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Regulation of IFN-τ Gene Expression by Uterine Factors: Interaction between the Conceptus and Maternal Environment During Early Pregnancy in Cattle and Sheep

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Abstract. Interferon-tau (IFNT) production is essential for the survival of the conceptus during early pregnancy in ruminant species. Early, rapid elongation of the trophoblast and the massive up-regulation of IFNT production from trophectoderm occur in parallel, vary seasonally and between individual animals, and probably depend on factors released in maternal uterine secretions that are induced in response to rising maternal serum progesterone levels. Growth factors in the uterine secretions are assumed to up-regulate signal transduction pathways that target IFNT genes (*IFNTs*) through activation of the transcription factor, Ets2. The latter, which is essential for functional differentiation of the mouse placenta, plays a central role in the transcriptional regulation of *IFNT*, and operates through an Ets2 binding element (-79 to -70) in the proximal promoter region of all known functional *IFNTs*. The Ets2-binding sequence overlaps an AP1 binding site (-71 to -64) and is two helical turns distal to a sequence (-54 to -46) that binds the homeobox transcription factor, Dlx3. The Ets2/AP1 enhancer element is required for activation for *IFNT* promoters via the Ras/MAPK pathway. Ets2 and Dlx3 act synergistically to drive *IFNT* promoter expression, and this combinatorial effect is massively increased by over-expression of a constitutively active form of PKA. Our model suggests that *IFNT* are activated by uterine growth factors operating through the MAPK and PKA pathways targeting the gene control elements associated with Ets2, Dlx3 and AP1 binding sites. It emphasizes the importance of a complex Ets-2 enhancer for expression of *IFNT* and suggests a means whereby the mother can exert control over conceptus growth and IFNT production, thereby coordinating the activity of the conceptus with her own physiological state.

Key words: Interferon-tau, Ets2, Dlx3, AP1, Protein kinase A, Mitogen-activated protein kinase

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Interferon-tau (IFNT) is a major signal for maternal recognition of pregnancy in the true ruminant species, such as cattle, sheep and deer. It plays a crucial role in maintaining pregnancy by suppressing the pulsatile release of the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterine endometrium and preventing the regression of the maternal corpus luteum (CL) [1–6]. IFNT is the major secretory product of conceptuses [7, 8] between day 13 and 21 of pregnancy in sheep [9, 10] and between day 14 and 24 in cattle [11, 12]. IFNT expression is confined to the trophectoderm, but its production is remarkably high, with a single, *in vitro* cultured, day-15 ovine conceptus able to release more than 100 μg/day of IFNT protein in 24 h [13–15].

In all ruminants so far examined, there are multiple IFNT genes (*IFNT*) [16-18]. In cattle they appear to be clustered with other type 1 IFN genes on chromosome 8. These genes are not expressed equivalently, and only a select few may be strongly transcribed [19, 20]. *IFNT* expression as assessed by Northern blotting [12, 21–23], RNase protection

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assays [20], *in situ* hybridization [12, 24, 25] and protein production [15, 26] is massively higher during the early phase of conceptus elongation than either earlier in development, e.g. at blastocyst formation, [27, 28] or after attachment to the uterine wall is initiated. It has been estimated that *IFNT* gene transcription may be up-regulated more than 1000 fold during the period when the conceptus first begins to elongate [6]. The regulation of *IFNT* is quite unlike that of other type 1 IFN, such as IFNA, since 1) the genes are not virally-inducible [29, 30]; 2) expression is confined to trophectoderm [12, 24, 25], and 3) although dynamic, *IFN* expression is sustained at extremely high levels over the several days when CL rescue occurs [6]. In this article we address the trophoblast specific regulatory control of *IFNT* responsible for these unique features of gene expression and implicate factors in the maternal uterine environment as agents that not only regulate *IFNT* promoter activity but coordinate communication between the conceptus and the mother.

