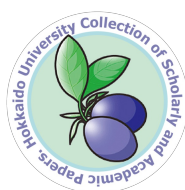




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—Original—

Expression and Potential Role of GATA6 in Ruminant Trophoblasts during Peri-implantation Periods

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Abstract: Expression of GATA6 has been found during embryonic development in many mammalian species. In mouse embryonic development, GATA6 is a primitive endoderm (PrE) marker. However, the expression and effect of GATA6 in ruminant ungulates has not been well characterized. In this report, the expression of GATA6 mRNA was examined in the uteri of ruminants. GATA6 mRNA was detected in days 17, 20, and 22 (day 0=day of estrus) bovine conceptuses and in days 15, 17, and 21 ovine conceptuses. In both cases, GATA6 mRNA increased on day 22 or 21 after conceptus attachment to the uterine epithelium. GATA6 mRNA was also detected in bovine trophoblast CT-1 or F3 cells and ovine trophoblast oTr cells. In transient transfection analyses using the upstream region of the bovine IFNT gene (bIFNT, IFN-tau-c1), GATA6 overexpression was effective in the up-regulation of the bIFNT construct that had been transfected into human choriocarcinoma JEG3 or bovine ear-derived fibroblast EF cells. The observation in which GATA6 increased IFNT transcription suggests that in addition to lineage specification, GATA6 may have acquired ruminant-specific functions involving the control of trophoblast gene expression.

Key words: GATA6, Trophoblast cells, Implantation, Ruminants

Introduction

Numerous factors that regulate trophoblast lineage specification have been identified; however, the regulation of trophoblast-specific gene expression has not been definitively characterized. GATA transcription factors contain a highly conserved DNA-binding domain, which bind to the consensus DNA sequence W (A/T) GATAR (A/G), resulting in transcriptional regulation of downstream genes [1–3]. In vertebrates, the GATA transcription factors comprise 2 subfamilies, GATA1–3 and GATA4–6. GATA1, GATA2, and GATA3 regulate development and differentiation of hematopoietic lineages [4–6], while GATA4, GATA5, and GATA6 are involved in cardiac development and endodermal derivatives [7–9]. Murine GATA6 expression has been reported to be restricted to pre-cardiac mesoderm, the embryonic heart tube, and the primitive gut. It is also expressed in the developing respiratory and urogenital tracts, arterial smooth muscle cells, bronchi, urogenital tract, and bladder [10–12]. In mice, GATA6 is expressed in embryonic stem (ES) cells of the inner cell mass (ICM) [13, 14], which later become the cells of the extra-embryonic endoderm (ExE) [8]. Gene targeting experiments have also shown that Gata6-null mice die shortly after implantation because of ExE defects [8].

Previously, we have shown that GATA2 and GATA3 are expressed in bovine conceptuses and regulate trophoblast-specific factors, including interferon tau (IFNT), a major cytokine involved in the maternal recognition of pregnancy in ruminants during the peri-attachment period [15, 16]. We also reported that GATA1 was expressed in ovine conceptuses after attachment to uterine endo-

metrial epithelial cells [17]. While we were studying the expression and function of these GATA factors, we also noticed that GATA6 was expressed in ruminant trophoblast cells.

The purpose of this study was to characterize the expression of GATA6 mRNA in ruminant trophoblast cells, as well as to examine the effect of GATA6 on *IFNT* expression. In this study, we demonstrated the expression of GATA6 mRNA in peri-attachment uteri and deduced that this expression could be involved in the regulation of *IFNT* transcription.

Materials and Methods

Animal sampling and uterine fixation

Whiteface crossbred ewes were maintained at the farm of the University of Tennessee, Knoxville, TN, and the protocol for sheep experimentation was reviewed and approved by the animal care committee at the University of Tennessee. Animal care, estrous synchronization procedures, and tissue collection were performed as previously described [18]. Hysterectomy was performed on days 15, 17, or 21. Day 15 and 17 conceptuses were collected by uterine flushing, while day 21 conceptuses were collected following longitudinal incision of the uterine horns in pregnant ewes. For uterine fixation, uteri from cyclic ewes on day 15 and pregnant ewes on days 15, 17, and 21 of gestation were removed, and subjected to whole uteri fixation immediately after slaughter [19]. Fixed whole uteri were serially dissected into proximal to distal uterine segments; each segment was paraffin-embedded and transferred to the Laboratory of Animal Breeding at the University of Tokyo, Japan.

