Transcriptional control of *IFNT* expression

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Abstract

Once interferon-tau (IFNT) had been identified as a type I IFN in sheep and cattle and its functions were characterized, numerous studies were conducted to elucidate the transcriptional regulation of this gene family. Transfection studies performed largely with human choriocarcinoma cell lines identified regulatory regions of the *IFNT* gene that appeared responsible for trophoblast-specific expression. The key finding was the recognition that the transcription factor ETS2 bound to a proximal region within the 5'UTR of a bovine *IFNT* and acted as a strong transactivator. Soon after other transcription factors were identified as cooperative partners. The ETS2-binding site and the nearby AP1 site enable response to intracellular signaling from maternal uterine factors. The AP1 site also serves as a GATA-binding site in one of the bovine *IFNT* genes. The homeobox-containing transcription factor, DLX3, augments *IFNT* expression combinatorially with ETS2. CDX2 has also been identified as transactivator that binds to a separate site upstream of the main ETS2 enhancer site. CDX2 participates in *IFNT* epigenetic regulation by modifying histone acetylation status of the gene. The *IFNT* downregulation at the time of the conceptus attachment to the uterine endometrium appears correlated with the increased EOMES expression and the loss of other transcription coactivators. Altogether, the studies of transcriptional control of *IFNT* have provided mechanistic evidence of the regulatory framework of trophoblast-specific expression and critical expression pattern for maternal recognition of pregnancy.

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Introduction

The function of the proteins that we now recognize as IFNT was established before their primary structures were known. The cloning and sequencing of the cDNAs reported thirty years ago caused considerable surprise, because type I IFN, which include the related IFNA and IFNB families, had always been assumed to be antiviral agents, expressed for a brief time in response to a viral infection and then secreted in order to stimulate viral resistance in target cells elsewhere in the body. What makes the IFNT unique, however, is not their ability to extend estrous cycle length in ruminants, since IFNA can accomplish this quite effectively (Green *et al.* 2005). Nor do the IFNT demonstrate reduced antiviral activity compared to their relatives; quite the opposite, they are quite potent in this respect and bind to the same receptors as all other type I IFNT. Instead, the uniqueness of the IFNT lies in their transcriptional control, which provides trophoblast-specific, time-specific, constitutive expression in the absence of a viral challenge. Additionally, production of IFNT is exceptionally high over the few days. As pointed out in the accompanying chapter of Ealy and coworkers (Ealy & Woolridge 2017), the relatively recent evolution of

the *IFNT* genes required the acquisition of distinctive regulatory sequences upstream of their transcription start sites. Although the topic of transcriptional control of *IFNT* expression has been reviewed previously (Roberts 2007, Roberts *et al.* 2008, Ealy & Yang 2009), the review presented here is more comprehensive than the earlier ones. Our goal herein is to update the reader with the most recent literature on the topic and to attempt to resolve some conflicting results.

Different transcriptional control of *IFNT* from other type I interferon family genes

IFNT is a member of the type I interferon family that is expressed exclusively in trophectoderm in the early stage of trophoblast development (Farin *et al.* 1990) in the suborder *Ruminantia* (Leaman & Roberts 1992, Roberts *et al.* 1992, Liu *et al.* 1996). As pointed out in the previous section, expression of *IFNT* is not inducible by viral infection but is sustained constitutively at high levels over several days during the preimplantation period (Cross & Roberts 1991, Roberts 2007). Additionally, the signaling pathways and transcriptional activators that promote the upregulation of other type I gene families, such as *IFNA* and *IFNB*, are not involved

in the regulatory control of the *IFNT* (Leaman *et al.* 1994). The realization that the transcriptional regulation of *IFNT* genes is distinct from that of other type I *IFN* genes (Roberts *et al.* 1992, Flint *et al.* 1994) represented an obvious challenge to those in the field.

Initial studies utilized up to -1675bp (originally referred BTP-1.8 (Cross & Roberts 1991) of the 5'-regulatory region of bovine *IFNT1A* gene, which was placed into a reporter plasmid (Cross & Roberts 1991, Leaman *et al.* 1994). Jar cells became the cell line of choice to study transcriptional control because fast-growing ovine trophoblast lines were not available. It was also reasoned that trophoblast cells, like Jar, despite their human origin might express much of the basic transcriptional machinery that would permit *IFNT* regulatory region to be active. Truncation of the longer BTP-1.8 construct quickly revealed that regions within 450bp from the transcriptional start site allowed constitutive expression. The finding is consistent with the observation that the more proximal regions of the bovine and ovine *IFNT* genes are highly conserved (Leaman & Roberts 1992). So-called 'mobility-shift electrophoresis' or 'gel-shift' experiments performed with crude nuclear extracts prepared from ovine conceptuses during the period of *IFNT* expression revealed a strong interaction of nuclear protein interaction with two regions, a proximal (-91 to -69bp from the transcriptional start site) and a more distal site (-358 to -322bp) (Leaman *et al.* 1994). These were assumed to represent major sites of transcriptional control. Such interactions were not observed with extracts from older conceptuses that were not expressing *IFNT* at the time they were recovered. These gel-shift experiments provided a useful beginning to subsequent transcriptional control studies as they mirrored *IFNT* expression over time and implicated particular control regions likely to interact with transcription factors.

