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—Original Article—

Embryonic Development and Implantation Related Gene Expression of Oocyte Reconstructed with Bovine Trophoblast Cells

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Abstract. The temporal progressive increase of interferon tau (IFNτ) secretion from the bovine trophoblast is a major embryonic signal of establishing pregnancy. Here, we cultured and isolated bovine trophoblast cells (BTs) from IVM/IVF oocytes and *in vitro* produced blastocysts, used them, for the first time, as donor cells for nuclear transfer and compared them with adult fibroblasts (AFs) as donor cells. BTs were reprogrammed in enucleated oocytes to blastocysts with similar efficiency to AFs (14.5% and 15.6% respectively, $P \leq 0.05$). The levels of IFNτ, CDX2 and OCT4 expression in IVF-, BT- and AF-derived blastocysts were analyzed using reverse transcription polymerase chain reaction and reverse transcription quantitative polymerase chain reaction (RT-PCR and RT-qPCR). IVF-produced embryos were used as reference to analyze the linear progressive expression of IFNτ through mid, expanded and hatching blastocysts. RT-PCR and RT-qPCR studies showed that IFNτ expression was higher in BT-derived blastocysts than IVF- and AF-derived blastocysts. Both IVF- and BT-derived blastocysts showed a progressive increase in IFNτ expression as blastocyst development advanced when it compared with AF-derived blastocysts. OCT4 was inversely related with IFNτ expression, while CDX2 was found to be directly related with IFNτ temporal expression. Persistence of high expression of IFNτ and CDX2 was found to be higher in BT-derived embryos than in IVF- or AF-derived embryos. In conclusion, using BTs expressing IFNτ as donor cells for bovine NT could be a useful tool for understanding the IFNτ genetics and epigenetics.

Key words: Bovine trophoblast, Interferon tau, Nuclear transfer, Real-time PCR, Reprogramming

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Since the first cloned lamb was born, nuclear transfer (NT) has been challenging in several species and has produced many cloned offspring [1–3]. To produce cloned offspring, fetal fibroblasts have been chosen as a preferential donor cell line for NT so far because they have high proliferative potentials [4–7]. In cattle, fetal and adult fibroblasts have been dominantly used for NT to produce cloned calves. Additionally, several types of cells, like granulosa, cumulus, oviduct epithelial cells, skin, tongue and other cells, have been used for NT [8, 9].

After fertilization of an egg with a sperm, the one-cell stage embryo grow up through several mitosis and reaches the preimplantation stage, becoming a blastocyst, which consists of an inner cell mass (ICM) that is capable of differentiation into all embryo organs and trophoblasts, which are the first differentiated cells from the embryo, and contributes formation of the placenta and fetal membranes but does not participate the formation of the fetus proper [7]. Some reports have demonstrated trophoblast isolation and its function *in vitro* in cattle [10–12]. In mice, living pups were born by nuclear transfer of trophectoderm cells from the expanded blastocysts into enucleated oocytes [13] as a trial to show the similarity in totipotency of both ICM and trophoblast cells from a single blastocyst. However, bovine trophoblast cell lines have not been employed in NT so far.

Over the past two decades, there has been much interest in interferon tau (IFNτ), which is produced by the trophectoderm during a defined period of peri-attachment in ruminant embryos. IFNτ is a type I IFN under a unique transcriptional control that limits its expression to ruminant trophoblasts prior to implantation [1, 14–16]. A major role of this cytokine is to mute the pulsatile release of prostaglandin $F_{2\alpha}$ from the maternal uterine endometrium, thereby, blocking luteolysis [17, 18].

