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—Original Article—

Regulation of Trophoblast-Specific Factors by GATA2 and GATA3 in Bovine Trophoblast CT-1 Cells

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Abstract. Numerous transcription factors that regulate trophoblast developmental processes have been identified; however, the regulation of trophoblast-specific gene expression has not been definitively characterized. While a new role of Gata3 in trophoblast development was being demonstrated in mice, we examined effects of GATA transcription factors on conceptus interferon tau (IFNT), a major trophectoderm factor in ruminants. In this study, expression patterns of trophoblast *ASCL2, CDX2, CSH1, ELF5, HAND1, IFNT*, and *TKDP1* mRNAs were initially examined, from which *ASCL2, CDX2, IFNT, and TKDP1* mRNAs were found to be similar to those of *GATA2* and *GATA3* in days 17, 20, and 22 (day 0=day of estrus) bovine conceptuses. A chromatin immunoprecipitation (ChIP) assay revealed that endogenous GATA2 and GATA3 occupied GATA binding sites on the upstream regions of *CSH1, IFNT*, and *TKDP1* genes and on the intron 1 region of *CDX2* gene in bovine trophoblast CT-1 cells. In transient transfection analyses of the upstream region of bovine *CSH1*, and *IFNT* or the intron 1 region of *CDX2* gene, over-expression of *GATA2* induced transactivation of these trophoblast-specific genes in bovine non-trophoblast ear fibroblast EF cells, but over-expression of *GATA3* did not substantially affect their transactivation. In CT-1 cells, endogenous *CDX2* and *IFNT* mRNAs were down-regulated by *GATA2* siRNA, while endogenous *ASCL2* and *CDX2* mRNAs were down-regulated by *GATA3* siRNA. Our results indicate that in addition to trophectoderm lineage specification, GATA2 and/or GATA3 are involved in the regulation of trophoblast-specific gene transcription in bovine trophoblast CT-1 cells.
Key words: Bovine, GATA factors, Gene regulation, Trophoblast cells

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During mammalian development, the first lineage decision is to establish the trophectoderm (TE) and inner cell mass (ICM). The outer TE layer attaches to and invades the maternal endometrium and eventually forms placental structures. It has been well established that caudal-type homeobox transcription factor 2 (Cdx2) serves as a lineage determining factor for the murine trophectoderm [1, 2]. Quite recently, one of the GATA transcription factors, Gata3, was found to specify the trophectoderm lineage [3].

In addition to these factors, there are numerous factors that are expressed in the trophoblasts during trophectoderm cell differentiation. These are E74-like factor 5 (Elf5), heart and neural crest derivatives expressed 1 (Hand1), a mammalian achaete scute-like homolog 2 (Ascl2), placental lactogens (PLs, CSHs) in many mammalian species, and interferon tau (IFNT) and trophoblast Kunitz domain proteins (TKDPs) in ruminant ungulates. Elf5 is necessary to maintain expression of the trophoblast stem cell genes Cdx2 and Eomes, and thereby reinforces trophoblast cell fate [4]. Hand1 and Ascl2, basic helix-loop-helix (bHLH) transcription factor genes, are essential for trophoblast proliferation and differentiation in mice [5, 6]. CSHs belong to the growth hormone (GH)/prolactin (PRL) gene family, and are expressed in trophoblast giant cells (TGC) in rodents or in trophoblast binucleate cells (BNC) in ruminants [7–11]. In ruminants, the embryonic trophectoderm produces the cytokine IFNT, which is known as a factor responsible for the prevention of luteolysis [12, 13]. IFNT and TKDPs are secreted by trophoblast mononuclear cells during days 13–21 of pregnancy in sheep [14]. Involvement of these factors in cell differentiation has been firmly characterized, but, with the exception of *IFNT* and *TKDP*s, their transcriptional regulation has not been well explored.

GATA transcription factors are a family of structurally related proteins that bind to the consensus DNA sequence W(A/T)GATAR(A/G) (GATA motif), resulting in transcriptional regulation of downstream genes [15–17]. Based on sequence homology and tissue distribution, these GATA factors have been divided into two subfamilies, GATA1-3 and GATA4-6. GATA1, GATA2 and GATA3 regulate the development and differentiation of hematopoietic lineages [18–20], while GATA4, GATA5 and GATA6 are involved in cardiac development and endodermal derivatives [21–23]. Of the six GATA factors, GATA2 and GATA3 are expressed in the mouse and human trophoblast cells [24, 25]. It was recently reported that GATA2 and GATA3 were also expressed in the bovine trophoblast cells, and that *IFNT* transcription was regulated through the expression of GATA2 and GATA3 transcription factors in bovine trophoblast CT-1 cells [26].

We hypothesized that if GATA motifs were present on the genes, which are expressed in a trophoblast-specific manner, these genes could be under the control of GATA2 and/or GATA3 binding to their transcription-regulatory regions. To characterize roles of GATA factors on the regulation of trophoblast-specific factor

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genes, we studied whether GATA2 and GATA3 were involved in the transcriptional regulation of these genes.