Transcription factors regulating *IFNT* expression

Regulatory mechanisms that upregulate *IFNT* gene expression

ETS2 enhancer region

To identify the factor(s) responsible for *IFNT* gene transcription, the proximal protein-binding region was employed as the bait sequence for a yeast one-hybrid screening with a day 13 ovine conceptus cDNA library, from which the transcription factor ETS2 was identified (Ezashi *et al.* 1998). ETS2 is a member of the ETS family of transcription factors that share a conserved ETS DNA-binding domain that recognizes a specific binding motif centered on a GGAA core. Active *IFNT* genes contain an ETS-binding site in the proximal site, whereas several ovine *IFNT* genes that are poorly expressed and could be 'pseudogenes' contain a mutated core motif

(TGAA) in the proximal binding site (Ezashi *et al.* 1998, Matsuda-Minehata *et al.* 2005). Overexpression of ETS2 strongly transactivated an *IFNT* reporter, while specific mutation of the ETS2-binding sequence substantially reduced both the basal and induced activity in the Jar cells (Ezashi *et al.* 2001, 2008, Ezashi & Roberts 2004, Das *et al.* 2008). When a 6bp CAGGAA ETS2 core binding sequence (Fig. 1C) was inserted into the equivalent region of the pseudogenes, the reporter still failed to confer higher ovine *IFNT* expression or induction with ETS2 overexpression. However, if the replacement nucleotide cassette was extended to 22bp on both ends from the ETS2 site and included

Figure 1 (A) Sequences of the ETS/AP1-binding sites of the murine *PLAU* and bovine *IFNT1A* control regions. The ETS core binding sequences are boxed. Conserved AP1-binding site is underlined. (B) Transcriptional activities of the *PLAU* and *IFNT* reporters by cotransfected ETS2 and HRAS expression plasmids (alone or in combination) into 3T3 cells. (A) and (B) are modified from Ezashi and Roberts (2004). (C) The 6bp and 22bp sequences of ETS-binding site of ovine *IFNT-o10* (*ovIFNT-o10*) used in a study of Matsuda-Minehata *et al.* (2005). The nonfunctional 6bp contains only the core of ETS-binding site. The functional 22bp has both ETS (blue) and adjacent AP1-binding site (orange).

an adjacent putative AP1 site from active ovine *IFNT* (*ovIFNT-o10*; Fig. 1C), full promoter activity of the ovine *IFNT* gene in response to ETS2 was restored (Matsuda-Minehata *et al.* 2005). This experiment indicated the importance of the neighboring sequences to the core ETS2-binding site. Finally, ETS2 was significantly more efficient than related ETS family transcription factors, such as ELF1, ETS1, FLI1, ETV4 and SPI1 and was concurrently expressed with IFNT in trophectoderm of day 15 ovine conceptuses (Ezashi *et al.* 1998).

ETS2 is a master regulator of trophoblast differentiation across species. In the mouse, *Ets2* is essential for trophoblast development (Yamamoto *et al.* 1998, Georgiades & Rossant 2006) and trophoblast stem (TS) cell self-renewal (Wen *et al.* 2007, Odiatis & Georgiades 2010). The ETS2 transcription factor controls many trophoblast signature genes including *Cdx2* (Wen *et al.* 2007) and *Hand1* (Odiatis & Georgiades 2010) in mice, *CGA* (Ghosh *et al.* 2005) and *CGB5* (Johnson & Jameson 2000, Ghosh *et al.* 2003) in humans and pregnancy-associated glycoprotein 2 (PAG2) (Szafranska *et al.* 2001) and trophoblast Kunitz domain protein-1 (TKDP1) (Chakrabarty & Roberts 2007) in ruminants. Secretion of PAG2 and TKDP1 begins concurrently with IFNT in ruminant trophectoderm (Green *et al.* 1998, MacLean *et al.* 2003).

