

—Original Article—

Estrous cycle stage-dependent manner of type I interferon-stimulated genes induction in the bovine endometrium

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Abstract. Interferon tau (IFN- τ) is a ruminant-specific type I IFN secreted by a conceptus before its attachment to the uterus. IFN- τ induces the expression of IFN-stimulated genes (ISGs) via the type I IFN receptor (IFNAR), which is composed of IFNAR1 and IFNAR2 subunits in the endometrium. However, expression patterns of IFNARs during the estrous cycle have not been reported. We hypothesized that the response to a type I IFN changes along with IFNARs and the IFN-regulatory factors (IRFs) driving transcription of IFN signal-related genes and modulating a type I IFN signal during the estrous cycle. We investigated the estrous cycle stage-dependent type I IFN induction of ISGs and expression patterns of IFN signal-related genes in bovine endometrial tissues. Endometrial tissue pieces collected from bovine uteri at each estrous stage (early, mid, and late) were cultured with or without recombinant bovine IFN- α or concentrated pregnant uterine flushing (PUF) on day 18 after confirming the presence of a conceptus. IFN- α and PUF each significantly increased the expression of ISGs in endometrial tissues. The induction levels of the typical ISGs (*MX1-a* and *ISG15*) were significantly higher at the mid stage and correlated with high expression of *IRFs* at the mid stage. The immunostaining of IFNARs showed strong fluorescence intensities in luminal and glandular epithelia at the early and mid stages. Collectively, these results suggest that the endometrium exhibits estrous cycle stage-dependent responsiveness to type I IFN that may be associated with the expression of IFNARs and IRFs for pregnancy recognition.

Key words: Cow, Interferon-stimulated gene (ISG), Type I Interferon (IFN), Type I interferon receptor (IFNAR), Uterus
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Interferon tau (IFN- τ) is secreted by the conceptus of ruminants at the preimplantation stage [1–4]. IFN- τ is essential for pregnancy recognition in order to maintain the corpus luteum (CL). The CL is necessary for continuous production of progesterone during pregnancy. In the estrous cycle, the CL regresses, and this is followed by the next follicular phase because the CL secretes oxytocin, which binds

to the oxytocin receptor expressed in the uterus and induces the CL regression factor: prostaglandin (PG) F₂ α [4, 5]. On the other hand, in the presence of a conceptus in the uterus, IFN- τ secreted by the conceptus inhibits the expression of oxytocin receptors. The synthesis of PGF₂ α is consequently inhibited, which prevents regression of the CL [4, 6]. Interferon-stimulated genes (ISGs) are strongly expressed in the uterus at this time [7–9]. Because the production of IFN- τ is limited to days 14–21 of pregnancy in cows [10], the expression of bovine Myxovirus resistance (*MX*) genes, typical ISGs, decreases immediately after implantation is completed, on days 25–40 [11].

The expression of ISGs is induced by the ISGF3 complex, which consists of phosphorylated and dimerized signal transducer and activator of transcription (STAT) 1/2 and IFN-regulatory factor (IRF) 9 in the IFN-stimulated Janus kinase (JAK)-STAT signaling pathway [12–14]. IFN- τ belongs to the type I IFN family, which includes

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IFN- α and - β , binds to type I IFN receptors (IFNARs) composed of IFNAR1 and IFNAR2 subunits [15], and subsequently activates the JAK/STAT signaling pathway [16, 17]. IFNAR1 and IFNAR2 mRNAs and proteins are strongly expressed in the endometrial luminal epithelium (LE), glandular epithelium (GE), and stromal cells in pregnant and nonpregnant ovine uteri [18]. However, only limited information is available on the expression of IFNAR in the bovine uterus. COP9 signalosome subunit 5 (*COPS5*)/Jun activation domain-binding protein 1—from a part of the COP9 signalosome complex that consists of eight subunits and plays a role in the ubiquitin proteasome system—was recently found to play a role in the inhibition of IFNAR1 proteolysis in human cells [19]. The data related to *COPS5* in the bovine uterus have not been reported.

Type I IFN is generally induced by viral infections, and ISGs show antiviral activity [20]. The transcription of some type I IFNs—IFN- α and - β —is regulated by IRF3 and -7 [13, 14]. On the other hand, the transcription of IFN- τ is regulated by other transcription factors such as ETS2 [21], AP1 [22], CDX2 [23], DLX3 [24], and GATA2 [25]. IFN- τ and IFN- α differ in the transcription-regulatory mechanism even though they are both type I IFNs. The luteotropic activities of IFN- α and IFN- τ have been explored in previous studies. Intrauterine and intramuscular administration of recombinant bovine IFN- α to cows has been shown to reduce the oxytocin-induced release of PGF2 α and to extend the lifespan of the CL [26–28]. Similarly, infusion of recombinant ovine IFN- τ or IFN- α to ewes extends the estrous cycle [29]. *In vitro*, recombinant bovine IFN- τ and IFN- α , recombinant ovine IFN- τ , and human IFN- α have been shown to suppress the synthesis of PGF2 α in bovine and ovine endometrial cells [30, 31]. Thus, IFN- α and IFN- τ have antiluteolytic activities. A comparative analysis of gene expression profiles in the bovine endometrium between early pregnancy (days 15 and 18) and the intrauterine application of human IFN- α revealed similar and differential gene expression patterns, suggesting that IFN- τ -specific gene induction takes place in the endometrium at the preimplantation stage in the uterus [32]. The typical ISGs—*MX1*, *MX2*, and *ISG15*—are expressed in early pregnancy and after application of IFN- α and show similar gene expression patterns. It currently remains unknown whether IFN- τ -induced ISGs play roles in immune responses or have pregnancy recognition-specific functions in the uterus at the preimplantation stage.