Japanese black cattle were maintained at Zen-Noh Embryo Transfer (ET) Center, Hokkaido, Japan, and experimental procedures for these cattle were approved by the Committee for Experimental Animals at Zen-Noh ET Center and the University of Tokyo. Conceptuses were collected non-surgically by uterine flushing on days 17, 20, or 22 ($n = 3$ each), as described previously [20] and were subjected to total RNA extraction.

Cell culture

Bovine trophoblast CT-1 cells [21] and ovine trophoblast oTr cells [22] were kindly provided by Dr. A. Ealy, University of Florida, Gainesville, FL. CT-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% (v/v) fetal bovine serum (FBS; Invitrogen) supplemented with 4.5 g/l D-glucose (Invitrogen), nonessential amino acids (Invitrogen), 2 mM glutamine (Invitrogen), 2 mM sodium

pyruvate (Invitrogen), 55 μ M β -mercaptoethanol (Invitrogen), and antibiotic/antimycotic solution (ABAM, Invitrogen). oTr cells were maintained in DMEM/Ham's F12 (Invitrogen) containing 10% FBS (Invitrogen), supplemented with 2 mM glutamine, 700 nM insulin, and ABAM (Invitrogen). Bovine trophoblast F3 cells [23], kindly provided by Dr. Christiane Pfarrer, were cultured in DMEM/Ham's F12 (Invitrogen) containing 10% FBS (Invitrogen), 2 mM glutamine, and ABAM (Invitrogen). Bovine ear-derived fibroblast (EF) cells were obtained from biopsied ear skin of a Japanese black bull (4 months old). EF cells were cultured in DMEM containing 5% FBS (Invitrogen) and ABAM (Invitrogen). Human choriocarcinoma JEG3 cells (HTB36; American Type Tissue Collection, ATCC) were grown in DMEM supplemented with 10% FBS (Invitrogen) and ABAM (Invitrogen). These cells were maintained at 37°C in air with 5% CO₂.

RNA extraction and analysis

Total RNA was extracted from bovine and ovine conceptuses ($n = 3$ for each day), trophoblast cells, JEG3 and EF cells with ISOGEN (Nippon Gene, Tokyo, Japan), according to the protocol provided by the manufacturer. For PCR and real-time PCR analyses, isolated RNA (total, 1 μ g) was reverse-transcribed to cDNA using ReverTra Ace qPCR RT Kit (Toyobo, Tokyo, Japan) including 5 $\times$ RT buffer, Enzyme Mix, and primer Mix in a 10- μ l reaction volume, and the resulting cDNA (RT template) was stored at 4°C until use. The cDNA reaction mixture was diluted 1:10 using DNase- and RNase-free molecular biology-grade water.

Reverse-transcribed cDNA (3 μ l) synthesized from conceptus cells, GATA6-transfected JEG3, and EF cells were subjected to PCR analysis as previously described [15]. Reverse-transcribed cDNA (3 μ l) synthesized from conceptus RNAs was subjected to real-time PCR amplification with 0.5 units of ExTaq HS polymerase (Takara Biomedicals, Shiga, Japan), 1 $\times$ ExTaq HS buffer, 0.2 μ M of the oligonucleotide primers listed in Table 1, 0.2 mM of dNTP, SYBR green (SYBR Green I Nucleic Acid Gel stain; Takara Biomedicals) as fluorescence intercalator and Rox reference dye (Invitrogen) in a final volume of 20 μ l. PCR amplification was carried out on an Applied Biosystems 7900HT real-time PCR System (Applied Biosystems, Tokyo, Japan) as previously described [24]. The thermal profile for real-time PCR was 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s. Average cycle threshold (Ct) values for bovine and ovine GATA6 mRNA were calculated and normalized to Ct values for *ACTB* mRNA. Each run was completed with a melting curve analysis to confirm the