The success obtained with yeast one-hybrid screening to identify ETS2 as a factor that associated with the proximal region of the *IFNT* 5'UTR was not so fruitful when applied to search the distal region (–358 to –322bp) for binding factors. Various gene products with generally weak interactions were initially identified, but after several rounds of the one-hybrid screening with different background threshold level, no consistent and plausible candidates for binding factors were found (T Ezashi and R M Roberts unpublished observation).

Although bovine and ovine *IFNT* genes have relatively conserved 5'UTR to around –400bp, their nucleotide sequences diverge considerably beyond that point (Ealy *et al.* 2001), suggesting that if control elements do exist they may be gene specific. In the ovine *IFNT* gene (*ovIFNT-o10*), a JUN/FOS (AP1) site was identified within the –654 to –555bp region and appeared to have a regulatory function (Yamaguchi *et al.* 1999). It formed a DNA–protein complex with nuclear extracts of JEG3 choriocarcinoma cells and mediated responses to phorbol 12-myristate 13-acetate (PMA) treatment by an ovine *IFNT*-SV40 hybrid reporter (Yamaguchi *et al.* 2001). This site appears to be involved with a coactivator, cAMP-response element-binding protein-binding protein (CREBBP) (Xu *et al.* 2003). However, different responses to the AP1 site mutations at –602 to –592 in the two bovine *IFNT* genes, *IFNT1* and *IFNT-c1* that appears to be homologous to the AP1 site in the ovine *IFNT* gene, were observed. In the latter gene, the AP1

mutation did not change the transcriptional activity (Sakurai *et al.* 2013b).

Closely juxtaposed ETS- and AP1-binding motifs in plasminogen activator urokinase (*PLAU*), and chorionic somatomammotropin hormone (*CSH2*) genes serve as a target of the RAS-mediated mitogen-activated protein kinase (MAPK) signaling pathway (Stacey *et al.* 1995, Sun & Duckworth 1999) (Fig. 1A and B). Signaling through this pathway leads to phosphorylation of a highly conserved threonine residue in ETS2 at threonine 72 (T72) and stimulates ETS2-driven transcriptional responses (Yang *et al.* 1996). The proximal regions of *IFNT* genes possess a similar consensus AP1 site at –71 adjacent to the ETS site (Figs 1A, C, 2B and 3), which is also responsive to RAS-mediated signaling (Ezashi & Roberts 2004). In all respects, the ETS/AP1-binding motif in the *IFNT* behaved identically to the one in the murine *PLAU* gene (Fig. 1B). Mutating T72 to alanine (A72) of ETS2 and mutation of the AP1 site in the ETS/AP1-binding motif (μ AP1, Fig. 2B) reversed the RAS effect on the *IFNT*

Figure 2 (A) ETS2 and potential six GATA-binding sites in the regulatory region of the bovine *IFNT* gene. Below is the transcriptional activity of the GATA site mutated *IFNT* reporters (mt1–mt6) with GATA2 expression plasmid. ‘Mock’ and ‘WT’ are readings of the wild-type –631 bp *IFNT* reporter construct without and with GATA2 expression plasmid, respectively. The reporter activities and the location of the six mutation sites are shown with arrows. Significant reduction ($P < 0.01$) of the transcriptional activity is noted with a red arrow and two asterisks on the mutation site, –70 to –63 (mt5). The image has been modified from Bai *et al.* (2009). (B) The bovine *IFNT* upstream region adjacent to the ETS-binding site (–79 to –70, blue) is shown as AP1 site (–71 to –65, orange) in Ezashi and Roberts (2004) or GATA site (–70 to –63, green, mt5 site in A) in Bai *et al.* (2009). Conserved core binding sequences are underlined. Introduced mutation sequences are shown in red.

promoter. This study also demonstrated the importance of the AP1 site adjacent to ETS2 at –71 relative to other potential AP1-binding sites in the –1675 bp region of 5'UTR control region, none of which were responsive to RAS-mediated signaling (Ezashi & Roberts 2004).

