Interferon-tau promotes luteal endothelial cell survival and inhibits specific luteolytic genes in bovine corpus luteum

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Abstract

Interferon-tau (IFNT), a maternal recognition of pregnancy (MRP) signals in domestic ruminants, suppresses the release of luteolytic pulses of uterine prostaglandin F_{2a} (PGF_{2a}), thus extending the corpus luteum (CL) life span. We hypothesized that IFNT also exerts anti-luteolytic actions in bovine CL. To examine the direct effects of IFNT on bovine CL, luteal slices and enriched luteal endothelial cells (LECs) were utilized. We found that recombinant ovine IFNT (roIFNT) markedly elevates interferon-associated genes (*STAT1*, *STAT2* and *IRF9*) and interferon-stimulated genes (ISGs: *MX2*, *ISG15* and *OAS1Y*) in both models. Furthermore, IFNT time-dependently induced *STAT1* phosphorylation in LECs without affecting total *STAT1*. roIFNT-stimulated viable LECs numbers and the knockdown of protein inhibitor of activated *STAT1* (*PIAS1*) abolished this effect, suggesting that *PIAS1* may mediate the proliferative effect of IFNT. IFNT significantly downregulated luteolytic genes such as *TGFB1*, thrombospondin-1 (*THBS1*), endothelin-1 (*EDN1*) and serpin family E member-1 (*SERPINE1*) in LECs. However, less robust effects were observed in luteal slices. Moreover, PGF_{2a} alone induced *THBS1*, *SERPINE1* and *EDN1* mRNA in CL slices whereas in the presence of IFNT, *THBS1* and *SERPINE1* stimulation was abolished. Collectively, these results indicate that IFNT acts via *STAT1*-*IRF9*-dependent and independent pathways and affects diverse luteal functions. Most interestingly, this study suggests the existence of an anti-luteolytic effect of IFNT in bovine CL, namely, inhibiting key PGF_{2a}-induced luteolytic genes. The proliferative effect of IFNT may constitute an additional mechanism that promotes luteal cell survival, thus, extending the luteal life span during early pregnancy in cows.

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Introduction

Maternal recognition of pregnancy (MRP) is a prerequisite for the successful establishment of pregnancy and maintenance of the corpus luteum (CL) function. In ruminants, the mononuclear trophectoderm of the conceptus secretes interferon-tau (IFNT) during the peri-implantation period, which serves as the MRP signal (Spencer *et al.* 1996, Roberts *et al.* 1999). Bovine IFNT transcripts were detected in uterine flushes in samples from days 12–28 (Takahashi H. *et al.* 2007), also prevents secretion of luteolytic pulses of prostaglandin f₂ alpha (PGF_{2a}) by uterine epithelia and blocks effects of exogenous estradiol and oxytocin to stimulate uterine release of PGF_{2a}. Expression of estrogen receptor 1 (*ESR1*) and oxytocin receptor (*OXTR*) mRNAs is either silenced or the receptors are not responsive to estradiol and oxytocin in endometria of both pregnant cows and cyclic cows treated with intrauterine injections of IFNT (Thatcher *et al.* 1989, Meyer *et al.* 1995). In this way,

it prevents the release of luteolytic pulses of PGF_{2a} from the endometrium and allows the prolongation of the CL life span and progesterone (P₄) production. Recent studies performed by Arosh and coworkers also emphasized the role of the prostaglandin transporter (PGT), in the transport of PGF_{2a} from the endometrium to the ovary (Banu *et al.* 2010, Lee *et al.* 2010).

In the absence of a functional/live conceptus in the uterus, the CL undergoes luteolysis (Niswender *et al.* 2000, Schams & Berisha 2004). Recent evidence highlighted certain genes such as endothelin1 (*END1*), transforming growth factor beta1 (*TGFB1*), thrombospondin1 (*THBS1*) and serpin family E member1 (*SERPINE1*) are specifically induced during luteolysis in the PGF_{2a}-responsive CL (Miyamoto *et al.* 1997, Kliem *et al.* 2007, Hou *et al.* 2008, Mondal *et al.* 2011, Romero *et al.* 2013, Farberov & Meidan 2016). Their gene products are expected to promote vascular instability, as well as apoptosis and matrix remodeling in the regressing CL. These genes are highly expressed

in luteal endothelial cells (LECs) (Levy *et al.* 2001, Zalman *et al.* 2012). Moreover, prostaglandin F receptor (PTGFR) that are present in LECs (Mamluk *et al.* 1998, Zannoni *et al.* 2007, Lee *et al.* 2010, Shirasuna *et al.* 2012), can mediate direct PGF2a actions on some of these genes (Girsh *et al.* 1996b, Shirasuna *et al.* 2008, Zalman *et al.* 2012).

