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Endocrine conceptus signaling in ruminants

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Abstract

The corpus luteum (CL) releases progesterone, which acts on the endometrium to induce release of histotroph that supports the free-floating conceptus and prepares for epithelial-chorial placentation. Two steroidogenic cell types, which are classified based on size, contribute to serum progesterone concentrations. Large luteal cells produce the bulk of progesterone because of constitutively active protein kinase A. Small luteal cells also contribute to serum progesterone concentrations through release of progesterone in response to luteotrophic stimuli. The CL is maintained in ruminants until endometrial-derived prostaglandin F2 alpha (PGF) initiates functional and structural regression. The decline in serum progesterone and loss of negative feedback on the hypothalamus and anterior pituitary sets up hormonal responses resulting in a new estrous cycle that is characterized by estrus, ovulation and formation of a new CL. If a conceptus is present, interferon tau (IFNT) is released from the conceptus, which binds receptors in the endometrium and prevents up-regulation of estrogen receptor (ESR1) and consequently oxytocin (OXT) receptor (OXTR). As a consequence, pulses of PGF are disrupted which results in rescue of the CL from luteolysis. In addition to these paracrine actions, early pregnancy also has direct endocrine action on the CL through inducing IFN-stimulated genes (ISGs) in the CL and resistance of the CL to PGF. Endocrine actions of IFNT have been described through detection of IFNT in uterine vein blood, induction of several ISGs in the CL during pregnancy, and following both *in vivo* (via miniosmotic pumps) and *in vitro* (in cultured small, large, and mixed luteal cells) delivery of recombinant ovine (ro) IFNT. These endocrine actions of IFNT might be applied to reducing embryo mortality and associated economic consequences in ruminants.

Keywords: corpus luteum, interferon-tau, luteolysis, pregnancy, prostaglandin F2 alpha.

Introduction

This review provides a brief overview of the ovine CL in context of general steroidogenic and luteolytic mechanisms, which serves as a prelude to more detailed discussion of endocrine action of pregnancy on the CL. It provides some background on anti-luteolytic paracrine actions of conceptus-derived IFNT on the endometrium, but primarily focuses on more recent studies describing an endocrine role for pregnancy through induction of ISGs in the CL, as well as peripheral blood cell mononuclear cells (PBMC). Described herein is evidence to suggest that IFNT is released into the uterine vein in biologically relevant concentrations and has direct endocrine action on the CL resulting in resistance to the luteolytic actions of PGF. The induction of ISGs in the CL in response to pregnancy is described for selected targets such as ISG15, oligoadenylate synthetase (OAS), and myxovirus (influenza virus) resistance proteins (MX1 and MX2), but also more globally in context of preliminary studies that are introduced herein using microarray approaches. An infusion model using miniosmotic pumps to deliver recombinant ovine (ro) IFNT into the uterine vein is described in context of induction of ISGs in the CL and protection of the CL from both endogenous (Bott *et al.*, 2010) and exogenous (Bott *et al.*, 2010; Antoniazzi *et al.*, 2013) challenge with PGF. The objectives of the review are to provide an overview of recent studies supporting the concept that endocrine signaling occurs through release of conceptus-derived IFNT and actions of this cytokine in inducing ISGs and resistance of the CL to lytic effects of PGF.

Luteal cells and luteolysis

Small and large luteal cells contribute to production of progesterone in the ovine CL (Fig. 1). Small luteal cells are more abundant than large luteal cells and are characterized by irregular shape, tapering cytoplasmic processes, predominantly smooth endoplasmic reticulum, mitochondria with tubular and lamellar

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cristae, and one or more Golgi complexes (O'Shea *et al.*, 1979). Large luteal cells synthesize and produce more progesterone, and are less responsive to luteinizing hormone (LH) than small luteal cells (Rodgers and O'Shea, 1982). Both large and small luteal cells have LH receptors, however large luteal cells release progesterone independently of LH action because of constitutively active protein kinase A and increased phosphorylation of steroidogenic acute regulatory protein (reviewed in Niswender *et al.*, 2007).

