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Author(s)	SHIROZU, Takahiro; SASAKI, Keisuke; KAWAHARA, Manabu et al.
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—Original Article—

Expression dynamics of bovine *MX* genes in the endometrium and placenta during early to mid pregnancy

Takahiro SHIROZU¹⁾, Keisuke SASAKI¹⁾, Manabu KAWAHARA¹⁾, Yojiro YANAGAWA²⁾, Masashi NAGANO²⁾, Nobuhiko YAMAUCHI³⁾ and Masashi TAKAHASHI¹⁾

¹⁾Laboratory of Animal Breeding and Reproduction, Department of Animal Science, Research Faculty of Agriculture, Hokkaido University, Hokkaido 060-8589, Japan

²⁾Laboratory of Theriogenology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Hokkaido 060-0818, Japan

³⁾Laboratory of Animal Breeding and Reproduction, Department of Animal and Marine Bioresource Sciences, Graduate School of Agriculture, Kyushu University, Fukuoka 812-8581, Japan

Abstract. *MX* belongs to a family of type I interferon (IFN)-stimulated genes, and the *MX* protein has antiviral activity. *MX* has at least two isoforms, known as *MX1* and *MX2*, in mammals. Moreover, bovine *MX1* has been found to have alternative splice variants—namely, *MX1-a* and *MX1B*. In ruminants, IFN- τ —a type I IFN—is temporarily produced from the conceptus before implantation and induces *MX* expression in the endometrium. However, the expression dynamics of *MX* after implantation are not clear. In the present study, we investigated the expression of *MX1-a*, *MX1B* and *MX2* in the endometrium and placenta before and after implantation along with the expression of IFN- α , type I receptors (*IFNAR1* and *IFNAR2*) and interferon regulatory factors (*IRF3* and *IRF9*). Pregnant uterine samples were divided into five groups according to pregnancy days 14–18, 25–40, 50–70, 80–100, and 130–150. Tissue samples were collected from the intercaruncular endometrium (IC), caruncular endometrium (C) and fetal placenta (P). Although all the *MX* expressions were significantly higher in the IC and C at days 14–18, presumably caused by embryo-secreted IFN- τ stimulation, their expressions were also detectable in the IC, C and P after implantation. Furthermore, IFN- α expression was significantly higher in the IC. RT-PCR indicated *IFNAR1*, *IFNAR2*, *IRF3* and *IRF9* mRNA in all the tissues during pregnancy. These results suggest that all the *MX* genes are affected by the type I IFN pathway during pregnancy and are involved in an immune response to protect the mother and fetus.

Key words: Cow, *MX*, Uterus

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In ruminants such as cows and sheep, a type I interferon (IFN)—IFN- τ —is secreted from the trophoblast before implantation [1]. IFN- τ is known as a pregnancy recognition signal that inhibits oxytocin receptor expression and the production of PGF2 α and maintains the corpus luteum and pregnancy [2]. Several IFN-stimulated genes (ISGs) are expressed in uterine tissue via IFN- τ stimulation [3–5]. The *Myxovirus resistance* (*MX*) genes are ISGs, and their levels of mRNA and protein increase in the endometrium at the time of implantation [6–11]. The *in vitro* expression of *MX1* and *MX2* mRNA in bovine epithelial cells is stimulated by IFN- τ [10, 12]. This IFN- τ signaling pathway is called the janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway, and it is activated via a transmembrane receptor composed of the IFNAR1 and IFNAR2 subunits [2–4, 13]. IFN- τ production occurs only at preimplantation, and it is not clear whether *MX* mRNA is induced by the IFN signaling pathway during pregnancy.

