Rapid communication

Title: **The localization of GATA2 in the nuclear and cytoplasmic regions of ovine conceptuses**

Running head: GATA2 localization in ovine conceptuses

Hanako BAI[1, 3], Toshihiro SAKURAI[1,4], Rulan BAI[1], James D. Godkin[2] and Kazuhiko IMAKAWA[1*].

[1] Laboratory of Theriogenology and Animal Breeding, Graduate School of Agricultural and Life Science, the University of Tokyo, Tokyo 113-8657, Japan

[2] Department of Animal Science, University of Tennessee, Knoxville, TN 37996, USA

[3] Present address: Mito Research Center, Meiji feed Co., Ltd. Ibaraki-Machi, Ibaraki 311-3123, Japan

[4] Present address: Dept. of Occupational and Environmental Health, Faculty of Pharmaceutical Science, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba, 278-8510 Japan

*Correspondence: Kazuhiko Imakawa, Ph.D.

Laboratory of Theriogenology and Animal Breeding, Veterinary Medical Sciences,

Graduate School of Agricultural and Life Sciences,

The University of Tokyo,

1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan.

Phone: +81-3-5841-5382; Facsimile: +81-3-5841-8180

Email: akaz@mail.ecc.u-tokyo.ac.jp

ABSTRACT

GATA transcription factors are emerging as critical regulators in trophoblast development and its gene regulation. The purpose of this study was to examine the expression and cellular localization of GATA2 in ovine conceptuses during the peri-implantation period. In Western blot analyses, GATA2 proteins were found in days 15, 17, and 21 ovine conceptuses (day 0 = day of estrus). Using immunohistochemistry and immunofluorescence analyses, we found that GATA2 was localized in days 15, 17, and 21 ovine conceptuses, and more importantly, GATA2 protein was detected in both nuclear and cytoplasmic regions of the trophectoderm. To our knowledge, the present study is the first to demonstrate that GATA2 is localized in two cellular compartments of the trophectoderm in ovine and any other mammalian species, and suggests that the difference in GATA2 location plays a role in the regulation of down-stream genes during early pregnancy period.

Key words*: GATA2, localization, ovine conceptuses.*

INTRODUCTION

Recently, transcription factors, previously not considered important, have been found to play roles in trophoblasts of early pregnancy. One of such factors is a family of the GATA transcription factors, which are expressed in hematopoietic cell lineages and in cardiac development, and regulate the expression of multiple genes in these cell types (Bai *et al*. 2013a). The GATA transcription factors consist of six structurally related proteins, GATA1-6, 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). Evidence accumulated indicates that GATA factors play important functional roles in trophoblast stem (TS) cells and in the trophoblast lineage specification (Home *et al*. 2009; Ray *et al*. 2009; Ralston *et al*. 2010). In addition, we found that GATA2 and GATA3 present in days 17, 20, and 22 bovine conceptuses regulate trophoblast cell-specific factors including interferon tau (IFNT), a major cytokine involved in the maternal recognition of pregnancy in the ruminants (Bai *et al*. 2009, 2011). Furthermore, we also reported that GATA1, 4, 5, and 6 were expressed in days 15, 17, and 21 ovine conceptuses during the same period (Bai *et al*. 2012, 2013b). Because of these findings, GATA factors should be considered as transcription factors essential for trophoblast development during peri-implantation periods in murine as well as ruminant species. However, we realized that expression and localization of ruminant GATA factors during the peri-implantation period has not been well studied. The aim of this study was to characterize the expression and cellular localization of GATA2 in days 15 (pre-attachment), 17 (attachment), and 21 (post-attachment) ovine conceptuses.

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).

Western blotting

To detect GATA2 protein, tissue lysates were prepared in RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1 mM Na_3VO_4, and 50 mM NaF). The lysates (10 μg/lane) were separated through 10% SDS-PAGE and were then transferred onto polyvinylidene difluoride (PVDF) membrane (n=3) (Millipore, Bedford, MA, USA). After blocking them with Block Ace reagent (DS Pharma Biomedical Co., Ltd, Tokyo, Japan), they were treated with rabbit polyclonal anti-GATA2 antibody (1: 250, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or rabbit monoclonal anti-ACTB antibody (for internal control, 1: 1000, Sigma-Aldrich, St Louis, MO, USA). The proteins were detected using the secondary antibody conjugated with horseradish peroxidase and ECL Western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). This analysis was performed three times with independent samples. Densitometric quantitation was done with the ImageJ softwere (http://rsbweb.nih.gov/ij/).

