Hot topic: Pregnancy-induced expression of interferon-stimulated genes in the cervical and vaginal mucosal membranes

Hiroki Kunii,* Keisuke Koyama,† Tsukino Ito,* Toshiyuki Suzuki,* Ahmed Z. Balboula,‡ Takahiro Shirozu,* Hanako Bai,* Masashi Nagano,§ Manabu Kawahara,* and Masashi Takahashi*¹

*Laboratory of Animal Breeding and Reproduction, Department of Animal Science, Research Faculty of Agriculture, Hokkaido University, Hokkaido 060-8589, Japan

†Dairy Cattle Group, Konsen Agricultural Experiment Station, Hokkaido Research Organization, Nakashibetsu, Hokkaido 086-1135, Japan

‡Theriogenology Department, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt

§Laboratory of Theriogenology, Department of Veterinary Clinical Science, Graduate School of Veterinary Medicine, Hokkaido University, Hokkaido 060-0818, Japan

ABSTRACT

In ruminants, IFN-tau (IFNT) is a pregnancy recognition signal secreted by the embryonic trophectoderm before implantation, and it induces the expression of IFN-stimulated genes (ISG) in the uterine endometrium and blood leukocytes. The expression of ISG in blood leukocytes could indicate the presence of a viable conceptus before return of the next estrus; however, expression levels have high variation for confirming pregnancy. We hypothesized that the secreted IFNT in the uterus would affect ISG expression in cervical and vaginal tissues because they are directly adjacent to the uterus. To prove the hypothesis, we investigated the expression of 3 ISG (*ISG15*, *MX1*, and *MX2*) in cervical and vaginal mucosal membranes collected from pregnant ($n = 12$) and nonpregnant ($n = 11$) lactating Holstein cows at 17 to 18 d after artificial insemination. Mucosal membrane samples of the cervical canal near the external os (cervix) and deep vaginal wall surrounding the external os (vagina) were collected separately by simply scraping with a curette on d 17 or 18 of pregnancy (d 1 = ovulation), at which time IFNT secretion into the maternal uterus is maximal. After pregnancy diagnosis on d 30 and 60, separately collected samples confirmed as pregnant and nonpregnant were used for evaluation of the expression of IFN-stimulated protein 15 kDa (*ISG15*) and myxovirus-resistance protein 1 and 2 (*MX1*, *MX2*) with quantitative real-time PCR. The collected mucosal membrane samples from cervix contained mostly cell clots showing membrane structure and a low content of blood cells. The expression levels of all 3 genes were significantly increased in pregnant cows compared with nonpregnant cows in both

cervical and vaginal samples. These results suggest that increased expression of ISG in the cervix and vagina is a pregnancy-associated phenomenon and is highly affected by IFNT secreted from the conceptus through the uterus.

Key words: pregnancy, interferon-stimulated genes, cervix, vagina

Hot Topic

Recently, the fertility of lactating cows has been decreasing, leading to substantial economic losses in the dairy industry. Moreover, the weakening of signs of estrus in the cow has made it difficult to detect estrus and perform AI with appropriate timing. Genetic selection for improving milk yield is considered a potential cause of infertility (Roelofs et al., 2010). Thus, it is important to detect nonpregnant cows before the return of estrus and perform AI without losing time. Interferon-tau (IFNT), produced by the ruminant trophectoderm, is a signal for the maternal recognition of pregnancy (Imakawa et al., 1987). It inhibits both the expression of oxytocin receptor and release of high-amplitude pulses of luteolytic PGF_{2α} in the uterine endometrium (Demmers et al., 2001) followed by the maintenance of corpus luteum. Production of IFNT is limited to the embryonic trophectoderm of ruminants during the peri-attachment period and it reaches its maximal level between d 17 and 19 of pregnancy (Ealy and Yang, 2009).

Induction of the expression of IFN-stimulated genes (ISG) is one of the most remarkable characteristics of IFNT. Interferon-stimulated genes such as IFN-stimulated protein 15 kDa (*ISG15*) and myxovirus-resistance protein 1 and 2 (*MX1*, *MX2*) are substantially induced in the uterine endometrium via the IFNT signaling pathway (Mansouri-Attia et al., 2009). Detection and measurement of these ISG could be a good indicator of early pregnancy determination that shows the existence

Received December 5, 2017.

Accepted May 22, 2018.

¹Corresponding author: mmasashi@anim.agr.hokudai.ac.jp

of a conceptus in the uterus; however, direct collection of uterine tissues for ISG detection is impractical because it has a harmful effect on the conceptus and uterine condition. Several reports have shown high ISG expression in blood leukocytes of pregnant cows, which is expected to be an indicator of early pregnancy diagnosis (Green et al., 2010; Matsuyama et al., 2012; Yoshino et al., 2018). Although a significant difference in gene expression levels of ISG can be observed between nonpregnant and pregnant cows around d 18, detection and evaluation of blood leukocytes currently lacks reliability because of the high variation in the expression levels of mRNA of ISG between nonpregnant and pregnant cows even when IFNT secretion becomes maximum (Gifford et al., 2007).

