Recombinant bovine interferon- τ enhances in vitro development of bovine embryos by upregulating expression of connexin 43 and E-cadherin

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ABSTRACT

Interferon- τ (IFNT), produced in ruminants by embryonic trophoblastic cells before implantation, is involved in the maternal recognition of pregnancy. It is a pleiotropic molecule that alters the synthesis of endometrial proteins and inhibits the proliferation of some cells. The present study investigated the effects of recombinant bovine IFNT on the development of early-stage bovine embryos and the molecular mechanism underlying this effect. This study demonstrated that expression of mRNA encoding type I IFN receptor subunits was detectable from d 4 to 8 in in vitro fertilized (IVF) bovine embryos. A considerable number of IVF ($n = 1,941$) and parthenogenetic activated ($n = 1,552$) bovine embryos demonstrated that supplementing the culture medium with IFNT (100 ng/mL) produced a greater percentage of blastocysts, and the total cell number within the resulting blastocysts was higher. In addition, IFNT upregulated the expression levels of both mRNA and protein for connexin 43 (GJA1) and E-cadherin (CDH1) and expression levels for granulocyte-macrophage colony-stimulating factor and insulin-like growth factor 2 mRNA but not for their proteins in d 8 embryos. However, IFNT inhibited mRNA expression for leukemia inhibitory factor (LIF), LIF receptor α , and the sodium/potassium-transporting ATPase subunit β -1. We concluded that IFNT promoted the development of bovine embryos by upregulating the expression of GJA1 and CDH1. Thus, supplementing embryo cultures or transfer medium with IFNT may stimulate embryo development and improve embryo transfer efficiency.

Key words: interferon- τ , embryo development, bovine

INTRODUCTION

Interferon- τ (IFNT), an important pregnancy factor, is secreted in bovines by mononuclear trophectoderm cells of elongating ruminant conceptuses from d 16 to d 26 (Bartol et al., 1985; Geisert et al., 1988). Interferon- τ exerts its biological activities by binding to the type I interferon receptor (IFNAR), which consists of 2 transmembrane chains, IFNAR1 and IFNAR2, associated with protein-tyrosine kinase 2 (Tyk2) and Janus kinase 1 (Jak1), respectively. The binding of IFN to the receptor complex induces activation of Jak-signal transducer and activator of transcription (STAT) or non-STAT signaling pathways (Payelle-Brogard and Pellegrini, 2010). Previously, IFNAR was thought to be expressed only in the endometrium (Han et al., 1997) and not in conceptuses until at least 15 d of pregnancy. However, a recent report demonstrated the expression of IFNAR1 at earlier stages in ovine conceptuses (Imakawa et al., 2002) and in bovine embryos from the morula to blastocyst stage (Takahashi et al., 2003). Our recent research revealed that IFNT acts as an autocrine factor to regulate ovine trophectoderm cell proliferation (Wang et al., 2013). However, the role and underlying mechanisms of IFNT effects in early bovine embryo development remain unclear.

Many genes are involved in regulating the early stages of bovine embryo development. Connexin 43 (GJA1; gap junction protein alpha-1, 43 kDa) is 1 of 4 connexins that contribute to the development of gap junctions in the preimplantation embryo (De Sousa et al., 1997). The increase in GJA1 was found to be dependent on the Jak-STAT signal transduction pathway in murine cortical astrocytes (Ozog et al., 2004). Bovine embryos cultured in vitro exhibited a lower expression of GJA1 and were associated with changes in embryo quality (Rizos et al., 2002). Similarly, E-cadherin (CDH1), a catenin-associated cell surface adhesion molecule, displayed a significantly lower level in bovine blastocysts cultured in vitro compared with those derived in vivo (Wrenzycki et al., 2001). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic

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regulatory molecule originally identified as a product of activated T lymphocytes involved in proliferation and differentiation of hematopoietic cells (Ruef and Coleman, 1990). The addition of recombinant bovine GM-CSF to serum-free medium 8 to 10 h after insemination increased the percentage of bovine oocytes that formed blastocysts (de Moraes and Hansen, 1997).

