary Table 8 which can be viewed online at www.reproduction-online.org/supplemental/). With the exception of *STAT1*, *RNF31*, *IRF7*, and *IRF9*, however, the relationship of these transcription factors to the action of Type 1 IFNs has not been explored.

IFNT promotes increased expression of inducers of apoptosis and intracellular proteolysis

A DAVID annotation analysis indicated that 24 genes ($P < 0.00006$) in Supplementary Table 1 were associated with control of apoptosis, with the majority up-regulated. We have listed some of these genes in Table 3. They include *BAD* (Bcl-2 antagonist of cell death), (Yang *et al.* 1995), *BID* (BH3-interacting domain death agonist), which induces caspases and apoptosis and counters the protective effect of Bcl-2 (Wang *et al.* 1996), and *BNIP3L* (Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3-like), a well-characterized inducer of apoptosis (Opferman & Korsmeyer 2003). The genes for caspase 8 (*CASP8*) and several proteasome components were also up-regulated. The ability of Type I IFNs to cause apoptosis (Chawla-Sarkar *et al.* 2003) and induce genes involved in apoptosis (Der *et al.* 1998, Leaman *et al.* 2003) is well established in human target cells and cultured epithelial cells from bovine endometrium (Wang *et al.* 2003) although we have not examined whether cell death resulted in the LE cells used here. Similarly, a link between IFN action and induction of proteasomal activity has been described by others (Li & Hassel 2001, Liu *et al.* 2003). These changes in gene expression may reflect a potential ability of conceptus IFNT to cause remodeling of the uterine epithelium and underlying stromal tissue prior to firm attachment of the trophoblast to the uterine wall.

Table 3 Regulated genes related to apoptosis.

Unigene cluster ID	Gene name	Gene symbol	8 h (10 ng/ml)	8 h (10 µg/ml)	24 h (10 ng/ml)	24 h (10 µg/ml)
Bt.42335	Protein 2-like (BNIP2L)	<i>LOC483190</i>	1.91	1.87	1.91	1.83
Bt.888	BCL2/adenovirus E1B 19 kDa-interacting protein 3-like (BNIP3L)	<i>BNIP3L</i>	1.12	1.47	1.22	1.4
Bt.7953	BCL2-antagonist of cell death protein (BAD)	<i>BAD</i>	1.62	1.47	1.6	1.28
Bt.3222	BH3-interacting domain death agonist isoform 1 (BID isoform 1)	<i>BID</i>	1.47	1.52	1.34	1.35
Bt.26611	CASP8-associated protein 2	<i>CASP8AP2</i>	1.29	1.68	1.32	1.28
Bt.41872	Caspase 8 isoform B	<i>CASP8B</i>	1.08	1.22	1.93	1.18
Bt.1793	Cell death regulator aven; programmed cell death 12	<i>AVEN</i>	1.6	1.4	1.4	1.32
Bt.21648	Cell death-inducing DFFA-like effector b	<i>CIDEb</i>	1.34	1.64	1.25	1.1
Bt.3682	Death-associated protein 6 (Fas-binding protein)	<i>FADD</i>	1.56	1.91	1.12	1.49
Bt.725	Platelet-derived growth factor receptor-like (PDGFRL)	<i>PDGFRL</i>	1.56	1.09	1.4	1.03

Interestingly, at least seven genes ($P < 0.005$) (*FADD*, *TNFSF10*, *SCOTIN*, *TRIM38*, *BST2*, *CXXC5*, *EEF1D*) associated with control of the NF- κ B cascade were up-regulated by IFNT under all four experimental treatments (Supplementary Table 1). Somewhat similar changes on genes playing a role in protein degradation and the NF- κ B cascade have been noted when comparing endometrium of pregnant and nonpregnant cattle (Bauersachs *et al.* 2006).