Materials and Methods

Collection of bovine conceptuses and cell culture

The experimental protocols for the use of Japanese Black cattle in this study were approved by the Committee for Experimental Animals at Zen-noh Embryo Transfer (ET) Center and the University of Tokyo. Estrous synchronization, super-ovulation and ET were performed as described previously [27]. Conceptuses were non-surgically collected from the uterus through uterine flushing on days 17, 20, and 22 (n=3 each) and subjected to RNA extraction.

Bovine trophoblast CT-1 cells, kindly provided by Dr A Ealy, University of Florida, Gainesville, FL, USA, had been established from *in vitro* matured and fertilized blastocysts [28]. CT-1 cells were maintained at 37 C in air with 5% CO_2 in Dulbecco's Modified Eagle's (DME) Medium (Invitrogen, Carlsbad, CA, USA) containing 10% (v/v) fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS, USA) supplemented with 4.5 g/l D-glucose, nonessential amino acids, 2 mM glutamine, 2 mM sodium pyruvate, 55 μM β-mercaptoethanol, and antibiotic/antimycotic solution (Invitrogen). Ear-derived fibroblast (EF) cells, obtained from biopsied ear skin of Japanese Black bulls (4 months old), were kindly provided by Dr M Takahashi of the National Agricultural Research Center for the Kyushu-Okinawa Region. EF cells were maintained in DME medium containing 5% (v/v) FBS (JRH Biosciences) and antibiotic/antimycotic solution (Invitrogen) at 37 C in air with 5% CO_2 and were used within six passages.

RNA extraction and analysis

RNA was extracted from bovine conceptus tissues (80–100 μg), or cultured cells (1×10^6 cells) using ISOGEN (Nippon gene, Tokyo, Japan) according to the protocol provided by the manufacturer. Isolated RNA (total 1 μg) was reverse-transcribed to cDNA using a ReverTra Ace qPCR RT Kit (Toyobo, Tokyo, Japan) including 5 × RT buffer, Enzyme Mix, and primer Mix in a 10 μl reaction volume, and the resulting cDNA (RT template) was stored at 4 C until use. The cDNA reaction mixture was diluted to 1:10 using DNase- and RNase-free molecular biology grade water, and 3 μl were taken for each amplification reaction. RT template (cDNA) was subjected to PCR or real-time PCR amplification using the primers shown in Table 1. Following 30 PCR cycles, amplification products were separated on 1.5% (w/v) agarose gels. Each PCR product was subcloned, and their nucleotide structures verified by DNA sequencing. Quantitative PCR reactions were performed using a SYBR Green kit (Takara Biomedicals, Tokyo, Japan) and Applied Biosystems thermal cycle system (7900HT, Applied Biosystems, Tokyo, Japan), as previously described [29, 30]. Average threshold (Ct) values for *GATA2*, *GATA3* and trophoblast-specific factor mRNAs were calculated and normalized to the Ct value for *GAPDH* mRNA.

Chromatin immunoprecipitation (ChIP) assay

A chromatin immunoprecipitation (ChIP) assay was performed as described previously [29, 30]. CT-1 cells were cultured in the medium described in the cell culture section. Protein-DNA complexes in these cells were cross-linked through the addition of 1% formaldehyde (Wako, Osaka, Japan) for 15 min, and were then sonicated to approximately 500 base pairs. The strength of sonication had been predetermined to generate DNA strands of approximately 500 bases long. The supernatant of sonicated cells was diluted 10-fold, and 1% of the diluted lysates was used for total genomic DNA as input DNA control. Immunoprecipitation was performed overnight at 4 C with rabbit polyclonal anti-GATA2 antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or mouse polyclonal anti-GATA3 antibody (1:50; Santa Cruz Biotechnology). For a negative control, normal rabbit serum (1:50; Santa Cruz Biotechnology) was used in place of specific antibody. For isolation of DNA, protein G magnetic beads (30 μl, Cell Signaling Technology, Danvers, MA, USA) were added and incubated for 1 h, and the beads were then washed with high or low salt buffer. Histone complexes were eluted from the antibody by freshly prepared elution buffer (1% SDS, 0.1 M NaHCO3, 10 mM DTT). Protein-DNA cross-links including the input DNA control samples were incubated with 5 M NaCl and proteinase K (Invitrogen, Carlsbad, CA, USA) at 65 C overnight. DNA fragments were extracted with a column (QIAquick PCR Purification Kit; Qiagen) following the protocol provided by the manufacturer, and resuspended in 60 μl elution buffer, from which 2 μl were used for PCR (35 cycles) or real-time PCR with the primers listed in Table 2. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide and were visualized under UV light.