DLX3

Having shown that *ETS2* was a major player in controlling *IFNT* expression, it was natural to examine other important transcription factors that had been implicated in trophoblast differentiation in the human and mouse. *DLX3* is a member of the distal-less family of non-Antennapedia homeobox genes. Its knock out in mouse embryos leads to embryonic death due to placental failure by about day 10 of pregnancy (Morasso *et al.* 1999). *DLX3* is also expressed in human, cow and mouse trophoblast cells (Panganiban & Rubenstein 2002, Ezashi *et al.* 2008). It is upregulated during formation of human syncytiotrophoblast (Yabe *et al.* 2016) and has a role in the control of human CGA (Roberson *et al.* 2001), placental growth factor (PGF) (Li & Roberson 2017a) and murine 3 β -hydroxysteroid dehydrogenase VI (*Hsd3b6*) expressions (Peng & Payne 2002, Berghorn *et al.* 2005). The *DLX3*-binding site (–54 GATAATGAG –46), which lies between the *ETS2*-binding site and the transcription start site of *IFNT* 5'UTR, is conserved in all *IFNT* genes examined (Fig. 3). Overexpression of *DLX3* in JAr cells transactivated the bovine *IFNT1A* 10 to 20-fold, whereas mutations introduced into the *DLX3*-binding site at –54 to –46 abolished the activation effect (Ezashi *et al.* 2008). In combination with *ETS2* overexpression, *DLX3* increased the *IFNT* reporter activity more than 250-fold (Fig. 4). However, if the *ETS2*-binding site (–79 to –70) was mutated (μ ETS2) or deleted, the effects of *DLX3* on reporter gene transactivation were abolished,

suggesting that a major portion of *DLX3* transactivation activity was dependent on the presence of *ETS2* at a site just two helical turns (~20bp) upstream of the *DLX3* site (Fig. 3). Suppression of bovine *DLX3* expression by siRNA led to reductions of both intracellular *IFNT* protein and antiviral activity released into the medium of bovine trophoblast CT-1 cell culture. Moreover, the two transcription factors (*ETS2* and *DLX3*) were co-immunoprecipitated together as a complex in the *IFNT*-expressing CT-1 cell line. Clearly, *DLX3* partners *ETS2* in a cooperative and dependent manner to regulate *IFNT* expression in trophoblast (Ezashi *et al.* 2008). *DLX3* also interacts with another transcription factor, *GCM1*, to regulate *PGF* expression in human trophoblasts (Li & Roberson 2017a,b).

GATA2 and GATA3

In the mouse placenta, *Dlx3* expression is controlled by GATA2 and -3 transcription factors (Home *et al.* 2017). These transcription factors are also regulators of mouse trophoblast differentiation (Home *et al.* 2009, Ray *et al.* 2009, Ralston *et al.* 2010). They are expressed in the bovine CT-1 cell line and in day 15–21 bovine and ovine conceptuses (Bai *et al.* 2009, 2014). GATA2 but not GATA3 overexpression also appeared capable of increasing expression from an *IFNT* reporter in non-trophoblast cells, namely bovine ear-derived fibroblasts (Bai *et al.* 2009). Similar experiments in JEG3 cells failed to reproduce the effect, possibly because JEG3 cells already express endogenous GATA2 or -3 in abundant quantities. This inference may be correct as siRNA silencing of GATA2 did decrease *IFNT-c1*-reporter expression (Bai *et al.* 2009). A search of six possible GATA sites as possible control elements for *IFNT* transcription identified only one (–70 to –63; Fig. 2A,

	-79	-70	-60	-50	-46	
<i>boIFNT1A</i>	ACAGGAAG	TGAGAGAGAA	TTTTTCGG	ATAATGAG		Hansen et al 1991
<i>boIFNT</i>	ACAGGAAG	TGAGAGAGAA	TTTTTCGG	ATAATGAG		Stewart et al 1990
<i>boIFNT1C</i>	ACAGGAAG	TGAGAGAGAA	TTTTTCGG	ATAATGAG		Ealy et al 2001
<i>boIFNT2B</i>	ACAGGAAG	TGAGAGAGAA	TTTTTCGG	ATAATGAG		Ealy et al 2001
<i>boIFNT3B</i>	ACAGGAAG	TGAGAGAGAA	TTTTTCGG	ATAATGAG		Ealy et al 2001
<i>boIFNT-c1</i>	CCAGGAAG	TGAGATAGAA	TTTTTGG	ATAATGAA		Chung & Seidel 2000
<i>ovIFNT-p7</i>	ACAGGAAG	TGAGGAGGA	TTTTTGG	ATAATGAG		Leaman & Roberts 1992
<i>ovIFNT-010</i>	ACAGGAAG	TGAGGAGGA	TTTTTGG	ATAATGAG		Nephew et al 1993
<i>Goat IFNT</i>	ACAGGAAG	TGAGGAGGA	TTTTTCGG	ATGATGAG		Leaman & Roberts 1992
<i>Giraffe IFNT</i>	ACAGGAAG	TGAGAGAGAA	TTTTTGG	ATAATGAG		Liu et al 1996
	ETS2	AP1/ GATA		DLX3		
<i>boIFNW</i>	TACAAGTACACAGAAATGACTAGAAAATGAAAC					Leaman & Roberts 1992
<i>ovIFNW</i>	TAAAAGTGCATAGAAATGACTAGAAAATGAAAC					Leaman & Roberts 1992