Insulin-like growth factor 2 (**IGF2**) is known as a mitogenic and differentiation-promoting growth factor (Harvey and Kaye, 1992), and its mRNA and protein are expressed in the preimplantation mouse embryo (Rappolee et al., 1992). Leukemia inhibitory factor (**LIF**) and the LIF receptor α (**LIFR**) play essential roles in early differentiation and implantation of embryos, and abundant levels of mRNA have been detected in bovine blastocysts produced in vitro (Niemann and Wrenzycki, 2000; Rizos et al., 2003). Both the α and β subunits in the sodium/potassium-transporting ATPase are expressed throughout early bovine development (Betts et al., 1997). Expression of the sodium/potassium-transporting ATPase subunit β -1 (**ATP1B1**) is required for blastocyst formation and normal assembly of trophectoderm tight junction-associated proteins (Madan et al., 2007).

Although culture medium supplemented with IFNT reportedly promotes the proliferation of ovine trophectoderm and in vitro development of bovine embryos (Takahashi et al., 2003; Wang et al., 2013), large quantities of embryos are required to confirm this hypothesis before applying this strategy to in vivo transfer. Therefore, our objectives were to investigate the effects of IFNT on the early development of and relative gene expression in bovine embryos.

MATERIALS AND METHODS

All chemicals and media were tested using cell culture by the suppliers and purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. All plasticware was from Nunc (Nalge Nunc International, Roskilde, Denmark). Recombinant bovine IFNT (1.13×10^8 IU of antiviral activity/mg) was generously provided by R. Michael Roberts (University of Missouri, Columbia).

Collection and In Vitro Maturation of Bovine Oocytes

Bovine ovaries were obtained from a local abattoir and shipped to the laboratory within 3 to 4 h after collection in a temperature-controlled flask (28–32°C) containing physiological saline solution (0.9% NaCl) with 75 μ g/mL penicillin and 50 μ g/mL streptomycin. The cumulus-oocyte complexes (**COC**) were aspirated from antral follicles (2–8 mm in diameter) with a sterile

18-gauge needle attached to a disposable syringe. All oocytes with a homogeneous cytoplasm and at least 3 intact layers of surrounding cumulus cells were selected for in vitro maturation. After rinsing the COC 3 times in prewarmed maturation medium consisting of TCM 199 with Earle's salts (Gibco/BRL, Grand Island, NY), 10% (vol/vol) fetal bovine serum (**FBS**, HyClone-Pierce, Shanghai, China), 10 μ g/mL FSH (Bioniche, Belleville, ON, Canada), 10 μ g/mL LH (Bioniche), 1 ng/mL epidermal growth factor, and 0.1 mg/mL cysteine, groups of 20 to 25 COC were cultured in 50- μ L medium droplets under mineral oil at 38.5°C and 5% CO₂ in air with maximum humidity for 22 to 24 h.

Effect of IFNT on Development of Bovine Embryos

After maturation, bovine COC were washed 3 times and then placed in a 50- μ L droplet of Brackett–Oliphant (**BO**) medium (Brackett and Oliphant, 1975) containing 10 mg/mL BSA under mineral oil for in vitro fertilization (**IVF**) as follows. Frozen semen (Beijing Dairy Cow Center, Beijing, China) were thawed in a water bath at 37°C for 30 s. The sperm cells were washed twice in BO medium with 2.5 mM caffeine, and the sperm pellet was resuspended in BO medium supplemented with 10 mg/mL BSA and 5 IU/mL heparin. The number of spermatozoa was counted using a hemocytometer and then diluted in BO medium to a concentration of 2×10^6 /mL. An aliquot (50 μ L) of this suspension was added into each 50- μ L droplet of BO medium containing 20 oocytes under mineral oil for insemination. The IVF was carried out in humidified air with 5% CO₂ at 38.5°C. After incubation for 6 to 8 h, loosely associated cumulus cells and spermatozoa were removed from the COC using gentle vortexing. The presumptive zygotes were first cultured in CR1aa medium supplemented with 1% EAA and 1% NEAA and 3 mg/mL BSA for 2 d and then continuously cultured in CR1aa medium containing 10% FBS at 38.5°C in a humidified atmosphere of 5% CO₂. The concentration of IFNT (100 ng/mL) was selected based on our previous study (Wang et al., 2013) and another article (Takahashi et al., 2003). The IFNT was added to the culture medium from d 4 to 8. Half of the culture medium per microdrop was refreshed every 2 d. On d 4, 6, and 8 after fertilization, embryos from each microdrop were harvested and used for gene expression and protein analyses.