PGF is recognized as the luteolytic hormone in sheep (McCracken *et al.*, 1972). Continued exposure of endometrium to progesterone during the late luteal phase causes down-regulation of progesterone receptor and, consequently, up-regulation of ESR1, OXT, OXTR, and pulsatile release of PGF (reviewed in Spencer *et al.*, 2007). During the early luteolytic phase (days 12-14), exposure of large luteal cells to limited pulses of PGF activates protein kinase C (PKC) and release of OXT (Wiltbank *et al.*, 1989). PGF inhibits steroidogenesis, but also induces cyclooxygenase (COX)-2 (Silvia *et al.*, 1984) which may increase the concentration of intraluteal PGF in large luteal cells. Intraluteal progesterone may prevent the actions of OXT on small luteal cells and ability of PGF to increase calcium in large luteal cells (Davis *et al.*, 1992). Later in the luteolytic process, by day 16, when release of progesterone has diminished by 80% and OXT binds to receptors on small luteal cells, secretion of progesterone is inhibited and intracellular levels of calcium increase which leads to apoptosis. Secretion of PGF from large luteal cells may be facilitated through a transporter (SLCO2A1) and through autocrine action on large luteal cells to further stimulate OXT release, PKC, and COX-2 activity. Intraluteal PGF acts on large cells through increasing intracellular calcium, which likely leads to apoptosis of these cells. PGF also activates PKC mediated increases in early growth response 1 (EGR1) and transforming growth factor β (TGFB1) during bovine luteal regression *in vivo* and *in vitro* (Hou *et al.*, 2008). Repression of insulin-like growth factor (IGF-1) and cell-survival responses by PGF have been demonstrated in *in vivo* and *in vitro* bovine CL models (Arvisais *et al.*, 2010). These luteolytic responses in large and small luteal cells are the focus of possible disruption through endocrine actions of pregnancy and more specifically IFNT. For example, cell-survival responses might be maintained in the CL during early pregnancy in response to IFNT, which counter apoptotic responses induced by PGF and are the focus of our present studies.

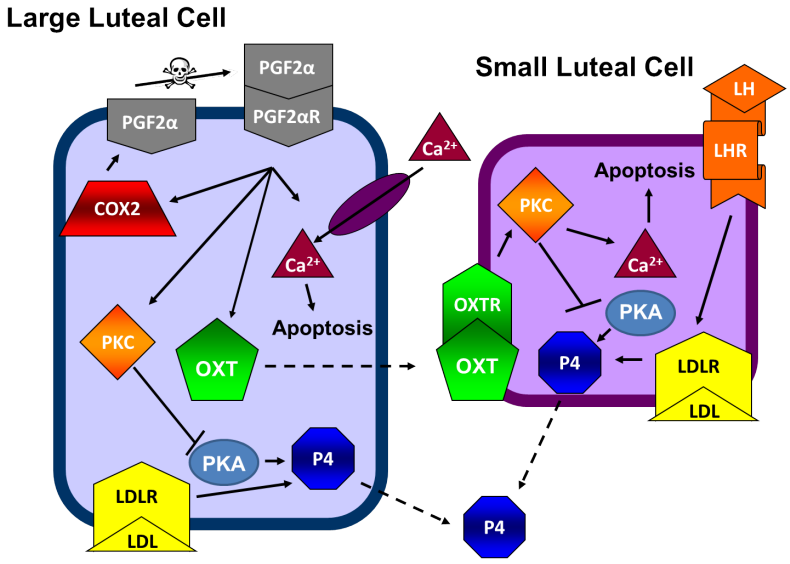

Figure 1. Small and large luteal cell responses during luteolysis (day 16) in ewes. See text above for detail. Diagram is adapted from Niswender *et al.* (2007), with permission. Abbreviations not defined in text are: low density lipoprotein (LDL) receptor (LDLR), progesterone (P4), protein kinase A (PKA).

Establishment of early pregnancy in sheep

Moor and Rowson (Moor and Rowson, 1966b) and Mapletoft and co-workers (Mapletoft *et al.*, 1976b) described local/ipsilateral effect of the conceptus in maintaining the CL during early pregnancy. These studies also were interpreted to mean that there is no systemic mediator of rescue of the CL, because ligation of the gravid horn protected the CL ipsilateral to the conceptus, while the contralateral CL regressed. However, these studies do not discount possible endocrine action of the conceptus on the CL in context of resistance to PGF and longer-term survival of the CL during early pregnancy. For example, several investigators have described the CL of pregnancy to be more resistant to lytic effects of PGF (Inskeep *et al.*, 1975; Mapletoft *et al.*, 1976a; Pratt *et al.*, 1977; Silvia and Niswender, 1984). Exactly why and how this

resistance to PGF occurs in the CL during pregnancy is unknown.