The unique pattern of IFNτ expression is regulated by promoter/enhancer regions that are distinct from those of other type I IFN genes [19, 20]. One key component of IFNτ expression is caudal-type homeobox 2 (CDX2), which stimulates IFNτ promoter activity in the presence of Ets-2 [21, 22]. In addition, the POU homeodomain protein (Oct-4), which is best known as a marker of pluripotency [23], blunts the ETS2-induced IFNτ promoter activity [14] in addition to inhibiting other factors during early pregnancy like CDX2 [24]. Recently, we showed the temporal interaction between OCT4 and CDX2 and their effects on IFNτ expression [25]. So this study, as a continuation of our previous work, was undertaken to (1) elucidate if BT, as an interferon tau secreting cell, can be reprogrammed in

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bovine enucleated oocyte; (2) determine the relative abundance of IFNτ expression in the resulting cloned preimplantation embryos; and (3) study the temporal gene interaction affecting IFNτ expression, especially the OCT4 and CDX2 genes.

Materials and Methods

Oocyte collection and in vitro maturation (IVM)

Cow ovaries were collected from a local abattoir, placed in saline at 35 C and transported to the laboratory within 2 h. Cumulus-oocyte complexes (COCs) from follicles 2–8 mm in diameter were aspirated using an 18-gauge needle attached to a 10 ml disposal syringe. The COCs with evenly-granulated cytoplasm that were enclosed by more than three layers of compact cumulus cells were selected, washed three times in HEPES-buffered tissue culture medium 199 (TCM-199, Invitrogen, Carlsbad, CA, USA) and supplemented with 10% FBS, 2 mM $NaHCO_3$ (Sigma–Aldrich, St. Louis, MO, USA) and 1% penicillin–streptomycin (v/v). For IVM, COCs were cultured in four-well dishes (30–40 oocytes per well; Falcon, Becton, Dickinson U.K., Plymouth, UK) for 22 h in 450 µl TCM-199 supplemented with 10% FBS, 0.005 AU/ml FSH (Antrin, Teikoku, Tokyo, Japan) and 1 µg/ml 17β-estradiol (Sigma–Aldrich) at 39 C in a humidified atmosphere of 5% CO_2.

Sperm preparation, in vitro fertilization (IVF) and in vitro culture of embryos (IVC)

Motile spermatozoa were purified and selected using a Percoll gradient [26]. Briefly, spermatozoa were selected from thawed semen straws by centrifugation on a Percoll discontinuous gradient (45–90%) for 15 min at 1500 rpm. A 45% Percoll solution was prepared with 1 ml of 90% Percoll and 1 ml of TALP medium. The sperm pellet was washed two times with TALP medium by centrifugation at 1500 rpm for 5 min. The active motile spermatozoa from the pellet were used for insemination of matured oocyte (at 22 h of IVM). Oocytes were inseminated (day 0) with $1–2\times10^6$ spermatozoa/ml for 18 h in 30 µl microdrops of IVF-TALP medium overlaid with mineral oil at 39 C in a humidified atmosphere of 5% CO_2. Presumptive zygotes were denuded and cultured in a two-step defined culture medium as described previously [27] and overlaid with mineral oil (Sigma–Aldrich).

Differential cell staining of blastocysts

The cell numbers of blastomeres, inner cell mass (ICM) and trophectoderm (TE) cells in blastocysts were counted after chemically defined staining as described by Thouas *et al.*[28]. Blastocysts were incubated in 500 µl of BSA-free, Hepes-buffered TCM-199 supplemented with 1% (v/v) Triton X-100 and 100 µg/ml propidium iodide for 30 sec. When the TE color visibly changed to red and shrank slightly during treatment, blastocysts were incubated at 4 C for overnight in 500 µl fixative solution consisting of 25 µg/ml bisbenzamide in absolute ethanol. The blastocysts were then treated in 99% (v/v) glycerol and mounted onto a glass microscope slide in a droplet of glycerol solution, and cell numbers of each parameter were counted using epifluorescence microscopy. The ICM cell nuclei labeled with bisbenzimide appeared blue, and TE labeled with both bisbenzimide and propidium iodide appeared pink.