Plasmid construction and luciferase reporter assay

Expression plasmids for GATA2 (pSG5-*GATA2*) and GATA3

Table 1. Oligonucleotide primers for RT-PCR and real-time PCR analyses

Name (GenBank accession No.)	Sequence
GATA2	F: 5'- GAGGACTGTAAGCGTAAAGG -3'
(XM_583307)	R: 5'- AAGAACCAAGTCTCCCCAT -3'
GATA3	F: 5'- ATGAAACCGAAACCCGATGG -3'
(NM_001076804)	R: 5'- TTCACAGCACTAGAGAGACC -3'
CDX2	F: 5'- GCCACCATGTACGTGAGCTAC -3'
(XM_871005)	R: 5'- ACATGGTATCCGCCGTAGTC -3'
ELF5	F: 5'- CGCTTGGGTGATGTTGGA -3'
(NM_001024569)	R: 5'- CATTTTTGTTCCTTTGTCCCC -3'
HAND1	F: 5'- CAAGGACGCACAGGCTGGCGA -3'
(NM_001075761)	R: 5'- CACTGGTTTAGCTCCAGCGC -3'
IFNT	F: 5'- CATCTTCCCCATGGCCTTCG -3'
(AF238613)	R: 5'- TCATCTCAAAGTGAGTTCAG -3'
ASCL2	F: 5'- GAGTACATCCGCGCCTTG -3'
(NM_001040607)	R: 5'- CCGACGAGTAGGCGGAAC -3'
CSH1	F: 5'- CTGCTGGTGGTGTCAAATCTAC -3'
(NM_001164321)	R: 5'- TGGTTGGGTTAATTGTGGGC -3'
TKDP1	F: 5'- CTCTGCCTGGAGCCTAAAGT-3'
(NM_205776)	R: 5'- TTGAGAGCAGGTCTTCATGC-3'
GAPDH	F: 5'- CCAACGTGTCTGTTGTGGATCTGA -3'
(NM_001034034)	R: 5'- GAGCTTGACAAAGTGGTCGTTGAG -3'

F: Forward R: Reverse.

Table 2. Oligonucleotide primers for RT-PCR analyses (ChIP)

Name	Sequence
CDX2 intron1-F1821	F: 5'- CCGATTACAGCCGCATAAAT -3'
CDX2 intron1-R 2040	R: 5'- GATAGCCAAGGGCCTTCATC -3'
CDX2 intron1-F3278	F: 5'- AGCTCTTGAGTTGGGGGATT -3'
CDX2 intron1-R3472	R: 5'- CAAACCCTGCCAGAGCTATC -3'
ELF5 Ups-972	F: 5'- CGACAAGTCCCCACTCTCTC -3'
ELF5 Ups-821	R: 5'- TGCATTTAAGCAGTCCAAGC -3'
ELF5 Ups-544	F: 5'- TGTTTGTGGGCACATTCACT -3'
ELF5 Ups-381	R: 5'- ATGGCCAGTAAGTGGCAAAG -3'
ELF5 Ups-277	F: 5'- GTGGAAGGCACTCAGAGGAG -3'
ELF5 Ups-61	R: 5'- GCAGGGCAACACTTGTTTTT -3'
HAND1 Ups-880	F: 5'- TCCTACTTGCCACCCATCTC -3'
HAND1 Ups-680	R: 5'- GAGCAGGATAGGCCCCTAAG -3'
HAND1 Ups-643	F: 5'- AGAGCAGATGGAGCTCAGGA -3'
HAND1 Ups-491	R: 5'- GGAAAGGAATCATGGAGCAA -3'
IFNTc1 Ups-877	F: 5'- GTGAAGAGTTGACTCATTGG -3'
IFNTc1 Ups-877	F: 5'- GTGAAGAGTTGACTCATTGG -3'
IFNTc1 Ups-644	R: 5'- TACACCTGTGGGCTTAGTTG -3'
IFNTc1 Ups-540	F: 5'- TGGCCCCAGTGAAATGTAAT -3'
IFNTc1 Ups-353	R: 5'- AACTGCATTTTTCCCTCCTT -3'
IFNTc1 ORF 272F	F: 5'- TTCCACACAGAGCACTCGTC -3'
IFNTc1 ORF 488R	R: 5'- CGCAGTCGCTGTATTCCTTC -3'
ASCL2 Ups-F280	F: 5'- GCCGCTCAAGGAAGACCT-3'
ASCL2 Ups-R89	R: 5'- CGGTCAAGTCTCAACGAAGA -3'
CSH1 Ups-840F	F: 5'- TGCATCTCCAGTGGCTAAGA -3'
CSH1 Ups-686R	R: 5'- GACAGGCTTCCTCTGACCTG -3'
CSH1 Ups-252F	F: 5'- GTCACTTGAAGGCCACCATC-3'
CSH1 Ups-58R	R: 5'- CCCAACAGGACTGAATGGAG-3'
TKDP1 Ups-691F	F: 5'- AACCAGGTGAGGGATGACTG -3'
TKDP1 Ups-481R	R: 5'- TTCCTACAACCATTGCCACA -3'

F: Forward R: Reverse.

