ISSN 2508-755X (Print) / ISSN 2288-0178 (Online)
J. Emb. Trans. (2018) Vol. 33, No. 4, pp. 195~203 https://doi.org/10.12750/JET.2018.33.4.195

Analysis of Bovine Interferon-tau Gene subtypes Expression in the Trophoblast and Non-trophoblast cells

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Abstract

Interferon-tau (IFNT) is known as a major conceptus protein that signals the process of maternal recognition of pregnancy in ruminants. Also, multiple interferon genes exist in cattle, However, molecular mechanisms of these bovine IFNT (*bIFNT*) genes whose expressions are limited have not been characterized. We and others have observed that expression levels of bovine subtype *IFNT* genes in the tissues of ruminants; thus, *bIFNT1* and other new type I (*bIFNTc1/c2/c3*) gene co-exist during the early stages of conceptus development and non-trophoblast cells. Its genes transcription could be regulated through *CDX2* and *ETS2* and *JUN* and/or cAMP-response element binding protein (CREB)-binding protein (*CREBBP*) expression, a transcription factor implicated in the control of cell differentiation in the trophectoderm. Bovine ear-derived fibroblast cells, were co-transfected with luciferase reporter constructs carrying upstream (positions -1000 to +51) regions of *bIFNT1* and other new type I gene and various transcription factor expression plasmids. Compared to each －1kb-*bIFNT1/c1/c2/c3*-Luc increased when this constructs were co-transfected with *CDX2*, *ETS2*, *JUN* and/or *CREBBP*. Also, Its genes was had very effect on activity by *CDX2*, either alone or with the other transcription factors, markedly increased luciferase activity. However, the degree of transcriptional activation of the *bIFNTc1* gene was not similar to that *bIFNT1/c2/c3* gene by expression plasmid. Furthermore, Sequence analyses also revealed that the expression levels of *bIFNT1/c2/c3* gene mRNAs expression were highest on day 17, 20 and 22 trophoblast and, Madin-Darby bovine kidney (MDBK), Bovine ear-derived fibroblast (EF), and endometrium (Endo) non-trophoblast cells. But, *bIFNTc1* mRNA had not same expression level, *bIFNTc1* lowest levels than those of *IFNT1/c2/c3* gene in both trophoblast and non-trophoblast cells. These results demonstrate that bovine subtype *bIFNT* genes display differential, in the trophoblast and non-trophoblast cells.

Received : 28 November 2018
Revised : 17 December 2018
Accepted : 19 December 2018

Key Words : Interferon-tau, Ruminants, Transcription factors, Trophoblast

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INTRODUCTION

Interferon-tau (IFNT) is known as a major conceptus protein that initiates the process of maternal recognition of pregnancy in ruminant ungulates (Godkin et al., 1982; Imakawa et al., 1987; Roberts et al., 1992). The expression of *IFNT* is subject to temporal and spatial limits since its production is restricted to TE during peri-implantation period (Farin et al., 1989; Guillomot et al., 1990; Demmers et al., 2001). *IFNT*, a major cytokine involved in the process of maternal recognition of pregnancy, is secreted into the uterine lumen by the mononuclear trophectoderm of the conceptus (Roberts et al., 1992; Imakawa et al., 2004). This factor is secreted from the embryo to mother around day 12 and the timeing of the signal production is very critical. Martal and coworkers have identified that days 14-16, not day 25 conceptuses. During the peri-implantation period of pregnancy in sheep, endometrial activity and their epithelial secretions are largely regulated by progesterone from the corpus luteum(CL) and cytokines/hormones from the conceptus such as interferon tau (*IFNT*) (Spencer et al., 2004, 2007).