IFNT may also act as an endocrine hormone, inducing the expression of several interferon-stimulated genes (ISGs; *ISG15*, *MX1* and 2, *OAS1Y* and others) in extra-uterine tissues such as the CL, peripheral blood cells and the mammary gland (Oliveira *et al.* 2008, Yang *et al.* 2010, Nitta *et al.* 2011, Matsuyama *et al.* 2012). Indeed, the uterine infusion or endocrine delivery of recombinant ovine (roIFNT) via the uterine or jugular vein extended the life span of CL in ewes (Oliveira *et al.* 2008, Bott *et al.* 2010, Antoniazzi *et al.* 2013). However, the underlying mechanism that mediates CL rescue has not been established yet. To gain a better understanding about IFNT-induced luteal events that may prevent luteolysis of bovine CL during MRP, we investigated the direct effects of IFNT on whole luteal tissue, using CL slices and on LECs, a specific cell type within the CL.

Materials and methods

Unless otherwise stated, all biochemicals were from Sigma-Aldrich and the cell culture materials were from Biological Industries (Kibbutz Beit Haemek, Israel).

Isolation and culture of LECs

The procedures for isolation and enrichment of LECs were detailed previously (Levy *et al.* 2001, Farberov & Meidan 2014, 2016). Briefly, CL was dispersed using sequential incubations with collagenase. Then cells were incubated with BS-1-coated magnetic beads. The adherent cells were washed and concentrated using a magnet until the supernatant was free of cells. BS-1-positive cells (enriched LECs fraction) were plated in DMEM/F-12 containing 10% fetal calf serum (FCS) on collagen type I-coated plates (Surecoat; Advanced BioMatrix). Colonies of LECs were trypsinized with 1% crystalline trypsin (Biological Industries), collected and reseeded; this process was repeated until homogenous cell cultures were visualized. Cell identity was verified using endothelial cell markers (*CD31*, *EDN1*) and the lack of contamination by smooth muscle cells (*ACTA2* expression) and fibroblasts (*COL1A1* expression) was evaluated. Cells from passages 7–10, derived from at least three different CLs, were used in the present study. The LECs were seeded in 12-well plates (70,000 cells/well) and cultured overnight in DMEM/F-12 containing 10% FCS. The next day, the cells were transferred to starvation medium (0.5% BSA in 0.1% FCS) for 24 h. Then, the LECs were incubated for the time indicated in the legends with or without roIFNT (0.01–10 ng/mL; a generous gift from Prof. Fuller W Bazer, Texas A&M University). At the end of the incubation period, the total RNA was extracted from the cells.

Experimental animals and synchronization

Holstein Friesian primiparous cows from commercial dairy farms in central Poland were utilized in this experiment. The experimental protocols and procedures were approved by the Local Ethics Committee. Cows received two intramuscular injections of PGF2a (5 mL dinolytic 5 mg/mL, Zoetis, Belgium) 11 days apart. Estrus was detected 24–72 h after the second PGF2a administration and reproductive tracts including ovaries were monitored by transrectal ultrasonography using a 5–8 MHz linear-array probe (MyLabOne VET, Italy) throughout the experiment. At mid-luteal phase (days 12–14 of the estrous cycle), ovaries with CL were surgically removed under anesthesia. Four CLs derived from individual cows were harvested and used for the experiment described below.

Culture of CL slices

CL (above) was cut into slices (300 µm thick, 8 mm diameter, 10–15 mg wet weight) using a Krumdick Tissue Slicer (K&F Research, Birmingham, AL, USA) as previously described (Przygodzka *et al.* 2014). Then, the slices (2 slices/well) were incubated for 12 h in M-199 supplemented with 0.1% bovine serum albumin (BSA; ICN Bio Medicals, Inc., Costa Mesa, CA, USA), antibiotics (penicillin-streptomycin; Sigma-Aldrich) and an anti-fungal drug (amphotericin B; Sigma-Aldrich) in the presence or absence of roIFNT (1 ng/mL) or PGF2a (Cayman Chemical; 30 nM) alone or in combination of roIFNT and PGF2a. Each treatment (for each cow) was examined in duplicates and averaged. At the end of the incubation, slices were collected, rapidly frozen in liquid nitrogen and stored at –80°C until total RNA isolation.