(pSG5-*GATA3*), and *IFNT*-reporter construct were described previously [26, 30]. Luciferase reporter constructs containing the upstream region of *CSH1*, *IFNT*, *TKDP1* or *CDX2* intron 1 were generated using the primers shown in Table 3. Upstream regions were each inserted into a pGL3 basic vector (Promega, Madison, WI, USA) and nucleotide structures of these constructs were confirmed by DNA sequencing. Transient transfection and the luciferase assay were performed as described previously [26]. In brief, EF cells were seeded on a 24-well plate. At 70–80% confluency, 2 μg of luciferase reporter constructs containing the 5'-upstream region of bovine *CSH1*, *IFNT*, or *TKDP1* and *CDX2* intron 1 along with pSG5-*GATA2* or pSG5-*GATA3* (0.5 μg) and 6 μl HilyMax (Dojin Chemicals) were prepared in 30 μl DME medium without supplements. The amount of reporter construct relative to that of the internal control Renilla luciferase pRL-TK (Promega) vector was 20:1. After incubation for 15 min, the plasmid mixture was added to EF cells and incubated at 37 C for 48 h under 5% CO_2 in air. These cells were then lysed through the addition of 100 μl Passive Lysis Buffer (Promega). The luciferase assay was performed with the Dual-Luciferase Reporter Assay System [26].

Table 3. Oligonucleotide primers for reporter construction

Name	Sequence
CDX2 intron1	F: 5'- GGTACCTTTTGCAAGTCTCTTGATTT -3'
	R: 5'- AGATCTCATGGTGGCGAGGGCCCGAC -3'
CSH1 Upstream	F: 5'- GGTACCGCACTGGTCACTTGAAGGC -3'
	R: 5'- AGATCTCATGAGGATTGGAGAGAAAT -3'
TKDP1 Upstream	F: 5'- GGTACCTTCATCACATGCCAGGTGCT -3'
	R: 5'- AGATCTCATCTTGGTGGCCTTGCAGG -3'

F: Forward R: Reverse.

Transient transfection of GATA2 or GATA3 siRNA

The nucleotide structures of *GATA2* siRNA and *GATA3* siRNA were designed through the siDirect program (RNAi, Tokyo, Japan), and the siRNAs were prepared commercially (Sigma-Aldrich, St. Louis, MO, USA). The nucleotide sequences of bovine *GATA2* (XM_583307) and *GATA3* (NM_001076804) were used to design the siRNA for *GATA2* and *GATA3* coding regions, respectively, while an unrelated sequence of *EGFP* (EU56363) was used as a negative control.

Bovine trophoblast CT-1 cells were cultured in the medium described in the cell culture section. To evaluate the effects of *GATA2* or *GATA3* siRNA on the abundance of endogenous trophoblast factor mRNAs, *GATA2* or *GATA3* siRNA (50 nM) was transfected into CT-1 cells using HilyMax as recommended by the manufacturer (Dojin Chemicals). The procedure for the transfection was essentially the same as previously described [26]. The concentrations of each siRNA had been predetermined before the experimentation. Total RNAs, extracted from CT-1 cells that had been transfected with *GATA2* or *GATA3* siRNA, were reverse-transcribed to cDNAs, which were then subjected to real-time PCR analysis with the primers listed in Table 1. These were used to determine mRNA levels for endogenous *GATA2*, *GATA3*, and trophoblast-specific factors. Average threshold (Ct) values for *GATA2*, *GATA3* and trophoblast-specific factor mRNAs were calculated and normalized to Ct values for *GAPDH* mRNA.

Statistical analysis

The real-time PCR data for mRNA levels were compared with the control by means of Student's *t*-test, and the real-time PCR data for the luciferase assays were analyzed by one-way ANOVA followed by Dunnett's test for multiple comparisons to compare those with the appropriate control using the StatView statistical analysis software (version 5; SAS Institute). Differences of $P<0.05$ were considered to be significant.

Results

Expression of GATA2, GATA3, and trophoblast-specific factor mRNAs in bovine conceptuses during the peri-implantation period

The amounts of *GATA2*, *GATA3*, and trophoblast-specific factor *ASCL2*, *CDX2*, *CSH1*, *ELF5*, *HAND1*, *IFNT*, and *TKDP1* mRNAs in days 17, 20 and 22 bovine conceptuses, and in bovine trophoblast CT-1 cells were examined by RT-PCR (Fig. 1A) or real-time