IFNs are divided into two groups, sud-resistant type I and acid-sensitive type II *IFNs* (Peskta et al., 1987). Type I *IFNs* is induced by virus infection and includes two families, *IFNA* and *IFNB*, which are produced by leukocytes and fibroblasts, respectively. *IFNG* is the only member in type II *IFN* and produced by T cells or NK cells under mitogen treatment. This conceptus *IFN* consists of four cysteine residues, which are conserved across Type I *IFN*, and therefore this *IFN* belongs to a family of type I *IFN*. However, the observations that this *IFN* is not secreted by blood cells and more importantly, it can be serologically distinguished from other Type I *IFNs*, lead to a new classification, ovine *IFNT* (Roberts et al., 1992; Yamaguchi et al 2001). *IFNA* consists of two subtypes as *IFNAI* and *IFNAII* (=*IFNW*). *oIFNT* cDNA shares 55-65% similarity to *IFNAI* and is the closest to *IFNW* with approximately 85% identity (Imakawa et al., 1987). *IFNT* gene was originated from a single duplication event from an *IFNW* gene about 36 million years ago in the mammalian lineage, leading to the present day pecoran ruminants, a suborder comprised of cattle, deer, giraffes and their relatives (Robertset al., 1997; Roberts et al., 1998). It has been continued to duplicate since then, and it has been estimated that there may be as many as ten *IFNT* in bovine, with all of them clustered within or in close proximity to the genetic locus that contains the other Type I *IFN* genes (Ryan et al., 1993). Presently, multiple ovine and bovine *IFNT* are transcribed during early pregnancy and encode proteins that can possess different biological activities (Ealy et al., 1998; Winkelman et al., 1999). But it has remained unclear how many are expressed, the extent of their variation, and whether different genes exhibit similar patterns of expression and code for proteins with similar biological activities. In ruminant ungulates, large amount of an anti-luteolytic substance secreted bythe bovine conceptus and primarily responsible for maternal recognition of pregnancy is bovine trophoblast protein-1 (*bIFNT1*), a new type I (*bIFNTc1*, *bIFNTc2*, and *bIFNTc3*) genes (Kim et al., 2016). However, molecular mechanisms of these *bIFN*T genes have not been characterized because of their expressionlevel. The objectives of the present study were, therefore, to determine the differences in *IFNT* gene expression, to identify differences in *IFNT* genes expression level, and trophoblast of cattle during the peri-implantation period and non-trophoblast cells.

MATERIALS AND METHODS

Animals and experimentation and sampling

Estrous synchronization, super-ovulation and embryo transfer processes were performed as previously described (Ideta et al., 2007). Seven-day embryos (Day 0=day of estrus) were collected from superovulated cattle. Twelve embryos derived from the superovulation were transferred nonsurgically into the uterine horn of three Holstein heifers (n=4 each), ipslateral to the CL on the day 7 of the estrous cycle. Elongated conceptuses were collected nonsurgically by uterine flushing on the days 17, 20, or 22.

DNA isolation and plasmids construction

Genomic DNA was isolated from pooled bovine trophoblast tissues using the Genomic DNA Purification Kit (Promega, Madison, WI), according to the protocol provided by the manufacturer. The quality and integrity of genomic DNA was determined by agarose gel (1%) electrophoresis and visualization under UV light after ethidium bromide staining. Bovine genomic DNA isolated from conceptus tissues was used as the template for amplifying the *IFNT* genes. In short, the upstream regions (positions -1000 to ☐51) of *bIFNT1*, *bIFNTc1*, *bIFNTc2*, and *bIFNTc3* (GenBank accession numbers *bIFNT1*; M60903: *bIFNTc1*; AF238613: *bIFNTc2*; AF238612:*bIFNTc3*; AF238611, respectively) were PCR amplified through PCR with specific primers in Table 1, and then inserted into the *Kpn*I/*Nhe*I sites of pGL3 basic vector (Promega, Madison, WI). *CDX2*, *ETS2*, *JUN* and *CREBBP*

Table 1. Oligonucleotides primer sequence used for probe

Gene Name	Accession Gene Bank	Primer(5'-3') Forword and Reverse
bIFNT1	M60903	F: aggtaccactgataccaaagctgaaac R: agctagcctgctgggctggctgagatgg
bIFNTc1	AF238613	F: aggtaccaatgatgctaaagctgaaac R: agctagcctgctgggctggctgagatgg
bIFNTc2	AF238612	F: aggtaccactgatgccaaagctgaaac R: agctagcctgctgggctggctgagatgg
bIFNTc3	AF238611	F: aggtaccactgatgccaaagctgaaac R: agctagcctgctgggctggctgagatgg

* Upstream regions

expression plasmids were described previously (Imakawa et al., 2006). All expression vectors, pSG5 (Agilent)-based constructs, were driven by the SV40 promoter/enhancer. These plasmid constructs were each confirmed to have expected nucleotide sequences by dideoxy sequencing.