Immunohistochemistry

Immunohistochemical analyses were performed on 10 μm frozen sections of day 15, 17, and 21 uterine tissues. Sections were fixed with 4% paraformaldehyde, and endogenous peroxidase was quenched by immersing in 0.3% (v/v) hydrogen peroxide/methanol. Streptavidin/Biotin Blocking Kit (VECTOR LABORATORIES, Inc. Burlingame, CA, USA) was used to block endogenous biotin. After 30 min incubation with 10% normal goat serum (Invitrogen-Life Technologies, Grand Island, NY, USA), the sections were incubated with polyclonal rabbit anti-GATA2 antibody (1: 50, Santa Cruz Biotechnology). Subsequently, the sections were incubated with goat anti-rabbit IgG biotin conjugate (1:500, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The immunoreactivity was visualized by means of avidin-peroxidase (Sigma-Aldrich) and AEC substrate kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For immunofluorescence analyses, immunoreactivity was visualized using Alexa Fluor 568-conjugated (Invitrogen) according to the manufacturer's protocol. The sections were examined under a light microscope (BX-51; Olympus, Tokyo, Japan).

RESULTS

Expression and localization of GATA2 proteins in ovine conceptuses

In the ovine pregnancy, conceptus attachment to the uterine epithelium begins on day 16. Using Western blotting, GATA2 protein expression was examined in days 15, 17, and 21 ovine conceptuses. GATA2 proteins were found in all days, and slightly decreased on day 21 after conceptus attachment to the uterine epithelium (Fig. 1). Immunohistochemical analysis was then performed to examine localization of GATA2

protein in days 15, 17, and 21 pregnant uteri. GATA2 positive cells were detected in trophoblast cells on days 15, 17, and 21. It appeared that GATA2 protein was localized in both nuclear and cytoplasmic regions (Fig. 2). To confirm the GATA2 localization in two cellular compartments, immunofluorescence staining was performed, which supported those found from the immunohistochemical analysis (Fig. 3). More importantly, localization of GATA2 exhibited two patterns; (i) both nuclear and cytoplasmic regions, and (ii) only cytoplasmic regions.

DISCUSSION

Unlike murine species, studies on GATA transcription factors in ruminants are limited. Previously, *GATA2*, *GATA3* and *IFNT* mRNAs in ruminant conceptuses were found to exhibit the same expression pattern; these are highly expressed before trophoblast attachment to the uterine epithelium and decrease after the attachment is initiated. We also found that GATA2 and GATA3 regulate trophoblast-specific factors, including IFNT, during the peri-attachment period in the bovine species (Bai *et al.* 2009, 2011), but GATA protein expression, particularly cellular localization had not been studied. In this study, GATA2 protein was found in days 15, 17, and 21 ovine conceptuses, of which expression was abundant on day 15 and 17, and slightly decreased on day 21 after conceptus attachment to the uterine epithelium (Fig. 1). In addition, we also reported that conceptus expression of GATA1 was up-regulated after its attachment to uterine endometrial epithelial cells, and that *GATA4, 5,* and *6* mRNAs were also present in ovine conceptuses and/or uteri (Bai *et al*. 2012, 2013b). Together, all six GATA factors are present in ovine and bovine conceptuses during early pregnant period.

In addition to the presence of GATA2 protein in days 15, 17, and 22 ovine conceptuses, the protein was localized in both nuclear and cytoplasmic regions (Figs. 2 and 3). It should be noted that cellular localization of GATA2 protein had two patterns; (i) both nuclear and cytoplasmic regions, and (ii) only cytoplasmic regions. It was demonstrated that the translocation of GATA3 from the cytoplasmic region to the nucleus determines the degree of down-stream Th2 cytokines genes, *IL4*, *IL5* and *IL13*, transcription (Maneechotesuwan *et al*. 2007). GATA3 nuclear translocation depends on its phosphorylation on serine residues by p38 MAPK, which facilitates interaction with the nuclear transporter protein importin-alpha. It was also found that following transcription and translation, GATA2 also goes through post-translational modification such as phosphorylation (Towatari *et al*. 1995), acetylation (Hayakawa *et al*. 2004), sumoylation (Chun *et al*. 2003), and ubiquitination (Minegishi *et al*. 2005). These may imply that GATA2 in the nucleus could be actively involved in transcriptions of down-stream genes whereas GATA localization only in the cytoplasmic region could not be involved in down-stream gene transcriptions. Results from these observations indicate that cellular localization of transcription factor is as important as that of DNA binding, protein stability and/or co-activator recruitment. These observations suggest that due to localization of two cellular compartments, the effectiveness of GATA2 in the regulations of down-stream genes depends on translational modification and/or location of its cellular compartment. Further studies are required to elucidate the function of cytoplasmic and nuclear GATA2 and possibly other GATA factors in the trophoblast lineage specification, maintenance, proliferation, differentiation and/or their down-stream gene regulation in ruminant conceptuses during peri-implantation periods.

