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Title: **Expression and *in situ* localization of *GATA4, 5,* and *6* mRNAs in ovine conceptuses and uterine endometria during the peri-implantation period**

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Running Head: *GATA4-6* transcripts in ovine uteri

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ABSTRACT

In vertebrates, six GATA transcription factors, GATA1 through GATA6, have been identified, and GATA1-3 are known to be involved in hematopoietic developments while GATA4-6 play roles in cardiac and endoderm developments. Recently, we and others have found that GATA2 and GATA3 found in the trophectoderm plays a role in gene expression specific to this cell type, but GATA4-6 have not been well characterized in early embryonic developments. Using quantitative polymerase chain reaction (qPCR) and *in situ* hybridization, we examined the expression of *GATA4*, *5*, and *6* mRNAs in ovine conceptuses and uteri during the peri-implantation period. In ovine conceptuses, *GATA4*, *5*, and *6* transcripts were present on days 15, 17, and 21 (day 0 = day of mating), and high *GATA5* and *6* mRNAs were found on day 21, most of which were localized in the trophectoderm and endoderm. Moreover, minute and substantial *GATA4* and *5* mRNAs were found in days 15 and 21 uterine endometria, respectively. Increase in *GATA4-6* transcripts in day 21 uteri indicates that in addition to GATA1-3, GATA4-6 may also play a potentially novel role in the development of ovine trophectoderm, endoderm and/or uterine endometria following conceptus attachment to the uterine epithelium.

Key Words*: GATA4-6, gene expression, ovine uterus, peri-implantation period .*

INTRODUCTION

In most mammals, the processes of conceptus implantation to the uterine endometrium consist of blastocyst hatching, migration, apposition/attachment, invasion and subsequent placental formation, during which nearly 50% of conceptuses fail to implant (Wilcox *et al*. 1988). Despite exhaustive studies thus far done to improve pregnancy outcome in large domestic animals, our knowledge is still too far from solving the problem. These indicate that factors and/or events classically studied may not be sufficient to reconstruct the phenomenon associated with conceptus implantation to the uterine endometrium, strongly suggesting that further, continuous studies are required on factors yet identified and those that have been overlooked.

In vertebrates, GATA transcription factors are a family of six structurally related proteins, GATA1 through GATA6, that bind to the consensus DNA sequence W(A/T)GATAR(A/G), resulting in transcriptional regulation of down-stream genes (Yamamoto *et al*. 1990; Ko & Engel 1993; Merika & Orkin 1993). These GATA factors have been divided into two subfamilies, GATA1-3 and GATA4-6, based on sequence similarity and expression pattern. GATA1, GATA2 and GATA3 found in hematopoietic lineages regulate their development and differentiation, while GATA4, GATA5 and GATA6 are involved in cardiac development and endodermal derivatives (Molkentin 2000; Patient & McGhee 2002). These studies show that members of GATA transcription factors play important roles, regulating cell lineage specification during vertebrate development.

In *Gata* gene ablation studies, lack of each *Gata* gene with the exception of GATA5 (Molkentin *et al*. 2000) results in mid-gestation lethality (Tsai *et al*. 1994; Pandolfi *et al*.

1995; Fujiwara *et al*. 1996; Molkentin *et al*. 1997; Morrisey *et al*. 1998). For this reason, GATA factors had not been considered important for early conceptus development. However, over the past four years, GATA3 has been found to assist trophectoderm differentiation in mice (Home *et al*. 2009), while we have shown that GATA2 and GATA3, expressed in bovine conceptuses, regulate trophoblast-specific factors during the peri-attachment period (Bai *et al*. 2009, 2011). In addition, we also reported that GATA1 expression increased in ovine and/or bovine trophoblast cells after conceptus attachment to the uterine epithelial cells, coincide with reduced *GATA2* and *GATA3* mRNA expression, suggesting the possibility that GATA1 may be involved in the down-regulation of *GATA2* transcription (Bai *et al*. 2012a). These results also suggest that even among hematopoietic group GATA1, 2 and 3, their expression patterns and possibly their roles may differ.

It should now be realized that GATA expression and role could be those in common among GATA family genes and those specific to each of GATA factors. Among the cardiac group of GATA4-6, *GATA6* was the only GATA factor studied in ovine conceptuses (Bai *et al*. 2012b). Studies from *GATA1-3* and *GATA6* suggest that the expression of other cardiac group of GATA factors, GATA4 and GATA5, along with GATA6 should carefully be evaluated *in utero* during the peri-implantation period. The aim of this study was to examine the expression of *GATA4*, *5*, and *6* mRNAs in ovine conceptuses and endometria during the peri-implantation period.