RNA extraction and RT-PCR

Total RNA (80-100 mg) was extracted from an individual trophoblast tissues on days 17, 20, and 22 with Madin-Darby bovine kidney (MDBK), Bovine ear-derived fibroblast (EF) and endometrium (Endo) non-trophoblast cells. Using the Isogen Reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The RNA extracted from each sample then underwent reverse transcription (RT) into cDNA using oligo (dT) 12-18 primers and SuperScript II (Gibco BRL Life Technologies, Rockville, MD) according to the protocol suggested by the manufacturer, and these RT products were used as templates for PCR analysis. The PCR mixture consisted of 1❍l of RT product, 1❍l of 10X PCR buffer, 0.4❍l each of forward and reverse primers (10 pM), 0.2❍l of dNTP mixture (10 mM), 0.3 ❍l of $MgCl_2$ (50 mM), 6.6❍l of ddH_2O, and 0.1❍l of Taq DNA polymerase (5U/❍l; Invitrogen, Carlsbad, CA). The PCR was performed under the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 1min, 57°C for 1min, and 72°C for 1min. The PCR product was analyzed by electrophoresis on 1% agarose gel stained with ethidium bromide. The cDNA fragment was extracted from agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Tokyo, Japan) and then cloned into pGEM T-easy vector (Promega, Madison, WI), and nucleotide sequences were determined by DNA sequencing (ABI-PRISM, Foster City, CA). β-actin mRNA was used as an internal control.

Sequencing and sequence analysis

The positive clones as well as the direct PCR product were sequenced using an ABI PRISM automatic sequencer (ABI-PRISM, Foster City, CA) using standard cycle conditions by Sanger's dideoxy chain termination method with standard sequencing primers (viz., M13F and M13R) and the primers employed for amplification, respectively. The sequences were subjected to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST). The nucleotides as well as deduced amino acid sequences of *bIFNT* were aligned with that of ruminant ungulate species available in the GenBank database using the Clustal method of MegAlign Program of Lasergene Software (DNASTAR). A phylogram was also constructed to analyze the evolutionary significance.

Cell culture

Ear-derived fibroblast (EF) cells were obtained from Animal Genetic Resources Research Center, Korean. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 5% FBS (JRH Biosciences) and antibiotics (Invitrogen) at 37°C in air with 5% CO_2.

Transient transfection

EF cells were cultured in the medium described in the cell culture section, and plated on a 24-well plate for subsequent transfection (Imakawa et al., 2006). At 60 - 80% confluence, transient transfection was performed using HilyMax reagents (Dojin Chemicals, Kumamoto, Japan) according to the manufacturer's protocol. In brief, 2 mg total plasmid DNAs including *bIFNT*-reporter (1.5 mg), expression plasmids (total of 0.5 mg) and 4ml HilyMax were prepared in 30ml DMEM with no supplements (plasmid mixture). Amounts of total plasmids for each transfection

were adjusted with the inclusion of pSG5 only (empty vector). After 15min, plated cells were overlaid with the plasmid mixture and incubated at 37°C for 48hr under 5% CO2 in air. Forty-eight hours after transfection, the cells were lysed by the addition of 100ml Passive Lysis Buffer (Promega). Luciferase assay was performed using Dual-Luciferase Reporter Assay System as described previously (Imakawa et al., 2006).

Statistical analysis

The results of luciferase assays were expressed as means ±SEM. Differences in fold activation (luciferase activity) were examined by ANOVA, followed by multiple comparison tests of Fisher's LSD.

RESULTS

Sequences analysis of *IFNT* subtype genes

To determine whether *IFNT* from different phylogenetic groupings had conserved or very different promoter sequences, particularly within the main enhancer regions, an extensive series of *IFNT* clones were analyzed after PCR amplification from genomic DNA. The primers were designed to provide approximately- 1000bp of DNA upstream of the transcription start site, the +51bp untranslated region and the entire open reading frame (Table 1). This upstream region is well conserved across ruminant ungulates (Leaman et al., 1992) and is both necessary and sufficient to provide full expression from IFNT promoters in Jar choriocarcinoma cells (Cross et al., 1991; Leaman et al., 1994). Four of the ten were bi-directionally sequenced. The upstream regions of these four genes are compared of the *IFNT* gene (GenBank Acc. No. *bIFNT1*; M60903: *bIFNTc1*; AF238613: *bIFNTc2*;AF238612: *bIFNTc3*; AF238611) in Figure 1. All sequences were very similar (GenBank Database). The *ETS2* promoter region at (between -70 to -63) (Ezashi et al., 1998, 2001) was completely conserved in all four genes analyzed. A modified TATA box (TATTTAA) starting at (between -33 to-26) bases promotor from the transcription start site (the position +1 adenine), 69 bases up from the ATG start codon; the consensus start site for transcription of *IFNT* was present in all clones.