Western blot analyses

Proteins were extracted by adding sample buffer (×2), separated by 7.5%–12.0% SDS-PAGE, and subsequently transferred to nitrocellulose membranes, as previously reported (Zalman *et al.* 2012). Membranes were blocked for 1 h in TBST (20 mmol/L Tris, 150 mmol/L NaCl and 0.1% Tween 20; pH 7.6) containing 3% BSA or 5% low-fat milk and then incubated overnight at 4°C with the following antibodies: goat polyclonal phosphorylated STAT1 (TYR701; 1:200; sc-7988R; Santa Cruz Biotechnology); goat polyclonal STAT1 (1:200; sc-346-G; Santa Cruz Biotechnology); mouse anti-THBS1 (1:500; ab1823; Abcam) and rabbit anti-p44/42 total MAPK (1:50,000; Sigma-Aldrich). The membranes were then incubated with peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) or goat anti-mouse immunoglobulin G (heavy + light) (ab6789; Abcam) for 1 h at room temperature. A chemiluminescent signal was generated with SuperSignal (Thermo Fisher Scientific), and the membranes were exposed. The signal was analyzed using the Gel-Pro Analyzer version 3.0 (Media Cybernetics, Inc., MD, USA) and normalized to total p44/42 MAPK in the same sample to correct for the amount of protein loaded.

Transfection of LECs

Cells were seeded for overnight in 24-well (16,000 cells/well) plates containing 500 µL of DMEM/F-12 medium comprising 10% FCS. The next day, cells were transfected with siRNA (50 nmol/L; GeneCust, Luxembourg; as previously described (Farberov & Meidan 2016)). siRNA targeting protein inhibitor of activated STAT1 (*PIAS1*) and scrambled siRNA (negative control; 50 nmol/L) using 3.6 µL of lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol at 1% FCS. The siRNA of *PIAS1* (siPIAS1) was sense (S) GAGACAAUCAGCACUAUAA dCdA, antisense (AS) UUAUAGUGCUGAUUGUCUCdCdT, corresponding to bases 1664–1684 (NM_001075396.2). Scrambled siRNA sequence – negative control was (S) UUCUCCGAACGUGUCACGUDtD and (AS) ACGUGACACGUUCGAGAAAdtD. After 24 h post transfection, LECs were transferred to starvation medium and the next day they were treated with or without IFNT (1 ng/mL) for 24 h.

Determination of the viable cell numbers

The viable cell numbers were estimated as previously described (Farberov & Meidan 2014), using the XTT kit (Biological Industries), which measures the reduction of a tetrazolium component by the mitochondria of viable cells. On the day of measurement, XTT was added to the culture media according to the manufacturer's instructions. Then, plates were incubated at 37°C for 2–3 h. Afterwards, the absorbance was read at 450 nm (reference absorbance, 630 nm).

RNA extraction and qRT-PCR

CL slices

Collected luteal tissue samples were homogenized in Tri Reagent (Molecular Research Center) with a FastPrep-24 instrument (MP Biomedical, Santa Ana, CA, USA). Total RNA was extracted by using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The integrity and quality of RNA were verified using the Agilent 2100 Bioanalyzer (Agilent Technologies) and NanoDrop (ThermoFisher). Genomic DNA contamination was removed according to the manufacturer's instructions (DNase I Kit, ThermoFisher). Gene expression was evaluated by one-step real-time PCR using the Applied

Biosystems 7900 HT Sequence Detection System (Applied Biosystems) with TaqMan assays (ThermoFisher; Table 1). As detailed previously, each qPCR was performed in duplicates on 384-well plates and was preceded by a reverse transcription reaction (15 min at 40°C) (Przygodzka *et al.* 2016). To select the most stable housekeeping gene among glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), beta-actin (*ACTB*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT*), the NormFinder algorithm was applied, based on the report and *GAPDH* was chosen as a housekeeping gene (Andersen *et al.* 2004). The expression of selective genes was normalized relative to the abundance of *GAPDH* mRNA. The threshold cycle number (*Ct*) was used to quantify the relative abundance of the gene; arbitrary units were calculated as $2^{-\Delta Ct} = 2^{-(Ct \text{ target gene} - Ct \text{ housekeeping gene})}$ (Livak & Schmittgen 2001).

LECs

Total RNA was isolated from cells using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. As previously described, total RNA was reverse transcribed and quantitative RT-PCR (qPCR) was performed using the LightCycler 96 system (Roche Diagnostics) with Platinum SYBR Green (SuperMix, Invitrogen) (Farberov & Meidan 2016). The sequence of primers used for quantitative RT-PCR (qPCR) is listed in Table 2. All primers were designed to have single-product melting curves, as well as consistent amplification efficiencies between 1.8 and 2.2 (Schmittgen & Livak 2008). All amplicons were verified by sequencing. To select the most stable housekeeping gene among glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein S26 (*RPS26*) in LECs, the NormFinder

Table 2 Genes, Genbank accessions and the sequences of primers used for real-time PCR performed in LECs.

