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—Original Article—

Bovine embryo induces an anti-inflammatory response in uterine epithelial cells and immune cells *in vitro*: possible involvement of interferon tau as an intermediator

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Abstract. Recent observations suggest that the bovine uterus starts to react to the early embryo immediately after its arrival from the oviduct. The present study aimed to investigate the effect of the early developing embryo on the immune-related gene profile in bovine uterine epithelial cells (BUECs) *in vitro*, and to further examine the impact of conditioned media (CM), either from embryo-BUEC co-culture or embryo culture alone, on gene expression in peripheral blood mononuclear cells (PBMCs). First, BUECs were co-cultured with morulae (n = 10) for D5-D9 (D0 = IVF), and gene expression in BUECs was analyzed. Subsequently, PBMCs were cultured in CM from embryo-BUEC co-culture or D5-D9 embryo culture, and gene expression was evaluated. In BUECs, the embryo induced interferon (IFN)-stimulated genes (*ISGs*: *ISG15*, *OAS1*, and *MX2*), a key factor for IFN-signaling (*STAT1*), and type-1 IFN receptors (*IFNAR1* and *IFNAR2*), with suppression of *NFkB2*, *NFkBIA* and pro-inflammatory cytokines (*TNFA* and *IL1B*). The embryo also stimulated *PTGES* and PGE2 secretion in BUECs. In PBMCs, both CM from embryo-BUEC co-culture and embryo culture alone induced *ISGs*, *STAT1* and *TGFB1*, while suppressing *TNFA* and *IL17*. Similarly, interferon tau (IFNT) at 100 pg/ml suppressed *NFkB2*, *TNFA* and *IL1B* in BUECs, and also stimulated *TGFB1* and suppressed *TNFA* in PBMCs. Our findings suggest that the bovine embryo, in the first four days in the uterus (D5-D9), starts to induce an anti-inflammatory response in epithelial cells and in immune cells. IFNT is likely to act as one of the intermediators for induction of the anti-inflammatory response in the bovine uterus.
Key words: Bovine, Embryo, Interferon tau (IFNT), Immunity, Uterus

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The female reproductive tract (FRT) is equipped with a unique and well-developed mucosal immune system that plays dual roles by accepting allogenic sperm and a semi-allogenic embryo/fetus, while providing protection against pathogens [1, 2]. After fertilization, the embryo stays in the bovine oviduct for about 4 days, and enters into the uterus at approximately the 16-cell or early morula stage [3, 4]. In the uterus, the embryo develops to blastocyst by D7, hatches from the zona pellucida between D9 and D10, and begins to elongate and form a filamentous conceptus that attaches to the uterine epithelium on D19 in cattle [4, 5]. Several studies have investigated the immunological interaction between embryo and endometrium in cattle after hatching of the embryo (D12-13), during maternal recognition of pregnancy (D16), and during the peri-implantation period (D18-19) [6–10]. In contrast, there are few studies in cattle that focus on the uterine immune response to the pre-implantation embryo, particularly during the period from the

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arrival of the morula into the uterus (D5) until the hatching of the blastocyst (D9). It was observed that nuclear factor kappa B (NFkB) protein, a central mediator of inflammatory and immune responses, was down-regulated in bovine uterine fluid on D8 of pregnancy, which may confer tolerance to the semi-allogenic embryo in the uterus [11]. It should be noted that the bovine blastocyst expresses the paternal antigen of MHC molecule I on D7 of pregnancy [12, 13], and it therefore could be considered as foreign to the FRT. However, the blastocyst somehow escapes from maternal immune attack and establishes pregnancy in the uterus.

In ruminants, conceptus-derived interferon tau (IFNT) is a pregnancy recognition signal that indirectly inhibits PGF2α release from the endometrial epithelium on D16, and thereby prevents the corpus luteum from regression and maintains pregnancy [14, 15]. Apart from its anti-luteolytic function, IFNT is considered as an immunosuppressive molecule that inhibits proliferation of lymphocytes, and thus may protect the semi-allogenic fetus from maternal immune attack [16]. Furthermore, the interferon-stimulated genes (*ISGs*) were activated in peripheral blood leukocytes during early pregnancy in cows [17, 18], suggesting that the resident immune cells in the FRT may also respond to IFNT. Until recently, there was little or no information on the involvement of IFNT in embryo-maternal communication before elongation of the embryo, which starts from D12-D13 in cattle. However, IFNT mRNA is expressed and its protein can be detected in the trophectoderm of non-hatched blastocysts on D7 [19, 20]. Therefore, it is possible that a small amount of IFNT from very early stage embryo could be involved in modulation of the local immune response in the bovine uterus.

To date, the molecular mechanism involved in the acceptance of the semi-allogenic embryo in the uterus is poorly understood in cattle. The present study aimed to investigate the effect of the early developing embryo on the immune-related gene profile in bovine uterine epithelial cells (BUECs) using an *in vitro* model, and to examine the impact of conditioned media (CM) either from embryo-BUEC co-culture or embryo culture alone on gene expression in immune cells. We further examined the direct effect of IFNT on both BUEC and immune cell gene expression.

Materials and Methods

Ethics statement

Animal experiments were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals Promulgated by Obihiro University of Agriculture and Veterinary Medicine, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit number 25–101).

Experimental model

We used an *in vitro* model (Fig. 1) to investigate the communication of the embryo with uterine epithelial cells and immune cells. First, bovine uterine epithelial cells (BUECs) were co-cultured with IVM-IVF-derived morulae (n = 10) for 4 days to mimic *in vivo* conditions, from the arrival of the embryo into the uterus (D5) until the hatching of the blastocyst (D9). BUEC culture without embryos served as the control. The gene expression in BUECs was compared in the presence and absence of the embryo. ELISA was performed to determine the PGE2 and IFNT concentration in the conditioned media (CM) of embryo-BUEC co-culture and BUEC culture alone (control). Subsequently, peripheral blood mononuclear cells (PBMCs) were cultured in CM from embryo-BUEC co-culture or BUEC culture alone (control), and gene expression in the PBMCs was evaluated. Next, morulae (n = 10) were cultured alone without BUECs for 4 days. At the same time, the fresh medium without embryos was incubated for 4 days. PBMCs were cultured in CM from embryo culture or in CM without embryos (control), and gene expression was analyzed in the PBMCs.