Effect of IFNT on the Development of Bovine Parthenogenetic Embryos

The activation of matured oocytes was conducted according to the following protocol. Cumulus cells

were removed from oocytes using 0.1% hyaluronidase after in vitro maturation. Oocytes were rinsed 3 times in H199 containing 10% FBS and pretreated in 5 μ M ionomycin at 38.5°C for 5 min. They were cultured in CR1aa supplemented with 2 mM 6-DMAP (6-dimethylaminopurine) for 4 h. The oocytes were first cultured in CR1aa medium with 3 mg/mL BSA for 2 d and then continuously cultured in CR1aa medium with 10% FBS for 4 d at 38.5°C in a humidified atmosphere of 5% CO₂. A total of 100 ng/mL of IFNT was added to the culture medium from d 4 to 8, and the culture medium was changed every 2 d. The embryos in each medium were recovered for experimentation on d 4, 6, or 8 after parthenogenesis activation.

Blastocyst Cell Count

The nuclei of 30 to 45 ($n = 3$ to 5 per experimental group) IVF blastocysts were stained with Hoechst 33342 (10 ng/mL) for 10 min and then washed 3 times in Dulbecco's PBS. Embryos with nuclei labeled under UV excitation were individually mounted on microscope slides in glycerol beneath a coverslip and examined as a whole-mount preparation.

Quantitative PCR

Total cellular RNA from d 4 embryos, d 6 early blastocysts, and d 8 blastocysts was extracted using an RNeasy Pure tissue kit (Qiagen Biotech, Beijing, China). The RNA content was quantified and purity was determined using a NanoDrop ND 1000 spectrophotometer (Wilmington, DE). For RNA extraction, 100 embryos (4–5 microdrops, 20–25 embryos per microdrop) from each treatment group were used. From each sample, total cellular RNA (500 ng) was used for cDNA synthesis. In the first step, secondary structures were removed and an oligo (dT)-18 primer was annealed to the mRNA by heating the sample at 70°C for 5 min. The reaction was quenched rapidly on ice and 5 $\times$ reverse transcriptase (RT) buffer, 10 mM deoxyribonucleotide triphosphates, 2 mM dithiothreitol, 20 U of RNase inhibitor (TaKaRa Biotech, Dalian, China), and 200 U of Moloney murine leukemia virus (M-MLV) RT (Qiagen Biotech) were added (total reaction mixture volume of 25 μ L). This mixture was heated at 42°C for 60 min and then at 70°C for 15 min to terminate the reaction. For each sample, a complete reaction without RT was also performed. Amplification reactions were conducted on a 96-well thermal cycler (Applied Biosystems, Foster City, CA) in a 20- μ L reaction volume containing 1 μ L of cDNA, 9 μ L of 2 $\times$ Master SYBR Green mix (Qiagen Biotech), 9 μ L of sterile water, and 0.5 μ L each of forward and

reverse gene-specific primers (10 μ M). The primers and GenBank accession numbers for each gene are shown in (Table 1). The relative expression of each gene was calculated using the 2^{− $\Delta\Delta$ Ct} method (Livak and Schmittgen, 2001).

Western Blot

In total, 100 embryos (4–5 microdrops, 20–25 embryos per microdrop) from each treatment group were lysed in Laemmle sample buffer (Bio-Rad Laboratories, Hercules, CA). Equal amounts of proteins were separated using SDS-PAGE (7.5% acrylamide running gel) and transferred to a nitrocellulose membrane (BioTrace NT, Pall Corp., Port Washington, NY). Nonspecific binding to the membrane was blocked with 5% nonfat milk in Tris-buffered saline-Tween (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) at room temperature for 1 h. The membranes were then incubated with primary antibodies (4°C, overnight), washed in Tris-buffered saline containing 0.1% Tween, and incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The antibodies against GJA1 (H-150, sc-9059) and anti- β -actin (sc-47778) were purchased from Santa Cruz Biotech (Dallas, TX), anti-CDH1 (07-697) was purchased from Millipore (Billerica, MA), and mouse anti-bovine-GM-CSF (MCA1828) was purchased from Serotec (AbD Serotec, Bio-Rad Laboratories). The protein bands were visualized using enhanced chemiluminescence detection reagents (Appligen Technologies Inc., Beijing, China) and X-Omat BT film (Kodak, Rochester, NY) according to the manufacturer's instructions. The films were digitized and densitometry analysis was performed with ImageJ 1.45s software (National Institutes for Health, Bethesda, MD; <http://imagej.nih.gov/ij/>). The relative intensity of the bands was quantified and normalized to the respective loading control.