Gene name	Sequence (5'–3')	Accession no.
<i>GAPDH</i>	f-gtcttcactaccatggagaagg r-tcatggatgacctggccag	NM_001034034
<i>RPS26</i>	f-ccaactgtgccgatgtg r-ttcccgagagcgattcctga	NM_001015561
<i>IFNAR1</i>	f-gtagtgggtggccgaaag r-tgctgctgtccacttcagga	NM_174552.2
<i>STAT1</i>	f-cagccagctcccaagtg r-gccaactcagcacctctg	NM_001077900.1
<i>IRF9</i>	f-agcagcaacagccctgag r-gccccgtttagatgaagg	NM_001024506.1
<i>PIAS1</i>	f-ggcatactgagtctccacatcaag r-tgctgcagcaaggaggaggtgt	NM_001075396.2
<i>MX2</i>	f-tggcaggtggaagagagc r-gagtcgatgaggtcaatgcag	NM_173941.2
<i>ISG15</i>	f-ggtatccgagctgaagcagtt r-acctccctgctgtaaggt	NM_174366.1
<i>OAS1Y</i>	f-tttggtctggctggattacc r-tagccctggaacatcaggtc	NM_001040606.1
<i>TGFB1</i>	f-caggacctgtgctactgtg r-gagccctggacaccaactac	NM_001166068
<i>THBS1</i>	f-atcatggctgactcaggac r-taagcccatggttcagaa	NM_174196
<i>SERPINE1</i>	f-cagaaggtgaagattgaggtg r-ggcccataacaggacagttcc	NM_174137
<i>EDN1</i>	f-ctgccacctggacatcatttg r-tctcacggtctgttgcctttg	NM_181010.2

Table 1 Genes, Genbank accessions and TaqMan Assays' ID used in real-time PCR for luteal tissue slices.

Gene name	TaqMan Assay ID	Accession no.
<i>GAPDH</i>	Bt03210919_g1	NM_001034034
<i>STAT1</i>	Bt03252661_m1	NM_001077900.1
<i>STAT2</i>	Bt04284638_m1	NM_001205689.1
<i>IRF9</i>	Bt03220019_m1	NM_001024506.1
<i>MX2</i>	Bt03211948_m1	NM_173941.2
<i>ISG15</i>	Bt03223508_m1	NM_174366.1
<i>OAS1Y</i>	Bt03211516_m1	NM_001040606.1
<i>TGFB1</i>	Bt04259488_m1	NM_001166068
<i>THBS1</i>	Bt03213209_m1	NM_174196
<i>SERPINE1</i>	Bt03212914_m1	NM_174137
<i>EDN1</i>	Bt03217448_m1	NM_181010.2

algorithm was applied, based on the report, *RPS26* was chosen as a housekeeping gene (Andersen *et al.* 2004). The expression of selective genes was normalized relative to the abundance of *RPS26* mRNA.

Statistical analyses

All statistical analyses were conducted using GraphPad Prism version 4.03 Software (GraphPad Software). Data are presented as the means of $\pm$ S.E.M.; LECs culture experiments were repeated at least three times; CL slices were collected from four different mid-luteal phase (days 12–14th of the estrous cycle) cows and incubated separately. The expressions of selective mRNA levels were normalized to the relative abundance of *GAPDH* (for luteal slices) and *RPS26* (for LECs). Data were analyzed by either Student's *t*-test or one-way ANOVA, followed by Bonferroni's *post hoc* multiple comparison test, when indicated. Differences were considered as significant at $P < 0.05$. Asterisks or different letters represent statistical significant differences ($P < 0.05$). Additional information is provided in legends to figures.

Results

IFNT induces ISGs, IFN-associated genes and *STAT1* phosphorylation in LECs

We first examined whether IFNT induces the expression of classical ISGs such as *ISG15*, *MX2* and *OAS1Y* mRNA in LECs. As shown in Fig. 1 (A, B and C), rolIFNT (0.01–10 ng/mL) elevated *MX2* ($P < 0.001$), *ISG15* ($P < 0.001$) and *OAS1Y* ($P < 0.001$) levels in LECs in a dose-dependent manner. Among these ISGs, *MX2* exhibited the highest induction by rolIFNT, followed by *ISG15* and *OAS1Y* (Fig. 1A, B and C).