MATERIALS AND METHODS

Collection of ovine conceptuses and uterine fixation

Whiteface crossbred ewes were maintained at the farm of the University of Tennessee, Knoxville, TN, and the protocol for sheep experimentation had been reviewed and approved by the animal care committee at the University of Tennessee. Animal care, estrous synchronization procedures, and tissue collections were done as previously described (Sakurai *et al*. 2010). Hysterectomy was performed on days 15, 17, and 21 (day 0 = day of estrus). Conceptuses from days 15 and 17 pregnant animals were collected by uterine flushing while conceptuses from day 21 pregnant ewes were collected following longitudinal incision of uterine horns. These samples were each frozen immediately and transported to the Laboratory of Animal Breeding, the University of Tokyo, Japan.

For uterine fixation, uteri from pregnant ewes on days 15, 17, and 21 of gestation (n=3 each) were removed and subjected to whole uterus fixation immediately after slaughter (Imakawa *et al*. 2002). Fixed whole uteri that were serially dissected into proximal to distal uterine segments were each paraffin-embedded and transferred to the Laboratory of Animal Breeding at the University of Tokyo.

RNA extraction and analysis

Total RNA was isolated from ovine conceptus tissues (n=3 from each day) with ISOGEN (Nippon Gene, Tokyo, Japan) according to the protocol provided by the manufacturer. For quantitative polymerase chain reaction (qPCR) analyses, isolated RNA (total 1 μg) was reverse-transcribed to cDNA using ReverTra Ace qPCR RT Kit (Toyobo, Tokyo, Japan) including 1 x 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 1:10 using DNase and RNase free molecular biology grade water and 3 μL were taken for each amplification reaction.

Reverse-transcribed cDNA (3 μL) synthesized from conceptus RNAs was subjected to qPCR amplification with 0.5 units of ExTaq HS polymerase (Takara Biomedicals), 1 x ExTaq HS buffer, 0.2 mM 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 intercalater and Rox reference dye (Invitrogen) in a final volume of 20 μL and PCR amplification was carried out on a real-time PCR System (7900HT; Applied Biosystems) as previously described (Sakurai *et al*. 2009). The thermal profile for real-time PCR was at 95 C for 10 min, followed by 40 cycles at 95 C for 10 sec, 60 C for 20 sec and 72 C for 30 sec. Average cycle threshold (Ct) values for ovine *GATA4*, *5*, and *6* mRNA were calculated and normalized to Ct values for *ACTB* mRNA. Each run was completed with a melting curve analysis to confirm the specificity of amplification and the absence of primer dimers. Delta-delta Ct method was used to calculate the data, and the results were shown as the mean +/- SEM.

In situ hybridization

DNA fragment of ovine *GATA4*, *5*, and *6* (GenBank ID: XM_004005334, XM_004014417, XM_004020625, respectively) was subcloned into pGEM-T Easy vector (Promega) and was used for generation of sense or anti-sense RNA probe. A 570 bp length of plant-derived nucleotide sequence was also used to generate an RNA probe to serve as the negative control (Genostaff Co., Ltd; Arao et al. 2010). Digoxigenin (DIG) labeled-RNA probes were prepared with DIG RNA labeling Mix

(Roche Diagnostics). Ovine uteri and conceptuses fixed and embedded in paraffin were sectioned at 5 μm, and at least two sections from different portions of uteri were examined. *In situ* hybridization was performed under contract with Genostaff Co., Ltd (Tokyo, Japan) as described previously (Bai *et al*. 2012a, b). 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).

Statistical analysis

The qPCR data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons to compare with the control of experimental group with the StatView statistical analysis software (version 5; SAS Institute, Inc., Cary, NC, USA). Differences of $P < 0.05$ were considered to be significant.

RESULTS

Expression of *GATA4, 5,* and *6* mRNA in ovine conceptuses

In the ovine pregnancy, the trophoblast begins to attach to the uterine epithelium on days 16-17. Adhesion of the trophoblast to the uterine epithelium progresses along the uterine caruncles and appears to be completed around day 22 (Boshier 1969; Guillomot *et al*. 1981). Amounts of *GATA4*, *5*, and *6* mRNA in days 15, 17, and 21 ovine conceptuses were examined by qPCR using primers listed in Table 1. Ovine *GATA4*, *5*, and *6* transcripts were found in all days examined. No significant difference was found in *GATA4* expression at this time period. Trace amounts of *GATA5* and *GATA6* mRNAs were found in day 15 ovine conceptuses, and higher expression of these mRNAs were found on day 21 after conceptus attachment to the uterine epithelium (Fig. 1). Note that

GATA5 mRNA on day 21 became more than 40 times the value of day 15.