ELISA

Bovine IGF2 content from 60 to 75 IVF blastocysts at d 6 or 8 (3 microdrops, 20–25 embryos per microdrop) was determined ($n = 3$ –5 per experimental group) using commercially available ELISA kits (Shanghai Yifeng Biochem Tech Co. Ltd., Shanghai, China). The ELISA was performed according to the manufacturer's instructions.

Statistical Analysis

Experiments were replicated on different days, using one or more microdrops of embryos per treatment on each day. Data were analyzed by least-squares ANOVA

Table 1. Primer pairs (F = forward; R = reverse) used for detection of mRNA

Gene ¹	PCR primer (5' to 3')	Fragment size (bp)	GenBank accession number
<i>IFNAR1</i>	F: CGACCCTGATGCTGGTCGCT R: TGCACATCTGGGGGACCAATCTG	376	NM174552.2
<i>IFNAR2</i>	F: TCCACGGAGGGTCTGAGGG R: GGGGTTGGTGAAGGGCAGCT	210	NM174553.2
<i>CDH1</i>	F: TTCCCGCCATCCTGGGGATCC R: GGGTGTTCATCTTCTGGGGGCGAGT	126	AB037667.1
<i>GJA1</i>	F: GCCCATGTCTCCTCCCGGGT R: GCTGGCTCTGCTGGAAGGCC	268	J05535.1
<i>GM-CSF</i>	F: GGGCACTGTGGTCTGCAGCT R: ATCCACATGCTGCCAGGGCC	76	U22385.1
<i>IGF2</i>	F: TGTGGGGACCGCGGCTTCTA R: CAGGGCCAGGTCGCAGCTTC	105	NM_174087.3
<i>LIF</i>	F: AGCAACGTGCTCTGCCGCTT R: AGGTGTCGGGGCCGTAGGTC	73	NM_173931.1
<i>LIFR</i>	F: TCTCCGGTGGCTGAGCGTCC R: TGCTCCCGGGTTCGCTAGCT	111	XM_005221613.1
<i>ATP1B1</i>	F: ATGGCCCGCGAAAAGCCAA R: GCCCGCCAGGCAGCCATAAA	114	NM_001035334.1
<i>GAPDH</i>	F: CGCCTGGAGAAACCTGCCAAGT R: CACCACCCTGTTGCTGTAGCCA	234	NM001190390

¹*IFNAR1* and *IFNAR2* = type I IFN receptor 1 and 2; *CDH1* = E-cadherin; *GJA1* = connexin 43; *GM-CSF* = granulocyte-macrophage colony-stimulating factor; *IGF2* = insulin-like growth factor-II; *LIF* = leukemia inhibitory factor; *LIFR* = LIF receptor α ; and *ATP1B1* = sodium/potassium-transporting ATPase subunit β -1.

using the general linear model procedure of SAS software (version 9.1, SAS Institute Inc., Cary, NC). For the analysis of embryo development, each microdrop was considered to be an experimental unit, and cleavage and blastocyst development rates were calculated within each microdrop. The data for relative expression of connexin 43, E-cadherin, GM-CSF, IGF2, LIF, LIFR, and ATP1B1 were analyzed using a general linear model and one-way ANOVA followed by post hoc tests using the MIXED procedure models of SAS. All data are expressed as means $\pm$ standard errors of the mean. The percentages were subjected to arcsine transformation before analysis. Differences were considered significant when the *P*-value was < 0.05 .