Transcription Factors Involved in the Regulatory Control of *IFNT*

Considerable evidence has accumulated to suggest that the transcription factor, Ets2, through its interaction with the proximal promoter region (-79 ACAGGAAGTG -70) of *IFNT* is the key transcription factor governing *IFNT* expression in trophectoderm [31, 32] (Fig. 1). Over-expression of Ets2 in JAr choriocarcinoma cells generally increases reporter gene activity driven by *IFNT* promoters containing the Ets2-binding element 10- to 50-fold or more, depending upon the transfection conditions, while mutation of that element largely reverses this effect [31–33]. Ets2, however, does not operate alone. As discussed below, it cooperates with at least two other transcription factors, Dlx3 and AP1 and responds to in-put from at least two signal transduction pathways to exert control over IFNT expression [33, 34]

Ets2 is a member of a large family of transcription factors that are characterized by their conserved Ets DNA-binding domain, which binds to a specific DNA motif, characterized by a core GGAA sequence (Fig. 2D). Importantly, not all sequences carrying GGAA either bind or respond to Ets2, and so the flanking sequences are crucial for providing specificity and protein-binding affinity. Ets2 is also essential for full placental development in the mouse, with deletion of the *Ets2* gene leading to embryonic mortality before day 8.5 of embryonic development [35]. Several ovine *IFNT* "pseodogenes" that are poorly expressed lack a consensus Ets2 binding sequence in the proximal promoter element [31]. Ovine *IFNT-o10* carries an Ets2 binding site that has identical sequence to the one in the bovine (Fig. 2D). In contrast, expression from some ovine *IFNT*, e.g. ovine *IFNT-o2* and *IFNT-o8* are extremely low. Both these genes carry mutations in the Ets2 binding site [19]. Replacement of the "mutated" and presumably defective Ets2 binding site in *IFNT-o8* with a 22 bp from the homologous region of *IFNT-o10*, restores full promoter activity [36]. Further evidence for the key involvement of Ets2 in *IFNT* expression is its presence in spherical, ovoid and filamentous bovine blastocysts at levels seemingly correlated with *IFNT* activity in the tissue [37] and nuclear localization in trophectoderm cells of ovine conceptuses during the period of peak IFNT production[31]. Clearly, Ets2 is in the right place and at the right time to influence *IFNT* expression.

Ets2 is known to up-regulate "signature" trophoblast genes of various species, including the genes for human chorionic gonadotropin-alpha (*CGA*) [38]and beta (*CGB*) [39, 40], matrix metalloproteinase-9 (*MMP9*) (gelatinase-B) [41], -3 (*MMP3*; stromelysin) [42, 43], and -1 (*MMP1*; collagenase 1) [44, 45], urokinase-type plasminogen activator (*uPA*) [46, 47], ovine P-450 side chain cleavage enzyme (*CYP11A1*) [48], porcine pregnancy associated glycoprotein 2 [49], rat placental lactogen II (r*PLII*)[50], and rat prolactin-related protein [51] (Fig. 1). These data suggest that Ets2 controls the expression of many genes associated with fully competent trophoblast and that it might be implicated in driving the differentiation process itself.

Although deletion of the murine *Ets2* gene leads to placental failure and embryonic death, possibly as the result of defects in the development of the ectoplacental cone and reduced expression of matrix metalloproteinases [35, 52], Ets2 is expressed widely in the adult mouse, just as it is in adult bovine tissues (see GenBank Accessions BE758238.1, AW668843.1; BE479476.1). Moreover, Ets2 continues to be expressed in bovine