Figure 3 Sequence alignment of upstream regulatory regions (–79 to –46) of *IFNT* and *IFNW* genes from various species, including bovine (bo) and ovine (ov). The *IFNT* sequences contained a conserved *ETS2*-binding site (blue), putative AP1-binding site (orange) or GATA-binding site (green) and *DLX3*-binding site (yellow). The corresponding regions of *IFNW* genes do not contain such sequence motifs. The references for the sequences are *boIFNT1A* (Hansen *et al.* 1991); *boIFNT* (Stewart *et al.* 1990); *boIFNT1C*, -2B and -3B (Ealy *et al.* 2001); *ovIFNT-p7*, goat *IFNT*, *boIFNW* and *ovIFNW* (Leaman & Roberts 1992); *ovIFNT-o10* (Nephew *et al.* 1993); and giraffe *IFNT* (Liu *et al.* 1996). The *boIFNT-c1* sequence is direct submission to GenBank (AF238613) by Chung and Seidel in 2000. A thymine (T) at –66 (bold letter) is unique to the *boIFNT-c1* gene that makes a putative GATA binding site but the base is not conserved in other *IFNT* genes.

Figure 4 Cooperation of ETS2 and DLX3 in the transactivation of *IFNT*. The -457 *boIFNT1A* construct was transfected into choriocarcinoma JAr cells in either the absence (-) or presence (+) of the DLX3 or ETS2 expression plasmids. Values on Y axis represent fold activations with the basal activity of the -457 reporter set as 1. Obtained from Ezashi *et al.* (2008).

red arrow) that, when mutated, reduced *IFNT* expression to that of mock-transfected cells. Interestingly, the -70 to -63 site overlaps the previously reported AP1 site (-71 to -64) adjacent to the ETS2-binding site at -79 to -70 (Ezashi & Roberts 2004) (Fig. 2B). However, the generality of these data implicating GATA2 and GATA3 in control of *IFNT* expression should be treated with caution. First, the positive effects of GATA2 were only noted in a non-trophoblast cell line. Second, of all the ovine and bovine genes studied, only bovine *IFNT-c1* has the unique thymine (T) at -66 instead of the common guanine (G) present in other *IFNT* genes (Fig. 3). Finally, an introduced GATA site mutation also disturbs the AP1-binding site sequence (Fig. 2B). Clearly, further studies are needed to address whether GATA factors directly interact with the *IFNT* regulatory regions or even modulate *IFNT* promoter activity in trophoblast. Another possibility is that they act indirectly through other GATA2 and -3 regulated genes, such as DLX3 (Home *et al.* 2017).

CDX2

Caudal-type homeobox transcription factor CDX2, like ETS2, AP1, DLX3 and GATA2 and -3, plays an essential role in trophoblast lineage emergence and maintenance in the mouse and possibly other species (Strumpf *et al.* 2005, Ralston & Rossant 2008, Roberts & Fisher 2011). When the relative expression of *IFNT*

Figure 5 (A) RT-PCR analysis of *IFNT*, *CDX2* and *EOMES* expressions in bovine conceptus at days 17, 20 and 22 by Sakurai *et al.* (2013a). Amplicons from *ACTB* served as loading controls. (B) CDX2-knockdown (KD) bovine embryos were compared by *IFNT* expression (Y axis) and embryo size (X axis) to matched nuclear transfer (nt) and wild-type (wt) embryos by Berg *et al.* (2011). *CDX2* expression was decreased by 72% in the KD embryos. Regression lines are solid for CDX2-KD nt, dashed for nt control and dotted for wt control embryos. Wt and nt generated control embryos expressed *IFNT* in proportion to their embryo size in length. Smaller sized CDX2-KD nt embryos expressed *IFNT* in amounts comparable to much larger WT embryos.