Culture of BUECs

The reproductive tracts of cows at luteal phase (D5-D7) were collected from a local slaughterhouse (Hokkaido Livestock, Doto Plant Tokachi Factory; Obihiro, Hokkaido, Japan) and transported to the laboratory in physiological saline containing 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA) and 1% amphotericin B (Gibco). The phase of the estrous cycle was identified as previously reported [21]. The uterine horn, ipsilateral to the corpus luteum, was used for isolation and culture of epithelial cells according to a previously described method [22], with minor modifications. Briefly, epithelial cells were cultured in DMEM/F12 (Gibco) supplemented with 2.2% $NaHCO_3$ (Sigma-Aldrich, Steinheim, Germany), 1% penicillin-streptomycin, 1% amphotericin B and 10% FCS (Bio Whittaker, Walkersville, MD). The cells were seeded in 25 cm^2 culture flasks (Nalge Nunc International, Roskilde, Denmark) and cultured at 38.5°C in a humidified atmosphere of 5% CO_2 in air. The medium was changed every 48 h until growing BUECs reached to 70–80% confluence, at which point cells were given a second passage. The cells were trypsinized (0.05% trypsin EDTA; Amresco, Solon, OH, USA), re-plated in 4- or 12-well plates (Nalge Nunc International) and cultured until sub-confluence. The BUECs from the second passage were supplemented with 5 ng/ml progesterone (P4) (Sigma-Aldrich) and 3 pg/ml estradiol 17β (E2) (Sigma-Aldrich) as described previously [23]. The purity of the epithelial cells was evaluated by immunofluorescence staining using a monoclonal antibody against cytokeratin (anti-cytokeratin 8+18; ab53280, Abcam, Tokyo, Japan) as previously described [24]. The purity of the cultured uterine epithelial cells was > 98% (Fig. 2a and b).

In vitro embryo production

The *in vitro* maturation (IVM) and fertilization (IVF) were performed according to a previously described protocol [25] with minor modifications. Briefly, bovine cumulus oocyte complexes (COCs) were used for *in vitro* maturation for 22 h, in a group of 30–40 in 500 µl of high performance-modified 199 medium (HP-M199; Research Institute for the Functional Peptides, Yamagata, Japan) supplemented with 10 µg/ml FSH (Follotropin-V, Bioniche Animal Health, Belleville, Ontario, Canada) and 5% FCS in 4-well plates under mineral oil (Sigma-Aldrich) at 39ºC in 5% CO_2 with humidified air. IVF was performed by co-incubation of 15 *in vitro*-matured COCs and sperm (5×10^6 /ml) in 50 µl droplets of fertilization medium IVF100 (Research Institute for the Functional Peptides) under mineral oil for 6 h at 39°C in 5% CO_2 in humidified air. Subsequently, the presumptive zygotes (n = 15) were transferred to 50 µl droplets of

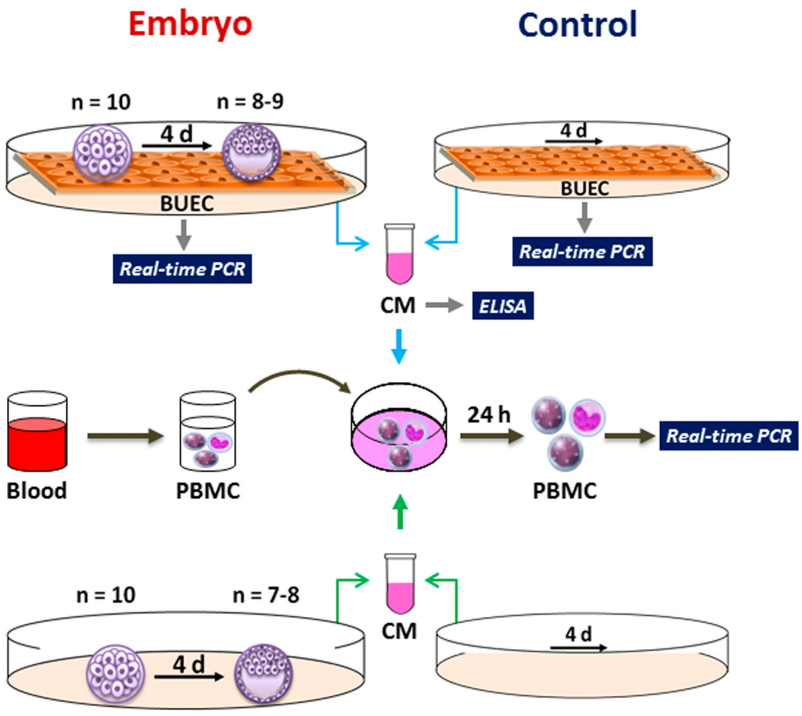

Fig. 1. Schematic representation of the experimental model. A BUEC monolayer was co-cultured with morulae (n = 10) or without (control) for 4 days. At the end of co-culture, morulae (n = 8–9) were developed into blastocysts. Gene expression was analyzed in BUECs. Specific ELISAs for PGE2 and IFNT were used for determination of their concentration in the conditioned media (CM). Then, PBMCs were cultured in CM from embryo-BUEC co-culture or BUEC culture without embryo (control) for 24 h, and the gene expression was evaluated in the PBMCs. Morulae (n = 10) were cultured alone without BUECs for 4 days. At the end of culture, morulae (n = 7–8) had developed into blastocysts. Subsequently, PBMCs were cultured in CM from embryo culture or in CM without embryo (control) and gene expression was analyzed in the PBMCs.