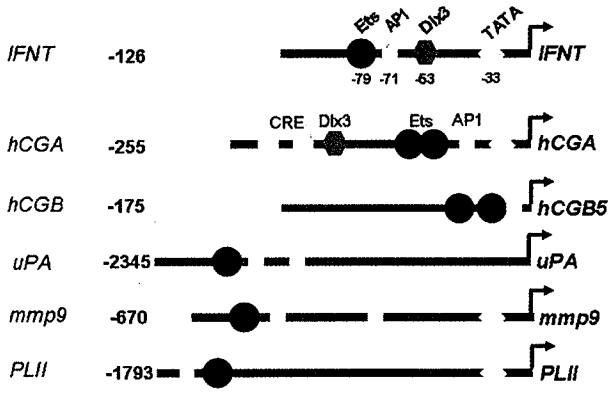
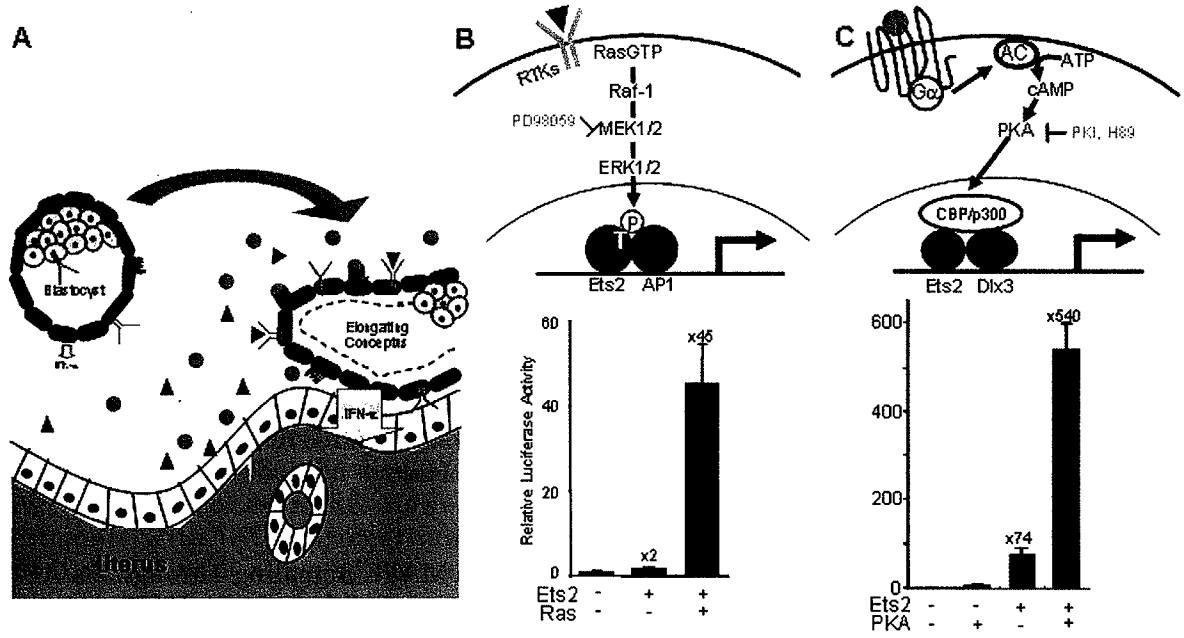


Fig. 1. Schematic presentation of the structures of the gene regulatory region that drive trophoblast specific expression of *IFNT* and other trophoblast-expressed genes. Shown are the important Ets (●), AP1 (■) and Dlx3 (hexagon) binding sites as well as the TATA box (◊). The figure compares (*top to bottom*) bovine *IFNT1* [33, 34], human *CGA* [38] with CREs (□), human *CGB* [40], human *uPA* [41, 105], human *MMP9* [41, 106] and rat *PLII* [50].



D

	-81	Ets2	AP-1	Dlx3	TATA -28
boIFNT1	aa**acaGGAAgTGAGaga**gaaattttcgg**aTAATgag**taccgtcttccc**TATTTA**				
oIFNT-o10	aa**acaGGAAg**TGAGgaggaatgtttggaTAATgagtaccgtcttccc**TATTTA**				

Fig. 2. (**A**) Depiction of conceptus development and *IFNT* expression. *IFNT*s are expressed within the first epithelium (trophectoderm) and not in the ICM of the conceptuses of ruminant ungulate species from the time that of blastocyst hatching to when the trophoblast attaches to the uterine wall. IFNT can first be detected around the time of blastocyst formation and production becomes high only as the conceptus begins to elongate. Growth factors (▲) and hormones (●) are synthesized by the uterine epithelium during pregnancy, and may be targeted to receptors resident on trophectoderm. (**B, C**) Models for *IFNT* gene regulation through the Ras/MAPK (**B**) and cAMP/PKA (**C**) signaling pathways. Many activated receptor tyrosine kinases (RTKs) stimulate Ras/MAPK signaling, which can lead to the phosphorylation of downstream targets, including Thr72 of Ets2. The bar graph shows activity of a transfected bovine *IFNT1*-luciferase (*luc*) reporter gene containing the entire -126/+50 gene control region and the stimulatory effects of Ets2 and Ras over-expression in 3T3 cells (see [33]. (**C**) The binding of a hormone to its G-protein-linked receptor leads to the activation of adenylyl cyclase (AC), an increase in intracellular cAMP, and activation of PKA. How this signal transduction pathway targets Ets2 is unclear, but there appears to be a requirement for a co-activator (probably p300 or its close relative CBP) [81]. CBP involvement in *IFNT* regulation has also been reported by others [107]. The bar graph shows the stimulatory effects of Ets2 and activated PKA expression on -126*luc* reporter activity in JAr cells. The -126*luc* reporter was co-transfected with expression plasmids for Ets2 and activated PKA (alone or in combination) into JAr cells. In both B and C, luc activity was normalized relative to β-galactosidase activity from the reference reporter pRSVLTR-βgal. The data are expressed as fold activation (means ± SEM; n=3) relative to controls. (**D**) Comparison of the proximal promoter sequences of bovine (*boIFNT1*) [108] and ovine IFNT (*oIFNT-o10*) [19]. The characterized regulatory elements are colored and the core sequences are bold.

trophoblast after *IFNT* have been down-regulated (Anindita Chakrabarty & R Michael Roberts, submitted manuscript). Since IFNT production is restricted to a single epithelial cell layer for just a few days in early pregnancy, Ets2 cannot be the only transcription factor involved in its regulatory control. As with most known genes, expression is dependent upon several transcription factors acting combinatorially.