We then examined *STAT1* phosphorylation by IFNT. As shown in Fig. 2, 1 ng/mL of rolIFNT significantly induced tyrosine 701 phosphorylation already within 15 min ($P < 0.05$). *STAT1* phosphorylation was evident between 15 and 60 min and its highest induction in response to rolIFNT was observed after 60 min of LECs treatment with IFNT ($P < 0.001$), the response markedly dropped after 120 min (Fig. 2). The content of total *STAT1* remained unchanged throughout treatment with IFNT (Fig. 2). The effect of 1 ng/mL rolIFNT on mRNA expression of the

IFN-associated genes *IFNAR1*, *STAT1*, *IRF9* and *PIAS1* in LECs is shown in Table 3. IFNT markedly elevated as compared to control treatment: *STAT1* (5.3-fold; $P < 0.01$), *IRF9* (8.6-fold; $P < 0.001$) and *PIAS1* (1.8-fold; $P < 0.01$) mRNA levels. However, *IFNAR1* levels in LECs remained unaffected after the treatment with IFNT.

IFNT regulates LECs function

To examine whether IFNT is functionally important for the survival of LECs, we assayed the cells viability. rolIFNT doubled viable LECs numbers as compared with the control group (cells cultured in basal medium; Fig. 3A). Since *PIAS1* is known to be involved in cell proliferation (Munarriz *et al.* 2004), we examined the effect of *PIAS1* silencing on IFNT-induced cell proliferation. IFNT maintained its stimulatory effect (1.5 folds) on viable cell numbers with scrambled siRNA transfection (Fig. 3C). However, *PIAS1* siRNA significantly reduced *PIAS1* mRNA (basal as well as rolIFNT-stimulated levels) by 60–70% (Fig. 3B). Concurrently with the decrease in *PIAS1* mRNA, cell viability was significantly reduced by *PIAS1* silencing (compared with cells transfected with a scrambled siRNA-negative control; Fig. 3C). Furthermore, the positive effect of rolIFNT on LECs viability was abolished in *PIAS1*-silenced cells (Fig. 3C). IFNT had an additional important effect on LECs, i.e., incubation of these cells for 24 h with 1 ng/mL of rolIFNT reduced the mRNA levels of luteolytic genes: *TGFB1* (~25%; $P < 0.01$), *THBS1* (~36%; $P < 0.001$), *EDN1* (~40%; $P < 0.001$) and *SERPINE1* (~50%; $P < 0.001$) (Fig. 4A). *THBS1* protein levels in LECs were also strongly reduced (by 60%) in cells incubated with rolIFNT (Fig. 4B).

Effects of IFNT, *PGF2a* and their combination in CL slices

CL slices were treated with 1 ng/mL of rolIFNT for 12 h as detailed in the materials and methods section. As shown in Fig. 5A, B, C, D, E and F, rolIFNT-elevated *STAT1* (2.2-fold; $P < 0.01$), *STAT2* (6.0-fold; $P < 0.01$), *IRF9* (6.1-fold; $P < 0.001$), *MX2* (230-fold; $P < 0.01$), *ISG15* (30-fold; $P < 0.01$) and *OAS1Y* (260-fold; $P < 0.001$) mRNA levels

Figure 1 Dose-dependent effect of IFNT on *MX2* (A), *ISG15* (B) and *OAS1Y* (C) mRNA expression in LECs. Serum-starved cells were incubated for 24 h with either basal media (control) or varying concentrations of rolIFNT (0.01–10 ng/mL). Cells were then harvested and mRNA expression was determined using quantitative PCR (qPCR). The results are presented as the means $\pm$ S.E.M. of 4 independent experiments. Asterisks indicate significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) statistical differences from their respective controls.

Figure 2 Time-dependent effect of IFNT on STAT1 tyrosine phosphorylation. Whole cell extracts from serum-starved LECs were incubated with IFNT (1 ng/mL) for different time points as indicated. Tyrosine phosphorylated STAT1 (p-STAT1) and total STAT1 proteins were analyzed using specific antibodies in western blotting. Phospho-STAT1 and total STAT1 were normalized relative to the abundance of total MAPK (p44/42). The results represent the means $\pm$ S.E.M. of 4 independent experiments. Asterisks indicate significant (* P < 0.05, ** P < 0.01, *** P < 0.001) statistical differences from time 0.

as compared to controls. To determine the anti-luteolytic effects of rolFNT in whole bovine CL tissue, slices were incubated with IFNT, PGF2a and their combination (Fig. 6). Similar to the responses observed in LECs, there was a significant downregulation of *TGFB1* (17%; P < 0.05), *THBS1* (25%; P < 0.05) and *SERPINE1* (30%; P < 0.001) mRNAs in rolFNT-treated CL slices (Fig. 6). PGF2a significantly stimulated *THBS1* (137%; P < 0.01), *SERPINE1* (138%; P < 0.01) and *EDN1* (134%; P < 0.01). Importantly, the stimulation of *THBS1* and *SERPINE1* induced by PGF2a alone was abolished in the presence of IFNT. *TGFB1* expression was significantly reduced by the combined treatment of IFNT and PGF2a (32%; P < 0.001), although there was no induction by PGF2a alone. Contrary to LECs, the expression of *EDN1* was stimulated by rolFNT alone or in presence of PGF2a (Fig. 6). Similar, but less pronounced, effects of IFNT were also observed in LECs cultured for 12 h (data not shown).