Recent studies have shown that a pregnancy-specific increase of ISG is present not only in uterine tissues and blood cells, but also in other organs, including high expression levels of *ISG15* in the ovine corpus luteum at d 15 of pregnancy (Oliveira et al., 2008), *ISG15* and *MX1* expression in the bovine liver at d 20 of pregnancy (Meyerholz et al., 2016), and protein and mRNA of *ISG15* in ovine bone marrow at d 16 (Yang et al., 2017). These studies suggest that organs other than the uterine tissue and blood leukocytes have pregnancy-specific responsiveness to type-I IFN that stimulates ISG expression through the bloodstream.

On the contrary, when focusing on the location of organs directly adjacent to the uterus, the cervix is physically open throughout the entire estrous and gestation period. This raises the possibility that factors secreted in the uterus move towards to the adjacent organs, causing some effects directly or indirectly. Therefore, we hypothesized that secreted IFNT in the uterus may affect extra-uterine tissues, such as cervical tissue, directly or indirectly through the reproductive tract. To prove the hypothesis, we investigated the expression of 3 ISG—*ISG15*, *MX1*, and *MX2*—in cervical and vaginal mucosal membranes collected from pregnant and nonpregnant lactating Holstein cows on d 17 to 18 after AI. We also measured the expression level of *ISG15* in peripheral blood leukocytes in pregnant and nonpregnant cows.

Lactating Holstein cows maintained at the Field Center for Northern Biosphere, Hokkaido University (Hokkaido, Japan) and Konsen Agricultural Experiment Station (Nakashibetsu, Hokkaido, Japan) were used for this experiment. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of National University Corporation Hokkaido University (approval no. 16-0019) and the Animal Care Committee for Laboratory Animals of the Konsen Agricultural Experiment Station (approval no. 20180312-3). Sampling was conducted on d 17 or 18 (d

0 = standing estrus and AI) of pregnancy by scraping the mucosal membrane with a curette (Figure 1B) from the cervical canal near the external os at approximately 3 to 5 cm deep (cervix; Figure 1A, white arrow) and in the deep vaginal wall surrounding the external os (vagina; Figure 1A, black arrow). Each sample was collected with a volume of approximately <100 μ L (Figure 1B). Samples were collected from the vagina first, followed by the cervix, changing the curette in between. The overall time required for sample collection was less than 20 min on average. Blood samples were collected from the jugular vein at the same time into tubes containing EDTA-2Na (Terumo, Tokyo, Japan). Some mucosal membrane samples were immediately placed into

Figure 1. Sampling points of the extra-uterine sites of cows: (A) The white arrow shows the external os of the cervix (cervical samples); the black arrow shows the deep vaginal wall surrounding the external os (vaginal samples). (B) Sampling curette and collected mucosal membrane sample from cervix. Color version available online.

1.5-mL tubes filled with 500 μ L of Isogen II (Nippon Gene, Tokyo, Japan), and the other samples were used for microscopic observation. Nuclei were stained with 1 μ L of ready-to-use Hoechst 33342 (ImmunoChemistry Technologies, Bloomington, MN). Blood samples were also mixed with Isogen-LS (Nippon Gene). Total RNA extraction was performed according to the manufacturer's instructions. At 30 and 60 d after AI, pregnancy diagnoses were performed twice by ultrasonography and cows were divided into 2 groups: pregnant and nonpregnant. After total RNA extraction and purification, the concentration of each RNA sample was measured by spectrophotometry (NanoDrop ND-2000, Thermo Scientific, Wilmington, DE), and cDNA was synthesized from total RNA using ReverTra Ace with gDNA Remover (Toyobo Life Science, Osaka, Japan).