One such transcription factor that appears to be a key player in controlling IFNT gene transcription is the homeobox protein, Distal-less 3 (Dlx3), a member of the Distal-less family of non-Antennapedia homeobox genes [53–56]. Dlx3 binds to a sequence (–54 GATAATGAG –46) in the *IFNT* proximal promoter two helical turns proximal to the Ets2-binding site [34] (Fig. 1, Fig. 2D). It is expressed in human, mouse and bovine trophoblast cells [34, 54–56], and *Dlx3 -/-* mice die between embryonic day 9.5 and 10 because of placental defects [54]. Dlx3 has a regulatory role in the expression of at least two other trophoblast-expressed genes, *hCGA* [56] and the one encoding murine 3β-hydroxysteroid dehydrogenase VI (3βHSD VI) [57], that are essential for survival of a pregnancy. There seems little doubt that Dlx3, although little studied, contributes in an important way to trophoblast function.

Over-expression of Dlx3 by transfection transactivates bovine *IFNT1* promoter/reporter constructs by about 20-fold in human JAr cells [34], well in excess of its ~2-fold effect of Dlx3 on the *hCGA* promoter in JEG3 cell line [56]. Interestingly, this effect is largely abolished if the neighboring Ets2-binding site is mutated. Co-expression of Dlx3 and Ets2 up-regulates wild type *IFNT*-luciferase constructs around 250-fold, indicating that the two transcription factors act synergistically to regulate *IFNT* [34].

Efficient transactivation by Ets transcription factors often requires the presence of an AP1 binding site adjacent to the Ets-responsive region in the control region of the targeted gene, which appears to be important for providing Ras-reponsiveness [58, 59]. Such complex enhancers have been observed for *uPA* [41, 46], *MMP-1, -3, -7, -9,* and *-13* [60, 61], and r*PLII* [50], all of which are characteristically expressed in trophoblast. As with the aforementioned Ets2-responsive genes, *IFNT* promoters also have a close-to-consensus AP1 site (-71 to -64), overlapping the functional Ets2-binding site (-79 to -70) (Fig.1, 2D). As discussed later in the paper, this Ets2/AP1element may play a role in allowing *IFNT* to react to maternally-produced factors that up-regulate IFNT production [33]. In particular, the AP1 binding site appears to be important for signaling through the Ras/MAPK signal transduction pathway. In common with Ets2 and Dlx3, at least some of the components of AP1 transcription factors are required for proper placental development in the mouse [62, 63].

Together these findings indicate that *IFNT* regulatory control requires several transcription factors that interact directly with the *IFNT* control region to bestow tissue specific expression and high expression capacity. Presumably, transcription rates will be governed by the availability of these and other transcription factors, including co-activators and repressors, and maximal output will only be achieved when the individual components are present in an optimal combination. These same transcription factors also have been implicated in the regulation of several signature genes of trophoblast in other species, e.g. *hCGA* in the human, indicating that the transcriptional control mechanisms may be well conserved across species. Finally, the same genes that confer *IFNT* expression appear to be required for functional differentiation of trophoblast.

Ras/MAPK Signaling Through Ets/AP1 Enhancer of the IFNT

Many transcription factors, probably the majority, must be modified covalently, e.g. by phosphorylation, in order to fulfill their function in gene regulation [64]. Frequently they are targeted by signal transduction cascades and hence responsive to the external environment of the cell. It is now becoming clear that hormones and growth factors secreted by the maternal endometrium may be required for the optimal production of IFNT (see Fig. 2A). For example, supplementation of culture medium for *in vitro*-produced blastocysts with uterine flushing from ewes in the secretory phase of the estrus cycle markedly raised the production of IFNT and also increased cell proliferation [27]. Since conceptus elongation occurs earlier in pregnant ewes that have a more rapid rise in progesterone during early pregnancy [65, 66] or that are administered supplementary progesterone

[67, 68], and these conceptuses produce IFNT earlier and in larger quantities than the smaller conceptuses flushed from control ewes, it seems likely that maternal uterine secretions contain factors that drive conceptus growth as well as increase IFNT production. Further evidence supporting this hypothesis comes from ewes with uteri that have failed to form normal glands, the main source of uterine secretions. Although such ewes can become pregnant, the conceptuses fail to elongate, produce little IFNT, and die [69].