On the other hand, IFN is typically produced in innate immune responses in mammals. When bacteria and viruses infect animals, pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and RIG-I-like receptors, recognize pathogen-associated molecular patterns and trigger the type I IFN signaling pathway [14]. IFN- α/β , a type I IFN, is induced by interferon regulatory factors (IRFs) 3 and 7 and stimulates the JAK-STAT signal pathway via type I IFN- α/β receptors composed of the IFNAR1 and IFNAR2 subunits [15, 16]. At that time, activated JAK1 and tyrosine kinase 2 bind to the intracellular domain of IFNAR1/2 and are responsible for phosphorylating and dimerizing STAT1/2, which serves as a transcription factor [15, 16]. Phosphorylated and dimerized STAT1/2 combines with IRF9 to form the ISGF3 complex, which activates the transcription of ISGs by binding to the IFN-stimulated responsive element after nuclear translocation [15, 16].

MX is known to suppress several viruses including the influenza virus and vesicular stomatitis virus (VSV) [17–20]. In cows, two isoforms of *MX* are present—*MX1* and *MX2*; additionally, bovine *MX1* has alternative splice variants, *MX1-a* and *MX1B* [9, 21]. Interestingly, *MX1-a* has antiviral activity against VSV, whereas *MX1B* does not inhibit VSV proliferation in the cytoplasm, and its own function is unknown [22, 23]. In fact, *MX1-a* localizes in the cytoplasm, but almost all of *MX1B* localizes in the nucleus. *MX2* also has antiviral activity against VSV [24], and its intracellular

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Correspondence: M Takahashi (e-mail: mmasashi@anim.agr.hokudai.ac.jp)

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localization is unknown [25]. As mentioned above, cows have three *MX* gene isoforms; however, the distribution of *MX1-a* and *MX1B* expression have not been analyzed. In addition, very little is known about *MX* expression in uterine and placental tissue after implantation.

In this study, to elucidate whether these *MX* genes are expressed after implantation in cows, we investigated to detect the expression dynamics of *MX1-a*, *MX1B* and *MX2* mRNA in uterine and placental tissues from early to mid pregnancy. Furthermore, we examined relative expressions of *IFN-α* mRNA and the expression of type I IFN receptor subunits (*IFNAR1* and *IFNAR2*) and interferon regulatory factors (*IRF3* and *IRF9*) in relation to the type I IFN signaling pathway.

Materials and Methods

Sample collection from pregnant uterine and fetal tissues

This study was conducted in accordance with the Hokkaido University guidelines for the care and use of animals. Pregnant uteri were collected from cows slaughtered between days 14 and 18 of pregnancy after embryo transfer on day 7 of the estrus cycle. Uteri at mid-pregnancy stages were collected from abattoirs. The collected pregnant uteri were divided into five groups: days 14–18 ($n = 3$), 25–40 ($n = 3$), 50–70 ($n = 3$), 80–100 ($n = 4$) and 130–150 ($n = 4$), confirming the existence of a conceptus on days 14–18 and assessing day of pregnancy in the other groups according to crown-rump length of fetuses from the top of the head to the bottom of the buttocks. The intercaruncular endometrium (IC) and caruncular endometrium (C) tissues from pregnant uterine horns, and fetal placenta (P) tissues were collected for RNA extraction.

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

Total RNA was extracted from the 3 tissue groups (IC, C and P) using ISOGEN II (Nippon Gene, Toyama, Japan), and cDNA was synthesized from total RNA by reverse transcription using the ReverTra Ace[®] qPCR RT Master Mix with gDNA Remover (Toyobo Life Science, Osaka, Japan) according to the manufacturer's protocol. PCR was performed with an Astec Program Temp Control System (PC-815 or 816, Astec, Fukuoka, Japan). Each cDNA sample concentration was measured by spectrophotometry (NanoDrop ND-2000, Thermo Scientific, Wilmington, DE, USA) and was adjusted to 100 ng/μl. All cDNA samples were stored in a freezer at -30°C .