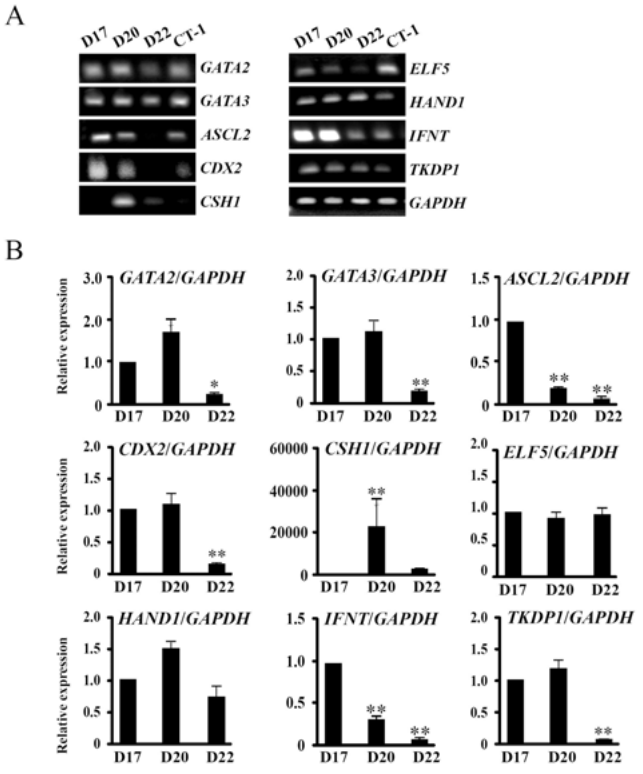

Fig. 1. Expression of *GATA2*, *GATA3* and trophoblast-specific factor mRNAs in the bovine conceptus during the peri-implantation period. A: Using RT-PCR and the primers shown in Table 1, the amount of *GATA2*, *GATA3* and trophoblast-specific factor (*ASCL2*, *CDX2*, *CSH1*, *ELF5*, *HAND1*, *IFNT* and *TKDP1*) mRNAs were evaluated in days 17, 20 and 22 (day 0 = day of estrus) bovine conceptuses and trophoblast CT-1 cells. The experiments were performed using three independent samples, and a representative one is shown. B: Using real-time PCR and the primers shown in Table 1, the amounts of *GATA2*, *GATA3* and trophoblast-specific factor (*ASCL2*, *CDX2*, *CSH1*, *ELF5*, *HAND1*, *IFNT* and *TKDP1*) mRNAs were evaluated in days 17, 20 and 22 (day 0=day of estrus) bovine conceptuses (n=3) and trophoblast CT-1 cells (n=4). Asterisks indicate statistically significant difference in mRNA levels ($P<0.05$) relative to the value on day 17. Note that the amount of *CSH1* mRNA on day 20 becomes more than 20,000 times the value on day 17.

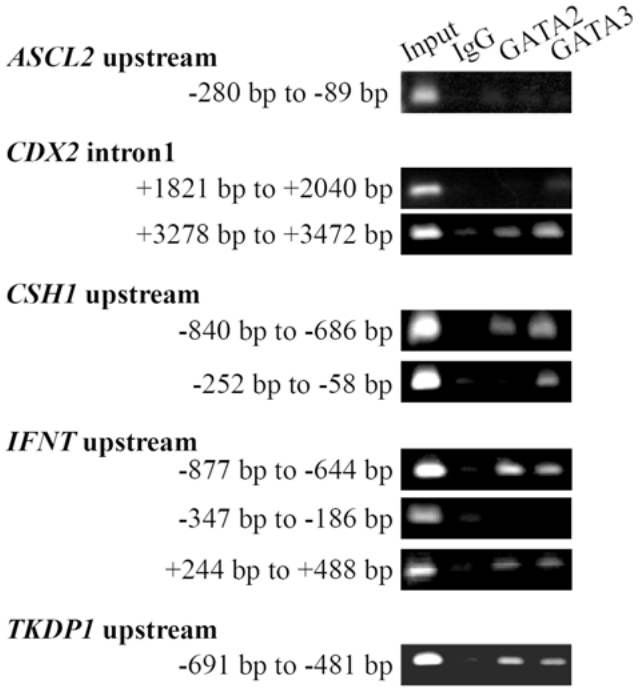

Fig. 2. Binding of GATA2 or GATA3 to regulatory regions of trophoblast-specific factor genes. Chromatin immunoprecipitation (ChIP) assay was used to show GATA binding to the regulatory regions of trophoblast-specific factor genes. In brief, bovine trophoblast CT-1 cells were treated with 1% formaldehyde, and the resulting protein-DNA complexes were sonicated. These complexes (approximately 500 bases) were then treated with antibody specific to GATA2, GATA3, or non-specific, negative control rabbit IgG. After precipitation and protein dissociation, DNAs were amplified for GATA binding regions in *ASCL2*, *CSH1*, *IFNT*, *TKDP1* and *CDX2* genes using the specific primers shown in Table 2.

PCR (Fig. 1B). *GATA2* and *GATA3* mRNAs decreased on day 22, after conceptus attachment to the uterine epithelium. Decreases in *ASCL2*, *CDX2*, *CSH1*, *IFNT* and *TKDP1* mRNAs were also found on day 22. Expression of *ELF5* and *HAND1* mRNA was detected on days 17, 20 and 22, but no significant change was found at pre- and post-conceptus attachment to the uterine epithelium. Among all factors evaluated, *CSH1* mRNA was not detected in CT-1 cells (Fig. 1A). Because of expression patterns similar to *GATA2* and *GATA3* mRNA in bovine conceptuses, *ASCL2*, *CDX2*, *CSH1 IFNT*, and *TKDP1* were chosen for subsequent studies.

Binding of GATA factors on the upstream regions of trophoblast-specific factor genes.

According to a motif search on the bovine genome, GATA binding sites are located on the upstream regions of *ASCL2*, *CSH1*, *IFNT*, and *TKDP1* genes. In addition, GATA binding sites are also found on the intron 1 region of the *CDX2* gene, which has been shown to be a regulatory element for *CDX2* transcription [3, 31, 32]. Targeting these locations, ChIP assays were performed to test the binding status of GATA2 or GATA3 to the upstream region of bovine trophoblast-specific factor genes in CT-1 cells (Fig. 2). The ChIP assays revealed that endogenous GATA2 or GATA3 occupied these GATA binding sites on the upstream regions of the *CSH1* (–840 bp to –686 bp and –252 bp to –58 bp), *IFNT* (*IFNTc1*, –877 bp to –644 bp and ORF region), and *TKDP1* (–69 bp to –481 bp) genes and on the intron 1 region of the *CDX2* (+3278 bp to +3472 bp) gene, but not on the upstream region of the *ASCL2* gene (Fig. 2). As a result, *ASCL2* gene was not subjected to luciferase studies.