and *CDX2* transcripts were compared in ovine (days 14–20) and bovine (days 17–22) conceptuses during the peri-implantation period, roughly similar expression patterns over time were observed (Sakurai *et al.* 2010, 2013a) (Fig. 5A), suggesting a possible link between the two. Perhaps of note, ovine *CDX2* expression became undetectable around day 20 at a time coincident with trophoblast attachment to the uterine epithelium and ovine *IFNT* silencing (Helmer *et al.* 1987, Ealy *et al.* 2001). Some co-transfection experiments performed on human choriocarcinoma JEG3 cells have also suggested a possible role for *CDX2* in the transactivation of *IFNT* genes (Imakawa *et al.* 2006). There is also an indication that *CDX2* in combination with a number of other transcription factors, including ETS2 and AP1 might also be capable of upregulating endogenous *IFNT* gene transcription in non-trophoblast bovine ear-derived fibroblast cells, which do not normally express either *IFNT* or *CDX2* (Fig. 6A) (Sakurai *et al.* 2009, 2013b). Possibly *CDX2* expression contributes to a partial trans-differentiation of the mesodermal fibroblast cells to trophoblast-like and a permissive environment for low levels of *IFNT* transcription.

Figure 6 (A) Bovine *IFNT1*-reporter activity with *CDX2*, *ETS2*, *JUN* (AP1) and *CREBBP* expression plasmids. The plasmids were transfected in bovine ear-derived fibroblasts. Copied from Sakurai *et al.* (2013b). (B) 62% *CDX2* reduction by siRNA in CT-1 cells reduces expressions of some genes including *IFNT* but increases *ETS2*. Copied from Schiffmacher and Keefer (2013).

Some confusion exists as to the location of the site of *CDX2* interaction with the 5'UTR of *IFNT*. One, apparently functional site, was reported between -581 and -575bp in an ovine *IFNT* gene (Imakawa *et al.* 2006). Later, potential *CDX2*-binding sites were noted in a more proximal site at -248 to -47bp of *boIFNT-c1* (Sakurai *et al.* 2009) and between -414 and -235bp in an ovine *IFNT* (Sakurai *et al.* 2010). *CDX2* knockdown in bovine trophoblast BT-1 (Sakurai *et al.* 2009) and CT-1 cells (Schiffmacher & Keefer 2013) (Fig. 5B) has also implicated a role for *CDX2* in controlling *IFNT* expression.

However, other investigators have presented contrasting interpretation on the effects of *CDX2* knockdown. For example, no effect was noted in with day 7.5 bovine blastocysts (Goissis & Cibelli 2014). Essentially negative results were also reported with day 14 conceptuses (Berg *et al.* 2011). For example, when *CDX2*-knockdown conceptuses were transferred to the uteri of synchronized recipient cows and recovered a week later, they were significantly shorter in the size than controls. However, these treated conceptuses expressed *IFNT* at levels equivalent to that of the control conceptuses (Fig. 5B). From these results, it would appear that *CDX2* is required for trophoblast development but not for *IFNT* expression. Clearly, further studies are required if this conundrum is to be solved.

Multiplicity of *IFNT* genes

Multiple *IFNT* genes exist in most ruminant species studied (Leaman & Roberts 1992). Those expressed in cattle have been categorized into three groups (Ealy *et al.* 2001, Walker *et al.* 2009), and it is clear that not all these genes are expressed equivalently in the same conceptus (Walker *et al.* 2009) and that relative expression of each can change over development (Walker *et al.* 2009, Sakurai *et al.* 2013b). Yet, most known *IFNT* genes have highly conserved sequences within the *ETS2*–*AP1*–*DLX3* enhancer region (-79 to -46) (Fig. 3). The sequences in Fig. 3 do not include those of ovine *IFNT* that are poorly expressed and contain a mutation in the *ETS*-binding site (Ezashi *et al.* 1998, Leaman & Roberts 1992, Nephew *et al.* 1993). All genes in the list contain the *ETS2*, the putative *AP1* and the *DLX3*-binding sites. However, the presence of a *GATA*-binding site is limited to the bovine *IFNT-c1*. Note also the lack of conservation in the corresponding region of *IFNW*. Little is known about the function of the latter, although more than 20 apparently functional *IFNW* are found in the bovine genome (Walker & Roberts 2009).

Mechanisms that upregulate *IFNT* gene transcription following blastocyst formation