RT-PCR and quantitative RT-PCR (qRT-PCR)

Specific primers for *MX1-a*, *MX1B*, *MX2*, *IFN-α*, *IFNAR1*, *IFNAR2*, *IRF3*, *IRF9* and *H2AFZ* mRNA expression were designed using Primer-BLAST. The primer details are shown in Table 1. Expression of *IFNAR1*, *IFNAR2*, *IRF3*, *IRF9* and *H2AFZ* was detected by RT-PCR analysis using an Astec Program Temp Control System (PC-815 or 816, Astec) and GoTaq[®] Hot Start Green Master Mix (Promega, Madison, WI, USA). The thermal cycling conditions were 1 cycle at 95°C for 5 min (initial denaturation); 35 cycles at 95°C for 30 sec (denaturation), 55°C for 1 min (primer annealing) and 72°C for 1 min (extension); and then 1 cycle at 72°C for 5 min (final extension). The PCR products were detected by electrophoresis using a 2.0% agarose gel with Midori Green (Nippon Genetics, Tokyo, Japan).

The expression levels of *MX1-a*, *MX1B*, *MX2* and *IFN-α* were investigated by qRT-PCR using a LightCycler[®] 480 System II (Roche Diagnostics, Basel, Switzerland) and THUNDERBIRD[™] SYBR[®] qPCR Mix (Toyobo Life Science). The 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). The relative expression levels of *MX1-a*, *MX1B*, *MX2* and *IFN-α* were calculated by the $\Delta\Delta\text{Ct}$ method using the expression of *H2AFZ* as the reference gene.

Statistical analysis

All data are shown as the mean $\pm$ standard error of the mean (SEM). The statistical significance of differences was assessed by one-way analysis of variance (ANOVA) followed by the Fisher's protected least-significant difference (PLSD) procedure as the multiple comparison test. P values of < 0.01 or < 0.05 were considered statistically significant. P values of < 0.1 was regarded as indicating a tendency.

Results

Expression of *MX1-a*, *MX1B* and *MX2* mRNA in uterine tissues in the pre- and postimplantation stages

The relative expressions of *MX1-a*, *MX1B* and *MX2* mRNA in pregnant uterine tissues (IC and C) were higher at days 14–18 than days 25–40 (Fig. 1A, C and E; $P < 0.01$) and the highest on days 14–18 compared with the mid-pregnancy stages, including days 50–70, 80–100 and 130–150 (Fig. 1A–F; $P < 0.01$). Although the expression levels of all the *MX* mRNAs markedly decreased immediately after the pregnancy stage of days 25–40 in the time of implantation, these *MX* genes were also expressed in the endometrium after implantation.

Expression of *MX1-a*, *MX1B* and *MX2* mRNA in uterine and fetal placental tissues at mid pregnancy

Comparison of the expression levels in IC, C and P tissues revealed that *MX1-a* expression was significantly higher in IC tissue than in C or P tissues at days 80–100 and higher than in P tissues on days 130–150 (Fig. 1B; $P < 0.05$). The C and P tissues showed different tendencies with respect to expression at days 80–100, and the IC and C tissues showed different tendencies at days 130–150 ($P < 0.1$). *MX2* expression in the IC tissues was higher than in the P tissue at days 50–70 and higher than in either C or P tissues at days 80–100 (Fig. 1F; $P < 0.05$). The expression of *MX2* in the IC tissues also tended to be higher at days 50–70 in the C tissue ($P < 0.1$). No significant difference was observed in *MX1B* expression (Fig. 1D).

Comparison of the levels at days 50–70, 80–100 and 130–150 revealed that *MX1-a* expression in the IC tissue increased significantly at days 80–100 followed by a significant decrease (Fig. 1B; $P < 0.05$). The expression pattern of *MX1-a* and *MX2* in the P tissue was similar; *MX1-a* expression in the P tissue at days 130–150 was significantly higher than at days 50–70 and 80–100 (Fig. 1B; $P < 0.05$), and *MX2* expression in the P tissue at days 130–150 tended to be higher than at days 50–70 and 80–100 (Fig. 1F; $P < 0.1$).