Only ruminant-specific IFN- τ has antiluteolytic activities for pregnancy recognition between the mother and embryo [33]. However, not having antiluteolytic activities, other type I IFN such as porcine and equine IFN- δ or human and murine IFN- ϵ are expressed in the endometrium and trophoblast cells [34–37] and have been suggested to play roles in achieving a successful pregnancy. Additionally, the expression of genes *IFNAR1*, *IFNAR2*, and *MxA* shows similar changes in the endometrium during the menstrual cycle in humans [38]. We hypothesized that the estrous cycle stage-dependent responsiveness to type I IFN in the bovine endometrium is accompanied by a change in the expression of IFNARs and IRFs. In the present study, we investigated type I IFN-mediated induction of ISGs and expression patterns of IFN signal-related genes in bovine endometrial tissue at each estrous stage: early, mid, and late.

Materials and Methods

Collection of samples of endometrial tissues

This study was conducted in accordance with the Hokkaido University guidelines for the care and use of animals. Uteri collected from abattoirs were subdivided into three stages; early (days 4–9), mid (days 10–15), and late (days 16–21) according to the luteal stages of the ovary [39] as well as the status and electrical impedance of uterine mucus [40]. Intercaruncular endometrial tissues were collected from the uterine horn ipsilateral to the CL. The collected tissues were embedded in Optimal Cutting Temperature compound (Sakura Finetek, Tokyo, Japan) in liquid nitrogen, and stored in a freezer at -80°C until section preparation. Moreover, the collected tissues were used in expression analyses or explant cultures. Noncultured tissue samples were stored in a freezer at -80°C until RNA extraction. All the experiments were conducted on the tissue samples of at least three cows at each stage.

Uterine flushing and bovine recombinant IFN- α

Pregnant uterine flushing (PUF) was collected from a cow, confirming the existence of a conceptus on day 18 of pregnancy after embryo transfer on day 7 of the estrous cycle. Nonpregnant uterine flushing (NPUF) was collected from a cow confirmed to not have a conceptus after embryo transfer on day 7 of the estrous cycle. Approximately 2 l of each collected uterine flushing was filtered through gauze to remove cell debris. Then, the flushing was concentrated to 20 ml by means of a dialysis membrane (Size 36, Wako, Osaka, Japan) and dehydrated using polyethylene glycol 20,000 (Merck Schuchardt OHG, Hohenbrunn, Germany) at 4°C overnight. Concentrated uterine flushing was finally filtered through a $0.45\text{-}\mu\text{m}$ membrane, and then stored in a freezer at -80°C . Bovine recombinant IFN- α was kindly provided by Novartis (Novartis Animal Health, Basel, Switzerland).

The IFN- τ protein was detected in PUF by a western blot analysis. The antiviral activities of PUF and IFN- α were measured by a viral resistance assay using Madin-Darby bovine kidney cells and were found to be 82,620 IU/ml and 602,290 IU/ml, respectively. Five microliters of PUF or $0.5\ \mu\text{l}$ of IFN- α was added to 1 ml of the medium, which was adjusted to approximately 400 IU/ml or 300 IU/ml. Five microliters of NPUF (the volume equivalent to that of PUF, $5\ \mu\text{l}$) was also added to the medium as a negative control.

Explant culture conditions

Collected tissues were placed in calcium- and magnesium-free Hanks' balanced salt solution (HBSS) (–) and cut into small pieces ($5 \times 5\ \text{mm}$). These pieces were preincubated in Dulbecco's Modified Eagle's medium – high glucose (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 0.06 g/l penicillin G potassium (Nacalai Tesque, Kyoto, Japan) and 0.1 g/l streptomycin sulfate (Nacalai Tesque) at 38.5°C [41, 42] for 1 h at 5% of CO_2 . The medium volume was 2 ml in Falcon 35-mm Easy Grip Style Not Treated Bacteriological Petri Dishes, Sterile (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). After the preincubation, endometrial tissue pieces were cultured with nonsupplemented DMEM containing 10% (v/v) of fetal bovine serum (FBS, ICN Bio-Source International, Camarillo, CA, USA) (Cont.) or DMEM containing 10% of FBS and supplemented with IFN- α (300 IU/ml), PUF (400 IU/ml), or

Table 1. Information on primer sequences used for qRT-PCR

Gene	Sequence (5'-3')	Accession No.	Product length (bp)
<i>MX1-a</i>	GCCAACTAGTCAGCACTACATTGTC GCTCTGGACTCCATATCTTCAC	NM_173940.2	139
<i>MX1B</i>	GTGATATCTCCAACAGTGAAGC AACTGATTTCGAGAAGCCAAG	AB_060169.1	94
<i>MX2</i>	CAGAGACGCCTCAGTCGAAG GAGACGTTTGCTGGTTCCATG	NM_173941.2	113
<i>ISG15</i>	TGAGGGACTCCATGACGGTA GCTGGAAAGCAGGCACATTG	NM_174366.1	72
<i>IFNAR1</i>	GCGAAGAGTTTCCGCAACAG TCCAAGGCAGGTCCAATGAC	NM_174552.2	275
<i>IFNAR2</i>	TCGTATGTTGCGCCTGTTCT GTCCGTCGTGTTTACCCACA	NM_174553.2	231
<i>COPS5</i>	GCCCTGGACTAAGGATCACC TTCCAGGTTGCCTCCTGATC	NM_001192139.1	100
<i>IRF1</i>	CACAGTTCGCAGCTACATGG AAGTTGTACAGGTCGCTGGT	NM_001191261.2	147
<i>IRF2</i>	GGAGCGTTGAAGGCAAACAG AGGAGCTGTTGTAAGGCACC	NM_001205793.1	150
<i>IRF3</i>	GCTCAACTGACGGGAAGTGG TGGTCTGGCCTAAGTGTGG	NM_001029845.3	116
<i>IRF9</i>	CAGTTCAGGAGTGTGCTG TATATCGCCAGGCCTTGAA	NM_001024506.1	125
<i>H2AFZ</i>	AGAGCCGGTTTGCAGTCCCG TACTCCAGGATGGCTGCGCTGT	NM_174809.2	116