IFNT regulates the expression of angiogenesis factors

The conceptus as it begins to form a close association with the uterine wall recruits a localized blood supply in the underlying maternal endometrium, which is accompanied by local edema in the regions where the trophoblast makes contact (Spencer *et al.* 2004). The formation of new blood vessels involves the coordinated actions of proteolytic enzymes, protease inhibitors, extracellular matrix components, and cell adhesion molecules. IFNA and IFNB are generally considered to be angiostatic, in part by down-regulating the production of FGF2 (Fidler 2000). On the other hand, IFNA has also been reported to increase expression of several angiogenic factors (de Veer *et al.* 2001). The response to IFNT in LE cells was also mixed (Table 4). Some genes were down-regulated, including *FGF2*, angiopoietin-1

(*ANGPT1*), VE-cadherin (*CDH5*), and *HIF1A* and *EPAS1* (hypoxia-inducible factor-2 α), which direct responses to low O₂ conditions, while other genes implicated in blood vessel formation were up-regulated, e.g. angiopoietin-2 and -4 (*ANG2* and *ANG4*), and vascular endothelial growth factors B and C (*VEGFB* and *VEGFC*). These ostensibly conflicting data are not easy to interpret and suggest complex effects of IFNT on angiogenesis in the endometrium. One possibility is that IFNT activates certain angiogenic pathways, such as the one that involves VEGF-VEGFR2 system, while at the same time it down-regulates pathways normally initiated by hypoxia. Such a complementary system for promoting angiogenesis has recently been reported in tumors (Casanovas *et al.* 2005). In the pregnant uterus, we propose that LE and possibly the upper, contiguous glandular epithelial cells, which are responsible for transducing signals received from the trophoblast to the underlying tissue, are stimulated to produce increased amounts of angiogenic factors such as VEGFs and therefore may direct the growth of new vessels from the existing capillary network bed.

In addition to its effects on genes associated with angiogenesis, IFNT up-regulated seven genes linked to blood coagulation (*CD93*, *F2RL2*, *PLAU*, *TAU1*, *ANXA5*, *PLSCR1*). The implications of such a link are unclear.

Table 4 Regulated genes related to angiogenesis.

Unigene cluster ID	Gene name	Gene symbol	8 h (10 ng/ml)	8 h (10 μ g/ml)	24 h (10 ng/ml)	24 h (10 μ g/ml)
Bt.13127	Angiopoietin-1 (ANG-1)	<i>ANGPT1</i>	0.97	1.02	0.82	0.89
Bt.137	Angiopoietin-2 (ANG-2)	<i>ANG2</i>	1.48	1.29	1.43	1.01
Bt.43747	Angiopoietin-4 precursor (ANG-4)	<i>ANG4</i>	1.48	1.46	1.48	1.33
Bt.4725	B-cell translocation gene 1, antiproliferative	<i>BTG1</i>	1.07	0.74	1.01	0.65
Bt.23246	Cadherin 5, type 2, VE-cadherin (vascular epithelium)	<i>CDH5</i>	0.57	1.37	0.56	1.4
Bt.6670	Cardiovascular basic helix-loop-helix factor 2	<i>CHF2</i>	1.92	1.54	2.24	1.43
Bt.5240	Connective tissue growth factor	<i>CTGF</i>	1.88	1.59	1.56	1.57
Bt.4353	Endothelial PAS domain protein 1	<i>EPAS1</i>	0.78	0.72	0.83	0.72
Bt.13998	FGF intracellular-binding protein isoform b	<i>FIBP</i>	0.82	0.8	0.7	0.81
Bt.12991	Fibroblast growth factor 10	<i>FGF-10</i>	0.62	0.72	0.79	0.56
Bt.4834	Fibroblast growth factor 2 (basic)	<i>FGF2</i>	0.81	1.09	0.64	0.4
Bt.21540	Fibroblast growth factor receptor 2	<i>FGFR2</i>	0.92	0.65	0.5	0.67
Bt.5038	Fibroblast growth factor, acidic (endothelial growth factor)	<i>RAB24</i>	1.62	1.3	1.33	1.23
Bt.279	Fibroblast growth factor-binding protein (FGF-BP)	<i>LOC281812</i>	1.03	1.1	0.6	0.77
Bt.4184	Hypoxia-inducible factor 1, α subunit (basic helix-loop-helix transcription factor)	<i>HIF1A</i>	0.8	0.77	0.79	0.83
Bt.18203	Junctional adhesion molecule 2 precursor (Vascular endothelial junction-associated molecule) (VE-JAM) (<i>Canis familiaris</i>)	<i>JAM2</i>	0.48	0.82	0.63	0.85
Bt.45242	Neuropilin 2 isoform 2 precursor	<i>NRP2</i>	0.77	0.91	0.78	0.89
Bt.9423	Platelet-activating factor receptor	<i>PAFR</i>	2.2	1.56	1.69	1.53
Bt.42912	Vascular cell adhesion molecule 1 isoform a precursor	<i>CD106</i>	1.54	1.83	0.69	0.96
Bt.5402	Vascular endothelial growth factor B	<i>VEGFB</i>	1.67	1.93	1.68	1.9
Bt.3913	Vascular endothelial growth factor C	<i>VEGFC</i>	1.44	1.16	1.21	1.21