In situ localization of *GATA4, 5,* and *6* mRNAs in ovine uteri

Using *in situ* hybridization, the presence of *GATA4, 5,* and *6* mRNAs was examined in days 15, 17, and 21 ovine conceptuses and uteri (Figs. 2-4). *GATA4* mRNA was localized in days 15, 17 and 21 trophectoderm and endodermal cells (Fig. 2). *GATA4* mRNA was also found in the border of day 15 uterine epithelial cells and stromal cells, and in day 21 stromal cells. *GATA5* mRNA was found in trophectoderm and endodermal cells of days 17 and 21 ovine conceptuses, and in days 15 and 21 uterine stroma (Fig. 3). *GATA6* mRNA was faintly detected in days 17 and 21 trophectoderm and endodermal cells, and in day 21 uterine stroma, but was not found in day 15 conceptuses, or day 15 or 17 endometria (Fig. 4). Similar staining was found in two different uterine sections/blocks from the same day. Moreover, no signal was detected in the serial section when the *in situ* hybridization experiment was performed with riboprobes of the sense *GATA4, 5* or *6*, or a 570 bp length of plant-derived sequence (Supple. Fig. 1).

DISCUSSION

In this study, transcripts of *GATA4, 5,* and *6* were detected in both trophectoderm and endodermal cells of ovine conceptuses, and *GATA4* and *5* mRNAs were localized in day 21 endometrial stroma. In the present and previous (Bai *et al*. 2009, 2012a, b) studies, we characterized that conceptus *GATA2* and *GATA3* expression decreased, whereas *GATA1* and *GATA4-6* expression increased following its attachment to the uterine endometrial epithelial cells. Although the role of GATA2 and GATA3 on the expression of IFNT, a factor essential for pregnancy establishment in ruminants (Imakawa *et al*. 2009), has been characterized (Bai *et al*. 2009), potential roles of other GATA factors, of

which expression coincides with conceptus attachment and subsequent placentation, have not been investigated or elucidated. Somewhat temporal and spatial expression of *GATA* transcripts in the conceptus and uterine stroma suggests that other than those already known such as blood and heart formation, GATA1-3 transcription factors play a role on the regulation of conceptus specific gene transcription (Home *et al*. 2009; Bai *et al*. 2013), whereas GATA4-6 may be involved in subsequent processes such as conceptus attachment and adhesion to uterine caruncles and/or initial placental formation.

Spatial and temporal expression pattern of GATA5 in the developing heart, lung, vasculature, and genitourinary system indicates its involvement in tissue specific transcriptional regulation in the mouse embryonic development (Morrisey *et al*. 1997). *GATA5* expressed in endodermal cells of ovine conceptuses during the peri-implantation period suggests that common to many species, GATA5 surely plays a role in the differentiation of endoderm cell lineages. In chick, *GATA5* is transcribed in the cardiac crescent prior to the formation of primordial heart tube (Laverriere *et al*. 1994). In zebra fish, however, a *gata5* null mutation results in embryonic lethality with similar phenotype to that observed in *Gata4* null mice (Reiter *et al*. 1999). Gene targeting and transgene expression in mice will undoubtedly continue to improve our understanding on functions of various genes. However, these results suggest that the functions of each of GATA factors may differ between developmental stages, cell/tissue types and/or vertebrate species.

Types of implantation (invasive and non-invasive) and placental structures differ among mammalian species, however, the processes leading to conceptus implantation to the

maternal endometrium are considered similar (Bazer *et al*. 2009). In addition, regardless of these differences, the placenta has the same function, which is to protect and nourish a fetus. For the acquisition of these functions, there must be common or similar factors functioning during the implantation and placentation periods, although they may differ in the degree and/or timing of their expression (Bazer *et al*. 2009; Imakawa *et al*. 2009). In addition to those already elucidated, the GATA factors are potential candidates for monitoring conceptus development and/or uterine environment. Thus, the studies of GATA factors may shed light on the regulation of various steps in successful implantation and placental formation in mammalian species.

Conclusion

The spatial and temporal expression pattern of *GATA4-6* transcripts in ovine conceptuses and uteri during the peri-implantation period suggests that these transcription factors play potential roles, including species-specific functions and those common to other mammalian species, in developing conceptuses and/or uterine endometria during the peri-implantation period. A next step in elucidating molecular events associated with implantation processes in ruminants would be to examine each of these GATA factors as well as their interactions, possibly resulting in the understanding of down-stream gene expressions that are yet unidentified, or may have been over-looked during the critical period of pregnancy establishment in ruminants.

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Figure Legends

Figure 1. *GATA4, 5, and 6* mRNA in ovine conceptuses during the peri-attachment period. Real-time PCR analysis was carried out to determine amounts of *GATA4*, *5*, and *6* mRNAs in days 15, 17, and 21 ovine conceptuses. Total RNAs extracted from frozen ovine conceptuses were reverse-transcribed to cDNA, and then subjected to real-time PCR analysis using primers shown in Table 1. Results with double asterisks differ at $P < 0.01$ (n = 3 each).