Effect of GATA2 or GATA3 over-expression on the transcriptional activity of trophoblast-specific factor genes

GATA binding sites on the upstream regions of trophoblast-specific factor genes are well conserved among several species. It was realized that GATA2 and/or GATA3 occupied with distal (–840 bp

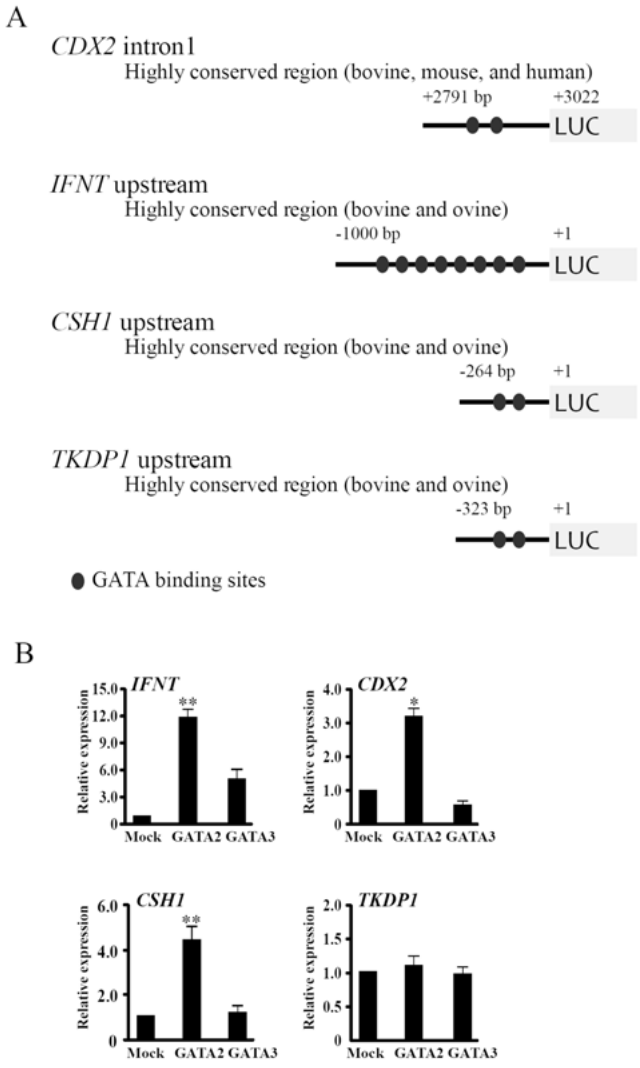

Fig. 3. Effects of GATA2 or GATA3 over-expression on transactivation of trophoblast-specific factor-reporter constructs. A: Schematic representation of the upstream regions of *CDX2*, *CSH1*, *IFNT*, and *TKDP1*, or the intron 1 region of *CDX2* [3, 31, 32] gene used for luciferase assay. Oligonucleotide primers used to amplify the upstream regions for reporter constructs are shown in Table 3. Note that the upstream region used as the promoter for bovine *CSH1* gene was described previously [33]. B: Transactivation of trophoblast-specific gene-reporter constructs when co-transfected with *GATA2* or *GATA3* expression plasmid into bovine EF cells. Note that GATA sites are well-conserved on the regulatory regions of trophoblast-specific factor genes. Data are presented as the mean ± SEM from four independent experiments, and single and double asterisks differ at P<0.05 and P<0.01 (n=3 each), respectively.