Isolation and culture of trophoblasts

Primary cultures of bovine trophoblasts were done as described previously [12]. Briefly, hatched blastocysts produced by IVF were plated into 4-well tissue culture dishes on day 10 to 11 of their development (Nunc, Thermo Scientific, Roskilde, Denmark). The dishes were coated with 0.1% gelatin and containing feeder layers of mouse embryonic fibroblasts treated with mitomycin C (Sigma-Aldrich). Blastocysts were cultured in 1 ml of DMEM-F12 (mixture of DMEM-F12 supplemented with 10% FBS, 0.1 mM, β-mercaptoethanol, 1% nonessential amino acids [Invitrogen], 2 mM GlutaMax, and 1% Penicillin/Steptomycin [Invitrogen]). Fresh medium was added to the primary cultures every 3–4 days. Secondary passage of the trophoblast cell cultures was performed by physical dissociation. Secondary and subsequent cultures were done by removing the monolayer of cells from the tissue culture plate surface followed by mechanical dissociation and chopping of the primary colonies and subculture of the small chops on new feeder plates. The cells were pelleted by centrifugation (1500 rpm for 2 min) in a 1.5 ml round-bottom centrifuge tube. The resulting relatively small clumps of cells were resuspended in 10% DMEM-F12 and plated onto a feeder layer, typically at a 1:4–6 split ratio. Incubation performed at 39 C in a humidified atmosphere of 5% CO_2 air.

Preparation of donor cells and nuclear transfer

For donor cells, BT, the cell sheets were carefully removed by pipetting, then transferred to washing medium (DMEM-F12 with 10% FBS) and washed 2 times. Trypsin-EDTA was added for 6–8 min with interval pipetting every 2 min. Cells were centrifuged (1500 rpm for 2 min) and washed with PBS 2 times and then suspended in PBS containing 0.5% FBS (v/v). For adult fibroblasts, confluent cell cultures were washed 2 times with PBS. Trypsin-EDTA was added for 2–3 min to detach the cells. The cells were centrifuged and washed with PBS 2 times and then suspended in PBS containing 0.5% FBS (v/v). A single trophoblast or adult fibroblast was deposited into the perivitelline space of enucleated oocytes. The couplets were subsequently placed in a fusion medium comprising 0.26 M mannitol, 0.1 mM $MgSO_4$, 0.5 mM HEPES and 0.05% BSA (Sigma) and transferred into a cell fusion chamber with a stainless steel wire electrodes (BTX 453, 3.2 mm gap; BTX, San Diego, CA, USA) after equilibration for 3 min. Fusion was induced by two DC pulses of 1.75 to 1.85 kV/cm for 15 µsec using a BTX Electro Cell Manipulator 200. Fusion of the donor cell and ooplast was observed 1 h after electric stimulation under a stereomicroscope. Only fused embryos were selected and cultured for 4 h in TCM 199 supplemented with 10% FBS. Reconstructed embryos were activated for 4 minutes with 5mM ionomycin (Sigma-Aldrich) followed by 4 h of culture in 1.9 mM 6-DMAP (Sigma-Aldrich) microdrops. Cloned embryos were cultured in 25 µl microdrops of a two-step defined culture medium overlaid with mineral oil (Sigma-Aldrich) for 7 to 8 days at 39 C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2.

Semi-quantitative and relative quantitative PCR

Three embryos from each stage, mid, expanded and hatching blastocysts (C6, C7 and C7H respectively according to the IETS embryo codes), were subjected to RT-PCR and qPCR with three repetitions per sample. A single embryo from each stage was washed

Table 1. Primers used for RT-PCR* and real-time qRT-PCR

Gene	Primer sequences (5'----3')	Annealing temperature (C)	Fragment size (bp)	GenBank accession number
IFNτ	F: TCCATGAGATGCTCCAGCAGT R: TGTTGGAGCCCAGTGCAGA	60	103	X65539
	F*: GACGATCTCTGGGTTGTTAC R*: GTG ATGTGGCATCTTAGTCA	55	565	
OCT4	F: GGTTCTCTTTGGAAAGGTGTTC R: ACACTCGGACCACGTCTTTC	60	314	AF022987
CDX2	F: GCCACCATGTACGTGAGCTAC R: ACATGGTATCCGCCGTAGTC	60	140	DQ126146
KRT8	F: CACCAGTTCCAAGCCTGTGG R: TCAGGTCTCCTGTGCAGATGC	55	176	NM_001033610.1
GAPDH	F: GGCGTGAACCACGAGAAGTA R: CCCTCCACGATGCCAAAGT	60	119	NM_001034034.1