Uterine flushings from either cattle or sheep contain a range of growth factors, among which are granulocyte-macrophage colony stimulating factor, GM-CSF [70–72], IGF-1 and -2 [73], CSF-1 [74], and FGF2 [75]. These and possibly others are clearly potential candidates for the factors that drive growth and IFNT production as progesterone concentrations rise during early pregnancy. Such growth factors trigger a range of different signal transduction pathways, including the PKC [76, 77], the Ras/MAPK [76, 78, 79] and the cAMP/PKA pathways [80–83]. We have chosen to concentrate on the latter two, since they are known to be capable of targeting Ets2.

Since composite Ets/AP1 binding sites have been identified as Ras-responsive enhancers (RRE) in various genes, we hypothesized the Ets/AP1 sites in *IFNT* would function as an RRE. However initial experiments with JAr choriocarinoma cells revealed no ability of activated Ras to increase either basal or Ets2-stimulated transcription from *IFNT* promoters [31]. One possible explanation was that JAr cells, which are derived from a tumor, already possessed a maximally activated Ras oncogene component [40]. Accordingly, we repeated the experiment in mouse 3T3 cells, which lack high endogenous Ras activity. In this case a combination of Ras and Ets2 over-expression resulted in a synergistic 50- to 100-fold increase in reporter activity from the bovine *IFNT* promoter [33] (Fig. 2B). Comparable data have been obtained with the *hCGB* promoter, which also possesses a potential RRE [40]. Not unexpectedly, mutation of Ets2 at Thr 72, the residue targeted by MAPK signaling [84], diminished *IFNT* reporter activity, as did the addition of PD98059, an inhibitor of the pathway.

Finally, *IFNT* promoter activity was up-regulated in response to CSF-1, a growth factor that acts through the Ras/MAPK signaling pathway, when the *IFNT* promoter was transfected into 3T3 cells expressing the CSF-1 receptor *c-fms* [33]. This response did not occur when either the AP1 site or the Ets2 binding sites were mutated. Nor was the response to CSF-1 observed in 3T3 cells expressing an inactive form of *c-fms* [33]. Taken together, these data suggested that the *IFNT* are responsive to Ras/MAPK, and that CSF-1 or some other maternal factor capable of activating the Ras/MAPK signaling pathway might direct conceptus *IFNT* expression during the critical time that the CL is wavering on the point of regression.

Imakawa and colleagues [85, 86] have drawn attention to the likely importance of an additional, upstream AP1 site (-654 to -555) in the transcriptional control of an ovine *IFNT* promoter and as a responsive site for phorbol 12-myristate 13-acetate (PMA) activation. This distal element is in a part of a region of *IFNT* genes that is poorly conserved across species and is not represented in the bovine gene control region used in our work. Although the *boIFNT1* carry AP1 or AP1-like binding sequences at -867, -695, -603, -403, -332, and -188, in addition to the one at the Ets/AP1 composite site, our experiments have failed to demonstrate that any of these except the most proximal (-71 to -64) is functional [31, 32, 87].

PKA Signaling through Ets2 and Dlx3 of the IFNT

The cyclic AMP signaling system is another key regulator of trophoblast differentiation and function [88]. Mononuclear cytotrophoblast cells aggregate and fuse to form multi-nucleated synctiotrophoblasts in the human placenta, and this behavior may be controlled by cAMP [89]. Single cytotrophoblast-like cells of the human choriocarcinoma (BeWo) cell line fuse and undergo extensive morphological differentiation to yield syncytia in the presence of a stimulator of adenylcyclase, forskolin, which leads to an increase in the intracellular levels of cAMP [89, 90]. Finally, although only about 5% of JAr cells normally produce measurable hCG, the addition of 8-bromo-cAMP increases the number of hCG-producing cells several fold, as well as stimulating hCG synthesis as a whole [91, 92]. These data suggest that the expression of the *IFNT*, like many other genes associated with trophoblast function, might

be regulated by the cAMP/PKA signal transduction pathway and hormones and growth factors that activate that pathway.