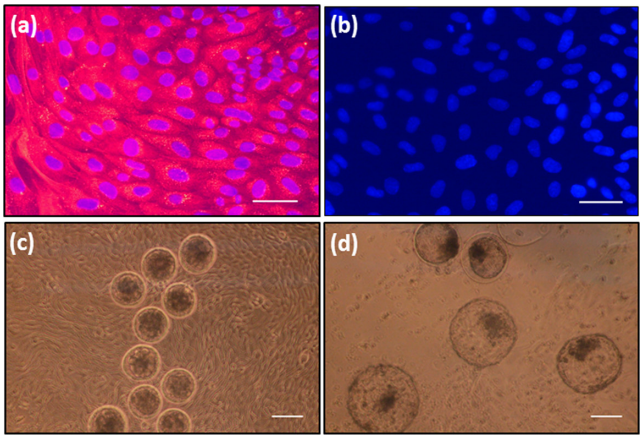

Fig. 2. (a) Immunofluorescence image of cultured BUECs with anti-cytokeratin antibody. Goat anti-rabbit IgG Alexa Flour 546 (red) was the secondary antibody, DAPI (blue) was used to visualize nuclei (b) Immunofluorescence image of cultured BUECs without anti-cytokeratin antibody as negative control. PBS-T (0.1% Tween-20 in PBS–/–) was used instead of primary antibody (c) Morulae on the BUEC monolayer at the start of 4 day co-culture, and (d) Blastocysts on the BUEC monolayer at the end of 4 day co-culture. Scale bar = 100 µm for a, b and 200 µm for c, d.

KSOMaa (Zenith Biotech, Guilford CT, USA) supplemented with 5% FCS under mineral oil at 38.5°C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for *in vitro* culture (IVC). At 96 h of IVC (102 h after IVF), developing morulae were transferred onto a BUEC monolayer for co-culture.

For production of conditioned media (CM) from embryo culture without uterine epithelial cells (Fig. 1), IVC was first performed using 25–30 zygotes in 400 µl KSOMaa medium supplemented with 5% FCS without mineral oil in 4-well plates for 4 days. The developing morulae (n = 10) were then transferred in 400 µl fresh KSOMaa medium supplemented with 5% FCS for a further 4-day culture, at which point the medium was collected (denoted CM from embryo culture).

Embryo-BUEC co-culture

The BUEC culture medium was completely replaced with 400 µl of KSOMaa with 5% FCS 6 h before adding the embryos. The morulae (n = 10 per well), at 102 h after IVF, were transferred on a BUEC monolayer in 4-well plates, and incubated for 4 days at 38.5°C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. Half of the medium was replaced after 48 h. At the end of co-culture, BUECs were lysed with Trizol, and stored at –80°C until RNA extraction. Supernatants were stored at –80°C for further use. The experiment was repeated six times and 80.0 ± 2.6% (48/60) morulae developed

into blastocysts, of which 35.0 ± 2.2% were hatched at the end of the 4-day co-culture (Fig 2c and d).

Isolation of PBMCs

PBMCs were isolated as previously described [17] with minor modifications. Heparinized blood (20 ml) from a multiparous Holstein cow during luteal phase was collected and mixed with an equal volume of PBS–/–, slowly layered over Ficoll-paque solution (Lymphoprep, Axis Shield, Oslo, Norway), and centrifuged at 1000 × *g* for 35 min at 10°C. The buffy coat (white layer containing mononuclear cells) was collected as PBMCs, mixed with hemolysis buffer (155 mM NH_4Cl, 9.9 mM $KHCO_3$, 96.7 µM EDTA) for 10 sec, and centrifuged at 500 × *g* for 10 min at 10°C to remove red blood cells. After centrifugation, the cell pellet was washed twice with PBS–/–. The purity of PBMCs as evaluated by flow cytometry was > 98%, and the viability as assessed by Trypan blue staining was around 99%.

Culture of PBMCs in conditioned media (CM) from embryo-BUEC co-culture

PBMCs (5×10^6 cells) were cultured in a 48-well plate (Nalge Nunc International) in 400 µl CM from embryo-BUEC co-culture or in CM from BUEC culture without embryos (control) for 24 h in a humidified atmosphere at 38.5°C in 5% CO_2. After 24 h of incubation, the supernatant was removed and the cells were collected, lysed using Trizol, and then stored at –80°C until RNA extraction.

Culture of PBMCs in CM from embryo culture

PBMCs (5×10^6 cells) were cultured in a 48-well plate in 400 µl CM from D5-D9 embryo culture or in CM without embryos (control) for 24 h in a humidified atmosphere at 38.5°C in 5% CO_2. After 24 h of incubation, the supernatant was removed and the cells were collected, lysed using Trizol, and then stored at –80°C until RNA extraction.

Treatment of BUECs and PBMCs with recombinant bovine IFNT (rbIFNT)

The sub-confluent BUEC monolayers were washed twice and cultured in medium supplemented with 0.1% FCS in combination with 100 pg/ml of rbIFNT (bIFNT 2B, specific activity = 4.15×10^7 U/mg; Zenoaq, Koriyama, Japan) for 24 h in a humidified atmosphere at 38.5°C in 5% CO_2. BUECs without IFNT treatment served as the control. The dose of 100 pg/ml IFNT was chosen on the basis of preliminary experiments where a similar magnitude of *ISG15* mRNA stimulation was observed in BUECs and PBMCs with this dose as with the embryo.