NPUF (5 µl/ml) for 12 or 24 h at 38.5°C and 5% CO₂ in duplicate. The medium volume was 0.5 ml/well in Thermo Scientific™ Nunc™ Cell-Culture Treated Multidishes with 24 round wells (Thermo Fisher Scientific, Roskilde, Denmark). Cultured tissues were stored in a freezer at -80°C until RNA extraction.

RNA extraction and quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from tissues homogenized using BioMasher® (Nippi, Tokyo, Japan) by NucleoSpin® RNA II (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's protocol. All RNA samples were stored in a freezer at -80°C. Each RNA sample's concentration was measured by spectrophotometry (NanoDrop ND-2000, Thermo Scientific, Wilmington, DE, USA), and cDNA was synthesized from 0.2 µg of total RNA by reverse transcription using the ReverTra Ace® qPCR RT Master Mix (Toyobo Life Science, Osaka, Japan) according to the manufacturer's protocol. PCR was run on an Astec Program Temp Control System (PC-815 or 816, Astec, Fukuoka, Japan). All cDNA samples were stored in a freezer at -30°C. Specific primers for *MX1-a*, *MX1B*, *MX2*, *ISG15*, *IDO1*, *IFNAR2*, *COPS5*, *IRF1*, *IRF2*, *IRF3*, *IRF9*, and *H2AFZ* were designed using Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Primer details are shown in Table 1. The relative expression levels of *MX1-a*, *MX1B*, *MX2*, *ISG15*, *IFNAR1*, *IFNAR2*, *COPS5*, *IRF1*, *IRF2*, *IRF3*, and *IRF9* were assessed by

qRT-PCR using a LightCycler® 480 System II (Roche Diagnostics, Basel, Switzerland) and THUNDERBIRD™ SYBR® qPCR Mix (Toyobo Life Science) added at the final concentration of each primer 0.5 µM. Thermal cycling conditions were 1 cycle at 95°C for 30 sec (denaturation), followed by 50 cycles at 95°C for 10 sec (denaturation), 55°C for 15 sec (primer annealing), and 72°C for 30 sec (extension). Relative mRNA abundance was calculated by the $\Delta\Delta C_t$ method using the expression of *H2AFZ* as a reference gene.

Immunohistochemical analysis of frozen tissue slices

Frozen tissue slices were prepared and mounted on slides (Kenis, Osaka, Japan) using a Leica CM3050 S Research Cryostat (Leica Biosystems, Nussloch, Germany) and fixed in 4% (w/v) paraformaldehyde in calcium- and magnesium-free phosphate-buffered saline (PBS) (-) for 15 min. The samples were then washed three times with PBS (-) for 5 min and permeabilized by incubation with PBS (-) containing 0.2% (v/v) Triton X-100 (PBS-T) for 10 min. After three washes with PBS (-) for 5 min, the samples were blocked with 1% (w/v) BSA (Sigma) in PBS-T for 1 h. After three washes with PBS (-) for 5 min, the samples were incubated with a primary rabbit polyclonal antibody to IFNAR1 (sc-845, Santa Cruz Biotechnology, Dallas, TX, USA) or IFNAR2 (GTX105770, GeneTex, CA, USA) diluted 1:500 with Solution A of Can Get Signal Immunostain Immunoreaction Enhance (Toyobo Life Science) at 4°C overnight. The samples were washed three times with PBS (-) for 5 min, and incubated for 1 h