with PBS three times, transferred into 5 µl of diethyl pyrocarbonate (DEPC)-treated water and stored at –80 C or used freshly for total RNA extraction using an RNeasy total extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer instructions. Reverse transcription was carried out at 50 C for 50 min. Individual RT reactions were performed using a random hexamer and SuperScript™ III Reverse Transcriptase (Invitrogen) in a 20 µl reaction. One to two microliters cDNA were subjected to reverse transcription-polymerase chain reaction (RT-PCR) using a Maxime PCR PreMix kit (i-starTaq) (Intron, Seoul, Republic of Korea). Primer sequences, annealing temperatures and approximate sizes of the amplified fragments are listed in Table 1. PCR amplification was carried out with one cycle of denaturation at 95 C for 5 min and subsequent cycles of denaturation at 95 C, annealing for 30 sec, extension at 72 C for 45 sec and final extension at 72 C for 5 min. Ten µl of PCR products were fractionated on 1% agarose gel (Intron) and stained with RedSafe™ (Intron). The expression level for each gene was determined densiometrically with the Image J software (Version 1.40g, NIH, Besthesda, MD, USA). Relative expression levels of each gene at specific stages of embryo development were represented as a ratio to GAPDH gene expression. Relative quantitative PCR (RT-qPCR) was done according to the Takara Bio guidelines. A 22 µl PCR reaction mix was made by adding 2 µl cDNA, 1 µl forward primer, 1 µl reverse primer, 8 µl SYBR Premix Ex Taq, 0.4 µl ROX Reference (Takara Bio, Shiga, Japan) and 9.6 µl of nuclease-free water (Ambion, Austin, TX, USA). The reaction was done using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the company instructions. The thermal profile for real-time PCR was 95 C for 10 min, followed by 40 cycles of 95 C for 10 sec, 60 C for 20 sec and 72 C for 40 sec.

Gene expression in blastocysts derived from different donor cells

Interferon tau (IFNT), CDX2 and OCT4 expression were studied by reverse transcription PCR of blastocysts derived from NT using bovine trophoblasts (BT) and adult fibroblasts (AF) or from *in vitro* fertilization (IVF). Relative quantitative PCR for these genes of different embryonic stages, mid (C6), expanded (C7) and hatching (C7H) blastocysts, were evaluated using the pixel intensity of the agarose gel bands and using imaging analysis program ImageJ. The relative expression of each specific gene was calculated and presented as a ratio to the same gene in the same stage of IVF-produced embryos as arbitatory units.

Relative progression of gene expression in embryos derived from different donor cells

According to our recent results [25], the stages of blastocyst expansion till blastocyst hatching showed variable behaviors in the temporal gene expression patterns, especially for the IFNτ, CDX2 and OCT4 genes. Here, we studied the relative temporal expression of these genes in mid, expanded and hatching blastocysts in relation to their expression in the morula stage within the same group as an internal reference in order to determine the relative progressive expression of each gene separately.

Statistical analysis

In each experimental group, presumptive zygotes were randomly distributed. All data were subjected to one-way ANOVA followed by Tukey's test to determine differences among the experimental groups using GraphPad (Version 4.0, Graphpad software, San Diego, CA, USA). Statistical significance was determined when a P value was less than 0.05.

Results

Isolation and culture of trophoblast cells

Primary culture of trophoblasts was done by placing of hatching or hatched blastocysts on a feeder layer. Outgrowths from the attached blastocysts were seen and left to expand for 3 weeks until reaching about 1 cm in diameter. Trophoblasts were morphologically large cuboidal cells (Fig. 1). Secondary and subsequent cultures were done by mechanical dissociation and chopping of the primary colonies and subculturing of the small chops on new feeder plates every 7 days after reaching a diameter of about 0.5 cm. After several passages, cells maintained the same morphology and strongly expressed mRNA of trophoblast markers, IFNτ, keratin (KRT8) and CDX2 (Figs. 1 and 2).