PBMCs (5×10^6 cells) were also cultured in a 12-well plate in 500 µl RPMI-1640 medium (Sigma-Aldrich) supplemented with 0.1% FCS in combination with 100 pg/ml of IFNT for 24 h in a humidified atmosphere at 38.5°C in 5% CO_2. PBMCs without IFNT treatment served as the control. At the end of culture of BUECs and PBMCs, the supernatant was removed and the cells were collected, lysed using Trizol, and then stored at –80°C until RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was extracted from BUECs and PBMCs using the Trizol reagent as previously described [26]. The RNA extracted from all samples was detected by ultraviolet (UV) spectroscopy (optical density, 260 nm) and the concentration was measured using a spectrophotometer (Eppendorf, Munich, Germany) at 260 and 280 nm absorbance values. The total extracted RNA was stored in RNA storage solution (Ambion, Austin, TX, USA) at –80°C until cDNA conversion. The cDNA synthesis was carried out according to a previously described protocol [27]. The synthesized cDNA was stored at –30°C.

Real-time PCR

Quantitative real-time PCR of target genes (Table 1) was performed using QuantiTect SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany) by an iCycler iQ (Bio-Rad Laboratories, Tokyo, Japan). The amplification program was set up according to a previously described protocol [27]. The calculated cycle threshold (Ct) values were normalized using *B-actin* as the internal standard by applying the Delta-Delta comparative threshold method [28] to quantify the fold change between samples.

ELISA for determination of PGE2 and IFNT concentration in conditioned media

We used a commercially available ELISA kit for determination of PGE2 (R&D systems, Minneapolis, MN, USA) and bovine specific IFNT (Cloud-Clone, Houston, TX, USA) concentration in conditioned media obtained from embryo-BUEC co-culture according to the manufacturers' instructions. The optical density (OD) value was detected using an ELISA microplate reader (Labsystem Multiskan MS 352, Labsystems, Finland) at 450 nm wavelength. Standard curves were prepared for PGE2 and IFNT in the range of 20–2500 pg/ml and 7.8–500 pg/ml, respectively.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed using SPSS software, version 14.0 (SPSS, Chicago, IL, USA). Student's t- test was applied to compare the data between two groups. P values < 0.05 were considered to be statistically significant.

Results

The effect of embryos on gene expression and PGE2 secretion in BUECs

The developing embryos significantly ($P < 0.01$) induced IFN-stimulated genes (*ISGs*; *ISG15*, *OAS1* and *MX2*) and a key factor for IFN-signaling (*STAT1*) with the stimulation of type-1 IFN receptors (*IFNAR1* and *IFNAR2*) in BUECs. In addition, the embryos significantly suppressed *NFkB2*, *NFkBIA* and pro-inflammatory cytokines (*TNFA* and *IL1B*) ($P < 0.01$) in BUECs. There was no significant ($P > 0.05$) change observed in the expression of anti-inflammatory cytokines (*TGFB1* and *IL10*) in BUECs in the presence of embryos. The developing embryos stimulated *PTGES* ($P < 0.01$) in BUECs. In accordance with the gene expression data, the presence of the embryo increased the amount of PGE2 secretion from BUECs by 20-fold when compared to that of the control ($P < 0.01$) (Fig. 3). We could not determine the IFNT concentration in conditioned media from embryo-BUEC co-culture by ELISA.

Table 1. Primers used in real-time PCR

Gene		Sequence of nucleotide (5'→3')	Accession no.
B-actin	Forward	TCACCAACTGGGACGACATG	AY141970.1
	Reverse	CGTTGTAGAAGGTGTGGTGCC	
ISG15	Forward	TCTGAGGGACTCCATGACGG	NM_174366
	Reverse	TTCTGGGCGATGAACTGCTT	
OAS1	Forward	TAGGCCTGGAACATCAGGTC	NM_178108
	Reverse	TTTGGTCTGGCTGGATTACC	
MX2	Forward	CTTCAGAGACGCCTCAGTCG	NM_173941
	Reverse	TGAAGCAGCCAGGAATAGT	
STAT1	Forward	CTCATTAGTTCTGGCACCAGC	AW289395
	Reverse	CACACGAAGGTGATGAACATG	
IFNAR1	Forward	GCGAAGAGTTTCCGCAACAG	NM_174552.2
	Reverse	TCCAAGGCAGGTCCAATGAC	
IFNAR2	Forward	TCGTATGTTGCGCCTGTTCT	NM_174553.2
	Reverse	GTCCGTCGTGTTTACCCACA	
PTGES	Forward	AAAATGTACGTGGTGGCCGT	NM_174443.2
	Reverse	CTTCTTCCGCAGCCTCACTT	
NFKB2	Forward	CCTGCTGAATGCTCTGTCTG	NM_001102101.1
	Reverse	TCCTCCTTCACCTCTGTGCT	
NFKBIA	Forward	AAGTGGTCCGCCAAGTGAAG	NM_001045868.1
	Reverse	CGATTTCTGGCTGGTTAGTGATC	
TNFA	Forward	CAAAAGCATGATCCGGGATG	NM_173966.3
	Reverse	TTCTCGGAGAGCACCTCCTC	
IL1B	Forward	AATCGAAGAAAGGCCCGTCT	NM_174093.1
	Reverse	ATATCCTGGCCACCTCGAAA	
TGFB1	Forward	CTTTCTTCAAATGCAGCATTGG	NM_001166068.1
	Reverse	GGGTCTGGGTGATACAACGAA	
IL10	Forward	GAGATGCGAGCACCCTGTCT	NM_174088.1
	Reverse	GGCTGGTTGGCAAGTGGATA	
IL17	Forward	CACAGCATGTGAGGGTCAAAC	NM_001008412
	Reverse	GGTGGAGCGCTTGTGATAAT	

The effect of CM from embryo-BUEC co-culture on gene expression in PBMCs

The CM from embryo-BUEC co-culture significantly induced *ISGs* and *STAT1* ($P < 0.01$) in PBMCs. CM from embryo-BUEC co-culture also stimulated *TGFB1* ($P < 0.05$), while suppressing *TNFA* and *IL17* ($P < 0.05$) in PBMCs (Fig. 4).