Table 1 Primers for GATA6

Name (GenBank accession No.)	Product length (bp)
For RT-PCR and Real-time PCR analyses	
<i>GATA6</i> (Bovine: XM001253596; Ovine: AB612145)	
F: 5'- AAGATGCTGACCAGACATCT -3'	
R: 5'- AGAGACCAGCTGCCTGGAAGT -3'	206
<i>Gata6</i> (Murine: NM_010258.3, for expression check)	
F: 5'- GCCAACTGTCACACCACAAC -3'	
R: 5'- TGTACCGGAGCAAGCTTTT -3'	189
<i>ACTB</i> (Bovine: BC102948; Ovine: NM_001009784)	
F: 5'- CTCTTCCAGCCTTCCTTCCT -3'	
R: 5'- GGGCAGTGATCTCTTCTGC -3'	178
For <i>in situ</i> hybridization probe	
<i>GATA6</i> (Ovine: AB612145)	
F: 5'- AAGATGCTGACCAGACATCT -3'	
R: 5'- TCAGGGAAGGGGCTGTGGGA -3'	406

F: Forward, R: Reverse

specificity of amplification and the absence of primer dimers. The $\Delta\Delta C_t$ method was used to calculate the data, and the results are shown as the mean $\pm$ SEM.

In situ hybridization

In situ hybridization, using serial sections, was performed under contract with Genostaff Co., Ltd. (Tokyo, Japan), as previously described [17]. Digoxigenin (DIG)-labeled RNA probes were prepared with DIG RNA labeling Mix (Roche Diagnostics Japan, Tokyo, Japan). Ovine uteri and conceptuses fixed and embedded in paraffin were sectioned at 4- μ m thickness. Tissue sections were deparaffinized, rehydrated, fixed with 4% paraformaldehyde, and then treated with Proteinase K in PBS at 37°C for 30 min. Hybridization was performed with probes in the Probe Diluent (Genostaff Co., Ltd.) at 60°C for 16 h [25]. A 570 bp long of plant-derived nucleotide sequence (Genostaff Co., Ltd.) was also used to generate an RNA probe to serve as the negative control [26]. After hybridization, the sections were washed, treated with 50% formamide and then followed by RNase treatment. The sections were then washed and subjected to 2-h incubation with anti-DIG alkaline phosphate (AP) conjugate (Roche Diagnostics Japan) diluted 1:1000 with TBST. Coloring reactions were performed with NBT-BCIP (Roche Diagnostics Japan), an AP color substrate. The sections were counterstained with Kernechtrot stain solution (Nuclear Fast Red; Muto Pure Chemicals, Tokyo, Japan), dehydrated, mounted with Malinol (Muto Pure Chemicals), and then examined under a light microscope (BX-51; Olympus, Tokyo, Japan).

Plasmid, transient transfection, and luciferase assay

To generate bovine *IFNT* (*bIFNT*, *IFN-tau-c1*)-luciferase reporter construct (*bIFNT*-Luc), the upstream region (−631 to +59 bp) was inserted into pGL3 basic vector (Promega, Madison, WI), as described previously [15, 24]. The *Renilla* luciferase-pRL-TK vector (Promega), driven by the herpes simplex virus-thymidine kinase (HSV-TK) promoter, was used to normalize the transfection efficiency [27]. Amount of reporter construct used relative to that of the internal control vector pRL-TK was 20:1. A mouse *GATA6* expression plasmid, constructed in pPyCAG-IP, was kindly provided by Dr. H. Niwa, Laboratory for Pluripotent Cell Studies RIKEN Center for Developmental Biology [28].