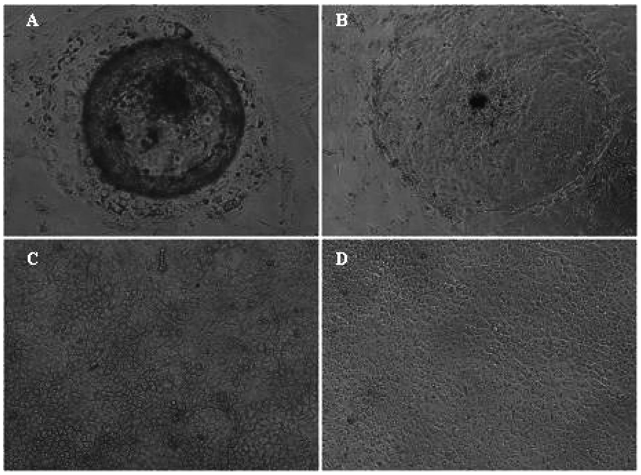

Fig. 1. Isolation of trophoblasts from *in vitro* fertilized embryos. The embryo, a hatched blastocyst, was put on a feeder layer (Day 0) (×40). After 4 (A) (×40) and 7 days (B), the embryo was attached and exhibited trophoblast outgrowth. Primary trophoblast cells, which are large cuboidal cells on the feeder cells (C) (×100), were stable after serial subculture (D) (×100).

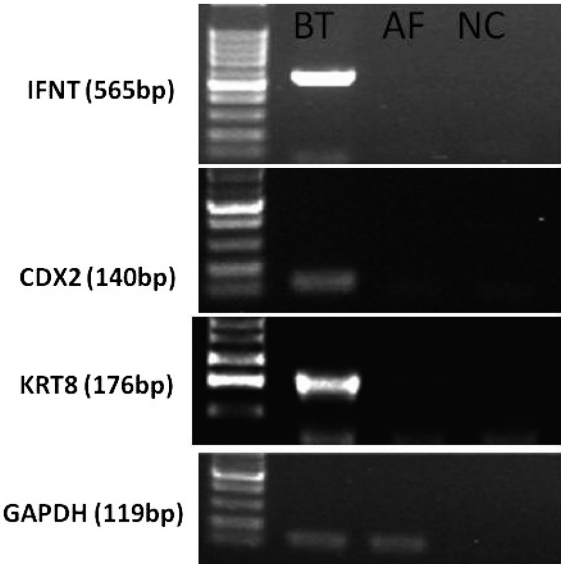

Fig. 2. Gene expression screening in bovine trophoblasts (BT) and adult fibroblast cell lines (AF). Isolated trophoblasts cells strongly expressed IFNτ, KRT8 and CDX2, but adult fibroblasts (AF) did not. NC: negative control cDNA template.

Trophoblast reprogramming and early embryo development

A trophoblast cell was injected into enucleated oocytes, and the oocytes and trophoblast cells were then fused, activated and cultured in a two-step defined culture medium. Fusion of BTs was lower than in AFs (71.3% *vs.* 93.1%, respectively), while there were no difference in cleavage, morula, blastocysts formation rates and ICM/TE ratio (71.3%, 30.6%, 14.5% and 28.92 ± 5.1%, respectively) between the two types of donor cell (Table 2). For more confirmation, we injected mouse embryonic fibroblasts as a negative control donor cell (the feeder cells of BT-1 in culture), and no reprogramming occurred (morula and blastocyst compaction were both 0%, n=60 oocytes), which provide that the reprogramming was solely occurred from the BT donor cells. Moreover, we used transgenic BTs derived from green fluorescence protein (GFP) embryos as donor cells for nuclear transfer, and the resultant embryos expressed GFP (Suppl eFig. 1: available at www.jstage.jst.go.jp/browse/jrd).