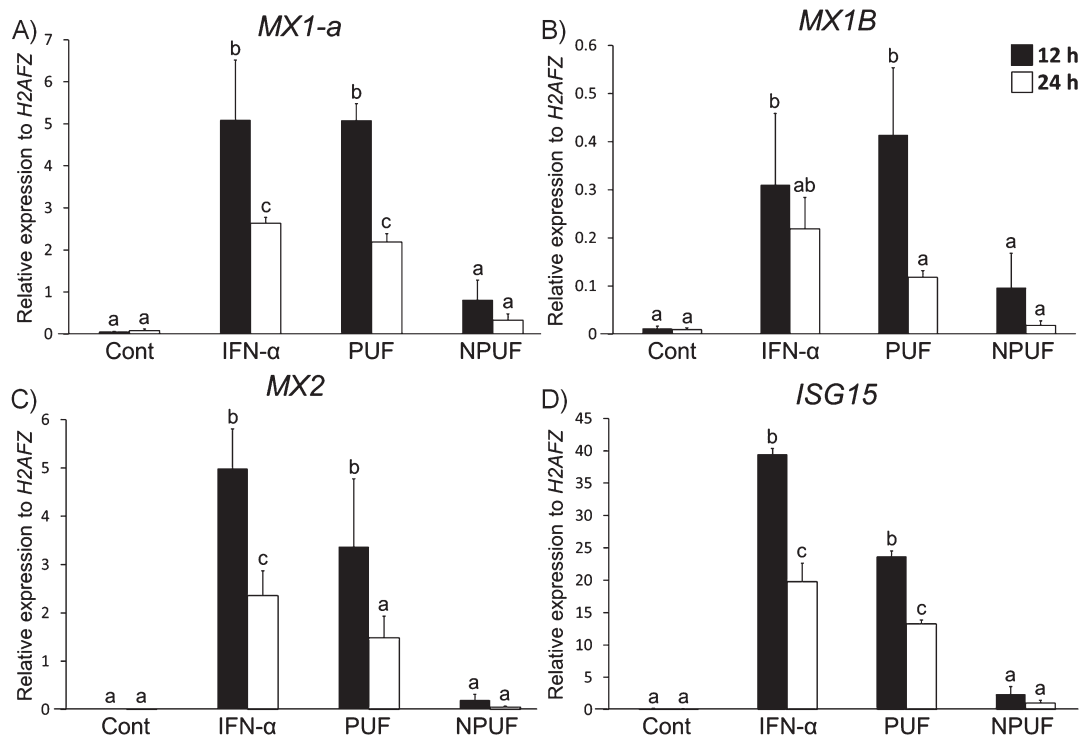


Fig. 1. Expression of ISGs in the bovine endometrium cultured with IFN- α , PUF, or NPUF at the mid stage of the estrous cycle. The vertical line shows the relative expression levels of mRNAs according to qRT-PCR, normalized to *H2AFZ* as a reference gene, while the horizontal axis indicates each culture condition for (A) *MX1-a*, (B) *MX1B*, (C) *MX2*, and (D) *ISG15*. Cont: nonsupplemented culture, IFN- α : addition of recombinant bovine IFN- α , PUF: addition of pregnant uterine flushing, and NPUF: addition of nonpregnant uterine flushing. All data are shown as the mean \pm standard error of the mean (SEM). Letters ($P < 0.05$) indicate significant differences in each culture condition or at each culture time point according to ANOVA followed by Fisher's PLSD procedure as a multiple-comparison test.

with a fluorescein-conjugated secondary antibody (Alexa Fluor® 568-conjugated donkey anti-rabbit IgG antibody, A10042, Thermo Fisher Scientific, CA, USA) diluted 1:200 with Solution A of Can Get Signal Immunostain Immunoreaction Enhance (Toyobo Life Science). The samples were washed three times with PBS (-) for 5 min, covered with 10 μ l of the mounting solution, Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA), and examined under a confocal microscope (Leica TCS SP5 II, Leica Biosystems).

Statistical analysis

All data are shown as the mean \pm standard error of the mean (SEM). The significance of differences was assessed by analysis of variance (ANOVA) followed by Fisher's protected least-significant difference (PLSD) procedure as a multiple-comparison test in the StatView statistical analysis software (version 5; SAS Institute, Cary, NC, USA). Data with P values of < 0.05 were considered significant. P values of < 0.1 were assumed to indicate a slight difference.

Results

Induction of ISG mRNAs in mid-stage endometrial tissues cultured with IFN- α or PUF

PUF stimulated the expression of ISGs as well as IFN- α did. The expression of *MX1-a* and *ISG15* significantly increased in tissues

cultured for 12 and 24 h with IFN- α or PUF (Fig. 1A and D; $P < 0.05$). The expression of *MX2* significantly increased in tissues cultured for 12 and 24 h with IFN- α or for 12 h with PUF (Fig. 1C; $P < 0.05$). The expression of *MX1B* significantly increased in tissues cultured for 12 h with IFN- α or PUF (Fig. 1B; $P < 0.05$). The expression of all ISGs was sufficiently induced in tissues cultured for 12 h with IFN- α or PUF, not with NPUF for 12 or 24 h.

Expression of ISG mRNAs stimulated by IFN- α or PUF in endometrial tissues at each stage

In tissues cultured for 12 h, the magnitude of induction of *MX1-a* by PUF was significantly greater at the mid stage than at the early and late stages (Fig. 2A; $P < 0.05$). Additionally, the induction of *MX1B* by PUF and *ISG15* by IFN- α or PUF was significantly stronger at the mid stage than at the late stage (Fig. 2B and D; $P < 0.05$). In spite of the absence of a significant difference, the expression of *MX1-a* under the influence of IFN- α from the mid to late stage and *MX1B* under the influence of PUF from the mid to early stage had a tendency to be high (Fig. 2A; $P = 0.079$, B; $P = 0.085$). No significant difference was observed in the expression of *MX2* among tissues of all stages cultured for 12 h with IFN- α or PUF.