IFNT is not secreted by morula stage conceptuses and is only modestly produced at the day 8 blastocyst

stage, but levels of its mRNA and total IFN production increase markedly as the conceptus begins to expand and then elongate (Farin *et al.* 1990, Kubisch *et al.* 1998, Ealy *et al.* 2001). ETS2, the master regulator of *IFNT* transcription, is expressed in trophoblasts of E5.0 stage mouse embryos and is a necessary factor for placental development (Yamamoto *et al.* 1998, Georgiades & Rossant 2006), TS cell (Wen *et al.* 2007, Kubaczka *et al.* 2015) and transcription of key placental hormones (Ghosh *et al.* 2003, 2005, Das *et al.* 2008). The initial low *IFNT* expression per cell in blastocyst stage ovine and bovine conceptuses has been speculated to be due to suppression by persistence of the POU domain class 5 transcription factor 1 (POU5F1 often known as OCT4) (Ezashi *et al.* 2001) that is expressed in early ruminant and primate trophoblast (Van Eijk *et al.* 1999, Niakan & Eggan 2013) (Fig. 7). Both CGA and CGB were efficiently silenced by overexpressing *POU5F1* in choriocarcinoma cells (Liu & Roberts 1996, Liu *et al.* 1997). We initially proposed that POU5F1 silenced *IFNT* by *quenching*, i.e., the silencer (=POU5F1 in this case) interferes with the ability of the DNA-bound transactivator (=ETS2) to interact with the basal transcriptional machinery (Ezashi *et al.* 2001). However, a more recent study of CGA silencing (Gupta *et al.* 2012) demonstrated a *squelching* mechanism, i.e., POU5F1 sequesters free ETS2 and prevents ETS2 from binding to the DNA. It can be speculated that this mechanism also operates for *IFNT* (Fig. 7). As trophoblast differentiation proceeds, POU5F1 becomes reduced in amount, and other trophoblastic gene regulators such as DLX3, CDX2 and GATA2/3 become upregulated. However, there are some conflicting observations that counter this hypothesis. When, for example, *POU5F1* was overexpressed in CT-1 cells, *IFNT* expression appeared not to be suppressed (Schiffmacher & Keefer 2013). In that experiment, overexpressed POU5F1 only changed *ASCL2* expression among the cohort of genes examined.

Because so few changes in trophoblast gene expression were observed when >60% of *POU5F1* transcripts had been knocked down, it remains unclear whether the expressed POU5F1 levels play a role in the gene regulatory network of CT-1 cells.

Regulatory mechanisms that downregulate IFNT gene transcription

Following the dramatic, short-term increase in *IFNT* transcription during the elongation phase of development, the expression of these genes is rapidly downregulated when the trophoblast attaches to the uterine epithelium (Helmer *et al.* 1987, Ealy *et al.* 2001). Precisely what mediates these events is unclear. However, downregulation of *in vivo IFNT* gene could be mimicked in CT-1 cells when the cells were cultured with bovine uterine epithelium cells (Sakurai *et al.* 2013a). The transcription factor T-box protein eomesodermin (EOMES) has been implicated in this silencing phenomenon, in part because its expression increases in trophoblast following the conceptus attachment (Fig. 5A) (Sakurai *et al.* 2013a). One explanation is that EOMES associates with the coactivator CREBBP and reduces its binding to AP1 (Xu *et al.* 2003), thereby disrupting transactivation of *IFNT* by ETS2/AP1 (Sakurai *et al.* 2013a) (Fig. 7). There are also suggestions that the relocation of TEAD2/4 from the nucleus to the cytoplasm leads to the downregulation of *IFNT* expression (Kusama *et al.* 2016), but this observation needs to be confirmed in future studies.

Epigenetic regulation

There is some limited evidence that epigenetic as well as genetic mechanisms might regulate *IFNT* expression. For example, endogenous *IFNT* expression was increased in bovine skin fibroblasts when CDX2 was overexpressed

Figure 7 Summary of speculative models for *IFNT* transcriptional control at prior to conceptus elongation (left), elongation phase (middle) and implantation (right). Before conceptus elongation, presence of POU5F1 in the early stage trophoblast suppresses *IFNT* expression through squelching ETS2. When the conceptus elongates, trophoblastic gene regulators DLX3, CDX2, and GATA2/3 become upregulated. Growth factors released from maternal endometrium activate MEK/ERK and PKA signaling pathways and increase *IFNT* expression through the transcription factors. When the trophoblast firmly attaches to the uterine epithelium in the implantation stage, the *IFNT* expression is down-regulated. The silencing is accompanied by EOMES association with CREBBP coactivator and reduction of histone acetylation of the *IFNT* gene upstream region.