Relative gene expression in blastocysts derived from different donor cells

IFNτ, CDX2 and OCT4 expressions in individual developing blastocyst (mid, expanded and hatching or C6, C7 and C7H, respectively, according to the IETS embryo codes) were compared among the different donor cell types (Fig. 3). RT-PCR showed that the IFNτ expression was increased in BTs than in AFs when the relative expressions of the same stages of IVF-produced blastocysts, stage C7 and C7H but not C6, were compared (Fig. 3). Similarly, in BT-derived blastocyts, CDX2 showed an increase in expression when compared with AF produced hatching blastocysts. On the other hand, OCT4 showed a decrement in expression in BT-derived blastocysts compared with AF-derived blastocysts in both hatching and hatched stages (Fig. 3).

Temporal progression of gene expression

Figure 4 illustrates the RT-qPCR comparison of IFNτ, CDX2 and OCT4 relative to the progression of the developmental stages of BT-, AF- and IVF-produced blastocysts in relation to their expression in the morula stage as an internal reference of the same group. IFNτ progressively increased along with blastocyst development, i.e., from the mid-blastocyst stage until hatching in both BT- and IVF-produced blastocysts; however, it showed a decrease in expression by advancement of blastocyst growth in AF-produced blastocysts. On the other hand, OCT4 expression progressively decreased along

Table 2. Development of embryos reconstructed by using an adult fibroblast or trophoblast as a donor cell

Type of donor cell	Total oocytes	Fused oocytes (%)	Cleaved embryos (%)	Morula (%)	Blastocyst (%)	Total blastocyst cell count on 7thday (ICM/TE)
Adult fibroblast	90	83 (93.1)*	63 (75.9)	24 (28.9)	13 (15.6)	74.02 ± 5.1 (31.27 ± 4.2%)
Trophoblast	87	62 (71.3)	49 (79.0)	19 (30.6)	9 (14.5)	76.71 ± 4.6 (28.92 ± 5.1%)

* The value is significant (P≤0.05).