The mRNA expression levels of *ISG15*, *MX1*, *MX2*, *H2A Family Z (H2AFZ)*, and β -actin (*ACTB*) were examined by quantitative PCR using a Light Cycler Nano System II (Roche Diagnostics, Basel, Switzerland) and Thunderbird SYBR qPCR Mix (Toyobo Life Science). The primers for qPCR were as follows: *MX1a* forward, 5'-GCCAACTAGTCAGCACTACATTGTC-3' and reverse, 5'-GCTCTTGGACTCCATATCTTCAC-3' (Accession No. NM_173940); *MX2* forward, 5'-CAGAGACGCCTCAGTCGAAG-3' and reverse, 5'-GAGACGTTTGCTGGTTTCCATG-3' (NM_173941); *ISG15* forward, 5'-TGAGGGACTCCATGACGGTA-3' and reverse, 5'-GCTGGAAAGCAGGCACATTG-3' (NM_174366); *H2AFZ* forward, 5'-AGAGCCGGTTTGCAGTTCCCG-3' and reverse, 5'-TACTCCAGGATGGCTGCGCTGT-3' (NM_174809); and *ACTB*

Figure 2. Microscopic observation of mucosal membrane samples collected from cervix of pregnant and nonpregnant cows (d 18 after AI). Bright field (A, B) and Hoechst-stained (C, D) images of mucosal membrane samples collected from cervix of nonpregnant (A, C) and pregnant (B, D) cows. Scale bar is 75 μ m. Color version available online.

forward, 5'-TGGACTTCGAGCAGGAGATG-3' and reverse, 5'-GTAGAGGTCCTTGCGGATGT-3' (AC_000182.1). The thermal cycling conditions were 1 cycle at 95°C for 30 s, followed by 45 cycles of 95°C for 10 s, 55°C for 15 s, and 72°C for 30 s. The relative expression levels of *ISG15*, *MX1*, and *MX2* to *H2AFZ* were respectively calculated by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001); *ACTB* was used as a reference gene for blood samples. All quantitative real-time (qRT)-PCR analyses were performed in duplicate.

Gene expression levels were described as fold change relative to that of the nonpregnant sample. All data are shown as the mean $\pm$ standard error of the mean (SEM). The statistical significance of differences in mRNA expression levels were assessed using an independent Student's *t*-test based on $2^{-\Delta\Delta C_t}$ values. $P < 0.05$ was considered statistically significant.

Microscopic observation indicated the presence of cell clots showing the mucosal membrane shape of the cervix in both pregnant and nonpregnant cows (Figure 2A and B). Total RNA collected from mucosal membrane was approximately 3 to 20 μ g.

The relative mRNA expression levels of *ISG15*, *MX1*, and *MX2* in both the cervical and vaginal mucosal membranes of pregnant cows were significantly higher than those of nonpregnant cows (Figure 3A, B, D, E, F, G). In particular, *ISG15* expression in the pregnant cervix and vagina was about 80-fold and 20-fold higher than that in the corresponding nonpregnant samples, respectively. In contrast, the relative expression level of *ISG15* in blood sample was about 5-fold higher in pregnant than in nonpregnant cows (Figure 3C). The pregnancy rate of cows that were sampled was 52% (12/23), whereas the pregnancy rate of cows not sampled was

Figure 3. Relative mRNA expression levels of *ISG15*, *MX1*, and *MX2* in the cervix, vagina, and peripheral blood leukocytes (*ISG15* only) of nonpregnant (np) and pregnant (p) cows. Values are shown as the mean $\pm$ SEM; differences between pregnancy status are given by * $P < 0.05$ and ** $P < 0.01$, as determined by Student's *t*-test.

30% (8/26). This suggests that the sampling process of the mucosal membrane from the cervix and vagina had little or no harmful effect on pregnancy. Considering these results, cervical or vaginal mucosal membrane samples could be used to detect the pregnancy-associated response to stimulation of ISG genes. Many studies have attempted to find and use reliable pregnancy markers that are less invasive, easy to measure, and have high accuracy. Detection and measurement of ISG in blood leukocytes is a good potential indicator to detect pregnant cows at 18 to 20 d before the return to estrus. In many reports, a significant increase of ISG in peripheral blood leukocytes appears from d 18 in pregnant compared with nonpregnant cows (Green et al., 2010; Matsuyama et al., 2012; Yoshino et al., 2018). In the present study, the approximately 5-fold change of leukocytes in pregnant cows was similar to the extent of change reported in previous studies (Green et al., 2010; Matsuyama et al., 2012; Yoshino et al., 2018). In contrast, high expression of ISG such as *MX1* (68-fold) and *MX2* (336-fold) in the uterus from pregnant cows on d 20 indicates the direct effect of secreted IFNT to uterine tissue (Mansouri-Attia et al., 2009). In the present study, we detected increases of 87-fold for *ISG15*, 17-fold for *MX1*, and 20-fold for *MX2* in the cervix and 17-fold for *ISG15*, 8-fold for *MX1*, and 8-fold for *MX2* in the vagina of pregnant cows on d 18. These substantial changes, especially in the cervical mucosal membrane, compared with those in peripheral blood leukocytes make it highly possible that the cervix is affected by type I IFN (i.e., IFNT).