The effect of CM from embryo culture on gene expression in PBMCs

The CM from embryo culture significantly induced *ISGs*, *STAT1*, *IFNAR1* and *IFNAR2* in PBMCs ($P < 0.01$). CM from embryo culture also increased *PTGES* and *TGFB1* ($P < 0.05$), but suppressed *TNFA* and *IL17* ($P < 0.01$) in PBMCs (Fig. 5).

The effect of IFNT on gene expression in BUECs and PBMCs

In BUECs, IFNT at 100 pg/ml induced *ISG15* and *PTGES*, while suppressing *NFkB2*, *TNFA* and *IL1B*, as well as stimulating *TGFB1* ($P < 0.05$) (Fig. 6a). On the other hand, in PBMCs, IFNT at 100 pg/ml stimulated *ISG15*, while down-regulating *TNFA* and up-regulating *TGFB1* and *IL10* ($P < 0.05$) (Fig. 6b).

Discussion

Successful pregnancy requires acceptance of a semi-allogenic embryo/fetus into the uterus by the maternal immune system, which is achieved by a series of complex interactions between the embryo and the maternal tract. In the present study, we used a simplified co-culture model to provide *in vitro* evidence that the uterine epithelial cells can recognize the IFNT signal from the bovine early embryo from morula (D5) to blastocyst (D9) stage, and generate an anti-inflammatory response to the embryo. Moreover, this study shows the first *in vitro* evidence that the early embryo can modulate gene expression of immune cells (PBMCs) towards suppression during this period of development, which might occur very early during pregnancy in cows, for the acceptance of a semi-allogenic embryo into the uterus.

In the present study, the developing embryos induced *ISGs*, *STAT1* and type-1 IFN receptors (*IFNAR1* and *IFNAR2)* in BUECs. Our

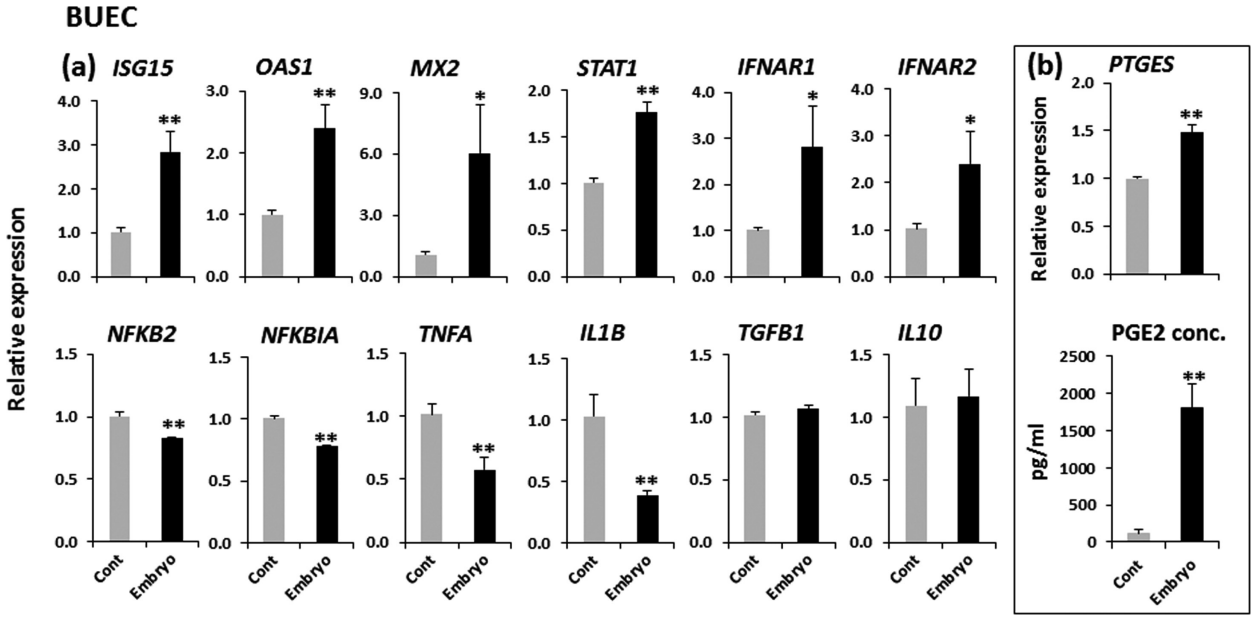

Fig. 3. The effect of developing embryos on gene expression and PGE2 secretion in BUECs. (a) Relative mRNA expression of IFN-stimulated genes (*ISG15*, *OAS1* and *MX2*), a key factor for IFN-signaling (*STAT1*), type-1 IFN receptors (*IFNAR1* and *IFNAR2*), key factors for the inflammatory response (*NFKB2* and *NFKBIA*), pro-inflammatory cytokines (*TNFA* and *IL1B*) and anti-inflammatory cytokines (*TGFB1* and *IL10*), and (b) Relative mRNA expression of an enzyme involved in prostaglandin E synthesis (*PTGES*) and secretion of PGE2 from BUECs after co-culture with the embryo. Data are presented as mean ± SEM of six experiments. Asterisks denote a statistically significant difference: * $P < 0.05$, ** $P < 0.01$, when compared to the control.