Pregnant and cyclic ewes have very different patterns of PGF release as reflected by PGF metabolite (PGFM) in the blood 12-16 days post ovulation (Thorburn *et al.*, 1972; Zarco *et al.*, 1988a). Peak production of PGF occurs at day 14-15, regardless of pregnancy status. Estrous cycling ewes release PGF in a pulsatile manner, while pregnant ewes have a pattern of more constant and slower increases in the release (Peterson *et al.*, 1976; Zarco *et al.*, 1988b). More PGF is found exiting the uterus through the uterine vein in day 13 pregnant vs estrous cycling ewes (Wilson *et al.*, 1972). The antiluteolytic antagonism of PGF release from the endometrium during pregnancy is regulated by conceptus-derived IFNT, which contributes to rescue of the CL. However, the release of PGF from the endometrium is not ablated completely and there is a possibility that the CL produces PGF (Silva *et al.*, 2000). Thus, mechanisms inducing resistance of the CL to PGF may need to be activated during early pregnancy in the ewe to prevent luteolysis. We propose herein that IFNT is released into the uterine vein and actually has direct endocrine actions on the CL to induce putative cell survival, anti-apoptotic and, consequently, antiluteolyic responses.

The lytic effect of the non-pregnant uterus can be blocked through transfer of ovine embryos into the uterus by day 12 (Moor and Rowson, 1966a, b). For this reason, day 10-12 ovine conceptuses were examined, which resulted in identification of IFNT (also called trophoblast proteins or protein X; Imakawa *et al.*, 1987, 1989) as the primary conceptus secretory protein (Godkin *et al.*, 1982) responsible for altering endometrial release of PGF (Zarco *et al.*, 1988a) and rescuing the CL during pregnancy in sheep. IFNT acts through silencing endometrial transcription of ESR1 and, consequently, OXTR, thereby disrupting pulsatile release of PGF in response to OXT (Spencer *et al.*, 1995b; Spencer and Bazer, 1996). Thus, IFNT acts through paracrine anti-luteolytic action on the endometrium to protect the CL during maternal recognition of pregnancy.

IFNT has been shown to stimulate Janus kinase signal transducers and activators of transcription (STATs; Binelli *et al.*, 2001; Thatcher *et al.*, 2001), IFN regulatory factors (IRFs; Perry *et al.*, 1999) and to modulate COX-2 and phospholipase A2 (Binelli *et al.*, 2000) in bovine endometrium. Also, pregnancy and more specifically IFNT induces many ISGs in the endometrium and in blood cells (for review see Hansen *et al.*, 2010a). One example of a pregnancy-associated ISG that is expressed both in the endometrium (Austin *et al.*, 1996; Johnson *et al.*, 1999b) and blood cells (Han *et al.*, 2006; Hansen *et al.*, 2010a) is ISG15, an ubiquitin homolog that resembles a tandem ubiquitin repeat which ends in carboxyl terminal LRLRGG. This C-terminal feature allows these post-translational modifiers and regulatory proteins to become covalently attached to proteins via a Gly:Lys isopeptide bond that ligates ubiquitin (Wilkinson and Audhya, 1981; Ecker *et al.*, 1987) and ISG15 (Loeb and Haas, 1992; Narasimhan *et al.*, 1996) to targeted proteins (reviewed in Haas, 2007). Polyubiquitinated proteins are thought to be targeted for degradation through the 26S proteasome, whereas ISGylated proteins might be stabilized or altered for specialized function such as RNA splicing, chromatin remodeling/polymerase II transcription, cytoskeletal organization and regulation, stress responses, translation and viral replication (Malakhova *et al.*, 2003; Giannakopoulos *et al.*, 2005; Zhao *et al.*, 2005; Takeuchi *et al.*, 2006). ISG15 serves as an excellent marker for IFNT action and is a conserved uterine response to pregnancy in primates (Bebington *et al.*, 1999a, b; Bebington *et al.*, 2000), mice (Austin *et al.*, 2003; Bany and Cross, 2006), and cattle (Austin *et al.*, 1996; Hansen *et al.*, 1997; Johnson *et al.*, 1998; Perry *et al.*, 1999; Thatcher *et al.*, 2001).