Conclusion

Here, we found that GATA2 was localized in both nucleus and cytoplasm in ovine conceptuses during the peri-implantation periods. As far as we can judge, there is no such knowledge of GATA2 expression and localization in two cellular components of the trophoblast cells in ovine or any other mammalian species.

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REFERENCES

Bai H, Sakurai T, Kim M-S, Muroi Y, Ideta A, Aoyagi Y, Nakajima H, Takahashi M, Nagaoka K, Imakawa K. 2009. Involvement of GATA transcription factors in the regulation of endogenous bovine interferon-tau gene transcription. *Molecular Reproduction and Development* **76**, 1143-1152.

Bai H, Sakurai T, Someya Y, Konno T, Ideta A, Aoyagi Y, Imakawa K. 2011. Regulation of trophoblast-specific factors by GATA2 and GATA3 in bovine trophoblast CT-1 cells. *Journal of Reproduction and Development* **57**, 518-525.

Bai H, Sakurai T, Konno T, Ideta A, Aoyagi Y, Godkin JD, Imakawa K. 2012. Expression of GATA1 in ovine conceptus and endometrium during the peri-attachment period. *Molecular Reproduction and Development* **79**, 64-73.

Bai H, Sakurai T, Godkin JD, Imakawa K. 2013a. Expression and potential role of GATA factors in trophoblast development. *Journal of Reproduction and Development* **59**, 1-6.

Bai H, Sakurai T, Godkin JD, Imakawa K. 2013b. Expression and in situ localization of *GATA4*, *5* and *6* mRNAs in ovine conceptuses and uterine endometria during the peri-implantation period. *Animal Science Journal* doi: 10.1111/asj.12156.

Chun TH, Itoh H, Subramanian L, Iñiguez-Lluhí JA, Nakao K. 2003. Modification of GATA-2 transcriptional activity in endothelial cells by the SUMO E3 ligase PIASy. *Circulation Research* **92**, 1201-1208.

Hayakawa F, Towatari M, Ozawa Y, Tomita A, Privalsky ML, Saito H. 2004. Functional regulation of GATA-2 by acetylation. *Journal of Leukocyte Biology* **75**, 529-540.

Home P, Ray S, Dutta D, Bronshteyn I, Larson M, Paul S. 2009. GATA3 is selectively expressed in the trophectoderm of peri-implantation embryo and directly regulates Cdx2 gene expression. *The Journal of Biological Chemistry* **284**, 28729-28737.

Ko LJ, Engel JD. 1993. DNA-binding specificities of the GATA transcription factor family. *Molecular and Cellular Biology* 13, 4011-4022.

Maneechotesuwan K, Xin Y, Ito K, Jazrawi E, Lee KY, Usmani OS, Barnes PJ, Adcock IM. 2007. Regulation of Th2 cytokine genes by p38 MAPK-mediated phosphorylation of GATA-3. *The Journal of immunology* **178**, 2491-2498.

Merika M, Orkin SH. 1993. DNA-binding specificity of GATA family transcription factors. *Molecular and Cellular Biology* **13**, 3999-4010.

Minegishi N, Suzuki N, Kawatani Y, Shimizu R, Yamamoto M. 2005. Rapid turnover of GATA-2 via ubiquitin-proteasome protein degradation pathway. *Genes to Cells: Devoted to Molecular & Cellular Mechanisms* **10**, 693-704.

Ralston A, Cox BJ, Nishioka N, Sasaki H, Chea E, Rugg-Gunn P, Guo G, Robson P, Draper JS, Rossant J. 2010. Gata3 regulates trophoblast development downstream of Tead4 and in parallel to Cdx2. *Development* **137**, 395-403.

Ray S, Dutta D, Rumi MA, Kent LN, Soares MJ, Paul S. 2009. Context-dependent function of regulatory elements and a switch in chromatin occupancy between GATA3 and GATA2 regulate Gata2 transcription during trophoblast differentiation. *The Journal of Biological Chemistry* **284**, 4978-4988.