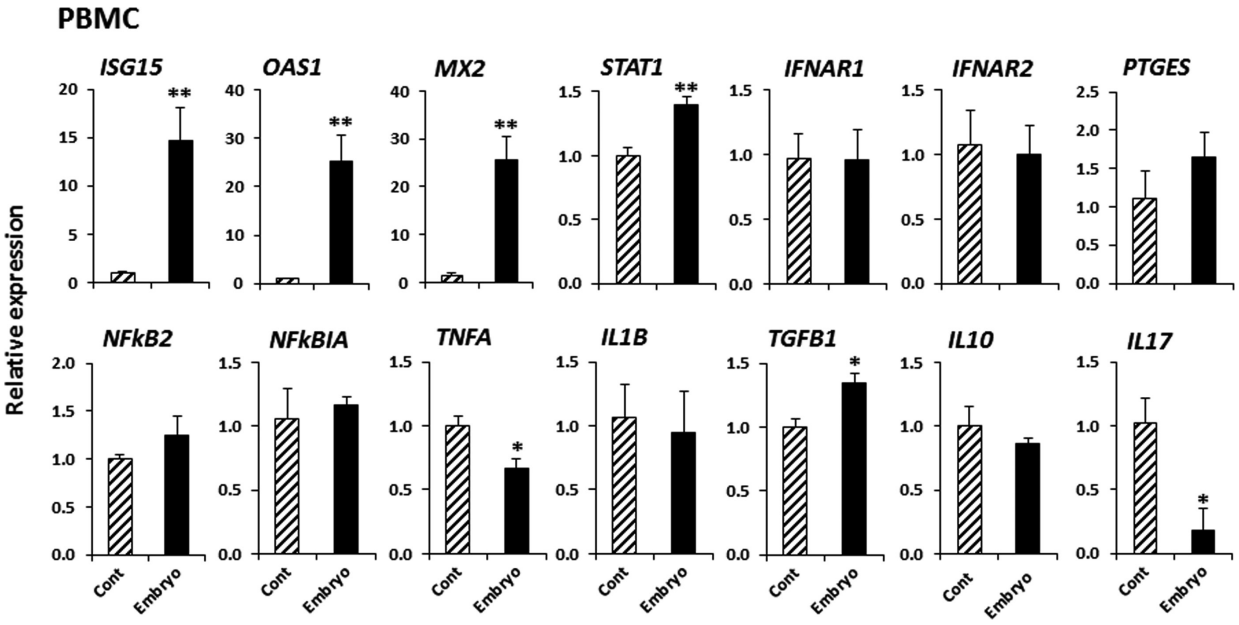

Fig. 4. The effect of conditioned media from embryo-BUEC co-culture on gene expression in PBMCs. CM from BUEC culture without embryos served as the control. Relative mRNA expression of IFN-stimulated genes (*ISG15*, *OAS1* and *MX2*), a key factor for IFN-signaling (*STAT1*), type-1 IFN receptors (*IFNAR1* and *IFNAR2*), an enzyme involved in prostaglandin E synthesis (*PTGES*), key factors for the inflammatory response (*NFKB2* and *NFKBIA*), Th1 cytokines (*TNFA* and *IL1B*), Th2 cytokines (*TGFB1* and *IL10*) and Th17 cytokine (*IL17*). Data are presented as mean ± SEM of six experiments. Asterisks denote a statistically significant difference: * $P < 0.05$, ** $P < 0.01$, when compared to the control.

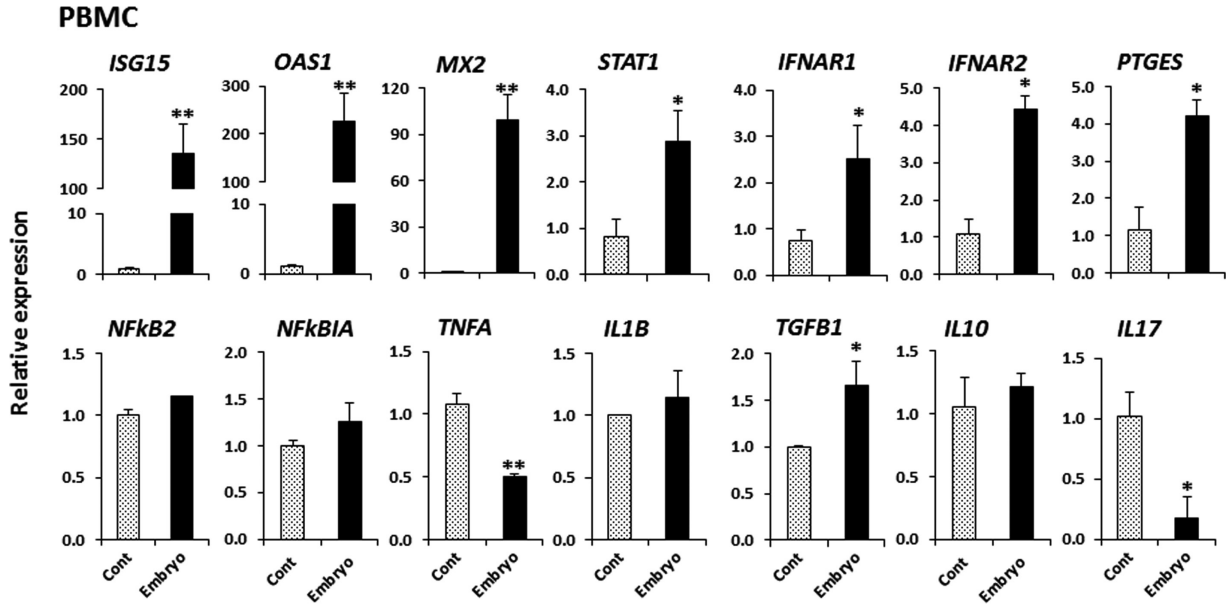

Fig. 5. The effect of conditioned media from D5-D9 embryo culture on gene expression in PBMCs. CM without embryos served as the control. Relative mRNA expression of IFN-stimulated genes (*ISG15*, *OAS1* and *MX2*), a key factor for IFN-signaling (*STAT1*), type-1 IFN receptors (*IFNAR1* and *IFNAR2*), an enzyme involved in prostaglandin E synthesis (*PTGES*), key factors for the inflammatory response (*NFKB2* and *NFKBIA*), Th1 cytokines (*TNFA* and *IL1B*), Th2 cytokines (*TGFB1* and *IL10*) and Th17 cytokine (*IL17*). Data are presented as mean ± SEM of three experiments. Asterisks denote a statistically significant difference: * $P < 0.05$, ** $P < 0.01$, when compared to the control.