Table 1. Information about the primer sequences used for RT-PCR or qRT-PCR

Gene	Sequence (5'-3')	Accession No.	Product length (bp)
<i>MX1-a</i>	GCCAACTAGTCAGCACTACATTGTC GCTCTTGACTCCATATCTTCAC	NM_173940.2	139
<i>MX1B</i>	GTGATATCTCCAACAGTGAAGC AACTGATTCGAGAAGCCAAG	AB_060169.1	94
<i>MX2</i>	CAGAGACGCCTCAGTCGAAG GAGACGTTTGCTGGTTTCCATG	NM_173941.2	113
<i>IFN-α</i>	CTAGAGAGCAGGTTACAGAGTC GCTGAGCAGCAACAGGGATAG	NM_001017411.1	106
<i>IFNAR1</i>	GCGAAGAGTTTCCGCAACAG TCCAAGGCAGGTCCAATGAC	NM_174552.2	275
<i>IFNAR2</i>	TCGTATGTTGCGCCTGTTCT GTCCGTCGTGTTTACCCACA	NM_174553.2	231
<i>IRF3</i>	GCTCAACTGACGGGAAGTGG TGGTCTGGCCTAAGTGTGG	NM_001029845.3	116
<i>IRF9</i>	CAGTTCCCAGGAGTGTGCTG TATATCGCCCAGGCCTTGAA	NM_001024506.1	125
<i>H2AFZ</i>	AGAGCCGGTTTGCAGTTCCCG TACTCCAGGATGGCTGCGCTGT	NM_174809.2	116

Expressions of the mRNA of IFN-α and IFN pathway-related genes (IFNAR1, IFNAR2, IRF3 and IRF9) in uterine and fetal placental tissues during pregnancy

Comparison of the expression levels of *IFN-α* mRNA among days 14–18, 25–40, 50–70, 80–100 and 130–150 revealed that *IFN-α* showed a scattered gene expression pattern; nevertheless, its expression in the IC tissue was significantly higher ($P < 0.05$) at days 50–70 than at days 14–18, 25–40 and 130–150 (Fig. 2A). The mRNA expression level of *IFN-α* in the C was not significantly different (Fig. 2B). In the P tissue, *IFN-α* was only minimally expressed during mid pregnancy (Fig. 2C).

The expression of type I IFN receptors (*IFNAR1* and *IFNAR2*) and type I IFN regulatory factors (*IRF3* and *IRF9*) was detected in all pregnant uterine and fetal placental tissues by RT-PCR analysis (Fig. 3). Thus, not only *IFN-α* but also IFN signaling pathway-related genes were expressed in IC, C and P tissues during pregnancy.

Discussion

In this study, to determine whether bovine *MX1-a*, *MX1B* and *MX2* genes were expressed in uterine and fetal placental tissues after implantation, we evaluated the expression of each *MX* mRNA in three different tissues (IC, C and P) during five pregnancy stages (days 14–18, 25–40, 50–70, 80–100 and 130–150) by qRT-PCR. *MX1-a*, *MX1B* and *MX2* were expressed not only in the endometrium (IC and C) but also in the P at each pregnancy stage. The relative expression levels of each *MX* mRNA in both the IC and C were higher at days 14–18 than at the other stages. The expression of *MX* genes is induced by stimulation with IFN- τ , which is secreted from the trophoblast of the conceptus cells during pregnancy days 14 to 21 in ruminants [1]. *MX* genes induced by IFN- τ at preimplantation may play a role in pregnancy recognition or uterine reception. It has

also been suggested that *MX1* is involved in the immune response and in cell adhesion at implantation [10]. Therefore, *MX* genes may play a role in cell adhesion mechanisms between the mother and fetus, such as conceptus attachment to the endometrium.