RESULTS

Bovine Embryos Express *IFNAR1* and *IFNAR2* from d 4 to 8 after IVF

To investigate the autocrine role for IFNT in the development of bovine embryos, we examined the mRNA

expression of *IFNAR1* and *IFNAR2* using RT-PCR on extracts from d 4, 6, and 8 IVF embryos. Expression of *IFNAR1* mRNA was detectable on d 4 and 6 and was sharply increased at d 8. However, expression of *IFNAR2* mRNA was detectable and stable from d 4 to 8 (Figure 1).

IFNT Promotes In Vitro Development of Bovine Embryos

The presumptive bovine zygotes from IVF or PA were randomly assigned to the control or IFNT treatment groups. When IFNT (100 ng/mL) was added to the culture medium from d 4, the embryos developed to the blastocyst stage at a significantly greater rate, and the number of cells within the resulting blastocysts was greater than that in controls after IVF (Table 2, Figure 2; $P < 0.05$) and PA (Table 3; $P < 0.05$) in d 8 embryos. However, the rate at which blastocysts hatched was unaffected ($P > 0.05$) by treatment. We concluded that IFNT promoted the early stages of embryo development in bovines.

Table 2. Effects of IFN- τ (IFNT) on the development (mean $\pm$ SEM) of bovine in vitro fertilized embryos

IFNT (ng/mL)	No. of microdrops ¹	Cleavage rate (%)	Blastocyst rate (%)	Hatched blastocyst rate (%)	Blastocyst cell number
0	40	73.5 $\pm$ 5.3	50.2 $\pm$ 2.2	44.0 $\pm$ 2.0	116.0 $\pm$ 3.0
100	40	75.8 $\pm$ 3.9	57.2 $\pm$ 6.1*	44.7 $\pm$ 1.9	130.4 $\pm$ 4.1*

¹Total oocyte number was 979 and 962 for 0 and 100 ng/mL treatments, respectively.

* $P < 0.05$.

Table 3. Effects of IFN- τ (IFNT) on the development (mean $\pm$ SEM) of bovine parthenogenetic embryos

IFNT (ng/mL)	No. of microdrops ¹	Cleavage rate (%)	Blastocyst rate (%)	Hatched blastocyst rate (%)	Blastocyst cell number
0	32	91.5 $\pm$ 4.3	53.5 $\pm$ 5.7	39.0 $\pm$ 3.9	58.4 $\pm$ 1.5
100	32	88.4 $\pm$ 5.1	66.9 $\pm$ 7.6*	40.2 $\pm$ 3.4	66.5 $\pm$ 2.4*

¹Total oocyte number was 772 and 780 for 0 and 100 ng/mL treatments, respectively.* $P < 0.05$.

Effect of IFNT on Gene and Protein Expression in Bovine IVF Embryos

We explored the mechanism underpinning the promoting effect of IFNT on embryonic development using quantitative RT-PCR to detect the mRNA expression of connexin 43, E-cadherin, *GM-CSF*, *IGF2*, *LIF*, *LIFR*, and *ATP1B1* in d 6 and 8 embryos. As shown in Figure 3, supplemental IFNT treatment increased the mRNA expression of *GJA1* and *GM-CSF* in d 8 embryos (Figure 3A and C; $P < 0.05$), and *CDH1* and *IGF2* in both d 6 and 8 embryos (Figure 3B and D; $P < 0.05$). However, IFNT treatment inhibited the expression of *LIF*, *LIFR*, and *ATP1B1* mRNA in d 8 embryos (Figure 3E, F, and G; $P < 0.05$).

The protein expression levels for these genes were also examined. As shown in Figure 4, IFNT treatment (100 ng/mL) significantly upregulated the levels of GJA1 and CDH1 (Figure 4A, B, and C; $P < 0.05$), whereas IFNT did not affect the protein level of GM-CSF (Figure 4A and D; $P > 0.05$) in d 8 bovine embryos. Moreover, the secretion of IGF2 was not significantly different in the control and IFNT-treated groups (Figure 4E; $P > 0.05$).