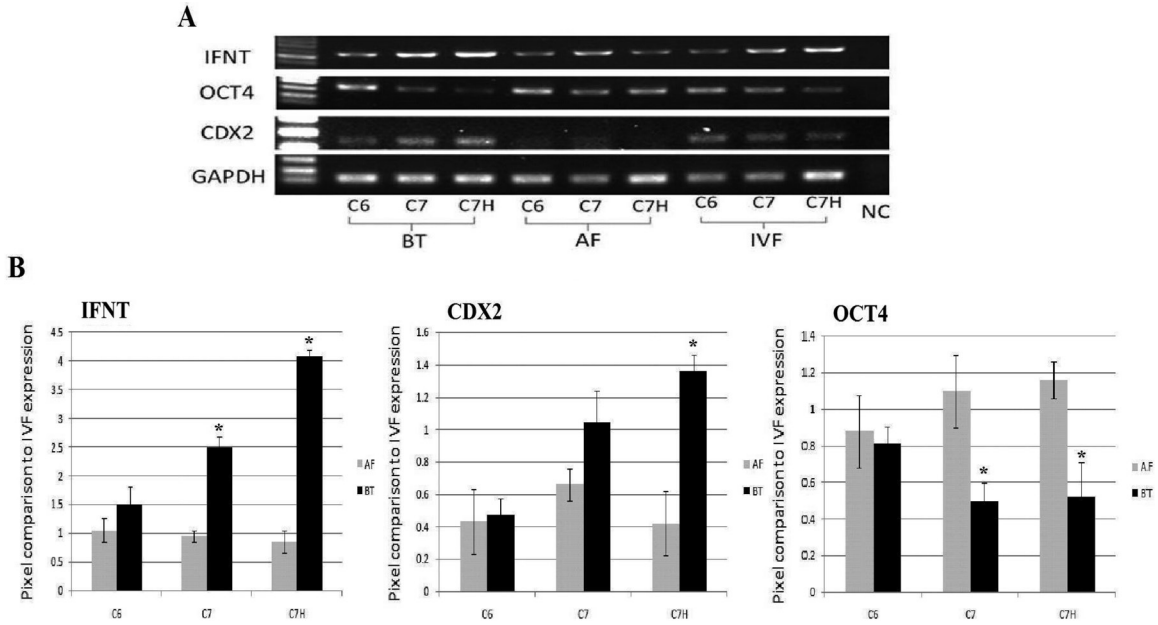

Fig. 3. Interferon tau (IFNτ), CDX2 and OCT4 expression by (A) reverse transcriptase PCR of blastocysts derived from NT by bovine trophoblasts (BT) and adult fibroblasts (AF) or by *in vitro* fertilization (IVF); (B) comparison of relative quantitative PCR of IFNτ, OCT4 and CDX2 in different stages of blastocysts, mid (C6), expanded (C7) and hatching (C7H), in BT- and AF-produced blastocyst. The values (mean ± SE) are presented as the relative expression to the same stage of IVF-produced blastocysts in arbitatory units. *The value is significant (P≤0.05).

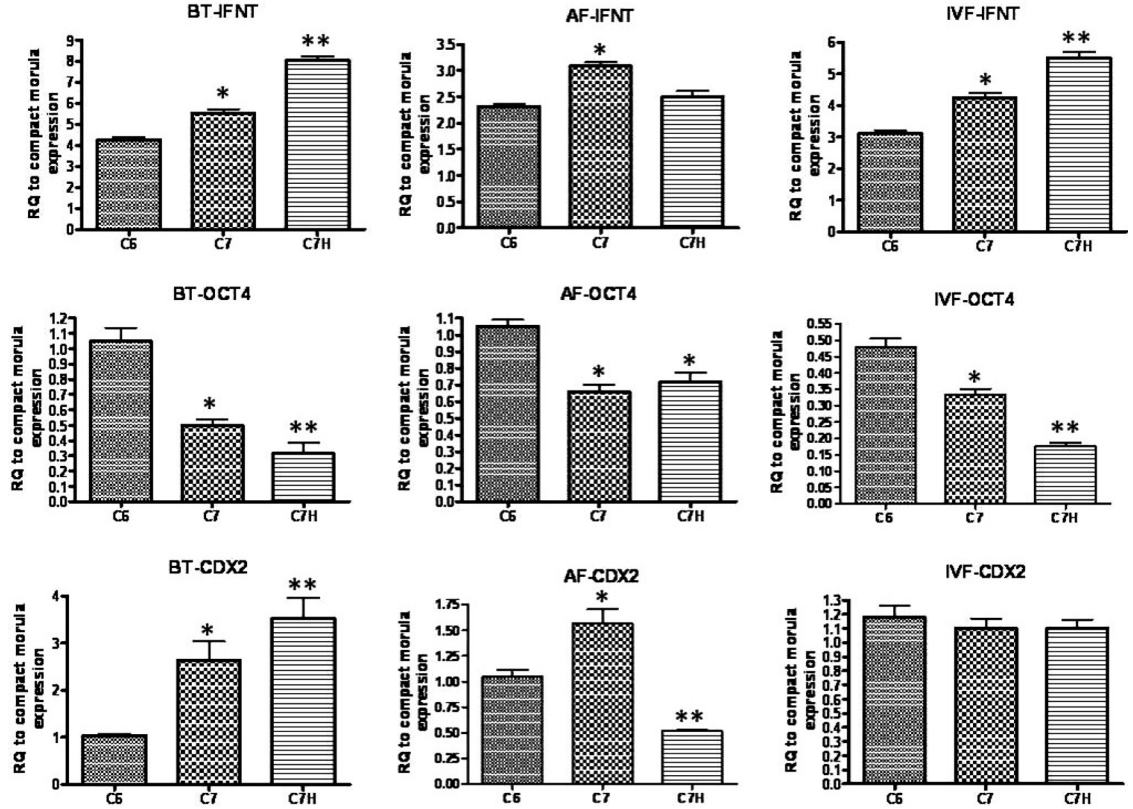

Fig. 4. Real-time PCR values show the temporal relative progress of IFNT, OCT4 and CDX2 expression along with embryo development in IVF-, BT- and AF-derived blastocysts. The values (mean ± SE) are presented as the relative quantitation (RQ) of the specific gene to its expression in the morula stage of the same group. * The value is significant (P≤0.05).

with blastocyst development in both BT- and IVF-produced blastocysts; however, it tended to be stable or slightly increased (but not significant) in conjunction with blastocyst hatching in AF-derived blastocyts. CDX2 showed a similar expression pattern of IFNτ in BT- and AF-derived blastocysts (i.e., progressive increase), while it did not show any significant change with the advancement of growth in IVF-produced blastocysts.