Pregnancy induces ISGs in peripheral tissues

Originally, IFNT was not thought to be released from the uterus and was believed to have only paracrine action on the endometrium because it was not detected in peripheral blood. Because ISG15 was localized to endometrial tissues subjacent to the luminal epithelium, including the myometrium (Johnson *et al.*, 1999a, b), the presence of surrogate mediators of IFNT, that induce these ISG responses, were suspected. Likewise, ISG mRNAs were found to be up-regulated in PBMC in response to pregnancy in both sheep (Yankey *et al.*, 2001) and cattle (Han *et al.*, 2006; Gifford *et al.*, 2007). The impact of pregnancy on induction of ISGs in blood cells was intriguing, especially in light of opinions that the conceptus acted locally in paracrine manner as an anti-luteolysin. Exactly how PBMC became activated to express ISGs was unknown. However, at least 674 genes were up-regulated and 721 genes were down-regulated in blood cells in response to pregnancy on day 18 in cattle (Hansen *et al.*, 2010a). Many of these genes were ISGs. In search of a mechanism to describe release of IFNT from the uterus and explain up-regulated ISGs in PBMC, we examined lymph nodes draining the uterus (iliac) and the head (submandibular) from day 15 pregnant ewes and found no difference in ISGs expression, suggesting that IFNT was not released into the uterine lymphatics and this was not a pathway through which IFNT induced ISGs in PBMC.

Uterine vein blood from pregnant or non-mated, non-pregnant sheep was examined for antiviral activity in order to evaluate the possibility that IFNT was released systemically from the uterus. Surprisingly, significant antiviral activity was found in uterine vein blood from day 15 pregnant sheep. IFN released from the uterus was in the amount of ~ 200 μg (2×10^7 U)/24 h when converted based on bioactivity of IFN standards

(Oliveira *et al.*, 2008). The uterine venous blood had 500- to 1000-fold higher concentrations of bioactive IFN than uterine arterial blood on day 15 of pregnancy. To determine if this antiviral activity was caused by IFNT or some other type I IFN, uterine vein blood was preadsorbed with antibody against IFNT and then tested in the antiviral assay against blood that was preadsorbed with non immune normal rabbit serum (Bott *et al.*, 2010; Fig. 2).

Preadsorption of uterine vein blood with normal rabbit serum had no effect on the high antiviral activity detected on day 15 of pregnancy. In contrast, preadsorption of uterine vein blood with antiserum against IFNT completely blocked antiviral activity found on day 15 of pregnancy. It was concluded that the major contributor to antiviral activity in uterine vein blood on day 15 of pregnancy was IFNT. This conclusion is further supported by detection of IFNT in uterine vein blood by mass spectroscopy (Romero and Hansen; Colorado State University, Fort Collins, CO; provisionally accepted in *Physiol Genomics*) and by using a specific and sensitive radioimmunoassay for IFNT (Antoniazzi and Hansen; Colorado State University, Fort Collins, CO; unpublished results).

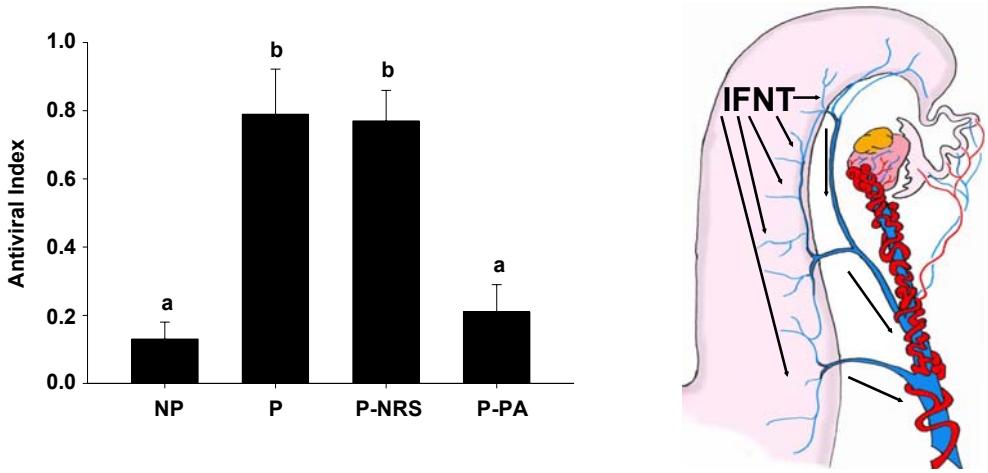

Figure 2. Preadsorption of uterine vein blood blocks antiviral activity detected in uterine vein blood from day 15 pregnant sheep. Left panel describes antiviral index and is adopted from Bott *et al.* (2010). NP: uterine vein blood from day 15 non pregnant sheep. P: uterine vein blood from day 15 pregnant sheep. P-NRS: uterine vein blood from day 15 pregnant sheep preadsorbed with normal rabbit serum. P-PA: Uterine vein blood from day 15 pregnant sheep preadsorbed with antiserum against IFNT. Based on these data it is concluded that the primary IFN released from the uterus into the uterine vein is IFNT (right panel).