Figure 2. Localization of GATA4 mRNA in the ovine uterus during the peri-implantation period. *In situ* hybridization analysis was carried out by using either anti-sense (A, C, E, G, I and K) or sense (B, D, F, H, J, and L) *GATA4* riboprobe to hybridize with serial sections of ovine conceptuses and uteri in days 15 (D15; A-D), 17 (D17; E-H), and 21 (D21; I-L) pregnant ewes (n=3 each day). The *in situ* hybridization was performed on more than two different uterine sections/blocks in three independent experiments containing uterine tissues from three animals, and two representative sections from a single animal are shown. En, Endoderm; LE, Luminal epithelium; St, Stroma; Tr, Trophectoderm. Scale bar: 50 μm.

Figure 3. Localization of *GATA5* mRNA in the ovine uterus during the peri-implantation period. *In situ* hybridization analysis was carried out by using either anti-sense (A, C, E, G, I and K) or sense (B, D, F, H, J, and L) *GATA5* riboprobe to hybridize with serial sections of ovine conceptuses and uteri in days 15 (D15; A-D), 17 (D17; E-H), and 21 (D21; I-L) pregnant ewes (n=3 each day). The *in situ* hybridization was performed on more than two different uterine sections/blocks in three independent experiments containing uterine tissues from three animals, and two representative

sections from a single animal are shown. En, Endoderm; LE, Luminal epithelial cells; St, Stroma; Tr, Trophectoderm. Scale bar: 50 μm.

Figure 4. Localization of *GATA6* mRNA in the ovine uterus during the peri-implantation period. *In situ* hybridization analysis was carried out by using either anti-sense (A, C, E, G, I and K) or sense (B, D, F, H, J, and L) *GATA6* riboprobe to hybridize with serial sections of ovine conceptuses and uteri in days 15 (D15; A-D), 17 (D17; E-H), and 21 (D21; I-L) pregnant ewes (n=3 each day). The *in situ* hybridization was performed on more than two different uterine sections/blocks in three independent experiments containing uterine tissues from three animals, and two representative sections from a single animal are shown. En, Endoderm; LE, Luminal epithelium; St, Stroma; Tr, Trophectoderm. Scale bar: 50 μm.

Supplemental Figure 1. *In situ* hybridization study of peri-implantation conceptus and uteri with positive (*ACTB*) and negative (plant-derived sequence) control probes. *ACTB* anti-sense riboprobe was used as a positive control (A, D, G) while *ACTB* sense riboprobe (B, E, H) and the riboprobe generated from a 570 bp length of plant-derived nucleotide sequence (C, F, I) were used as negative controls. A representative *in situ* hybridization from three independent experiments containing uterine tissues from three animals is shown. En, Endoderm; LE, Luminal epithelium; Tr, Trophectoderm. Scale bar: 50 μm.

和文抄録

着床周辺期ヒツジ子宮内における転写因子 GATA4、GATA5、GATA6 mRNA の発現と細胞局在

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要旨

転写因子GATAファミリーは、機能遺伝子群上流域のGATA結合配列（WGATAR）を認識して結合し、それらの遺伝子発現を制御する。哺乳類での GATA ファミリーはGATA1からGATA6の6因子からなり、GATA1-3は血液系細胞で、GATA4-6は主に消化管や心臓組織の発生過程において機能している。最近我々は、GATA2およびGATA3が反芻動物の胚・栄養膜細胞の着床周辺期においてインターフェロン・タウ（IFNT）を含む栄養膜細胞特異的な遺伝子群の発現制御に関与していることを明らかにした。しかしながら、GATA4-6に関しては、反芻動物の胚・栄養膜細胞を含む子宮内における機能や発現は検証されていなかった。そこで本研究では、PCR 法および in situ hybridization 法を用い、ヒツジ栄養膜細胞が子宮内膜上皮細胞に接着を開始（妊娠 16~17 日）する着床周辺期（妊娠 15 日、17 日、21 日）の *GATA4, 5, 6* mRNAs 発現を精査した。これらの解析により、*GATA4, 5, 6* mRNAs は着床期ヒツジ胚において発現が確認でき、特に *GATA5, 6* mRNAs は、胚が子宮内膜上皮へと接着を完了する妊娠 21 日に強い発現がみられた。さらに、これら *GATA* 因子は、栄養膜細胞だけではなく内胚葉系の細胞での発現が確認できた。一方、子宮内膜における *GATA4, 5* mRNA は妊娠 15 日や 17 日では発現が弱く、妊娠 21 日にて強い発現が確認された。こうした時期・細胞特異的な発現は、転写因子 GATA4-6 が着床後から初期胎盤形成期の胚栄養膜や子宮内膜細胞において未だに見つけられていない機能を持っていることを示唆している。

Key Words: GATA4-6; 遺伝子発現、ヒツジ子宮、着床期