Table 5 Regulated genes in the insulin-like growth factor system.

Unigene cluster ID	Gene name	Gene symbol	8 h (10 ng/ml)	8 h (10 µg/ml)	24 h (10 ng/ml)	24 h (10 µg/ml)
Bt.32572	Insulin-like growth factor-binding protein-like 1	<i>IGFBPL1</i>	1.43	0.93	1.22	0.89
Bt.5237	Insulin-like growth factor-binding protein 4 (IGFBP-4)	<i>IGFBP4</i>	2.31	1.79	1.12	1.35
Bt.422	Insulin-like growth factor-binding protein 3 (IGFBP-3)	<i>IGFBP3</i>	9.84	7.67	6.04	6.83
Bt.23176	Insulin-like growth factor 2 (IGF2)	<i>IGF2</i>	0.88	0.69	0.81	0.79

IFNT effects on the IGF system

IGF2 is believed to play a major role in controlling nutrient balance between the mother and the conceptus (Reik *et al.* 2003). Its gene is paternally imprinted, i.e. expressed in a mono-allelic manner from the paternal chromosome. Interestingly, *IGF2* was down-regulated in LE cells in response to IFNT (Table 5), a result consistent with that reported by us for *in vivo* effects (Chen *et al.* 2006). By contrast, *IGFBP2*, *IGFBP3*, and *IGFBP4* became up-regulated in the LE cells. One possibility is that IFNT from the conceptus functions to repress or deregulate the local maternal IGF2 activity, possibly to gain control of maternal nutrient supply and angiogenesis.

IFNT regulates expression of MMPs, TIMPs, and extracellular matrix components

Proteins of the matrix metalloproteinase (MMP) family are involved in the degradation of extracellular matrix during many normal as well as pathological processes (Fata *et al.* 2000) and are also able to promote cell growth indirectly through interactions with cytokines and growth factors (Curry & Osteen 2003). Tissue inhibitors of metalloproteinases (TIMPs) counteract and therefore modulate the activities of the MMPs and may also act as autocrine/paracrine factors in cellular

proliferation and differentiation. IFNT down-regulated genes for several MMPs, up-regulated *TIMP1*, *TIMP2*, *TIMP3*, and *TIMP4*, and increased transcript concentrations for a number of genes encoding extracellular matrix components (Table 6). Others have demonstrated that IFNT inhibits production of MMPs in primary cultures of bovine endometrial cells (Salamonsen *et al.* 1994, Hashizume *et al.* 2003). In addition, these observations suggest that IFNT promotes extracellular matrix accretion rather than breakdown in the ovine uterus during the preimplantation period. By contrast, matrix degradation appears to play a role in the decidualization process during implantation in mouse (Das *et al.* 1997). Decidualization does not occur in species, such as the sheep, with a minimally invasive trophoblast. Conceivably, the action of IFNT is to prevent decidualization rather than to induce it.

Other genes implicated in the luteoprotective action of IFNT

As noted in our earlier *in vivo* work (Chen *et al.* 2006), IFNT can rapidly down-regulate *OTR*, which is consistent with the work of others over the last decade (Mirando *et al.* 1993, Lamming *et al.* 1995, Spencer *et al.* 1995a, 1995b). On the other hand, we failed to obtain a

Table 6 Genes related to extracellular matrix.