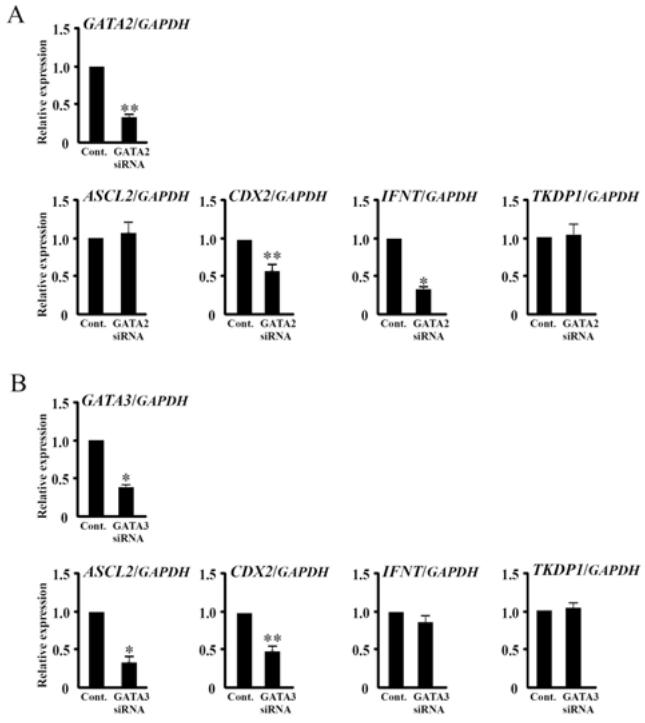

Fig. 4. Effects of *GATA2* or *GATA3* siRNA on transcript levels of endogenous trophoblast-specific factor genes in CT-1 cells. A: Endogenous transcript levels of trophoblast-specific factor genes following *GATA2* siRNA transfection into CT-1 cells. Amounts of trophoblast-specific factor mRNAs relative to that of a respective control in CT-1 cells were examined by means of real-time PCR and the primers shown in Table 1. B: Endogenous transcript levels of trophoblast-specific factor genes following *GATA3* siRNA transfection into CT-1 cells. Real-time PCR was used to examine amounts of trophoblast-specific factor mRNAs in CT-1 cells when transfected with *GATA3* siRNA. Data from real-time PCR were analyzed by Student's *t*-test, and results with single and double asterisks differ at P<0.05 and P<0.01 (n=3 each), respectively.

to –686 bp) and proximal (–252 bp to –58 bp) promoter regions of *CSH1* gene. Only the proximal promoter region was chosen for *CSH1*-reporter construction because the sequences required for ovine *CSH1* gene transcription have been characterized [33]. To examine GATA binding to these sites, the regulatory regions of approximately 500 bases in length were segmented adjacent to GATA binding sites (Fig. 3A). A luciferase assay was performed to examine whether GATA2 or GATA3 over-expression was effective in transactivation of *IFNT*, *CDX2*, *CSH1* or *TKDP1* reporter construct. Reporter constructs for *CDX2*, *CSH1*, and *IFNT* were transactivated when GATA2 was over-expressed (Fig. 3B). However, these constructs were not transactivated through GATA3 over-expression. Moreover, *TKDP1* reporter construct was not transactivated by either GATA2 or GATA3 over-expression (Fig. 3B).

Effect of GATA2 or GATA3 down-regulation on the degree of endogenous trophoblast-specific factor mRNAs

Changes in trophoblast-specific factor mRNAs were examined when *GATA2* siRNA (Fig. 4A) or *GATA3* siRNA (Fig. 4B) was transfected into CT-1 cells. Treatment with *GATA2* or *GATA3* siRNA was effective in reducing *GATA2* or *GATA3* mRNA. Expression of endogenous *CDX2* and *IFNT* mRNAs was reduced when *GATA2* siRNA was transfected into CT-1 cells. Expression

Gene name	Promotor location (bp)	ChIP Assay CT-1 cells		Luciferase Assay EF cells		siRNA Treatment CT-1 cells	
		GATA2	GATA3	GATA2	GATA3	GATA2	GATA3
ASCL2	-280 to -89	-	-			→	↓
CDX2	+1821 to -2040	-	-	↑	→	↓	↓
	+3278 to +3472	+	++				
CSH1	-840 to -686	+	+	↑	→	N.D.	N.D.
	-252 to -58	-	+				
IFNT	-877 to -644	+	+	↑ ↑	↑	↓	→
	-347 to -186	-	-				
	+244 to +488	+	+				
TKDP1	-691 to -481	++	+	→	→	→	→

Fig. 5. Experimental summary. Columns from left to light: gene name, promoter location (bp), ChIP assay (CT-1 cells), Luciferase assay (EF cells), and siRNA treatment (CT-1 cells). In each treatment, response to GATA2 or GATA3 is shown: –, no GATA binding; +, GATA binding; ↑, up-regulation; ↓, down-regulation; →, no change; and N.D., not detected.

of *ASCL2*, and *CDX2* mRNAs was reduced when *GATA3* siRNA was transfected into CT-1 cells (Fig. 4). The data from Fig. 2 to Fig. 4 are summarized in Fig. 5. Furthermore, *HAND1* and *ELF5*, excluded from the luciferase assay, were also examined for their GATA binding states and the effect of *GATA2* or *GATA3* siRNA on their mRNA levels. Expression of *ELF5* mRNA was reduced when *GATA3* siRNA was transfected into CT-1 cells, while *HAND1* mRNA was reduced through the use of *GATA2* siRNA (Supplemental Fig. 1).

Discussion

Multiple studies show that Gata2 and/or Gata3 are highly expressed in mouse trophoblast lineages, and regulate expression of multiple genes in trophoblast cells [3, 24, 34]. Although GATA factors are expressed in bovine trophoblast cells [26], except for *IFNT*, the identification of GATA's target genes has been rather limited. Because GATA motifs were found on the regulatory regions of trophoblast-specific factor genes, we hypothesized that these genes could also be under the control of GATA2 and/or GATA3. Here, we show that the expression patterns of several trophoblast-specific factor mRNAs in bovine conceptuses were similar to those of *GATA2* and *GATA3* during the peri-implantation period (Fig. 1). This agrees with previous findings that the expression pattern of GATA's target genes is often similar to those of GATA factors themselves [24, 25].