JEG3 or EF cells were cultured as described in the cell culture section and plated in 24-well plates for subsequent transfection. At 60–80% confluence, transient transfection was performed using HilyMax reagent (Dojin Chemicals, Kumamoto, Japan) according to the manufacturer's protocol. In brief, 2 μ g total plasmid DNA, including *bIFNT*-Luc (1.5 μ g), expression plasmids (total of 0.5 μ g), and 4 μ l HilyMax were prepared in 30 μ l DMEM containing no supplements (plasmid mixture). The amount of total plasmid DNA for each transfection was adjusted using pSG5 (an empty vector). After 15 min, plated cells were overlaid with the plasmid mixture and incubated at 37°C for 48 h under 5% CO₂ in air. At 48 h after transfection, the cells were lysed by adding 100 μ l Passive Lysis Buffer (Promega). A luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega) as described previously [27].

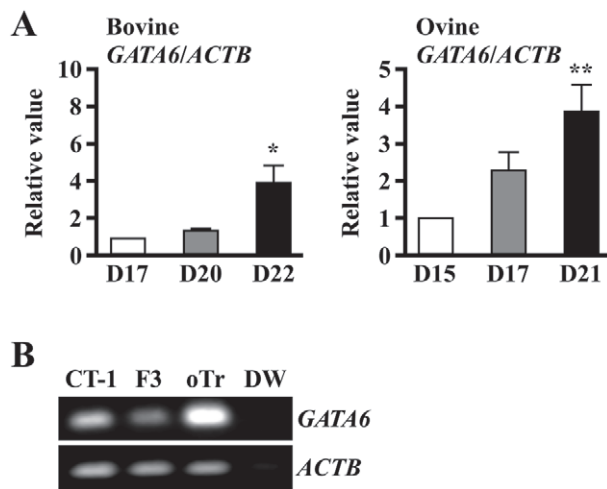


Fig. 1. GATA6 mRNA expression in bovine and ovine conceptuses and trophoblast cell lines.

A: Real-time PCR analysis was carried out to determine the amounts of GATA6 mRNA in days 17, 20, and 22 bovine conceptuses and in days 15, 17, and 21 ovine conceptuses. RNA extracted from frozen conceptuses was subjected to real-time PCR analysis using the primers shown in Table 1. Results with single and double asterisks differ at $P < 0.05$ and $P < 0.01$ ($n = 3$ each), respectively. **B:** Total RNAs isolated from bovine trophoblast CT-1 and F3 cells and ovine trophoblast oTr cells were reverse-transcribed to cDNA and then subjected to PCR analysis using the primers shown in Table 1. A representative agarose gel from 3 independent experiments is shown.

Statistical analysis

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

Results

GATA6 mRNA expression in bovine and ovine conceptuses and trophoblast cell lines

In bovine and ovine pregnancy, conceptus attachment to the uterine epithelium begins on day 19 and day 16, respectively. Amounts of GATA6 mRNA in days 17, 20, and 22 bovine conceptuses and days 15, 17, and 21 ovine conceptuses were examined by real-time PCR using the primers listed in Table 1. Minute amounts of GATA6 mRNA were found in day 17 bovine and day 15 ovine

conceptuses; these amounts increased after conceptus attachment to the uterine epithelium (Fig. 1A).

GATA6 mRNA levels in bovine and ovine trophoblast cell lines were then examined. GATA6 mRNA was found in bovine trophoblast CT-1 and F3 cells, as well as in ovine trophoblast oTr cells (Fig. 1B). These data indicate that GATA6 mRNA is expressed in bovine and ovine trophoblast cells.

Localization of GATA6 mRNAs in ovine conceptuses

To localize GATA6 mRNA in day 15, 17, and 21 ovine conceptuses, *in situ* hybridization was performed (Fig. 2). Although GATA6 mRNA could not be detected on day 15, GATA6 mRNA was found in trophectoderm cells in day 17 and 21 ovine conceptuses, which was in agreement with the data shown in Fig. 1. No signal was detected in the serial sections when the experiment was performed with the sense GATA6 riboprobe or with 570-bp plant-derived sequences as a negative control.