Although *MX* expressions decreased from days 14–18 to days 25–40, these expressions continued to change during mid pregnancy. Since IFN- τ secretion by the conceptus decreases after establishment of implantation, it was thought that *MX1-a*, *MX1B* and *MX2* mRNA after implantation might be induced by another type I IFN such as IFN- α/β . To determine whether *MX* expression correlated with the type I IFN signaling pathway, we focused on the mRNA of *IFN-α*, type I IFN receptors (*IFNAR1* and *IFNAR2*) and type I IFN regulatory factors (*IRF3* and *IRF9*). *IFN-α* mRNA was expressed in all pregnancy tissues. In particular, *IFN-α* expression in IC tissue was significantly higher at days 50–70. This result correlated with the upregulation of *MX1-a* and *MX2* expression at days 80–100. IFN- α is transcribed by IRF3 and affects the JAK-STAT signaling pathway via IFNAR1 and IFNAR2 receptors, which is followed by expression of ISGs induced by IRF9 [15, 16]. Detection of *IFNAR1*, *IFNAR2*, *IRF3* and *IRF9* mRNA indicates that this cascade stimulates *MX1-a*, *MX1B* and *MX2* mRNA even during pregnancy for the regulation of immune tolerance.

MX1-a, *MX1B* and *MX2* mRNA were expressed in the IC, C, and P on days 50–70, 80–100, and 130–150 during the placental formation phase. When comparing tissues (IC, C and P), the relative expression levels of *MX1-a* (days 80–100 and 130–150) and *MX2* (days 50–70 and 80–100) were significantly higher in the IC tissue than the P tissues. Thus, there was a tendency for the expression of the above genes to decrease in the P of fetal tissue than the IC of maternal tissue. Immune tolerance is required for successful pregnancy in any viviparous animal. In cows, decrease of expression of major histocompatibility proteins by the trophoblast, recruitment