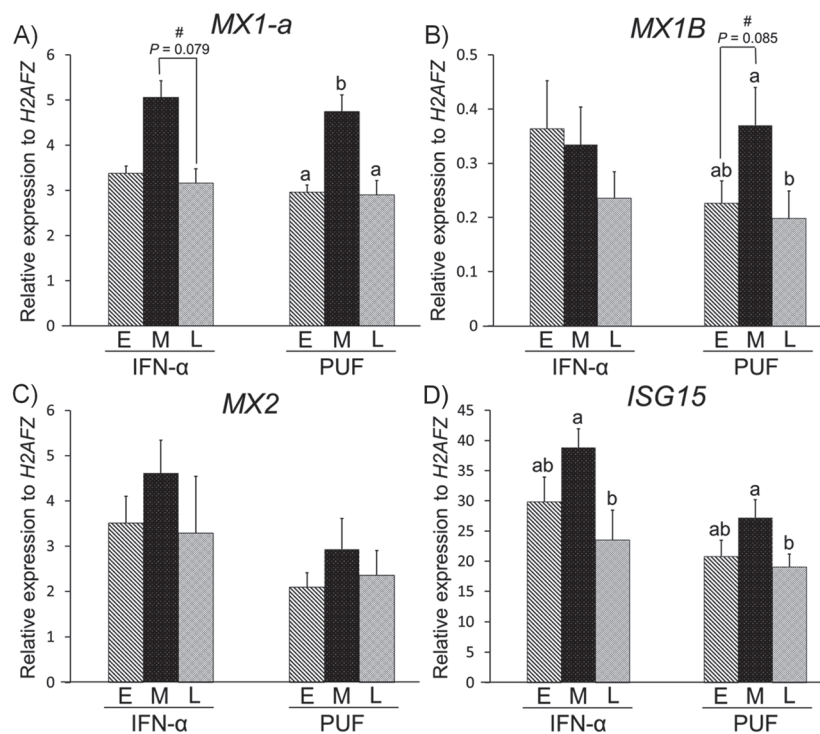


Fig. 2. Expression of ISGs in bovine endometrial tissues cultured with IFN- α or PUF at different estrous stages. The vertical line shows the relative expression levels of mRNAs according to qRT-PCR, normalized to *H2AFZ* as a reference gene, while the horizontal axis indicates each culture condition and estrous stage for (A) *MX1-a*, (B) *MX1B*, (C) *MX2*, and (D) *ISG15*. IFN- α : addition of recombinant bovine IFN- α , PUF: addition of pregnant uterine flushing, E: early stage, M: mid stage, and L: late stage. All data are shown as the mean \pm standard error of the mean (SEM). Letters ($P < 0.05$) indicate significant differences among stages according to ANOVA followed by Fisher's PLSD procedure as a multiple-comparison test. The number sign (#) indicates the tendency ($P < 0.1$).

Changes in the expression of *IFNAR1*, *IFNAR2*, *COPS5*, *IRF1*, *IRF2*, *IRF3*, and *IRF9* mRNAs during the estrous cycle

In noncultured tissues, *IFNAR1*, *IFNAR2*, and *COPS5* mRNAs were strongly expressed at the early stage. The expression of *IFNAR1* mRNA was slightly stronger at the early stage than at the mid stage (Fig. 3A; $P = 0.057$). The expression of *IFNAR2* mRNA was significantly stronger at the early stage than at the other stages (Fig. 3B; $P < 0.05$). The expression of *COPS5* mRNA was slightly stronger at the early stage than at the late stage (Fig. 3C; $P = 0.093$). *IRF1*, *IRF2*, *IRF3*, and *IRF9* mRNAs were strongly expressed at the mid stage. The expression of *IRF1* and *IRF2* mRNAs was slightly stronger at the mid stage than at the early stage (Fig. 3D and E; $P = 0.065$ and 0.055). The expression of *IRF2* and *IRF3* mRNAs was significantly stronger at the mid stage than at the late stage (Fig. 3E and F; $P < 0.05$). The expression of *IRF9* mRNA was significantly stronger at the mid stage than at the early and late stages (Fig. 3G; $P < 0.05$).

Expression and localization of *IFNAR1* and *IFNAR2* proteins in endometrial tissues at each stage

IFNAR1 and *IFNAR2* proteins were detected in the LE and GE in each tissue slice (Fig. 4A and Fig. 5A). The fluorescence intensities of *IFNAR1* and *IFNAR2* were strong at the early and mid stages and weak at the late stage. The localization of *IFNAR1* in GE was clearer

at the mid stage. The relative fluorescence intensities of *IFNAR1* in LE and GE significantly decreased from the early to late stage (Fig. 4B and Fig. 5B; $P < 0.05$), whereas those of *IFNAR2* in LE and GE remained stable from the early to mid stage and were slightly weaker at the late stage (Fig. 4C and Fig. 5C; $P < 0.05$ and $P = 0.087$).

Discussion

The present study demonstrated that the type I IFN response is strong at the mid stage of the estrous cycle according to the induction levels of ISG mRNAs in endometrial tissues cultured with a type I IFN. Additionally, this result was associated with the expression of *IFNARs* and *IRFs* in the endometrium. These data suggest that the endometrium at the mid stage of the estrous cycle requires specific conditions to respond to IFN- τ for pregnancy recognition between a mother and fetus in cows.

In other studies, IFN- τ has been detected in the PUF of cows, whereas other type I IFNs were not [43, 44]. Sakurai *et al.* [45] applied PUF as an alternative to IFN- τ and reported promotion of the processes of attachment of bovine trophoblast cells to endometrial epithelial cells, thereby mimicking the uterine environment at the peri-implantation stage *in vivo*. In the present study, the bovine endometrial reaction to a type I IFN was investigated by the experimental method of an explant culture with the PUF.