Induction of ISGs by IFNT and pregnancy in the CL

Pregnancy (the conceptus) has endocrine effects through inducing ISGs in extrauterine tissues such as the CL (Oliveira *et al.*, 2008; Bott *et al.*, 2010). For example, ISG15 mRNA concentrations were up-regulated in CL from day 15 pregnant compared to non-pregnant ewes. Likewise, ISG15 protein and its ISGylated protein targets also were up-regulated in CL in response to pregnancy. Immunohistochemical staining using an anti-ISG15 monoclonal antibody (clone 5F10; Austin *et al.*, 2004) revealed that ISG15 was predominantly localized to large luteal cells on day 15 of pregnancy, with diminished, but significant localization to small luteal cells. To confirm that these ISGs were pregnancy-associated and induced by IFNT, small, large, and mixed luteal cells which were isolated on day 10 of the estrous cycle and cultured with roIFNT for 24 h demonstrated significant induction of ISG15 (Antoniazzi *et al.*, 2013). Similar cultures of day 10 CL with prostaglandin E2 revealed no impact on production of ISG15. In order to more globally examine induction of ISGs in the CL by pregnancy, we collected CL on day 12 or 14 of pregnancy or the estrous cycle and isolated and screened mRNA using the bovine Affymetrix microarray. The specific days of pregnancy/estrous cycle were selected based on serum progesterone levels, which did not change in pregnant but started to decline from day 12 to 14 with a significant decline by day 15 of the estrous cycle (Fig. 3). There were 21 differentially expressed genes in CL from day 14 pregnant compared to day 12 non-pregnant ewes and 734 differentially expressed genes in CL from day 14 pregnant compared to day 14 non-pregnant CL (Romero and Hansen; Colorado State University, Fort Collins, CO, provisionally accepted in *Physiol Genomics*). Many of the genes differentially expressed in response to pregnancy were ISGs (type I IFN signaling).

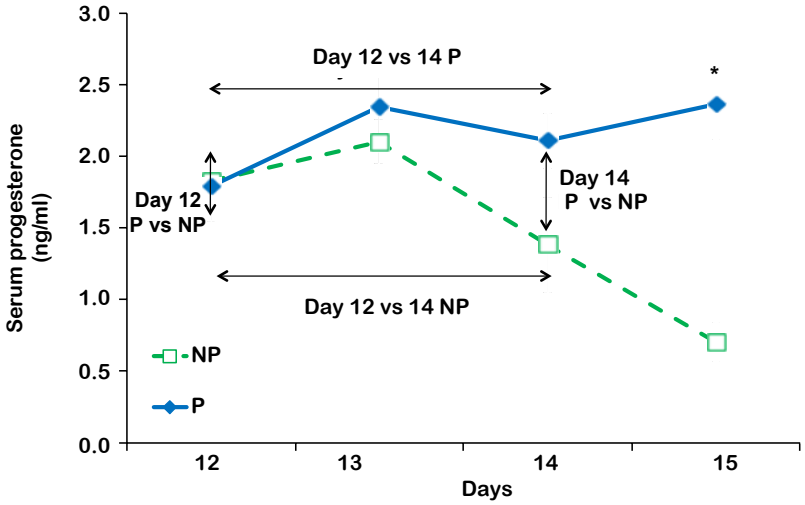

Figure 3. Serum progesterone and experimental design for microarray analysis of CL on days 12 and 14 of the estrous cycle and pregnancy. Microarray analysis revealed induction of many ISGs in response to pregnancy/IFNT. Data adapted from Romero *et al*. (provisionally accepted in *Physiol Genomics*). Note that day 0 in these studies is day of estrus.

Systemic delivery of IFNT

Many groups have examined infusion of roIFNT into the uterine lumen as well as subcutaneous injections and other systemic delivery approaches (Vallet *et al.*, 1988; Mirando *et al.*, 1991; Spencer *et al.*, 1995a, 1999). A general overview of these studies is that a paracrine effect was noted in altering the ESR1/OXTR/PGF system with little reported impact directly on the CL (Vallet *et al.*, 1988; Martal *et al.*, 1990; Green *et al.*, 2005). These studies were interpreted to mean that IFNT acted in paracrine action to extend the luteal phase.