Luciferase activity of upstream regions by *bIFNT* subtype

The wild-type 5'-upstream sequences of *bIFNT1*, *c1*, *c2*, and *c3* genes were examined for the degrees of transactivation using a transient transfection method into EF cell and luciferase activities were measured (Figure 2). In these data sets, similarly to its expression in vivo, the reporter plasmid of the upstream region of the *bIFNT1*, *c2*, and *c3* genes exhibited high luciferase assay whereas the c1 gene had very low activity, less than of that expressed by *bIFNT1*, *c2*, and *c3* (Figure 2).

Transactivation of *bIFNT* subtype genes in EF cells

To examine the responsiveness of *bIFNT1*, *c1*, *c2*, and *c3*-Luc constructs to transcription factor *JUN*, *ETS2*, *CREBBP* and/or *CDX2* the *bIFNT1*, *c1*, *c2*, and *c3*-Luc reporter plasmid with each or combination of transcription factor, was cotransfected into EF cells, and luciferase activities were measured (Figure 3). When EF cells were cotransfected with the *bIFNT1*, *c1*, *c2*, and *c3*-Luc constructs and *JUN*, *ETS2*, *CREBBP* and/or *CDX2* expression plasmid, Luc activity was enhanced approximately 10 and 18 fold more than twice of those cotransfected with *bIFNT1*, *c1*, *c2*, and *c3*-Luc construct and PSG5 empty plasmid (Con) (Figure 3). Furthermore, The degree of transcriptional activation by a combination of the *JUN*, *ETS2*, *CREBBP* and/or *CDX2* expression vectors was similar to that of *CDX2* along plasmid. However, expression patterns of these Luc activity differented. The degree of transcriptional activation of the *bIFNT1* gene was similar to that *c2* and *c3* genes by a combination of *JUN*, *ETS2*, and/or *CDX2* expression plasmid. Whereas *bIFNTc1*-Luc showed lowest antivity had than *bIFNT1*, *c2*, and *c3*-Luc reports. Although, lowest antivity had of the *bIFNTcl* – Luc report, cotransfected with the *bIFNTc1*-Luc construct and *JUN* or/and *ETS2* expression plasmid, Luc activity was enhanced approximately 2 and 4-fold more than the *bIFNT1*, *c2*, and *c3*-Luc. Furthermore, along *CDX2* expression factor had high effect on activity of *bIFNT1*, *c2*, and *c3*-Luc reporter than the *c1* gene in EF cells.

Expression levels of *bIFNT* subtype mRNA in trophoblast and non-trophoblast cells.

The regions of these four form genes are compared of the *IFNT* gene in Figure 1. For assay levels expression of *bIFNT1*, *c1*, *c2*, and *c3* mRNA in bovine day 17, 20, and 22 bovine trophoblasts and non-trophoblast cells, oligonucleotides identical sequence primer used for *bIFNT1*, *c1*, *c2*, and *c3* mRNAs levels analysis (Table 2). Relative amounts of *bIFNT1*, *c1*, *c2*, and *c3* mRNAs on day 17, 20, and 22 bovine trophoblasts and non-trophoblast cells. RNA extracted from frozen bovine trophoblasts