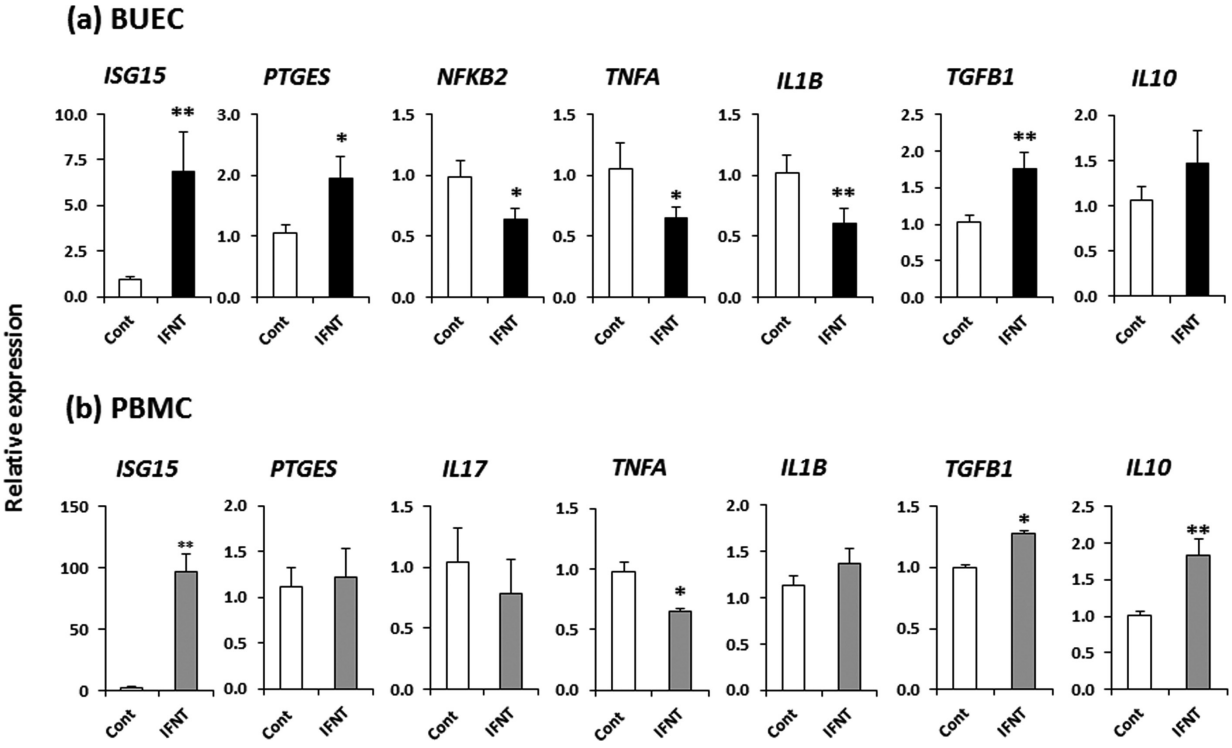

Fig. 6. The effect of IFNT (100 pg/ml) on gene expression in BUECs and PBMCs. (a) Relative mRNA expressions of *ISG15*, *PTGES*, *NFkB2*, pro-inflammatory cytokines (*TNFA* and *IL1B*) and anti-inflammatory cytokines (*TGFB1* and *IL10*) in BUECs, and (b) Relative mRNA expressions of *ISG15*, *PTGES*, Th17 cytokine (*IL17*), Th1 cytokines (*TNFA* and *IL1B*) and Th2 cytokines (*TGFB1* and *IL10*) in PBMCs. Data are presented as mean ± SEM of three experiments. Asterisks denote a statistically significant difference: * $P < 0.05$, ** $P < 0.01$, when compared to the control.

findings suggest that the morula (D5) to blastocyst stage (D9) embryo secretes significant amount of IFNT that can eventually activate interferon-signaling cascades in BUECs. In contrast with our findings with D5-D9 embryos, it has been observed that the embryo induces *ISGs* expression in the bovine endometrium as early as D13 of pregnancy in cattle [7]. Failure to detect *ISGs* expression in the endometrium before elongation of the embryo (D5 or D7 of pregnancy) in cattle could be due to the small-size of the embryo, which might elicit a very local effect on the endometrium, and may not be detected by transcriptome analysis of a large endometrial sample. In the present study, we applied 10 embryos onto the BUEC monolayer, which might have amplified the embryo-derived IFNT signal and thus the *ISGs* expression in BUECs. Our results suggest that "very early" maternal recognition of pregnancy could occur around D8 of pregnancy in cattle. In fact, we could not determine IFNT concentration in the conditioned media from embryo-BUEC co-culture. This may be because we used 5% FCS in the media for embryo-BUEC co-culture, which appears to disrupt the sensitivity and specificity of the ELISA. It should be noted that biological activity of IFNT was detected in embryo-conditioned medium, where *in vivo* derived blastocysts at D8.5-9.5 were cultured for 24–48 h [29], and this strongly supports our finding.