Systemic delivery (Nephew *et al.*, 1990; Martinod *et al.*, 1991; Schalue-Francis *et al.*, 1991; Davis *et al.*, 1992) of mg quantities of IFNT through intramuscular or subcutaneous injection also was studied to determine if it extended inter-estrous interval as well as ability to increase fertility. In some cases the amounts of IFNT used actually induced hyperthermia, and had no effect or caused a decline in fertility (Niswender *et al.*, 1997; Ott *et al.*, 1997). These doses of roIFNT have been reduced to 2 x 10^7 U (200 µg) in intrauterine deliveries to avoid hyperthermia and high death loss of ewes (Spencer *et al.*, 1999). Induction of ISG15 has been reported in the CL following subcutaneous (Spencer *et al.*, 1999) and intramuscular injections (Chen *et al.*, 2006) of roIFNT given between days 11-17. Chen *et al.* (2006) described inter-estrus interval of 32.7 days in ewes that received intrauterine infusions of 200 µg roIFNT, but an average interval of only 17 and 22 days in ewes that were injected (i.m.) with 200 µg or 2 mg roIFNT, respectively. The induction of ISG15 in the CL occurred in response to infusion and injection of 2 mg roIFNT, but not following injection of 200 µg roIFNT. None of these systemic methods of roIFNT treatment induced a pseudopregnant state that continued for more than a few days. However, both methods of roIFNT delivery were able to induce ISGs in the endometrium.

A new model was developed to deliver IFNT into the uterine vein by using surgically implanted miniosmotic pumps (Fig. 4). This was done to allow for systemic delivery of IFNT but also to provide an opportunity for the IFNT to have access to the utero-ovarian plexus in the event that it happened to cross over to the ovarian artery from the uterine vein. Delivery of 200 µg over 24 h was selected as an appropriate amount based on estimate of antiviral activity and relevant concentration of IFNT determined through this bioassay (see Hansen *et al.*, 2010a for more detailed rationale). Considering blood volume in sheep it is estimated that systemic levels in circulation will stabilize around 2.4 ng/ml.

After infusing 200 µg IFNT into the uterine vein per day, it was clear that this was enough to cause induction of ISG15 mRNA in ipsilateral and contralateral CL as well as in endometrium and liver (Oliveira *et al.*, 2008; Bott *et al.*, 2010). This systemic delivery, when continued from day 10 to day 17 of the estrous cycle, caused a delay in the return to estrus in all ewes with normal serum progesterone profiles prior to insertion of the miniosmotic pumps on day 10. All control (bovine serum albumin; BSA)-infused ewes returned to estrus by day 19, whereas all IFNT infused ewes had a delay in return to estrus that may have been extended beyond day 32, which is when this study was terminated and necropsy was performed to confirm presence of the original CL. It was concluded from these studies that endocrine delivery of IFNT into the uterine vein was able to block luteolysis from endogenously produced PGF if delivery occurred from days 10 to 17 of the estrous cycle. However, these studies did not directly test the ability of IFNT to protect the CL from lytic actions of PGF.

The first study to use miniosmotic pump delivery to investigate this was completed by Bott *et al.*

(2010) where 200 μg roIFNT was delivered into the uterine vein starting on day 10 (prior to endogenous action of PGF) for 12 h, at which time a single injection of PGF (Lutalyse; 4 mg/58 kg i.m.) (Silvia and Niswender, 1984, 1986; Silva *et al.*, 2000; Bott *et al.*, 2010) was administered and exposure to roIFNT continued for another 12 h at which time ewes were necropsied. Serum progesterone concentrations declined significantly in BSA-infused ewes following injection with PGF. In roIFNT-infused ewes, serum progesterone declined slightly following the PGF injection but to levels that were not different from BSA-infused controls. More recently we modified this experiment and demonstrated that infusion of only 20 μg/day into the uterine vein for three days from day 10 to 13 was able to significantly protect the CL from lytic action of PGF exogenously administered on day 11 (Fig. 5; Antoniazzi *et al.*, 2013).