Discussion

Many cloned offspring have been produced using several types of donor cells including fibroblasts to date [1, 3, 29]. Most embryonic or somatic cells are reprogrammed in enucleated oocytes and develop into preimplantation embryos with different efficiencies. Although embryonic cells, particularly, ICM cells, which are pluripotent, have been well applied to NT for producing cloned offspring in mice, trophoblast cells have not been given much attention. In ruminants, trophoblasts cells at the implantation stage strongly expressed IFNτ, a type I IFN that is considered to be an important signal for antiluteolysis in early pregnancy [30]. Here, we proved the hypothesis that trophoblasts cells expressing IFNτ would be reprogrammed in enucleated oocytes and develop into cloned blastocysts. In addition, IFNτ expression increased along with development from early to hatching blastocysts.

For this study, we isolated trophoblast cells from *in vitro* fertilized embryos. Hatched blastocysts were attached to a mouse feeder layer, and BT cells with a cuboidal morphology [12] were maintained for further culture (Fig. 1). The BT cell line had high proliferative capability, and outgrowth into visible colonies occurred within 5 to 7 days after subculture (Fig. 1). IFNτ was well expressed in these cells but not in adult fibroblasts (Fig. 2). In the current study, the blastocysts derived from AFs or BTs showed no difference in developmental competence parameters, including cleavage, blastocyst formation and total and differential blastocyst cell counts, while they did show a significant difference in membrane fusion that might have been due to the large cell size of the BTs compared with the AFs [31, 32].

Hernandez-Ledezma *et al.* [15] suggested that the best way to study IFNτ expression as an indicator of embryo quality is to examine the temporal expression rather than the absolute expression at a particular stage because the latter is known to vary widely. Therefore, the progressive increase in IFNτ expression from IVF- or BT-derived blastocysts indicates the similarity in behavior of IFNτ transcripts between the two groups, while its level was found to be decreased as growth of blastocysts produced with AFs progressed, reflects the blastocyst quality.

OCT4 was found to be inversely affected the IFNτ expression in all experimental groups, confirming our previous results that OCT4 is the major dominant gene affecting IFNτ expression. CDX2 expression was found to be directly related to IFNτ expression along with the embryo development until the hatching stage in both BT- and AF-derived blastocysts suggesting that CDX2 is a potent regulator of IFNτ expression [22, 33, 34] while it showed no change in IVF-produced blastocysts.

The significant increase in IFNτ expression in BT-derived blastocysts compared with IVF-produced blastocysts might be because of the increased expression of CDX2 and the decrease in OCT4. We speculate that the increase in IFNτ might be the result of a retained epigenetic status of the donor trophoblast (high expression of IFNt and CDX2) cell because it was found that the epigenetic state of the donor nucleus was retained in cloned embryos and that it affected the reprogramming and development of the embryos [35, 36]. In addition, reprogramming by NT keeps Oct-4 and Nanog stably silenced by methylation [35, 36], which is another factor increasing IFNτ expression. Further investigations are required in this regard.

This is the first report to show successful reprogramming of bovine trophoblast cells expressing IFNτ by NT into preimplantation embryos and that the embryos expressed a progressive increase in IFNτ along with development from early to hatching blastocysts. Also, it showed the persistence of high expression of IFNτ and CDX2 in embryos derived from a cell in which these genes are highly expressed. Finally, use of BT cells in NT will be one resource for further understanding of the interaction between OCT4 and CDX2 genes in the regulation of IFNτ expression and epigenetic regulation of this gene.

Acknowledgments

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