Table 3 The expression of *IFNAR1*, *STAT1*, *IRF9* and *PIAS1* in LECs cultured for 24 h with rolFNT (1 ng/mL).

Genes	IFNT	<i>P</i> value
<i>IFNAR1</i>	0.84 $\pm$ 0.08	0.203
<i>STAT1</i>	5.32 $\pm$ 0.87	0.01
<i>IRF9</i>	8.66 $\pm$ 0.36	0.00001
<i>PIAS1</i>	1.88 $\pm$ 0.77	0.007

Results are the means $\pm$ S.E.M. (n = 4); the expression of each gene is relative to its respective controls designated as 1.

Discussion

The present study demonstrates that IFNT affects diverse luteal functions in cows: it stimulated the expression of ISGs, IFN-associated genes, STAT1 phosphorylation, LECs survival and suppressed key luteolytic genes. These actions together may contribute to the maintenance of the bovine CL during early pregnancy.

It was previously shown that several ISGs are stimulated in the CL of ovine and bovine species during early pregnancy (Oliveira *et al.* 2008, Magata *et al.* 2012), or after IFNT infusion in ewes (Bott *et al.* 2010). A direct *in vitro* effect of IFNT on various luteal cell types was also demonstrated in several studies (Oliveira *et al.* 2008, Yang *et al.* 2010, Nitta *et al.* 2011, Antoniazzi *et al.* 2013, Romero *et al.* 2015, Shirasuna *et al.* 2015). In the present study, we showed that rolFNT dose-dependently stimulated ISGs (*MX2*, *ISG15* and *OAS1Y*) expression in cultured luteal slices as well as in LECs of bovine CL. Taken together, these results support the contention that IFNT acts directly on bovine CL to stimulate ISGs expression.

IFNT is a multifunctional cytokine that exhibits biological activity similarly to other type-1 IFNs. It acts via binding to the cell surface receptors IFNAR1 and IFNAR2 (Rosenfeld *et al.* 2002). It is well established that IFNT activates the classical JAK-STAT-IRF signaling pathway in the ruminant endometrium (Staggs *et al.* 1998, Vitorino Carvalho *et al.* 2016). Based on these studies, a model has been proposed in which the tyrosine phosphorylation of STAT1 elicited by IFNT leads to the formation of STAT1-STAT2-IRF9 heterotrimers, also known as IFN-stimulated gene factor 3 complex (ISGF3), which is translocated to the nucleus and binds to the interferon-stimulated response element (ISRE) present within the promoter of ISGs and regulates their expression (Vitorino Carvalho *et al.* 2016). However, IFNT signaling has not yet been validated in tissues other than the endometrium. Findings reported in the present study clearly showed that rolFNT can induce temporal STAT1 phosphorylation and that rolFNT stimulates the expression of interferon-associated genes such as *STAT1*, *STAT2* and *IRF9* in LECs and luteal tissue slices. Thus, it seems likely that IFNT-dependent induction of ISGs in the bovine CL occurs via the STAT1-IRF9 pathway.

PIAS proteins are a family of four multifunctional proteins (PIAS1-4), which are known to be important regulators of cytokine actions (Sharrocks 2006). PIAS1 regulates the JAK/STAT pathway by interfering with the DNA binding activity of activated STAT1 (Liu *et al.* 2004). PIAS1 also acts in a STAT-independent manner to promote mammalian cell proliferation by inhibiting p53 and p73 transcriptional activity (Munarriz *et al.* 2004). Using LECs, we showed that rolFNT induced PIAS1 expression and increased viable cell numbers. Moreover, PIAS1 silencing abolished rolFNT-elevated cell proliferation without affecting IFNT-induced ISGs