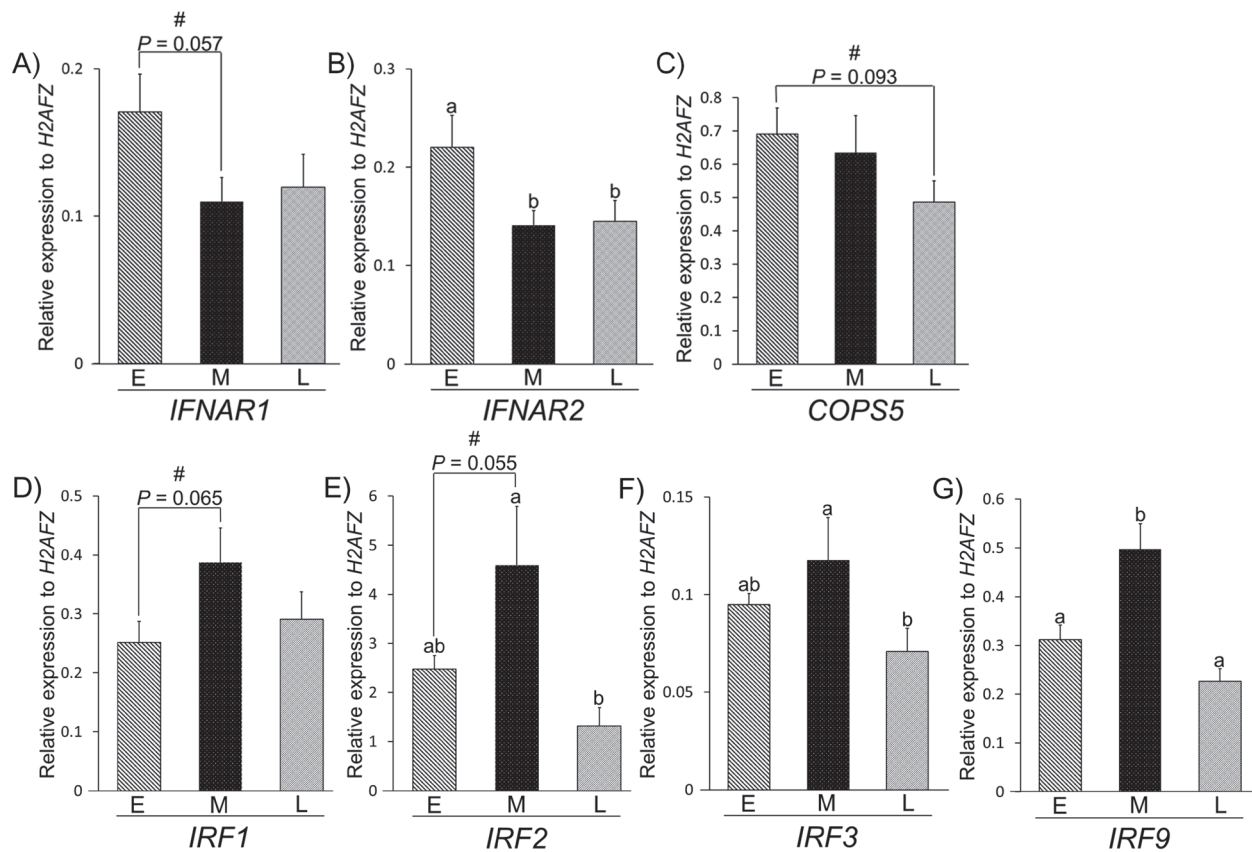


Fig. 3. Expression of *IFNAR1*, *IFNAR2*, *COPS5*, *IRF1*, *IRF2*, *IRF3*, and *IRF9* mRNAs in the bovine endometrium at different estrous stages. The vertical line shows the relative expression levels of mRNAs according to qRT-PCR, normalized to *H2AFZ* as a reference gene, while the horizontal axis indicates each estrous stage for (A) *IFNAR1*, (B) *IFNAR2*, (C) *COPS5*, (D) *IRF1*, (E) *IRF2*, (F) *IRF3*, and (G) *IRF9*. E: early stage, M: mid stage, and L: late stage. All data are shown as the mean \pm standard error of the mean (SEM). Letters ($P < 0.05$) indicate significant differences among stages according to ANOVA followed by Fisher's PLSD procedure as a multiple-comparison test. The number sign (#) indicates the tendency ($P < 0.1$).

We initially attempted the explant culture at IFN- α concentrations of 300 IU/ml and 3000 IU/ml at the mid stage (when pregnancy recognition occurs), as reported previously for a bovine embryo culture [46], and evaluated the relative expression of ISGs (*MX1-a* and *MX1B*) in cultured tissues. Both concentrations similarly induced *MX1-a* and *MX1B* mRNA expression (Supplementary Fig. 1: online only). These results indicate the absence of significant differences in type I IFN induction levels within an antiviral activity range between 300 IU/ml and 3000 IU/ml. PUF and IFN- α both sufficiently stimulated the expression of ISGs in the tissues cultured for 12 h (Fig. 1). Accordingly, we compared the expression of ISGs in tissues cultured for 12 h with PUF or IFN- α at each stage: the early, mid, and late stages.

In tissues cultured with PUF or IFN- α , the expression of *MX1-a*, *MX1B*, and *ISG15* was high at the mid stage (Fig. 2A, B and D). *MX* and *ISG* genes have been shown to suppress viral proliferation in the innate immune system [14, 47, 48]. When progesterone is dominantly produced in livestock, the suppression of immune functions in the uterus enhances the risk of uterine infection [49]. In humans, progesterone was found to suppress IFN- α -induced *MxA* expression in peripheral blood mononuclear cells infected with hepatitis C

virus [50]. ISGs may prevent infection at the preimplantation stage.

The expression of *IFNAR1* and *IFNAR2* mRNAs was strong at the early stage (Fig. 3A and B). A small amount of IFN- τ is known to be produced *in vitro* by hatched blastocysts (days 8–10) [51]. IFN- τ -mediated signal transduction prior to the peak in IFN- τ production may be necessary for achieving a successful pregnancy. The results of the immunohistochemical analysis showed strong fluorescence intensities of IFNAR1 and IFNAR2 from the early to mid stage and their weak intensities at the late stage (Figs. 4 and 5). Furthermore, the IFNAR1 protein was clearly localized to the cytoplasm or cell membrane in GE at the mid stage; therefore, the IFNAR1 protein may be more functional at the mid stage. In addition, the expression of *COPS5* decreased slightly from the early to late stage (Fig. 3C). *COPS5* has been shown to stabilize IFNAR1 protein levels by regulating ubiquitination and degradation [19]. These findings suggest that IFNAR is strongly expressed at the early stage because the uterus needs to prepare to respond to IFN- τ for pregnancy recognition at the mid stage. On the other hand, in the absence of a conceptus, the IFNAR protein is degraded from the mid to late stage.

All *IRF* genes were strongly expressed at the mid stage (Fig. 3D–G).