Sakurai T, Bai H, Konno T, Ideta A, Aoyagi Y, Godkin JD, Imakawa K. 2010. Function of a transcription factor CDX2 beyond its trophectoderm lineage specification. *Endocrinology* **151**, 5873-5881.

Towatari M, May GE, Marais R, Perkins GR, Marshall CJ, Cowley S, Enver T. 1995. Regulation of GATA-2 phosphorylation by mitogen-activated protein kinase and interleukin-3. *The Journal of Biological Chemistry* **270**, 4101-4107.

Yamamoto M, Ko LJ, Leonard MW, Beug H, Orkin SH, Engel JD. 1990. Activity and tissue-specific expression of the transcription factor NF-E1 multigene family. *Genes and Development* **4**, 1650-1662.

Figure legends

Figure 1 Expression of GATA2 protein in the ovine conceptuses during the peri-attachment period.

Upper: The presence of GATA2 protein in days 15, 17, and 21 (D15, D17, and D21, respectively, n=3 each day) ovine conceptuses was examined by Western blot analysis. Rabbit polyclonal anti-GATA2 antibody was used to detect the protein and ACTB was used as an internal control. A representative PVDF membrane data from three independent experiments containing protein samples from different animals is shown.

Lower: Densitometric quantitation of Western blots was done using the ImageJ software (http://rsbweb.nih.gov/ij/). The relative densities of the blots were determined by normalization against ACTB levels.

Figure 2 Localization of GATA2 protein in the ovine conceptuses during the peri-attachment period.

Immunohistochemical detection of GATA2 protein in ovine conceptuses and uteri in days 15 (D15; A, B), 17 (D17; C, D), and 21 (D21; E, F) pregnant ewes (n=3 each day) was carried out. Instead of the rabbit polyclonal anti-GATA2 antibody, rabbit pre-immune serum was used as a negative control. The pre-immune serum was applied to a serial section (inset in A) of day 15 ovine uteri. Representative immunohistochemistry from three animals is shown. GATA2 was localized in nuclear (▲) and cytoplasmic (△) regions. LE, Luminal epithelium; St, Stromal cells; Tr, Trophoblast cells.

Figure 3 Immunofluorescence analysis of GATA2 in day 17 ovine trophoblasts.

Day 17 ovine conceptuses were treated with GATA2 antibody (D17; A, B) or pre-immune serum (negative control, D17; C, D). GATA2 expression is seen in red and nuclei were counterstained with DAPI (B, D). Three conceptuses from day 17 pregnant animals (n=3) were subjected to immunofluorescence analyses and a representative one is shown. GATA2 was localized in both nuclear (solid white triangle) and cytoplasmic (white line triangle) regions.

和文抄録

ヒツジ胚・栄養膜細胞の核および細胞質における転写因子 GATA2 の局在

唄花子[1,3]、櫻井敏博[1,4]、白汝嵐[1]、James D. Godkin[2]、今川和彦[1]

[1] 東京大学大学院　農学生命科学研究科　獣医繁殖育種学研究室

〒113-8657　東京都　文京区　弥生 1-1-1

[2] Department of Animal Science, University of Tennessee, Knoxville, TN USA

[3] 現在の連絡先: 明治飼糧株式会社　水戸研究牧場

〒311-3123　茨城県　東茨城郡　茨城町　若宮 870

[4] 現在の連絡先: 東京理科大学　薬学部薬学科　環境労働衛生学研究室

〒278-8510　千葉県　野田市　山崎 2641

略表題: 栄養膜細胞の GATA2 発現

連絡者: 今川和彦

所属: 東京大学大学院　農学生命科学研究科　獣医繁殖育種学研究室

tel: 03-5841-5382　　fax: 03-5841-8180

e-mail: akaz@mail.ecc.u-tokyo.ac.jp

和文抄録

近年、転写因子 GATA は胚・栄養膜細胞の分化や遺伝子発現に重要な役割を果たすことが分かってきた。本研究では、反芻動物（ヒツジ）の着床周辺期胚・栄養膜細胞において、転写因子 GATA2 タンパク質の発現と細胞内局在をウエスタンブロッティング法および免疫染色法にて調べた。その結果、妊娠15日、17日、21日（発情日を0日とする）のヒツジ胚において GATA2 タンパク質が発現していた。興味深いことに、GATA2 タンパク質は胚・栄養膜細胞の核と細胞質の両方に局在していた。本研究以外に、胚・栄養膜細胞における GATA2 の細胞内局在についての知見は見当たらない。しかしながら、本研究は、こうした局在の違いが胚・栄養膜細胞特異的な遺伝子群の発現制御に影響を与えていることを示唆している。