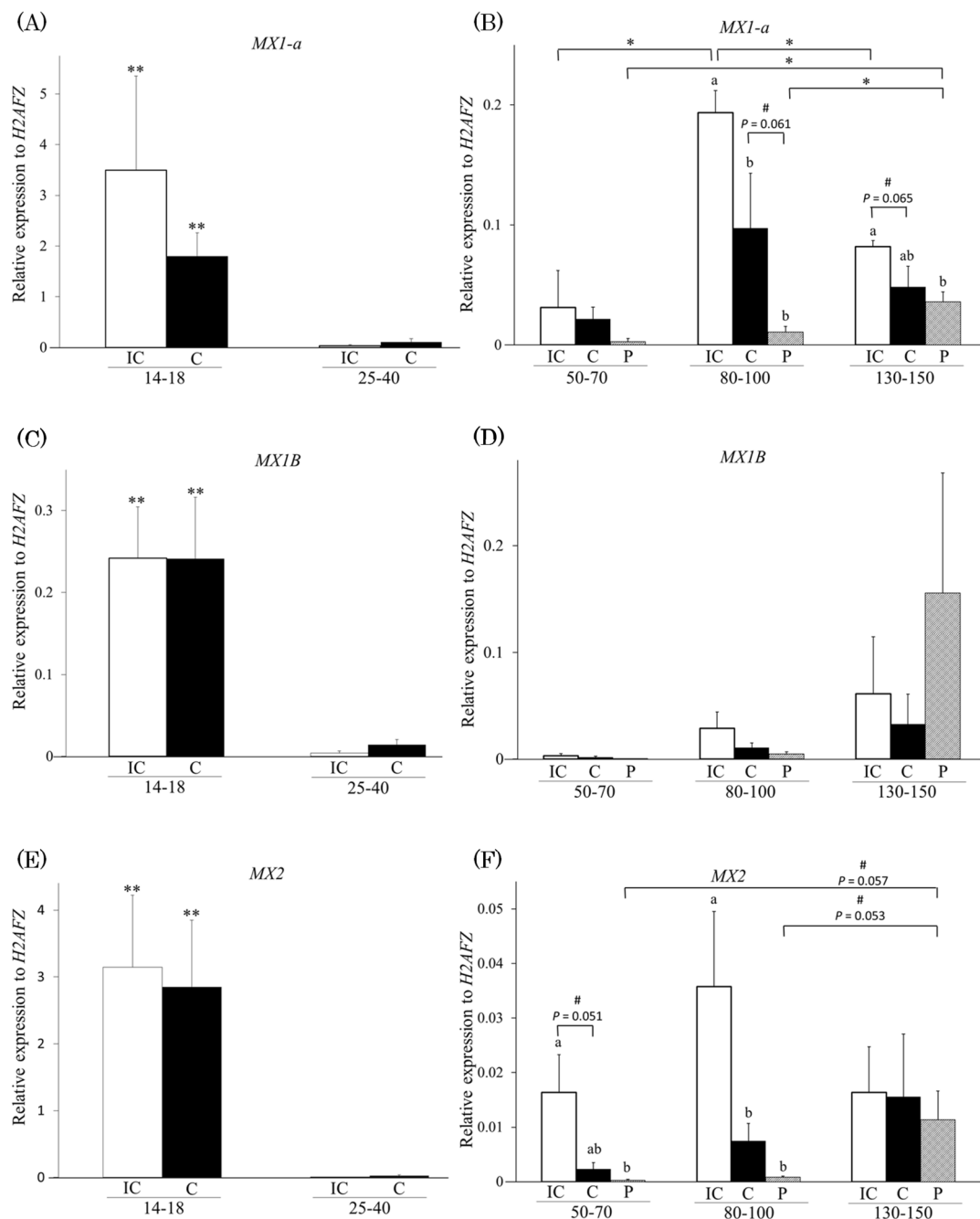


Fig. 1. Expression of *MX1-a*, *MX1B*, and *MX2* mRNA in the endometrium and fetal placenta in the pre- and postimplantation stages. The vertical line shows the relative expression levels of mRNA using qRT-PCR, standardized with the reference gene *H2AFZ*, whereas the horizontal axis indicates the stages of pregnancy and types of tissue samples for the genes (A and B) *MX1-a*, (C and D) *MX1B*, and (E and F) *MX2*. IC, intercaruncular endometrium; C, caruncular endometrium; P, fetal placenta. The numbers 14–18, 25–40, 50–70, 80–100, and 130–150 indicate the number of pregnancy days for each subgroup. All data are shown as the mean $\pm$ standard error of the mean (SEM). Asterisks indicate significant differences (** $P < 0.01$; * $P < 0.05$) among pregnancy stages, and letters indicate significant differences ($P < 0.05$) among three tissues as determined by ANOVA, followed by the Fisher's PLSD procedure as the multiple comparison test. Additionally, a number sign (#) indicates a tendency for significant differences ($P < 0.1$).

of macrophages to the uterus and modulation of immune-related genes contribute to the uterine immunosuppressive environment [26]. Immune tolerance between the mother and fetus is unbalanced when

the uterus is infected with pathogens [27–29]. During pregnancy as well as non-pregnancy conditions, the TLRs—a major family of PRRs—are expressed in the uterus. The levels of TLR2, 3, 4, 6