Figure 1. Bovine in vitro fertilized embryos express type I IFN receptor 1 and 2 (IFNAR1 and IFNAR2) from d 4 to 8. Bovine embryos were collected at d 4, 6, and 8 after in vitro fertilization, and mRNA expression levels of *IFNAR1* and *IFNAR2* were detected using reverse transcription-PCR followed by agarose gel electrophoresis; *GAPDH* served as an internal control.

DISCUSSION

An early study reported that IFN had no negative effects on the development of mice embryos in vitro (Carthew et al., 1986). Survival and implantation rates of the mouse embryo are promoted by perfusion of IFNT into the abdominal cavity (Assal-Meliani et al., 1995). The rate of blastocyst development for the bovine is also significantly increased by IFNT treatment (100 ng/mL) in in vitro culture medium (Takahashi et al., 2003), suggesting that development of the embryo is regulated by IFNT in an autocrine manner during the preimplantation stage. In our study, supplemental IFNT treatment (100 ng/mL) not only increased the percentage of bovine embryos developing to the blas-

Figure 2. Interferon- τ (IFNT) promotes bovine embryo development to the blastocyst stage and increases cell number. Photomicrographs (phase contrast microscopy) of d 8 blastocysts in control (A) and IFNT-treated groups (C); scale bar = 200 μ m. Total cell numbers of d 8 blastocysts were counted after Hoechst 33342 staining; blastocysts of the same diameter (350 μ m) were selected for cell number counting in control (B) and IFNT-treated groups (D); scale bar = 100 μ m. Color version available in the online PDF.

Figure 3. Interferon- τ (IFNT) affects expression of connexin 43 (GJA1), E-cadherin (CDH1), granulocyte-macrophage colony-stimulating factor (GM-CSF), insulin-like growth factor-II (IGF2), leukemia inhibitory factor (LIF), LIF receptor α (LIFR), and sodium/potassium-transporting ATPase subunit β -1 (ATP1B1) in bovine embryos. Bovine embryos were treated with IFNT from d 4 after in vitro fertilization and collected at d 6 and 8. The graph demonstrates the quantitative reverse transcription-PCR results of (A) *GJA1*, (B) *CDH1*, (C) *GM-CSF*, (D) *IGF2*, (E) *LIF*, (F) *LIFR*, and (G) *ATP1B1* mRNA expression levels. Values represent mean $\pm$ SEM of 3 experiments; * $P < 0.05$.

Figure 4. Effect of interferon- τ (IFNT) on the expression of connexin 43 (GJA1), E-cadherin (CDH1), granulocyte-macrophage colony-stimulating factor (GM-CSF), and insulin-like growth factor-II (IGF2) in d 8 embryos. (A) GJA1, CDH1, and GM-CSF protein expression detected with Western blot (a representative blot is shown). The graph demonstrates the results of the densitometric analysis of (B) GJA1, (C) CDH1, and (D) GM-CSF protein levels. The production of IGF2 in d 6 and 8 embryos was quantified with ELISA kits (E). Values represent mean $\pm$ SEM of 3 experiments; * $P < 0.05$.

tocyst stage, but also increased the total number of cells within the resulting blastocysts. In addition to its antiluteolytic actions, IFNT acts on the endometrium to induce or enhance the expression of several genes (IFNT-stimulated genes) that are hypothesized to regulate uterine receptivity to implantation and conceptus development (Hansen et al., 1999, Spencer et al., 2004).

Interferon- τ is a pivotal pregnancy signal in ruminants that is mediated through IFNAR (Li and Roberts, 1994). The expression of *IFNAR1* mRNA has been detected in d 5 morulas, d 7 early blastocysts, d 8 blastocysts, and expanded blastocysts of bovine embryos (Takahashi et al., 2003). Similarly, the present study found that both *IFNAR1* and *IFNAR2* mRNA

were detected at the blastomere stage (d 4) and that the level of the *IFNAR1* mRNA expression was increased from d 6 to 8 (Figure 1), indicating a possible role for IFNAR in regulating the mechanism underlying the autocrine function of IFN.