and the cultures were treated with a histone deacetylase inhibitor (Sakurai *et al.* 2010). Further evidence came from chromatin immunoprecipitation (ChIP) assays performed on elongating ovine conceptuses (days 14, 16, 16.5 and 20) to determine the acetylation status of histone H3 at the lysine-18 residue (H3K18) (Sakurai *et al.* 2010). Elevated H3K18 acetylation of the ovine *IFNT* gene was observed on days 14 and 16 but declined when the conceptus had initiated attachment to the uterine wall (Fig. 7). The authors also inferred H3K18 association with the –414 to –235 bp of the 5'UTR and the CDX2-binding sites (–300 to –294 bp and –293 to –287 bp) within the ovine gene (Sakurai *et al.* 2010). In addition, CDX2 overexpression in bovine ear-derived fibroblast cells increased histone acetylation and led to concurrent recruitment of CREBBP, a nuclear factor that has histone acetyltransferase activity. Acetylation of H3K18 was also detected in the proximity of the CDX2-binding region of a bovine *IFNT* gene in IFNT-secreting CT-1 cells. Conversely, when *CDX2* expression was inhibited by a small interfering RNA, H3K18 acetylation was decreased (Sakurai *et al.* 2010). These findings suggest that CDX2 regulates *IFNT* expression through CREBBP recruitment, which leads to greater H3K18 acetylation at CDX2-binding sites.

Maternal factors and intracellular signaling that control *IFNT* expression

Secretions from maternal endometrial glands including hormones and growth factors may play a promotive role for both induction of *IFNT* expression and conceptus growth (Gray *et al.* 2002, Satterfield *et al.* 2006, Roberts *et al.* 2008). For example, IFNT production from cultured blastocyst can be increased by supplementation of the medium with uterine flushing (Kubisch *et al.* 2001), which are known to contain colony-stimulating factor 2 (CSF2 also known as GM-CSF) (Imakawa *et al.* 1993), interleukin 3 (IL3) (Imakawa *et al.* 1995) and fibroblast growth factor-2 (FGF2) (Michael *et al.* 2006, Ocon-Grove *et al.* 2008, Rodina *et al.* 2009). Each of these cytokines can individually increase transcription from *IFNT*, presumably through cell signaling pathways initiated by the cognate receptor complexes. For example, CSF1, which is also present in uterine secretions, and its cognate receptor CSF1R (c-fms), expressed in the bovine trophoblast, can both exert MEK/ERK pathway transduction and increase *IFNT* expression (Ezashi & Roberts 2004) (Fig. 7). Additionally, overexpression of the catalytic subunit of protein kinase A (PKA) in combination with ETS2 overexpression induced a 500-fold upregulation of *IFNT* (Das *et al.* 2008). Presumably, growth factors released from maternal endometrium in combination with PKA, MEK/ERK and PKC signaling pathways and a combination of necessary transcription factors can

provide the massive upregulation of IFNT observed in the elongating conceptus (Fig. 7).

Conclusion

The *IFNT* genes diverged from those encoding its closest type I IFN family, *IFNW*, about 36 million years ago when the ruminant species started to emerge within the artiodactyl order (Roberts *et al.* 1997) and was probably initiated when a progenitor *IFNW* gene exchanged in some manner its viral response elements for ones that favored trophoblast expression. The acquired transcriptional control elements drive *IFNT* expression in trophoblast, which is then sustained over several days without viral infection (Roberts *et al.* 2008). The studies on *IFNT* gene regulation first focused on what regions of the gene were responsible for trophoblast expression and what transcription factors were involved. Later studies emphasized the role of external factors and signaling pathways and why the high-level expression became silenced after only a few days. More recently, emphasis has switched to epigenetic regulation. In particular, it was noted that many of the transcription factors that regulate trophoblast emergence and development elucidated in mouse studies play a role in trophoblast-specific *IFNT* gene expression. The studies confirm that there is a core group of transcription factors that probably operate across all mammals to initiate trophoblast emergence. However, these same factors may also guide unique aspects of the trophoblast phenotype, such as production of hormones and other signature events that are peculiar to a particular taxonomic group. Representative findings of *IFNT* gene control are summarized in three stages of conceptus development (Fig. 7). Modest *IFNT* expression in early trophoctoderm prior to conceptus elongation can be explained by a presence of POU5F1, which suppresses ETS2 transactivation. Species-specific onset patterns of trophoblastic gene expression, including *IFNT*, might be linked to a time length of persisted POU5F1 protein in trophoblasts that is very limited in rodents. Large increases of *IFNT* expression in the elongation phase appears to be combinatory by upregulated trophoblastic gene regulators (DLX3, CDX2, GATA2/3) and growth factors released from maternal endometrium that activate MEK/ERK and PKA signaling pathways in trophoblasts. The downregulation mechanism of *IFNT* expression at implantation phase has been anticipated by decreased histone acetylation of the regulatory region of *IFNT* gene and the association of EOMES and CREBBP that lead to disruption of a transactivation complex of *IFNT* gene.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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