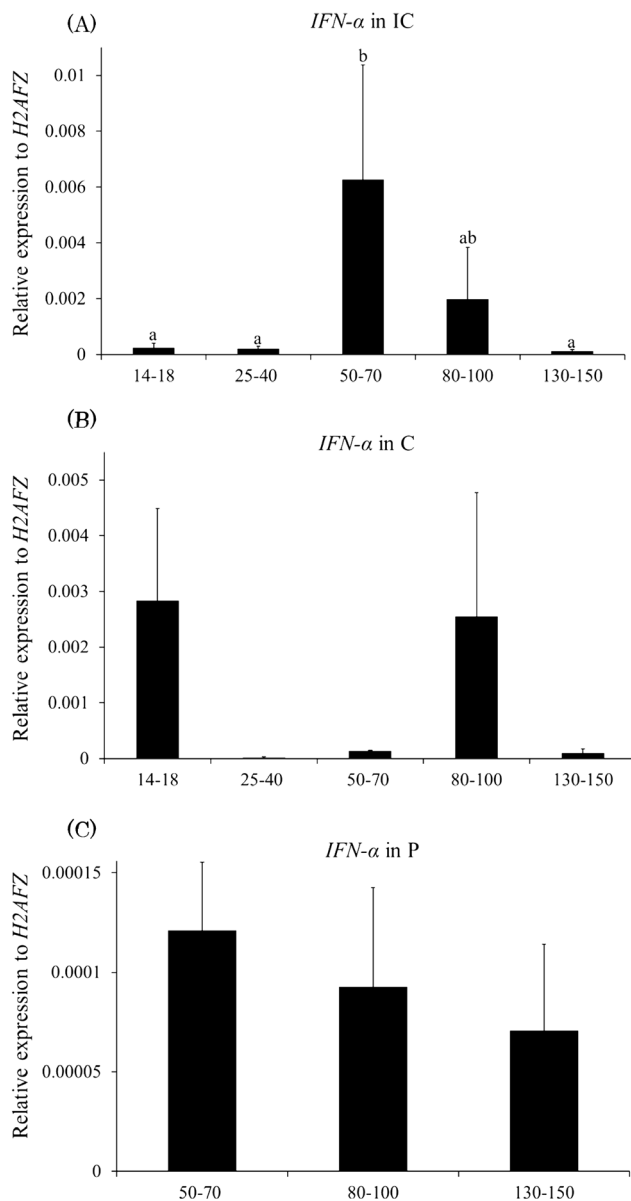


Fig. 2. Expression of *IFN-α* mRNA in the endometrium and fetal placenta in the pre- and postimplantation stages. The vertical line shows the relative expression levels of mRNA using qRT-PCR, standardized with the reference gene *H2AFZ*, whereas the horizontal axis indicates the stages of pregnancy for (A) *IFN-α* in IC tissues, (B) *IFN-α* in C tissues and (C) *IFN-α* in P tissues, respectively. IC, intercaruncular endometrium; C, caruncular endometrium; P, fetal placenta. The numbers 14–18, 25–40, 50–70, 80–100 and 130–150 indicate the number of days of pregnancy for each subgroup. All data are shown as the mean $\pm$ standard error of the mean (SEM). Letters ($P < 0.05$) indicate significant differences determined by ANOVA, followed by the Fisher's PLSD procedure as the multiple comparison test.

placental tissues, where maternal tissue is in contact with fetal tissue; in contrast, a stronger immune response in the intercaruncular tissue that is not in contact with fetal tissue would protect the mother and fetus from pathogens.

Moreover, *MX1-a* and *MX2* expression in the P tissue had a tendency to increase with the progress of pregnancy, although a significant difference was not recognized for *MX1B* expression, which has no antiviral activity against VSV. This indicates that immunity of the fetus may develop with the progression of pregnancy. Fetal immunity is formed from 80 to 120 days of pregnancy; hence, immune system maturation is not completed until about 120 days of gestation [30, 31]. Infection with bovine viral diarrhea virus prior to sufficient development of the fetal immune system causes abortion or calving of a persistent infected calf, which results in immunological tolerance [30, 31]. The expression of *MX1-a* in IC tissue increased at days 80–100, followed by a decrease at days 130–150. We suggest that the maternal innate immune response is active in the presence of an undeveloped fetal immune system until fetal immunity develops, at which time the maternal immune response then weakens.