Transient transfection analyses of bovine IFNT in human choriocarcinoma JEG cells and bovine ear-derived fibroblast EF cells

Co-transfection experiments with the *bIFNT*-reporter construct and GATA6 expression plasmids were performed in human choriocarcinoma JEG cells and in bovine ear-derived fibroblast EF cells. In either cell type, transfection of mGata6 resulted in high levels of GATA6 mRNA expression in JEG and EF cells. Results from GATA6 over-expression on *bIFNT* transcription in co-transfected human choriocarcinoma JEG cells or bovine non-trophoblast, ear-derived fibroblast EF cells, are shown in Fig. 3. In both cases, *IFNT* transcription, measured as luciferase activity, increased when GATA6 was over-expressed (Fig. 3).

Discussion

We have examined expression of GATA6 mRNA in bovine and ovine conceptuses during peri-implantation periods herein, as well as in previous studies. Minute amounts of GATA6 mRNA were detected in day 17 bovine and day 15 ovine pre-attachment conceptuses; the levels increased on day 22 and 21, respectively, following conceptus attachment to the uterine epithelium, and GATA6 mRNA was present also in bovine trophoblast CT-1 cells [15]. The expression of GATA6 has been reported in the bovine embryo [29]. The presence of GATA6 mRNA in bovine embryogenesis is also available in raw microarray data (GEO accession number: GSE12901). However, in our previous study [15], GATA6 was not chosen as a

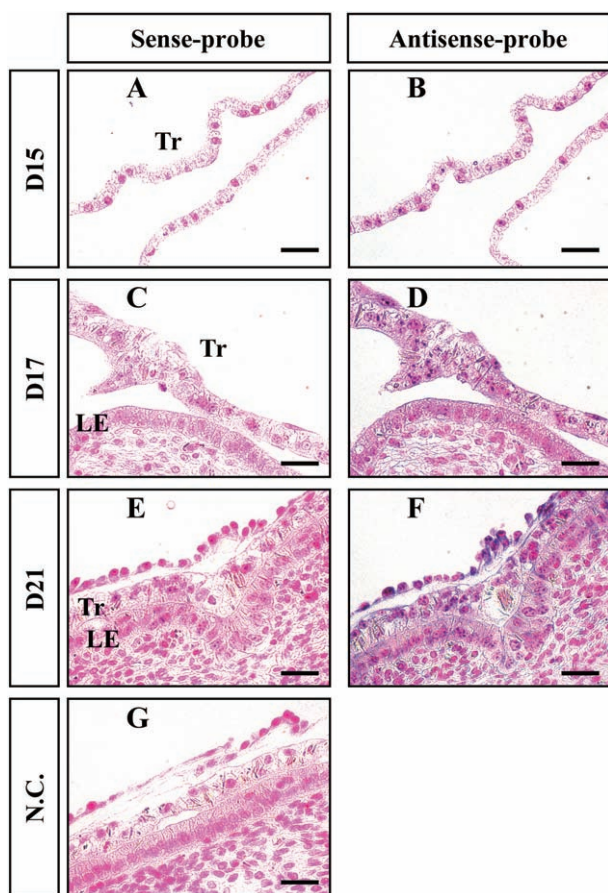


Fig. 2. Localization of *GATA6* mRNA in the ovine uterus during the peri-attachment period.

In situ hybridization analysis was carried out using either sense (A, C, and E) or anti-sense (B, D, and F) *GATA6* riboprobe (Table 1) to hybridize to serial sections of ovine conceptuses and uteri from day 15 (D15; A, B), 17 (D17; C, D), and 21 (D21; E, F) pregnant ewes ($n = 3$ for each day). The riboprobe generated from a 570-bp plant-derived nucleotide sequence was used as a negative control (N.C., D21; G). A representative *in situ* hybridization from 3 independent experiments containing uterine tissues from 3 animals is shown. LE, luminal epithelial cells; Tr, trophoblast cells. Scale bar: 50 μ m.

potential candidate dictating trophoblast-specific expression of *bIFNT* because (a) the *GATA6* mRNA expression pattern differed from that of *bIFNT*, *GATA2*, and *GATA3*, and (b) during early mouse embryonic development, *GATA6*, a well-known primitive endoderm (PrE) marker, is expressed in the ExE lineage [10]. In the present study, we demonstrated that the expression of *GATA6* mRNA in the trophoblast, as well as co-transfection of a *GATA6* expression construct, was effective in the up-regulation of the *bIFNT*-reporter construct transfected into human