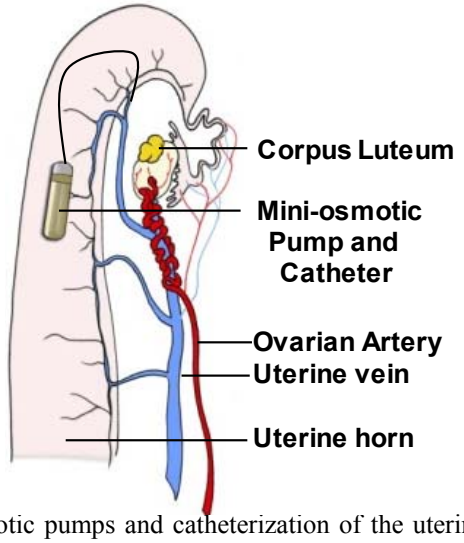

Figure 4. Description of miniosmotic pumps and catheterization of the uterine vein upstream of the utero-ovarian plexus for endocrine delivery of roIFNT. See Oliveira *et al.* (2008); Bott *et al.* (2010); Hansen *et al.* (2010a); Antoniazzi *et al.* (2013). Adapted from Bott *et al.* (2010).

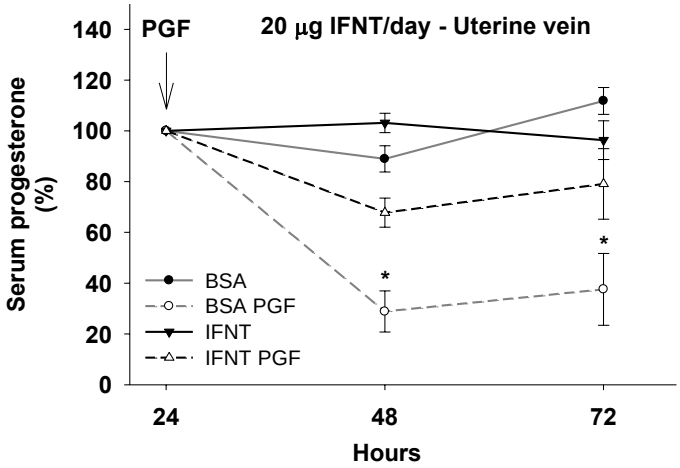

Figure 5. Serum progesterone concentrations following three day (10-13) infusion of BSA, BSA with PGF injection on day 11, IFNT or IFNT with PGF injection on day 11. Data were normalized to serum progesterone levels in ewes on day 11 (i.e., 24 h). Note that infusion of 20 μg roIFNT/day into the uterine vein protected the CL from lytic action of exogenously administered PGF. Adapted from Antoniazzi *et al*. (2013).

Conclusions

The ovine conceptus releases IFNT into the uterine lumen, where it has paracrine action on luminal epithelium to modify pulsatile release of PGF. In addition to well-studied action on the ESR1/OXTR/PGF axis, IFNT also induces many ISGs in the endometrium. These ISGs may be involved with preparing the uterine endometrium for epitheliochorial placentation and formation of placentomes. However, they may also contribute to a peripheral resistance to infection through upregulation of innate immune responses. For example, many of the ISGs induced in endometrium and in blood cells are major cellular responders to viral infection. ISGs such as ISG15, IRFs, ubiquitin activating enzyme E1-like, MX2, retinoic acid-inducible gene 1, OAS are

upregulated in response to pregnancy in the endometrium and the blood and also are now described in the CL. Currently, it is not known why these ISGs are so massively and systemically upregulated in sheep. Perhaps ruminants have developed this provoked and upregulated innate response machine to help protect the fetus from maternal infection with virus, which in some cases can result in persistent infection in the fetus (Fig. 6; Hansen *et al.*, 2010a, b). The induction of ISGs in the CL may contribute to protection (resistance) of the CL to PGF.

The induction of ISGs in the CL in addition to several other pregnancy-associated genes may confer resistance in the CL to lytic action of PGF. Infusion of roIFNT into the uterine vein for seven days caused significant delay in return to estrus and may have not only had an impact on endometrial PGF release, but also on the resistance of the CL to PGF. The later was directly tested through delivering IFNT into the uterine vein followed by an injection of exogenous PGF. Pre-treatment with IFNT for 12 h tended to block and with 24 h infusion of roIFNT blocked the decline in serum progesterone caused by injection of PGF. This direct action of IFNT in protecting the CL was replicated by using 10-fold lower concentration of roIFNT (20 instead of 200 µg/day) delivered into the uterine vein. Exactly how IFNT protects the CL from PGF is the focus of future experiments designed to examine signal transduction mechanisms coupled to type I IFN receptors, the role of ISGs, but also disruption of lytic-cell death responses to PGF. Through uncovering these relationships, it may become possible to apply this knowledge to improving embryo survival and fertility.