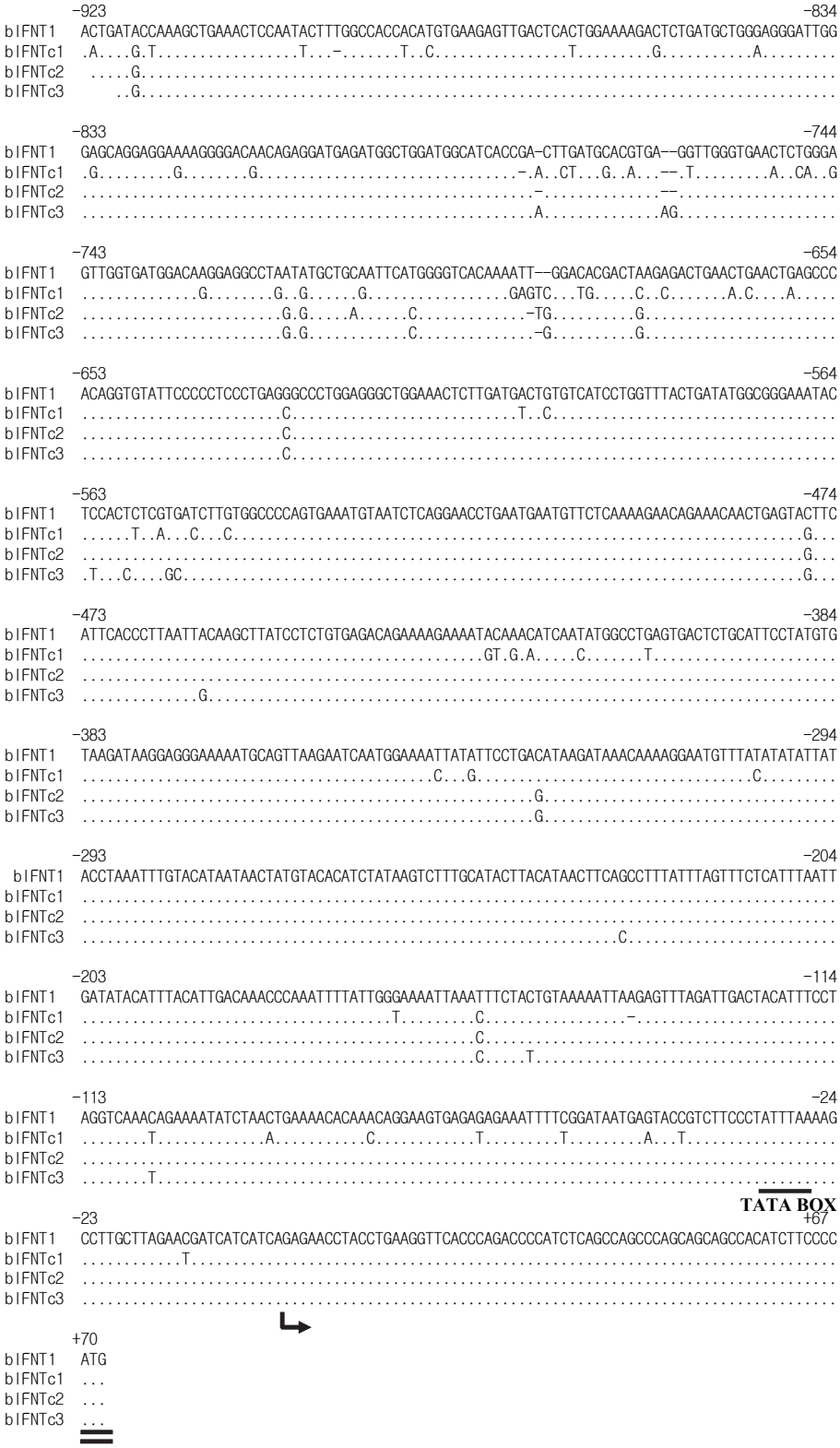

Figure 1. Differences in nucleotide structures between 4 subunits. Comparison of nucleotide sequences of the *bIFNT1* promoter/enhancer region and related type *bIFNTc1* and *bIFNTc2* and *bIFNTc3* genes. Multiple alignment of all known members of the bovine *IFNT* subtype. Alignment of nucleotides represents the open-reading frame only. This sequence was originally deposited to GenBank an accession number *bIFNT1*;M60903: *bIFNTc1*;AF238613: *bIFNTc2*;AF238612: *bIFNTc3;AF238611.* TATA box are underlined. Transcription start site (), and initiation codon (=) are indicated.

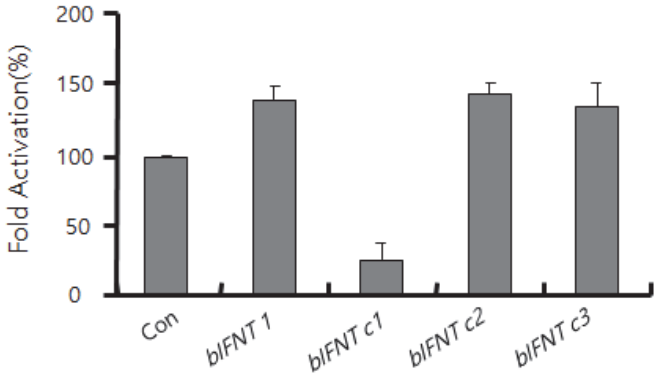

Figure 2. Change in the degree of the *bIFNT1*-reporter and related type *bIFNTc1* and *bIFNTc2* and *bIFNTc3* repoters -transcription in bovine non-trophoblast EF cells. Luciferase-reporter plasmid (-1000-Luc) containing the upstream region (-1000 to +51) of the wild type *bIFNT1* and *bIFNTc1* and *bIFNTc2* and *bIFNTc3* genes were transfected into ear-derived fibroblast (EF) cells, and luciferase activity was determined. Results were expressed as relative luciferase activity to that of the *bIFNT1*-reporter and related type *bIFNTc1* and *bIFNTc2* and *bIFNTc3*-reporter constructs without any expression plasmid. Luci activities are expressed relative to that of the control (pGL3-Control vecter) and the data are show as means±SEM (n=4 each) Data from *bIFNT*-reporter constructs are presented as means±SEM from four independent experiments.