To our knowledge, this is the first report showing pregnancy-associated high *ISG* mRNA expression in the cervical and vaginal mucosal membrane from pregnant cows, which might be triggered by IFNT secreted from the conceptus, although it is still unknown whether IFNT leaks directly to the cervix and vagina from the uterus or whether it circulates in the blood and affects the tissues. Thus, the effects of type I IFN appear to be more wide reaching than previously thought, because type I IFN affects not only the uterus but also the adjacent reproductive organs in the preimplantation period. Importantly, high expression of ISG in the cervical and vaginal tissues could be a useful indicator for the presence of a conceptus in the early pregnancy period.

ACKNOWLEDGMENTS

We thank the staff of the Field Center for Northern Biosphere, Hokkaido University (Hokkaido, Japan), who provided helpful assistance with sample collection.

We thank Editage (www.editage.jp) for English language editing. We also thank Abdus Shabur Talukder (Laboratory of Animal Breeding and Reproduction, Department of Animal Science, Research Faculty of Agriculture, Hokkaido University, Hokkaido, Japan) for help with the analysis. The present research was funded by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (KAKENHI, 15H04579; Tokyo, Japan).

REFERENCES

- Demmers, K. J., K. Derecka, and A. Flint. 2001. Trophoblast interferon and pregnancy. *Reproduction* 121:41–49.
- Ealy, A. D., and Q. E. Yang. 2009. Control of interferon-tau expression during early pregnancy in ruminants. *Am. J. Reprod. Immunol.* 61:95–106.
- Gifford, C. A., K. Racicot, D. S. Clark, K. J. Austin, T. R. Hansen, M. C. Lucy, C. J. Davies, and T. L. Ott. 2007. Regulation of interferon-stimulated genes in peripheral blood leukocytes in pregnant and bred, nonpregnant dairy cows. *J. Dairy Sci.* 90:274–280.
- Green, J. C., C. S. Okamura, S. E. Poock, and M. C. Lucy. 2010. Measurement of interferon-tau (IFN-tau) stimulated gene expression in blood leukocytes for pregnancy diagnosis within 18–20d after insemination in dairy cattle. *Anim. Reprod. Sci.* 121:24–33.
- Imakawa, K., R. V. Anthony, M. Kazemi, K. R. Marotti, H. G. Polites, and R. M. Roberts. 1987. Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm. *Nature* 330:377–379.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25:402–408.
- Mansouri-Attia, N., J. Aubert, P. Reinaud, C. Giraud-Delville, G. Taghouthi, L. Galio, R. E. Everts, S. Degrelle, C. Richard, I. Hue, X. Yang, X. C. Tian, H. A. Lewin, J. P. Renard, and O. Sandra. 2009. Gene expression profiles of bovine caruncular and intercaruncular endometrium at implantation. *Physiol. Genomics* 39:14–27.
- Matsuyama, S., T. Kojima, S. Kato, and K. Kimura. 2012. Relationship between quantity of IFNT estimated by IFN-stimulated gene expression in peripheral blood mononuclear cells and bovine embryonic mortality after AI or ET. *Reprod. Biol. Endocrinol.* 10:21.
- Meyerholz, M. M., K. Mense, H. Knaack, O. Sandra, and M. Schmicke. 2016. Pregnancy-induced *ISG15* and *MX1* gene expression is detected in the liver of Holstein-Friesian heifers during late peri-implantation period. *Reprod. Domest. Anim.* 51:175–177.
- Oliveira, J. F., L. E. Henkes, R. L. Ashley, S. H. Purcell, N. P. Smirnova, D. N. Veeramachaneni, R. V. Anthony, and T. R. Hansen. 2008. Expression of interferon (IFN)-stimulated genes in extra-uterine tissues during early pregnancy in sheep is the consequence of endocrine IFN- τ release from the uterine vein. *Endocrinology* 149:1252–1259.
- Roelofs, J., F. López-Gatius, R. H. Hunter, F. J. van Eerdenburg, and Ch. Hanzen. 2010. When is a cow in estrus? Clinical and practical aspects. *Theriogenology* 74:327–344.
- Yang, L., B. Liu, X. Yan, L. Zhang, F. Gao, and L. Zhichao. 2017. Expression of *ISG15* in bone marrow during early pregnancy in ewes. *Kafkas Univ. Vet. Fak. Derg.* 23:767–772. <https://doi.org/10.9775/kvfd.2017.17726>.
- Yoshino, H., N. Toji, K. Sasaki, K. Koshi, N. Yamagishi, T. Takahashi, T. Ishiguro-Oonuma, H. Matsuda, T. Yamanouchi, Y. Hashiyada, K. Imai, Y. Izaike, K. Kizaki, and K. Hashizume. 2018. A predictive threshold value for the diagnosis of early pregnancy in cows using interferon-stimulated genes in granulocytes. *Theriogenology* 107:188–193.