We have recently examined the role of the PKA signal transduction pathway in regulating *IFNT* expression through the activation of the key transcription factor, Ets2. Although overexpression of the catalytic subunit of PKA or the addition of 8-bromo-cAMP had a modest ability to up-regulate *boIFNT1* reporter constructs (usually no more than 4-fold), co-expression with Ets2 led to a large increase in gene expression, which, in the case of PKA, exceeded 500-fold [81] (Fig. 2C). In order to define which region of the *IFNT* promoter was responsible for the ability of PKA to act synergistically with Ets2 to provide the large up-regulation, progressive truncation of reporter constructs indicated that the responsive region lay between -126 and -67 (Fig. 1), lacked a classical CRE but included the Ets2/AP1 element (Fig. 1, Fig. 2D). Specific mutation of the Ets binding site reduced the PKA/Ets2 effects by more than 97%, whereas mutation of the AP1 binding site adjacent to the Ets2 site or pharmacological inhibition of the MEK2 kinase (PD98059) led to a doubling of the combined Ets2/PKA effects (to nearly 1000 fold), suggesting there is competition between the Ras/MAPK pathway and the PKA signal transduction pathway for Ets2 [81].

We then asked whether the Dlx3 binding site was important for the PKA effects. Mutation of the Dlx3 binding site reduced the PKA/Ets2 effects by about 87%, and activity was virtually abolished when both the Ets and Dlx3 binding sites were mutated (Das, Ezashi & Roberts unpublished results). These results suggest that bothDlx3 and Ets2 must be bound to the proximal promoter for PKA to activate transcription. We are currently studying how PKA exerts its effects. Our data indicate that Ets2 itself is not a substrate for PKA and are testing the hypothesis that the effect is indirect and involves one or more co-activators (Fig. 2C).

The PKA pathway plays a pivotal role in the expression of many trophoblast-specific genes, including *hCGA* and *hCGB*, *17β-HSD*, *CYP11A1* or *CYP19* [93–96]. Some of these genes, for example *hCGA* and *CYP19* have a classical cAMP response element (CRE) in their promoter region [97, 98], whereas others, such as *hCGB*., do not possess a such a CRE [39, 99]. Although Ets2 is not a well established target of the cAMP/PKA signal transduction pathway, 8-bromo-cAMP and over-expressed PKA also are able to up-regulate the *hCGB* promoter activity primarily through a previously unrecognized proximal Ets2 enhancer [40]. Interestingly, the Ets2 binding sites on the promoter for the *hCGB* partner gene, *hCGA*, are also crucial for mediating cAMP/PKA effects on *hCGA* gene expression [38]. Mutation of the Ets2 binding sites, for example, virtually abolishes cAMP responsiveness of *hCGA* and *hCGB* promoters. The concept that Ets2 is a target, albeit indirect, for PKA activation may explain why many genes lacking a CRE are responsive to cAMP and hence why they respond to endocrine factors that signal through the cAMP/PKA pathway.

Our observations clearly suggest that the Ets2/AP1 composite element is responsive to both the Ras/MAPK and cAMP/PKA signal transduction pathways and hence to factors that operate through these pathways. Interestingly, the two do not work together either additively or synergistically. Instead, we have noted that that inhibition of the MAPK pathway by MEK2 kinase inhibitors and mutation of the AP1 binding site adjacent to the Ets2 site significantly enhance PKA-dependent activation of the *IFNT* promoter [81]. Crosstalk is known to exist between these two signaling pathways [100, 101], and the inhibitor, PD98059, has been reported to up-regulate CRE-dependent gene activity [102]. In addition, activation of PKA tends to lower MAPK activity in cells [103], while inhibition of PKA increases growth factor-promoted MAPK activity [104]. One explanation for this phenomenon may be the ability of the cAMP pathway to target, and presumably inactivate, c-Raf, a component of Ras/MAPK signaling [101]. Although physiological significance of crosstalk between PKA and Ras/MAPK signalings in *IFNT* regulation remains unanswered, the process may help to balance the eventual output of IFNT in a manner that is consistent with maternal in-put into the process.

Together, these data suggest that factors present in uterine secretions are capable of up-regulating *IFNT* gene expression, thereby coordinating the growth and activity of the conceptus with the hormonal state of the mother. A reasonable inference is that these factors bind to receptors on the surface of trophectoderm and activate signal transduction pathways that up-regulate the already constitutively active *IFNT* (Fig. 2).

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