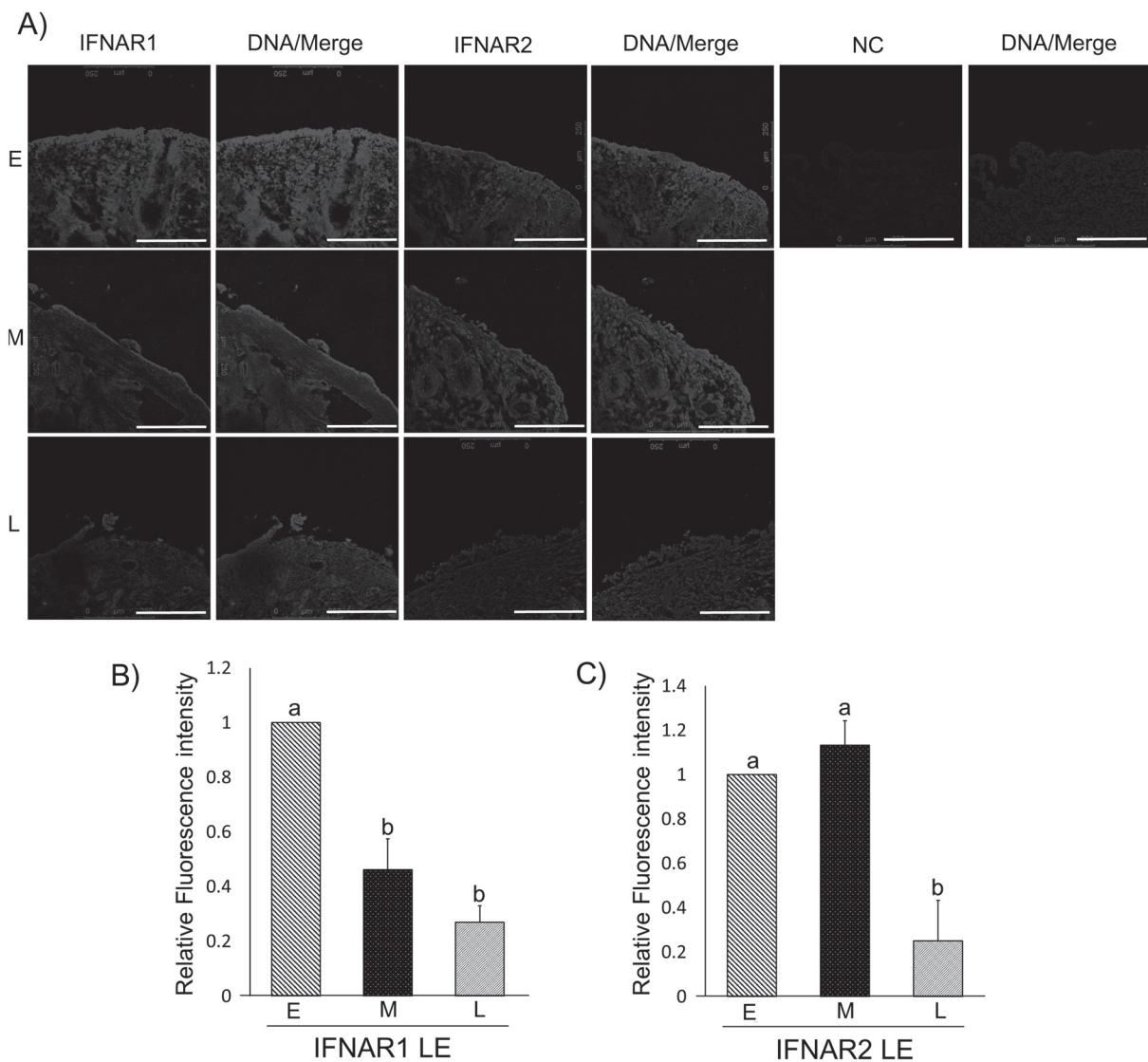


Fig. 4. Localization and relative expression of IFNAR1 and IFNAR2 in the luminal epithelium of the bovine endometrium at different estrous stages. Immunostaining images are shown at magnification $\times 20$. The white scale bar is 250 μm . (A) The localization of IFNAR1 and IFNAR2 and merged images of DNA and each target protein in the luminal epithelium (LE). NC means incubation with PBS (-) instead of the primary antibody. (B and C) The relative fluorescence intensity of the target protein to each fluorescence intensity at the early stage. (B) IFNAR1 in LE, (C) IFNAR2 in LE. E: early stage, M: mid stage, and L: late stage. Graph data are shown as the mean \pm standard error of the mean (SEM). Letters ($P < 0.05$) indicate significant differences among stages according to ANOVA followed by Fisher's PLSD procedure as a multiple-comparison test.

IRF1 regulates DNA damage and apoptosis in addition to activating immune responses including type I IFN signaling [13, 52]. One study suggested that IFN- τ induces apoptosis in bovine endometrial cells [53]. IRF1 activates the induction of IFN- τ -stimulated genes and may regulate the apoptosis in the endometrium for uterine tissue remodeling at the peri-implantation stage. IRF2 has been shown to inhibit the type I IFN signaling pathway [13, 54]. It may also suppress excessive responses to a type I IFN in the endometrium because a successful pregnancy requires immune tolerance. IRF3 is a transcriptional regulator of IFN- α and - β in the innate immune system [13, 14]. This observation suggests that not only the production of IFN- τ but

also the expression of type I IFNs such as IFN- α and - β in maternal uterine tissues is necessary for pregnancy recognition. IRF9 drives the transcription of ISGs [13, 14]. The strong expression of *IRF9* at the mid stage may enable endometrial tissues to positively respond to IFN- τ , and consequently, the expression of *MX1-a*, *MX1B*, and *ISG15* is strong at the mid stage. As described above, *IRF* genes may be involved in the type I IFN response for pregnancy recognition; in particular, the expression of *IRF1* and *IRF9* is more important than that of *IFNAR* because they serve as the activator and transcription factor in type I IFN signaling after binding of IFN- τ to IFNAR.