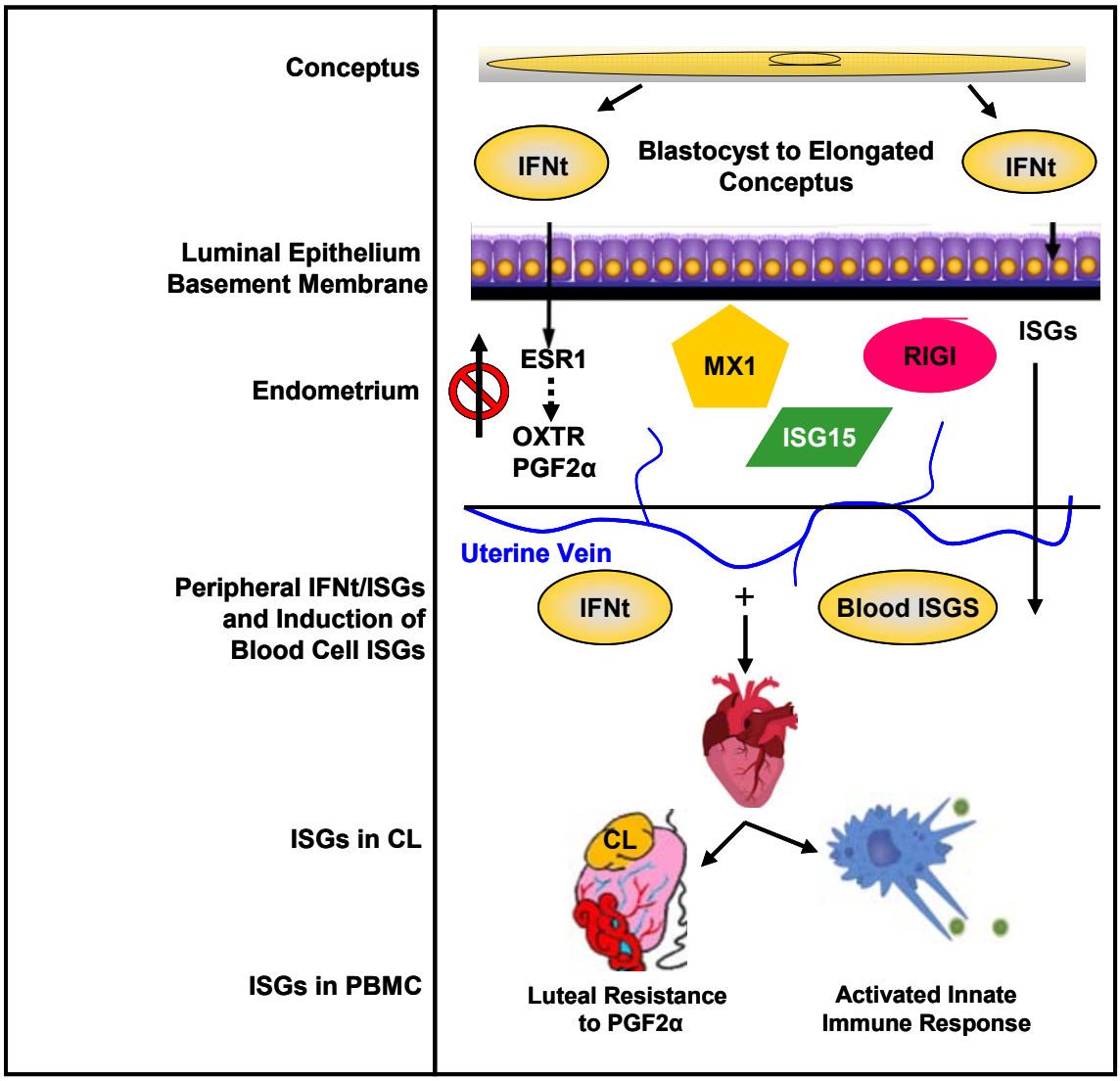

Figure 6. Paracrine and endocrine mechanism of IFNT action. IFNT is released from trophectoderm of the expanding blastocyst, binds receptors, regulates the ESR1, OXTR and PGF axis and also induces massive ISGs response in the endometrium. IFNT also has endocrine action based on detection in uterine vein blood and induction of ISGs in peripheral tissues such as PBMC, liver and CL. Why these ISGs are induced in peripheral tissues like the CL is unknown. However, the ISGs do have very clear and documented roles in the innate immune response to viral expression.

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