Unigene cluster ID	Gene name	Gene symbol	8 h (10 ng/ml)	8 h (10 µg/ml)	24 h (10 ng/ml)	24 h (10 µg/ml)
Bt.23318	α 1 type III collagen	<i>COL3A1</i>	1.91	1.4	1.25	1.23
Bt.41478	α 1 type VII collagen precursor	<i>COL7A1</i>	1.71	1.54	1.65	1.93
Bt.5273	Integrin, α V	<i>ITGAV</i>	1.82	1.65	1.97	1.72
Bt.23172	Integrin, β 5	<i>RPS23</i>	1.8	1.98	1.8	1.91
Bt.17999	Intercellular adhesion molecule-2	<i>ICAM2</i>	1.47	1.61	1.33	1.34
Bt.39	Matrix metalloproteinase 13 (collagenase 3)	<i>MMP13</i>	0.29	0.72	0.64	0.55
Bt.26938	Matrix metalloproteinase 17	<i>MMP17</i>	0.5	0.44	0.83	0.43
Bt.7179	Matrix metalloproteinase 23B	<i>MMP23B</i>	0.71	0.81	0.41	0.88
Bt.43352	Matrix metalloproteinase 28 preproprotein isoform 1	<i>MMP28</i>	0.79	0.63	0.66	0.6
Bt.4714	Matrix metalloproteinase 9	<i>MMP9</i>	0.58	0.83	0.86	0.91
Bt.632	Tissue inhibitor of metalloproteinase 1 (TIMP-1)	<i>TIMP1</i>	1.21	1.49	1.34	1.76
Bt.5136	Tissue inhibitor of metalloproteinase 3 (TIMP-3)	<i>TIMP3</i>	1.56	1.19	1.77	1.7
Bt.42237	Tissue inhibitor of metalloproteinase 4 (TIMP-4)	<i>TIMP4</i>	1.76	1.96	1.76	2.13
Bt.435	Tissue inhibitor of metalloproteinase 2 (TIMP-2)	<i>TIMP2</i>	1.32	2.05	1.61	1.21

sufficiently strong hybridization signal for the mRNA of either ESR1 or ESR2 to determine whether these genes were significantly regulated in our system by IFNT. One well-accepted model for the luteoprotective action of IFNT is that the cytokine causes reduction of *OTR* number on the uterine epithelium indirectly as an outcome of preventing the normal up-regulation of ESR1 (Spencer *et al.* 1995a, 1996), whereas in the nonpregnant cycle the rise in *OTR* allows the hormone, oxytocin to mediate PGF release (Robinson *et al.* 1999). Clearly, our results are consistent with one part of this model, namely the down-regulation of *OTR* in response to IFNT, but could not address the second.

Conclusions

The present study provides a comprehensive, descriptive profiling of gene expression in ovine LE cells in responsive to IFNT. Out of more than 15 000 genes examined, we identified over 1200 genes that were regulated ($P < 0.01$). Of these, we concentrated on the genes whose transcripts changed most significantly ($P < 0.001$). Many of the genes identified in the present study as IFN responsive have been noted earlier by others in human cells in response to IFNA and IFNB (Der *et al.* 1998, Kim *et al.* 2003a, Yan *et al.* 2004). However, our list is more comprehensive and has also expanded our understanding of the likely biological activities of IFNT on a physiologically relevant cell type. Our results suggest that IFNT, like other Type I IFN, up-regulates many genes that undoubtedly play a role in the antiviral activities of this class of cytokine. However, it is also clear that IFNT action on the uterus likely involves effects on prostaglandin metabolism, apoptosis, extracellular matrix accretion, angiogenesis and blood coagulation, inflammation, and growth factor production and activity. The recognition that such processes are likely to be modulated as the conceptus begins to attach to the uterine wall provides new insights into how IFNT might act during early pregnancy and the likely complexity of its role in maternal recognition of pregnancy in ruminants.

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