Figure 3 Role of PIAS1 in IFNT-induced LEC viability. (A) Non-transfected LEC. (B) *PIAS1* mRNA expression and (C) LECs numbers in cells transfected with *PIAS1* siRNA (siPIAS1) or scrambled siRNA. (A) Overnight serum-starved LECs were treated with rolIFNT (1 ng/mL) for 24 h followed by cell viability determination using XTT. B and C-LECs were transfected with either scrambled siRNA or specific siPIAS1. 24 h post transfection, cells were starved overnight, followed by incubation with or without IFNT (1 ng/mL) for additional 24 h. Control—cell cultured in basal media (without IFNT). The results are presented as the means $\pm$ S.E.M. from 4 independent experiments. Asterisks indicate significant statistical differences from their respective controls at *** P <0.001 (Student's *t*-test). The different letters indicate significant statistical differences at P <0.05 analyzed using ANOVA followed by Bonferroni's *post hoc* multiple comparison test.

(data not shown) thus, suggesting STAT-independent action of IFNT in the bovine CL. These findings underscore the importance of PIAS1 in mediating the stimulatory effect of IFNT on LECs survival. Antoniazzi and coworkers (Antoniazzi *et al.* 2013) demonstrated that endocrine delivery of IFNT upregulated cell survival genes such as BCL2-like 1 (BCL2L1 or Bcl-xL), serine/threonine kinase (AKT), X-linked inhibitor of apoptosis (XIAP) in ewes CL. These genes were inhibited during luteolysis by PGF2 α (Antoniazzi *et al.* 2013). These findings together with the current data support the role of IFNT as survival factor for luteal cells.

It was shown that the luteolytic cascade, initiated by PGF2 α , involves the luteal stage and the time-dependent induction of several key genes within the CL: *EDN1*, *THBS1*, *TGFB1* and *SERPINE1* are among the most extensively studied (Girsh *et al.* 1996a, Shirasuna *et al.* 2004, Choudhary *et al.* 2005, Maroni & Davis 2012, Romero *et al.* 2013, Meidan *et al.* 2017). These factors act in concert to inhibit P4 production, angiogenic support, cell survival and extracellular matrix (ECM) remodeling to achieve CL regression as described below. EDN1 and P4 curves showed a close, inverse relationship in response to luteolytic dose of PGF2 α (Ohtani *et al.* 1998)

suggested that luteal EDN1 may be the local mediator of PGF2 α in reducing P4 output. Indeed, EDN1 was shown to directly inhibit basal and LH-induced P4 in many species, acting via selective EDNRA (Miyamoto *et al.* 1997, Apa *et al.* 1998, Meidan *et al.* 2005). During early pregnancy luteal *EDN1* was inhibited (Costine *et al.* 2007, Przygodzka *et al.* 2016). PGF2 α administration at mid-cycle elevated the expression of several anti-angiogenic, pro-apoptotic genes, including *THBS1* and *TGFB1* (Hou *et al.* 2008, Mondal *et al.* 2011, Zalman *et al.* 2012, Romero *et al.* 2013, Berisha *et al.* 2016, Farberov & Meidan 2016). *THBS1* was shown to be a physiological activator of latent TGFB1 in LECs (Farberov & Meidan 2016). Both *THBS1* and *TGFB1* triggered apoptosis, but *THBS1* was significantly more effective in causing cell death and activating caspase-3. *THBS1* and *TGFB1* also differed in their activation of p38 mitogen-activated protein kinase (Farberov & Meidan 2016). Similarly to *THBS1* and *TGFB1*, *SERPINE1* showed marked luteal stage specific expression with extensive responses to PGF2 α at mid-cycle (Smith *et al.* 1997, Kliem *et al.* 2007, Berisha *et al.* 2016, Farberov & Meidan 2016). *SERPINE1* is known to be a downstream target of TGFB1; it encodes endothelial PAI-1, a member

Figure 4 The effect of rolIFNT on (A) *TGFβ1*, *THBS1*, *EDN1* and *SERPINE1* mRNA and (B) *THBS1* protein in LECs. (A) Serum-starved cells were incubated with either basal medium alone (control) or with rolIFNT (1 ng/mL) for 24 h. (B) Cells were incubated without (basal) or with rolIFNT (1 ng/mL) for 48 h, *THBS1* protein was determined in cell extracts by Western blotting and normalized relative to the abundance of total MAPK (p44/42). Results are presented as the means $\pm$ S.E.M. from 4 and 3 independent experiments for A and B respectively. Asterisks indicate significant (* P <0.05, ** P <0.01, *** P <0.001) differences from their respective controls.