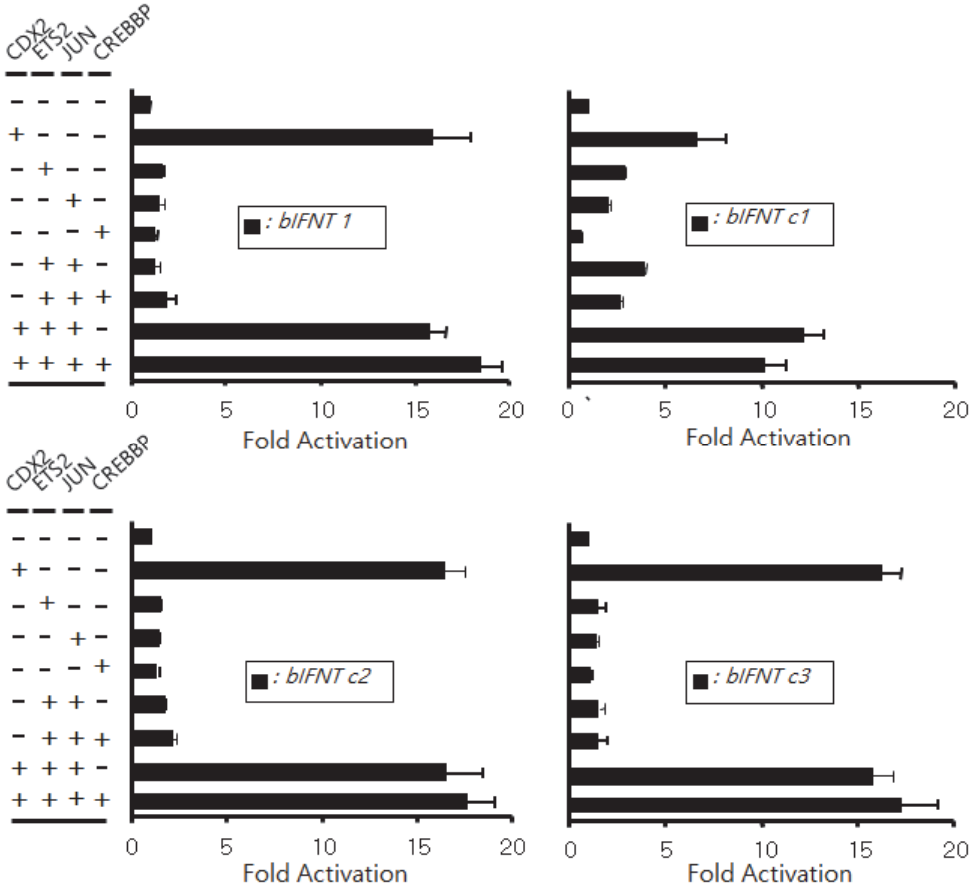

Figure 3. Change in the degree of the *bIFNT1* promoter/enhancer region and related type *bIFNTc1* and *bIFNTc2* and *bIFNTc3* gene repoter transactivation by *CDX2*, *ETS2*, *JUN* and/or coactivator *CREBBP*. Luciferase-reporter plasmid (-1000-Luc) containing the upstream region (-1000 to +51) of the wild type *bIFNT1* and *bIFNTc1* and *bIFNTc2* and *bIFNTc3* genes was cotransfected into EF cells with *CDX2*, *ETS2*, *JUN*, *CREBBP* and *ETS2* plus *JUN* and *ETS2* plus *JUN* plus *CREBBP* and *CDX2* plus *ETS2* plus *JUN* and *CDX2* plus *ETS2* plus *JUN* plus *CREBBP*. Left; Presence (+) or absence (-) of expression plasmids. Luci activities are expressed relative to that of the control (-1000-Luc) and the data are show as means±SEM (n=4 each).