In conclusion, herein we demonstrated that the endometrium

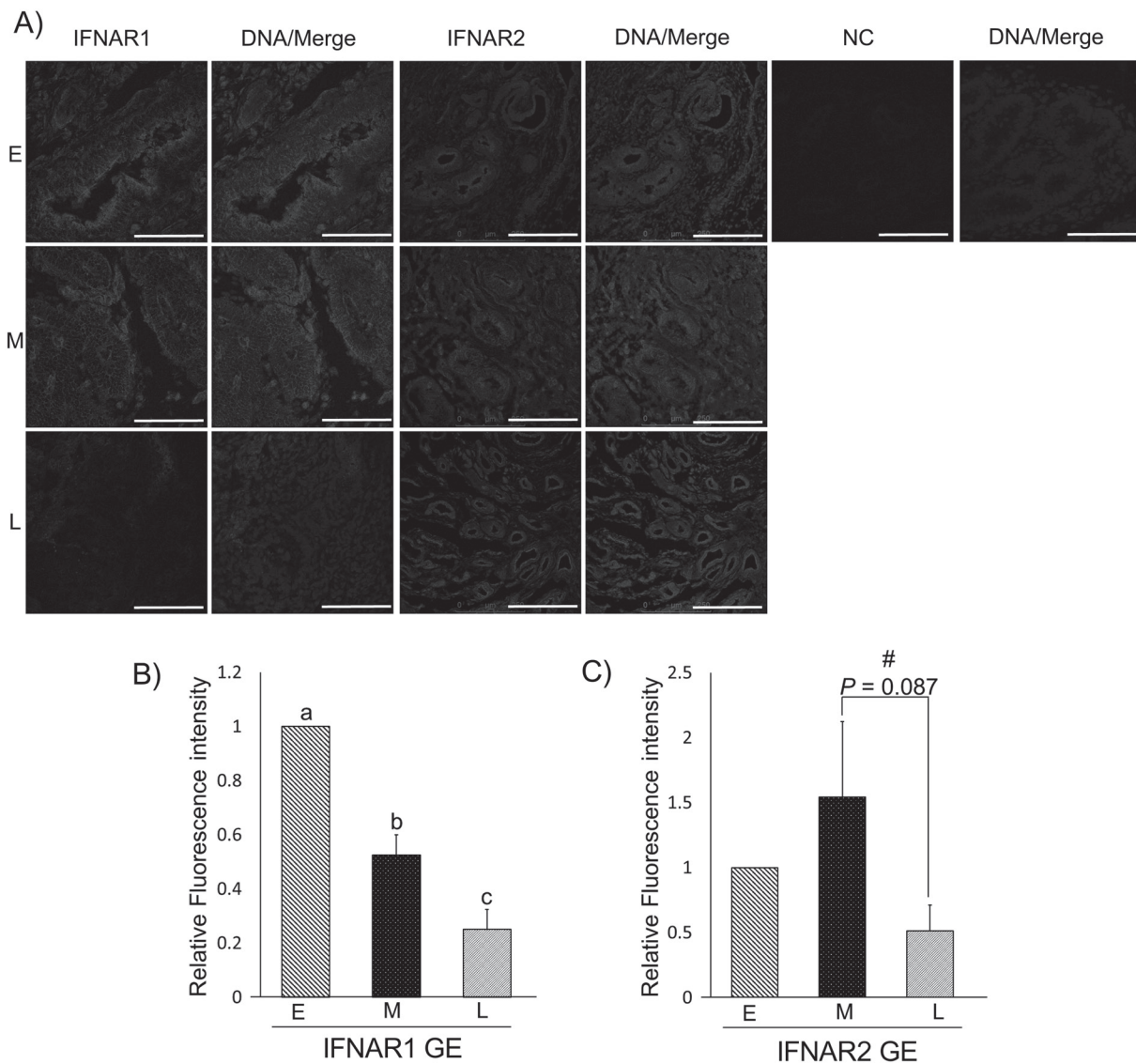


Fig. 5. Localization and relative fluorescence intensities of IFNAR1 and IFNAR2 in the glandular epithelium of the bovine endometrium at different estrous stages. Immunostaining images are shown at magnification $\times 20$. The white scale bar is 250 μm . (A) The localization of IFNAR1 and IFNAR2 and merged images of DNA and each target protein in the glandular epithelium (GE) (A). NC means incubation with PBS (-) instead of the primary antibody. (B and C) The relative fluorescence intensity of the target protein toward each fluorescence intensity at the early stage, (B) IFNAR1 in GE, (C) IFNAR2 in GE. E: early stage, M: mid stage, and L: late stage. Graph data are shown as the mean \pm standard error of the mean (SEM). Letters ($P < 0.05$) indicate significant differences among stages according to ANOVA followed by Fisher's PLSD procedure as a multiple-comparison test. The number sign (#) indicates the tendency ($P < 0.1$).

responds to a type I IFN specifically at the mid stage of the estrous cycle. To the best of our knowledge, this is the first study to examine stage-dependent differences in the induction of ISGs by a type I IFN in bovine endometrial tissue. The expression of IFNARs and IRFs is key to the type I IFN response in the bovine endometrium during the estrous cycle. These results suggest that the bovine endometrium prepares for pregnancy recognition via IFN- τ and easily responds to IFN- τ at the mid stage. ISGs induced by IFN- τ may play important roles in the endometrium for successful implantation, for example, protection from viral and other infections.

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