Recently, Gata3 was found to be a lineage-specific factor for trophectoderm cells in mice [35]. Numerous transcription factors have been found as markers that are specifically associated with trophoblast cell differentiation, i.e., Hand1 for trophoblast giant cell lineage [36, 37], and Ascl2 for spongiotrophoblasts in mice and for cytotrophoblast columns of the human placenta [38, 39]. However, molecular mechanisms by which these marker gene expressions are regulated have not been firmly established. In this study, we demonstrated that transcription of trophoblast-specific factor genes was regulated by GATA2 and/or GATA3 in bovine trophoblast CT-1 cells. In particular, GATA2 was found on the upstream regions of *IFNT* and *CDX2* genes in the ChIP assay, co-transfection of GATA2 enhanced *IFNT*- and *CDX2*-reporter activity in EF cells, and *GATA2* siRNA treatment reduced *IFNT* or *CDX2* mRNA in CT-1 cells. These results indicate that *IFNT* and *CDX2* genes are under the control of GATA2. Likewise, the regulation of *CDX2* and *ASCL2* transcription appears to be under the control of GATA3.

The response exhibited by ASCL2 is somewhat mysterious because there was no GATA factor binding to the upstream region of *ASCL2* gene, but *GATA3* siRNA was effective in reducing *ASCL2* mRNA in CT-1 cells (Fig. 5). Throughout the ChIP experimentations, the upstream regions for trophoblast-specific genes used were for those that have been well studied [31, 32, 33], conserved and/or identified through a motif search (www.motif.genome.jp/). Data from the present study suggest that the upstream sequence (–280 bp to –89 bp) of *ASCL2* may not be a solo region functioning in GATA binding sites in *ASCL2* gene transcription. If GATA3 binding sites were located on upstream regions other than the sequences currently examined, GATA binding sites could not be found in our ChIP assays. Finding GATA2 or GATA3 binding regions on trophoblast-specific genes is beyond the scope of the present investigation.

GATA2 or GATA3 binding to the regulatory regions was similar among the genes for trophoblast-specific factors examined in this study. It was recently reported that switching in chromatin occupancy between GATA2 and GATA3 is an important mechanism for the transcriptional regulation of *GATA2* gene expression and trophoblast differentiation [40]. These results indicate that changes and/or a balance in expression levels of GATA2 and GATA3 may be important in determining the expression of trophoblast-specific factor genes. It is likely that both GATA2 and GATA3 are involved in the regulation of trophoblast-specific factor genes, and that a molecular switch between GATA2 and GATA3 coordinates the transcriptional repression and activation of trophoblast-specific factor genes, respectively. It is possible that elucidation of molecular mechanisms associated with GATA switches in trophoblast cells may provide a new insight into controlling the expression of trophoblast-specific factor genes in addition to trophoblast cell differentiation.

In our transient transfection assays, *IFNT*-, *CDX2*- and *CSH1*-reporter constructs were transactivated when GATA2, not GATA3,

was over-expressed in non-trophoblast EF cells (Fig. 3). It should be noted that transcripts for *IFNT*, *CDX2*, or *CSH1* were not detected in EF cells. When each of the *IFNT*-, *CDX2*- or *CSH1*-reporter construct and *GATA2/GATA3* expression plasmid were co-transfected into EF cells, high Luciferase activity was detected. However, GATA2/GATA3 over-expression did not result in endogenous IFNT, CDX2 or CSH1 protein production in EF cells (unpublished observation). Thus, GATA2/GATA3 over-expression was effective in enhancing *IFNT*-, *CDX2*- or *CSH1*-reporter transactivation as determined by Luciferase activity, but did not result in the transcription of endogenous *IFNT*, *CDX2* or *CSH1* genes. It is possible that in addition to GATA factors, these trophoblast-specific factors are under the control of other transcription factors such as CDX2 and CREBBP [30]. It was recently reported that Gata3 and Cdx2 act in concert to induce the expression of common and independent target genes in the trophoblast lineage [35]. Moreover, transcription factors including GATA3 occupy regulatory regions of a number of target genes in TS cells [41]. GATA factors may interact alone or in combination with other transcription factors at the regulatory regions of trophoblast-specific factor genes, which in turn determine timing and degree of trophoblast-specific factor expression in trophoblast cells. Together, these data suggest that the transcription of trophoblast-specific genes is not entirely regulated through GATA2 and/or GATA3 transcription factors, rather their epigenetic status along with GATA2/GATA3 and possibly other transcription factors are involved in the transcription of endogenous genes in CT-1 cells.

In conclusion, we demonstrated that trophoblast-specific *ASCL2*, *CDX2*, *CSH1*, *ELF5*, *HAND1*, and *IFNT* genes were regulated by GATA2 and/or GATA3 in bovine cells. Because these trophoblast-specific factors exist in many mammalian species, it is likely that regulation of these genes by GATA2 and/or GATA3 could be similarly conserved. Therefore, effects of GATA2 and/or GATA3 on the transcriptional regulation of trophoblast-specific factors demonstrated in this study may not be limited to the ruminant ungulates.

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