Figure 5 IFNT-induced *STAT1* (A), *STAT2* (B), *IRF9* (C), *MX2* (D), *ISG15* (E) and *OAS1* (F) mRNA in CL slices. Slices were incubated with either basal medium alone (control) or rolIFNT (1 ng/mL) for 12 h. Results are presented as the means $\pm$ S.E.M. from 4 individual cows. Asterisks indicate significant (* P < 0.05, ** P < 0.01, *** P < 0.001) differences from their respective controls.

of the serine protease inhibitor family. PAI-1 inhibits tissue plasminogen activator and urokinase plasminogen activator, thus, *SERPINE1* is a potent pro-fibrotic factor (Ghosh & Vaughan 2012). In ewes, *THBS1* and *SERPINE1* were also shown to be concomitantly elevated during luteal regression, and inhibited during MRP (Romero *et al.* 2013). Furthermore, all of these genes (*EDN1*, *THBS1*, *TGFB1* and *SERPINE1*) were reported to be highly expressed in bovine LECs (Levy *et al.* 2001, Zalman *et al.* 2012, Farberov & Meidan 2016). In vitro response of luteal cells to IFNT was studied before by Romero and coworkers (Romero *et al.* 2013) using enriched ovine small, large and mixed luteal cells. The results obtained were not consistent with the expected effects of IFNT or pregnancy. For instance, neither *SERPINE1* nor *IL6* which were elevated during luteolysis in ewes and inhibited in the early pregnant CL, were not

significantly affected by IFNT *in vitro* in either of these cell preparations. The current study, using specifically enriched bovine LECs, showed that IFNT reduced *EDN1*, *THBS1*, *TGFB1* and *SERPINE1* mimicking the effect of early pregnancy (Costine *et al.* 2007, Romero *et al.* 2013, Przygodzka *et al.* 2016). Importantly, in CL slices IFNT also abolished the stimulatory effect of PGF2a on *THBS1* and *SERPINE1*. The reason why IFNT elevated *EDN1* in slices remains enigmatic, especially as *EDN1* is reduced during early pregnancy (Costine *et al.* 2007, Przygodzka *et al.* 2016).

Pregnant ruminants have higher levels of basal PGF2a than cyclic animals do (Wilson *et al.* 1972, Zarco *et al.* 1988). However, oxytocin-induced PGF2a, released from endometrium, was significantly inhibited during pregnancy or IFNT infusion (Vallet *et al.* 1989, Vallet & Lamming 1991, Meyer *et al.* 1995). This may be because IFNT can inhibit the majority of PGT-mediated release of PGF2a, but not the simple diffusion of PGF2a from endometrial luminal epithelial cells, as suggested by Banu and coworkers (Banu *et al.* 2010). These studies suggest that PGF2a can reach the CL during early pregnancy, thus requiring an anti-luteolytic mechanism at the level of the CL. In this context, our results demonstrating that IFNT can suppress luteolytic genes are physiologically important.

Precision-cut luteal slices and LECs were utilized to study the direct and functional roles of IFNT on bovine CL. Precision-cut CL slices represent an *in vitro* model that closely resembles the multi-cellular complexity as well as the structural and functional features of whole tissue. Use of precision-cut luteal slices to study luteal function has been validated in pigs (Przygodzka *et al.* 2014). However, luteal slices have limitations such as restricted incubation times. In addition, slices are also an unsuitable model to study functional assays such as cell proliferation and cell transfection. CL is a highly

Figure 6 Luteolytic gene expression in CL slices in response to rolIFNT (1 ng/mL), PGF2a (30 nM) or their combination. Levels of *TGFB1*, *THBS1*, *EDN1* and *SERPINE1* were determined in CL slices incubated in basal media (control) or with treatments for 12 h. Results are presented as the means $\pm$ S.E.M. from 4 CLs each derived from individual cow. The different letters indicate significant statistical differences at P < 0.05 analyzed using ANOVA followed by Bonferroni's *post hoc* multiple comparison test.

vascular gland, where LECs comprise more than 50% of all cell types within it (O'Shea *et al.* 1989). But in addition to their abundance, these cells are known to be active participants in the endocrine functions of the CL (Levy *et al.* 2001, Meidan *et al.* 2005), as highlighted again in this study. The stronger downregulation of luteolytic genes by IFNT in LECs may suggest that these cells are the primary target for IFNT to mediate its anti-luteolytic effects in bovine CL. However, the anti-luteolytic effects of IFNT on other bovine luteal cell types have not been studied yet and cannot be ruled out.

Collectively, the findings reported in the present study suggest that IFNT acts in the bovine CL via STAT1-IRF9-dependent and independent pathways to elicit ISGs, type 1-associated genes and anti-luteolytic mechanisms. The increased viability of LECs induced by IFNT may constitute an additional mechanism to promote luteal cell survival, thus extending the luteal life span of cows during early pregnancy.

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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