Recently, it was shown that ovine MX1 protein was located within exosomes secreted by uterine epithelial cells [32]. Exosomes are extracellular vesicles containing and transporting proteins, mRNA and miRNA and are necessary for intercellular communication. Exosomes are mainly produced by immune, epithelial and tumor cells [33]. In humans, it has been shown that the syncytiotrophoblast cells in the placenta constitutively secrete exosomes during pregnancy [34, 35]. These exosomes, derived from the human placenta, are secreted into the maternal bloodstream and enable cross talk between the mother and child—namely, transport of substances such as proteins and RNA molecules [35]. In sheep, the exosomes secreted by uterine epithelial cells incorporate the MX1 protein [32]. This might also be applicable to cows in ruminants; bovine MX1 protein transported inside exosomes may play a role in fetal-maternal communication as well as in humans. The MX protein belongs to the dynamin superfamily of GTPases, and therefore, it is suggested that MX may play a role in intracellular transport and endocytosis in addition to having antiviral activity [36–39]. Ovine MX1 protein in uterine glandular epithelial cells interacts with tubulin β ; this suggests that MX1 could play a role in transporting proteins or vesicles via secretion and mitosis [40]. MX may function in exosome secretion by uterine epithelial cells or serve as transporter.

Our findings reveal that three isoforms of bovine *MX* genes as well as the IFN signaling pathway-related genes, *IFN-α*, type I IFN receptors and type I IFN regulatory factors, are expressed in the IC, C and P tissues throughout pregnancy up to day 150. In the mid-pregnancy stages, the IC showed comparatively higher expressions of *MX* genes. Hence, in IC tissue that does not form a P, the mother regulates the immune response to defeat infections to protect the uterus and fetus. Consequently, it has been suggested that bovine *MX* genes are induced by *IFN-α* instead of *IFN-τ* after implantation, which indicates the need for the immune response to protect both the mother and embryo even during the placental development phase and maternal immune suppression.

and 9 are higher in the interplacentomal endometrium than in the placentome [26]. The present results suggest that pregnant cows may weakly modulate the immune response in the caruncular and

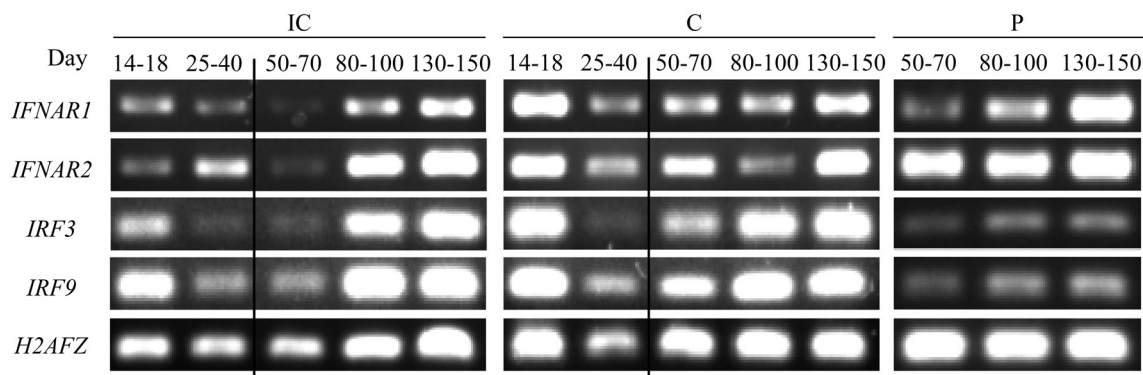


Fig. 3. Expression of *IFNAR1*, *IFNAR2*, *IRF3* and *IRF9* in the endometrium and fetal placenta in the pre- and postimplantation stages. The figure shows the expression of type I IFN receptor (*IFNAR1* and *IFNAR2*) and type I IFN regulatory factor (*IRF3* and *IRF9*) mRNA in pregnant uterine and placental tissues by RT-PCR. IC, intercaruncular endometrium; C, caruncular endometrium; P, fetal placenta. The numbers 14–18, 25–40, 50–70, 80–100 and 130–150 indicate the number of days of pregnancy for each subgroup. *H2AFZ* was used as the internal control gene.

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