Niemann and Wrenzycki (2000) reported that the *GJA1* gene is crucial for the maintenance of compaction in bovine embryos. After an injection of *GJA1* double-stranded RNA at the zygote stage, the percentage of bovine embryos developing to the blastocyst stage was decreased by 18.4% in vitro (Tesfaye et al., 2007). Connexin 43 transcripts are detected in bovine morulas and blastocysts grown in vivo. By contrast, although the early developmental stages from COC to morulas expressed connexin 43 in vitro, no detectable *GJA1* mRNA was found in the in vitro blastocysts and hatched blastocysts (Wrenzycki et al., 1996, 1998). However, in our study, *GJA1* mRNA was detected at both the morula (d 6) and blastocyst (d 8) stages, and IFNT treatment significantly increased the expression of *GJA1* mRNA in bovine blastocysts. This may be due, in part, to the addition of serum to our culture medium because the expression of *GJA1* mRNA virtually disappeared from the 8- to 16-cell stage and on but reappeared in the hatched blastocysts in the serum-replete medium (Wrenzycki et al., 1999). Connexin 43 protein level was greater in the IFNT-treated group than that in the control group. As a type-I interferon, IFNT functions through the activation of the Jak-STAT signal transduction pathway (Darnell et al., 1994; Darnell, 1997) in bovine endometrial cells. Connexin 43 is reportedly activated by ciliary neurotrophic factor via the Jak-STAT pathway in murine cortical astrocytes (Ozog et al., 2004), indicating that the IFNT-promoted expression of *GJA1* may also be via the Jak-STAT pathway in the bovine blastocyst.

Blastocyst formation is driven by the expression of specific sets of gene products that direct the acquisition of cell polarity within the trophectoderm. Critical genes controlling this event include mainly *CDH1* and sodium-potassium ATPase (Watson and Barcroft, 2001). Mice embryos carrying a homozygous mutation in the *CDH1* gene were initially able to compact but did not sustain the compacted state (Riethmacher et al., 1995). Moreover, injection of *CDH1* double-stranded RNA decreases either the relative expression of *CDH1* in bovine morulas and blastocysts or the percentages of embryos developing to the blastocyst stage (Tesfaye et al., 2007). We also demonstrated that the levels of the *CDH1* mRNA and protein expression in the bovine blastocyst were greater in the IFNT-treated group than in the control group in d 8 embryos, indicating that *CDH1* may be the key factor mediating the promoting

effect of IFNT. The sodium-potassium ATPase drives the movement of water through aquaporins across the epithelium into the extracellular space of the blastocyst to form the fluid-filled blastocoel during blastocyst formation (Watson and Barcroft, 2001). Transcripts encoding the ATP1B1 isoform were detected within zygotes, morulas, and blastocyst stages, but not in 2-, 4-, or 8-cell embryos, raising the possibility that the β subunit performs an essential role in coordinating the onset and progression of cavitation in the mouse embryo (Watson et al., 1990). In our study, the addition of IFNT did not affect the expression of ATP1B1 in d 6 embryos, and significantly decreased its expression in d 8 embryos compared with the appropriate control groups.

Supplementing with IGF2, GM-CSF, and LIF significantly accelerates bovine embryonic development, especially in the change from the expanded blastocyst to hatched blastocyst stage (Neira et al., 2010). The addition of IGF2 to the culture medium appears beneficial because it increases the number of embryos that develop to the blastocyst stage in bovine embryo production (Block et al., 2008). The expression of GM-CSF is stimulated by IFNT in both bovine leukocytes and endometrial stromal cells (Emond et al., 2004). The cytokine LIF facilitates the development of the morula to the blastocyst, improves hatching rate, and increases the cell number in the inner cell mass (Martal et al., 1998). Consistent with those observations, we demonstrated that the supplementation of IFNT in the culture medium resulted in a significant increase in the expression of GM-CSF mRNA in d 8 embryos. However, IFNT had no effect on the expression of the GM-CSF protein at the same stage, suggesting that GM-CSF might not be directly involved in the promoting effect of IFNT. We also found that the hatched blastocyst rate was not affected by the addition of IFNT, which might be because IFNT induced an increase in the expression levels for *IGF2* and *GM-CSF* mRNA but not their proteins, and because IFNT inhibited the expression of *LIF*, *LIFR*, and *ATP1B1* mRNA in d 8 embryos. Further study on the mechanisms of the IFNT regulation of important candidate genes is required to fully elucidate embryonic development and differentiation in mammals.

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