Table 2. Oligonucleotides primer sequence used for *bIFNTs*-ORF assay

Name (Accession Gene Bank)	Primer(5'-3') Forword and Reverse	Length(bp)	cycle
bIFNT1 (M60903) *bIFNTc1* (AF238613) *bIFNTc2* (AF238612) *bIFNTc3* (AF238611)	F: cagaaaagactttggtcttcc R: agagagggctctcatcatctc	364	30

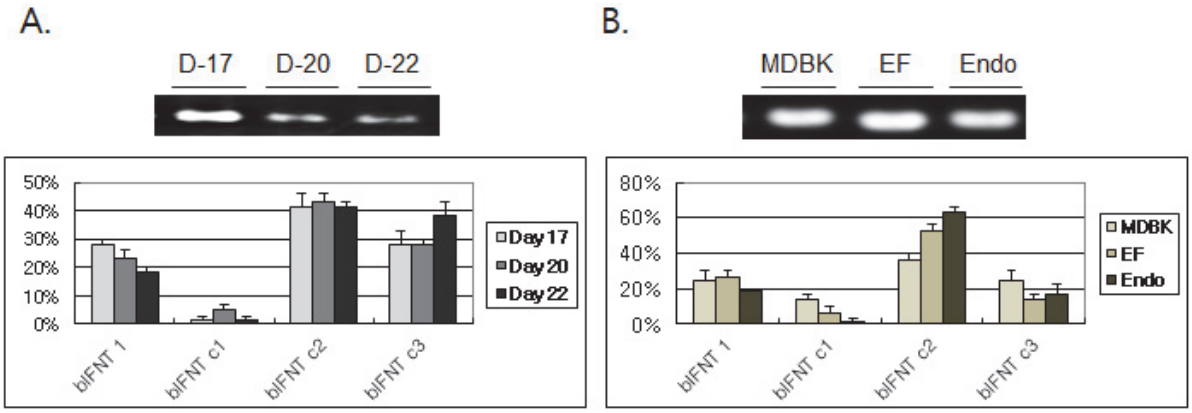

Figure 4. Levels of expression of *bIFNT* subtypes, mRNA. Expression of of *bIFNT1*, *bIFNTc1*, *bIFNTc2*, and *bIFNTc3* mRNA in bovine trophoblast and non-trophoblast cells. Relative amounts of *bIFNT1*, *bIFNTc1*, *bIFNTc2*, and *bIFNTc3* mRNAs in on the days 17, 20, and 22 bovine trophoblast and, Madin-Darby bovine kidney (MDBK), Ear-derived fibroblast (EF), and endometrium (Endo) non-trophoblast cells. RNA extracted from frozen bovine trophoblast and non-trophoblast cells (n=3 each), were analyzed for the presence of these transcripts using RT-PCR. Changes in *bIFNT1* and related type *bIFNTc1*, *bIFNTc2*, and *bIFNTc3* genes mRNAs levels were determined in mRNAs isolated from on trophoblast and non-trophoblast cells. Sequence analyses of *bIFNT1* and related type *bIFNTc1*, *bIFNTc2*, and *bIFNTc3* genes by Escherichia coli cells (n=3 each).

(n=3 each), was analyzed for the presence of these transcripts using RT-PCR. Agarose gel electrophoresis of the amplicon expected to reveal an amplification of a fragment of approximately 364 bp. The size of the amplicon was further confirmed by the nucleotide sequencing. A total of thirty prospective recombinant colonies that contained the 364 bp insert were sequenced. Sequence analyzed of *bIFNT1* and related type *c1*, *c2*, and *c3* genes by *Escherichia coli* cells (n=3 each). In order to characterize the difference on the levels of expression of *bIFNT1*, *c1*, *c2*, and *c3*, the *IFNT* mRNA in the nucleotides region of *bIFNT1*, *c1*, *c2*, and *c3* the *IFNT* were analyzed.

The levels of expression of *bIFNT1*, *c2*, and *c3* were shown higher on day 17, 20, and 22 bovine trophoblasts. However, the other *bIFNTc1* gene transcripts were expressions of transcripts became weaker on day 17, 20, and 22 bovine trophoblasts (Figure 4A). In addition, Sequence analyzed of *bIFNT1*, *c1*, *c2*, and *c3* in MDBK, EF, and Endo of non-trophoblast cells. The *bIFNT1*, *c1*, *c2*, and *c3* mRNA were present in the tree type non-trophoblast cells. The levels of expression of *bIFNT1* and *c2* were shown higher on the tree type non-trophoblast cells, weakly detectable on *bIFNT c1* and *c3* (Figure 4B).