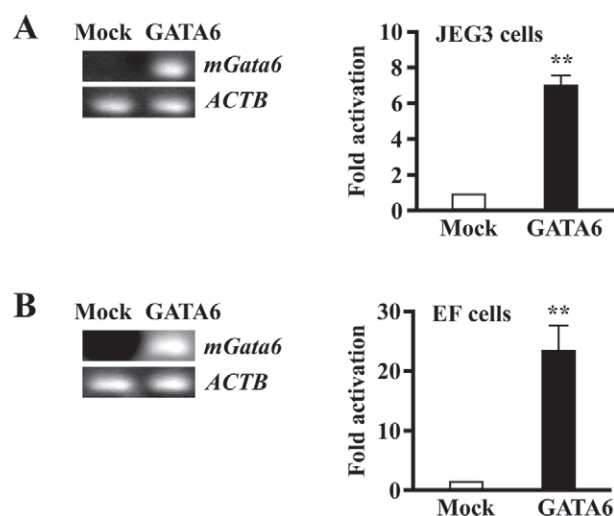


Fig. 3. Effects of *GATA6* overexpression on transactivation of the *bIFNT* gene-reporter construct.

Transactivation of the *bIFNT*-reporter construct, when co-transfected with a *GATA6* expression plasmid into human choriocarcinoma JEG3 cells or bovine ear-derived EF cells. Left: JEG3 cells and EF cells were transfected with pPyCAG-IP-*Gata6*, or pSG5-empty (mock construct) and cultured for 48 h. Total RNA was extracted from these cells, reverse-transcribed to cDNA, and subjected to RT-PCR analysis for the examination of *GATA6* mRNA expression. Right: Results are expressed as luciferase activity relative to that of cells harbouring the *bIFNT* gene-reporter construct but no expression plasmid (mock). Data are presented as mean $\pm$ SEM from 5 independent experiments; single and double asterisks differ at $P < 0.05$ and $P < 0.01$, respectively.

choriocarcinoma JEG cells or bovine non-trophoblast EF cells (Fig. 3).

The numerous transcription factors found thus far as regulators of *IFNT* genes include ETS2 [30, 31], activating protein 1 (AP-1; official symbol JUN) [32], CDX2 [24, 27], homeobox protein distal-less 3 (DLX3) [33], and co-activators cAMP-response element binding protein (CREB)-binding protein (CREBBP) [34] and p300 [35]. Recently, we found that *GATA2* and *GATA3* could also increase *IFNT* transcription in our transient transfection system [15, 16]. During the peri-attachment period, changes in *IFNT*, *GATA2*, and *GATA3* expression are similar, and the expression of these proteins declines following conceptus attachment to the uterine epithelium [15, 16]. In our experiment, *GATA6* expression increased after conceptus attachment to the uterine epithelium (Fig. 1A), when *IFNT* expression declined. Although *IFNT* expression declines, approximately 5–10 μ g *IFNT* are still produced when the conceptus is cultured *in vitro* [36]. These re-

sults indicate the possibility that GATA6 is not related to the initiation or cell-specific expression of IFNT, although it is involved in the maximum expression of IFNT during the pre-attachment period, or that GATA6 may support IFNT expression following conceptus attachment to the uterine epithelium when the reduced GATA2 and GATA3 levels can no longer support *IFNT* transcription.

It should be noted that GATA6 expression differs from that of GATA2, GATA3, and IFNT. The observation that GATA6 increased *IFNT* transcription in JEG3 or bovine EF cells indicates that similar to the findings previously demonstrated for GATA2 and GATA3 [15], GATA6 could have the ability to increase *IFNT* transcription during the early post-attachment period when other transcription factors may not be available to sustain lowered, but sufficient, levels of *IFNT* transcription. These results suggest that in addition to lineage specification, GATA factors may have acquired a species-specific ability to control trophoblast-specific gene expression in ruminant ungulates.

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