PTGES expression and PGE2 secretion were stimulated in BUECs by early developing embryos. Our result is in agreement with other studies, which have reported up-regulation of *PTGES* expression in the uterus in the presence of a viable embryo on D6-7 of pregnancy in cattle [30, 31]. It has also been reported that PGE2 is secreted from both conceptus and maternal endometrium at the feto-maternal interface throughout the pregnancy, which may be an important immunomodulatory agent to protect the semi-allogenic fetus from maternal immunological attack in cows and ewes [32]. The exact mechanism by which the embryo stimulates PGE2 secretion from BUECs is not known; however, there is evidence that IFNT could stimulate PGE2 secretion in the bovine endometrium [33]. Thus, in the present study, embryo-derived IFNT might contribute to stimulation of PGE2 secretion from BUECs.

The developing embryos suppressed *NFkB2* and *NFkBIA* expression in BUECs, accompanied by decreased expression of pro-inflammatory cytokines (*TNFA* and *IL1B*). A very recent study demonstrated that IFNT plays an anti-inflammatory role in endometritis in mice, through suppression of the NFkB pathway and inhibition of TNFA and IL1B production [34]. Therefore, the reduction in expression of pro-inflammatory cytokines in BUECs is likely to be as a result of down-regulation of the NFkB/IkBa signaling pathway [35, 36], mediated by embryo-derived IFNT.

Likewise in BUECs, the CM from embryo-BUEC co-culture induced *ISGs* and *STAT1* in PBMCs. This finding suggests that IFNT is present in the conditioned media of embryo-BUEC co-culture, although we could not determine the IFNT concentration by ELISA. Successful pregnancy has long been reported to be a Th2 phenomenon [37], while Th1 dominance is observed in pregnancy failure [38]. The Th1/Th2 paradigm has recently extended into a new Th1/Th2/Th17/ regulatory T (Treg) paradigm. In the present study, the presence of the embryo stimulated PGE2 secretion from BUECs, and the CM from embryo-BUEC co-culture and embryo culture alone both significantly increased the expression of *TGFB1* in PBMCs. It is well known that TGFβ synergistically works with PGE2 to induce the differentiation of naïve T cells (Th0) to regulatory T cells for immune suppression and tolerance [39]. On the other hand, TGFβ also enhances the differentiation of Th0 to Th17 cells, which express *IL17*, in the presence of pro-inflammatory cytokines such as IL1 or IL6 [40, 41]. In the present study, the expression of *IL17* and *TNFA* was significantly suppressed in PBMCs by both CM from embryo-BUEC co-culture and embryo culture alone. Therefore, the embryo induced *TGFB1* in PBMCs and PGE2 secretion from BUECs may synergistically play a role in the induction of anti-inflammatory and immune tolerance conditions in the bovine uterus.

In comparison with conditioned media from embryo-BUEC co-culture, the CM from embryo culture alone induced a relatively higher expression of *ISGs*, with stimulation of *IFNAR1* and *IFNAR2* expression in PBMCs. These findings suggest that the CM from embryo culture alone contains a higher amount of IFNT than that of embryo-BUEC co-culture. There is a possibility that a low amount of IFNT is present in CM from embryo-BUEC co-culture, due to binding of IFNT to type-1 IFN receptors of the uterine epithelial cells. Moreover, we cannot exclude the possibility that BUECs might act on the embryo to decrease IFNT secretion in the co-culture model used in the present study, both of which requires further investigation.

In the present study, we stimulated BUECs with different concentrations of IFNT (10, 100 and 1000 pg/ml, data not shown) for 24 h, and found that IFNT at 100 pg/ml induced a similar trend in gene expression (e.g. stimulated *PTGES,* but suppressed *NFkB2*, *TNFA*, and *IL1B*) in BUECs as the D5-D9 embryo. However, unlike the embryo, IFNT also stimulated *TGFB1* in BUECs. In PBMCs, IFNT at 100 pg/ml induced a similar response as the embryo, with *TNFA* suppression and *TGFB1* up-regulation observed. However, IFNT did not suppress *IL17* but stimulated *IL10* in PBMCs, unlike the embryo. All together, these results indicate that the gene expression response to IFNT in BUECs and PBMCs followed a similar trend to those observed with the embryo, suggesting that IFNT may be one of the intermediators from the embryo to BUECs and immune cells. Clearly, further investigation such as neutralization of IFNT or detection of IFNT using a sensitive ELISA, is required to clarify the role of IFNT on gene expression in PBMCs. Importantly, our findings are in agreement with others who demonstrated that early pregnancy favors an anti-inflammatory (Th2) response in the bovine endometrium on D13-16 of pregnancy [9], while the embryo is also known to induce expression of *ISGs* in the endometrium in pregnant cows [7]. In addition, other factors derived from the embryo may also play important roles in the immunological interaction observed in this study. It was proposed in human and mice that the early pre-implantation embryo produces certain embryo- and species-specific soluble factor(s) which are recognized by resident immune cells in the FRT, and cause the maternal immune system to undergo functional changes during the very early stages of pregnancy [42, 43].

Taken together, our findings support the hypothesis that the developing embryo, in the first four days in the uterus (D5-D9), starts to secrete IFNT and induces an anti-inflammatory response in epithelial cells, with activation of *ISGs*. In addition, during this period the early embryo regulates the gene expression of immune cells towards anti-inflammatory action. A small amount of IFNT from the very early stage embryo is likely to be involved in modulation of

this "local" immune response in the bovine uterus. Further study is needed to understand the molecular mechanisms involved in crosstalk between the embryo and the immune cells in the uterus during very early stages of pregnancy in cattle.

Note added in proof

After submission of this article, a paper was published by the Mario Binelli group (Sponchiado *et al*., 2017; *PLoS One* 12 (4): e0175954) describing that the IFN-signaling was detectable only in the utero-tubal junction (UTJ) and anterior region of uterine horn ipsilateral to the CL in cattle, where a D7 embryo was found. This *in vivo* investigation strongly supports our hypothesis that early developing embryo, in the first four days in the uterus (D5-D9), communicates "locally" with the uterine epithelial cells in cattle.

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