DISCUSSION

The present study, four forms of bovine *IFNT* transcripts in trophoblast and non- trophoblast cells were found through the use of sequence. It was previously predicted that several *IFNT*cDNAs of cattle could exist. There have been 12 *IFNT* cDNAs already identified (Ealy et al., 2001). Moreover, evaluation by (Walker et al., 2009) found six more *IFNT* cDNA variants from bovine conceptuses and trophophectoderm cells, indicating that at least 18 different *IFNT* cDNAs exist. At present, there are 4 novel sequences that we have been working to identify different *bIFNT* sequences andexpression levels. To date, none of the four full-length *bIFNT* genes isolated and three genes have the similar nucleotide sequence. Like its progenitor *IFNW*, the 585bp ORF of the *IFNT*gene encodes for a 195 amino acid pre-protein of which the first 23 residues is the signal peptide that is cleaved off to yield a mature protein of 172 amino acids (Roberts et al., 1997). Therefore, even though the expressed bIFNT represent a rapidly evolving group of genes, it is likely that the majority of them are represented in all bos taurus breeds. Their uniformity contrasts with the diversity of the ovine

IFNT, which differ as much as 10% in nucleotide sequence (Ealy et al., 1998). It is well known that *IFN* type I genes are found in various mammals, but *IFNT*genes are found only in ruminants (Robert et al., 1992). These *IFN* type I genes are known to be located tandemly in the chromosome 8, suggesting that duplication events lead the emergence of several *IFNT*genes in the bovine genome (Walker et al., 2009, Sakurai et al., 2013).

In this study, the relative abundance of transcription factor transcripts, which have been demonstrated to regulate four forms *IFNT* gene transcription, were evaluated. Luciferase activity assay resulted from the upstream region of wild type *bIFNT1*, *c1*, *c2*, and *c3* genes transfected to EF cell reflected the expression levels of these genes in the utero. These results indicated that the expressions of *bIFNT1*, *c1*, *c2*, and *c3* genes are regulated at transcriptional level and transient transfection analyses using EF cell and also demonstrated that high and low expression levels from *bIFNT1*, *c1*, *c2*, and *c3* genes, respectively. *CDX2*functions as a transcription factor that is required for *IFNT* transcription (Imakawa et al., 2006; Sakurai et al., 2009). Although the molecular mechanisms by which *CDX2* gene transcription subsides are unknown, similar regulatory mechanism might exist between *bIFNT1, c1, c2,* and *c3* genes with *CDX2* factor in the bovine. Importantly, *CDX2* exhibited effects on *bIFNT1, c1, c2,* and *c3* genes transcription only when *CDX2* was transfected intoEF cells. As *CDX2* expression increases, *CDX2* binds, allowing *ETS2* binding to the *JUN*, CREBBP complex formation and the activation of *bIFNT1, c1, c2,* and *c3* genes transcription.

In addition, the sequence analysis also revealed that only fourkinds of bovine *IFNT* transcripts were found in day 17, 20 and 22 trophoblastsof the peri-implantation period, and in MDBK, EF, and Endo ofnon-trophoblast cells. Coexpression of the four forms *bIFNT* mRNA was evident (trophoblast and non-trophoblast), *bIFNT1*, *c2*, and *c3* were highly expressed on day 17, 20, and 22 bovine trophoblasts. However, the transcrips of other *bIFNTc1* gene was weakly detectable on bovine trophoblasts. Also, expression of *bIFNT1*, *c1*, *c2*, and *c3* in MDBK, EF, and Endo of non-trophoblast cells. The *bIFNT1*, *c1*, *c2*, and *c3* werepresent in the tree type non-trophoblast cells. The levels of expression of *bIFNT1* and *c2* were shown higher on the tree type non-trophoblast cells, weakly detectable on *bIFNT c1* and *c3*. Collectively, perhaps more interestingly, a trophoblast and non-trophoblast cells temporal pattern and levelof gene expression was observed in this study.

In conclusion, Co-expression of the four forms *bIFNT* mRNA was evident in trophoblast peri-implantation period and non-trophoblast cells. The subtype *IFNT* genes had similar activity, regulated by the transcription factor complex and/or maternal factors, however, not the same, resulting in different degree of their expression.

ACKNOWLEDGEMENTS

This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project title: Development of data gathering and evaluation system for reproduction and milk component traits on dairy cattle in Korea, Project No. PJ01268901)" Rural Development Administration, Republic of Korea and supported by 2018 Postdoctoral Fellowship Program of National Institute of Animal Science, Rural Development Administration, Republic of Korean.

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