The local immune response to early embryo in bovine oviduct and uterus *in vitro*

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ウシ卵管と子宮の体外培養系における

初期胚に対する局所免疫応答

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Dedication

My mother, my wife Ananya & my son Ariyaan

In the memory of my dear father…

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Abbreviations

BOECs	Bovine oviduct epithelial cells
BSA	Bovine serum albumin
BUECs	Bovine uterine epithelial cells
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CL	Corpus luteum
CM	Conditioned media
COCs	Cumulus oocyte complexes
DCs	Dendritic cells
DMEM/F12	Dulbeccos's Modified Eagle Medium: Nutrient Mixture F-12
E2	Estrogen
ELISA	Enzyme-linked immunosorbant assay
FCS	Fetal calf serum
Foxp3	Forehead box p3
FRT	Female reproductive tract
FSH	Follicle stimulating hormone
HBSS	Hank's balanced salt solution
HP-M199	High performance-modified 199
IDO1	Indolamine 2,3-dioxygenase 1
IFN	Interferon
IFNAR	Interferon alpha receptor
IFNT	Interferon tau
IL	Interleukin
IL1B	Interleukin 1B
ISG15	Interferon-stimulated gene-15
ISGs	Interferon-stimulated genes
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
KSOMaa	Potassium simplex optimization medium amino acid
MHC	Major hiscompatibility complex
Mo	Monocyte
MX2	Myxovirus resistance 2
МФ	Macrophage

NFkB	Nuclear factor kappa B
NFkBIA	Nuclear factor kappa B inhibitor alpha
OAS1	2′-5′ oligoadenylatesynthetase 1
OCM	Oocyte collection medium
P4	Progesterone
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F-2alpha
PMNs	Polymorphonuclear neutrophils
PTGES	Prostaglandin E synthase
Real-time PCR	Real-time polymerase chain reaction
RNA	Ribonucleic acid
SIRPA	Signal regulatory protein alpha
STAT1	Signal transducer and activator of transcription 1
TGFB	Tissue growth factor beta
Th	helper T cells
TLRs	Toll-like receptors
TNFA	Tumor necrosis factor apha
Treg	T regulatory cells

General Introduction

The ultimate goal of a dairy farm is optimal milk production, which heavily relies on the reproductive performance of the cows. Unfortunately, there are several factors responsible for reproductive failure in cows. The immune system plays an important role in reproduction of the dairy cows. There are significant interactions between the immune system and the female reproductive tract (FRT) that are essential for establishment and maintenance of a successful pregnancy, as well as protection of FRT from microbial infection. Any dysregulation of immune response in the female reproductive system may lead to reproductive failure that negatively affects the economy of a dairy farm.

1. Female reproductive tract in the cow

The major structures of the female reproductive tract of a cow include the ovaries, oviducts, uterus, cervix, vagina and external genitalia- vulva. In cattle, the FRT is anatomically positioned below the rectum (Fig 1-1). The main functions of the FRT include production and transportation of gametes, fertilization, development of the embryo/ fetus, and delivery of the newborn at term. Besides, the FRT possesses an active immune system to protect itself from microbial challenge.

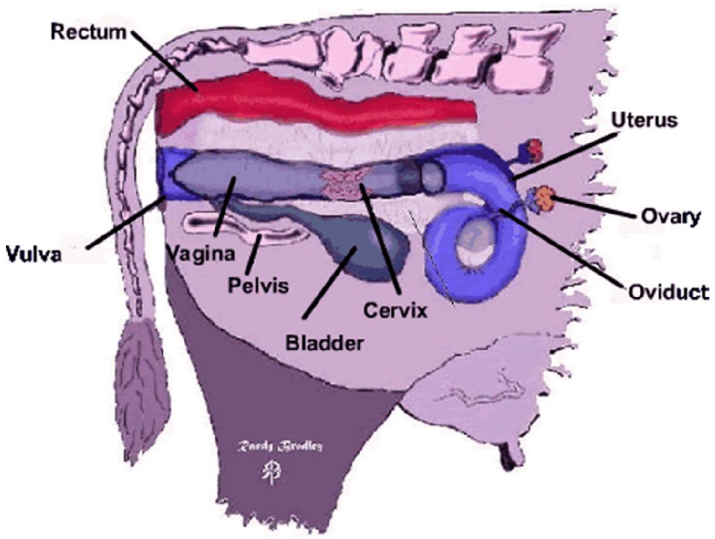

Fig. 1-1. Illustration of the female reproductive tract of a cow (taken from website: http://animalsciences.missouri.edu/reprod/Notes/female/anatomy.htm)

2. Immune system in the FRT

The upper reproductive tract (oviduct and uterus) is lined with a simple columnar epithelium which consists of ciliated and secretary cells. The ciliated cells help in transport of the gametes and embryo, and secretary cells secrete several proteins and growth factors, which contribute to the formation of oviductal and uterine fluid that play essential roles in sperm capacitation, fertilization, and early embryo development. Furthermore, epithelium of the FRT acts as a physical barrier and regulates the local immunity through release of several molecules containing antimicrobial peptides, cytokines, and chemokines etc., which influence the functions (recruit and activate or suppress) of underlying resident immune cells.

The FRT possesses a unique and well-equipped mucosal immune system to meet the sexually transmitted pathogens, allogenic sperm and immunologically distinct embryo/fetus [1]. Unlike mucosal immunity in other systems, mucosa of FRT undergoes through a hormonal changes during the different phases of estrous cycle in cows. It is known that the sex steroid hormones such as estrogen (E2) and progesterone (P4) are involved in regulation of local immune system in the FRT [2]. The epithelial cells and immune cells in the FRT provide immunological protection in part through the Toll-like receptors (TLRs).

2.1 Pathogen

The FRT of cows is frequently exposed to pathogens *via* vagina and uterus during insemination, parturition, and postpartum period under physiological *milieu*. Moreover, microorganisms can invade mucosal surface of the reproductive tract *via* follicular fluid and peritoneal cavity through opening of the infundibulum of the oviduct (Fig. 1-2). The FRT can also be contaminated during pathological conditions. Previous work from our laboratory and others showed that bovine oviduct epithelial cells induce a pro-inflammatory response (*TNFA*, *IL1B* and *NFkB1*) to bacterial lipopolysaccharides (LPS) *via* TLR4/TLR2 [3, 4]. Similarly, it has been demonstrated that the bovine uterine epithelial cells play important roles in innate immunity and generate an inflammatory response to bacterial lipopetides *via* TLR2, TLR1 and TLR6 [5].

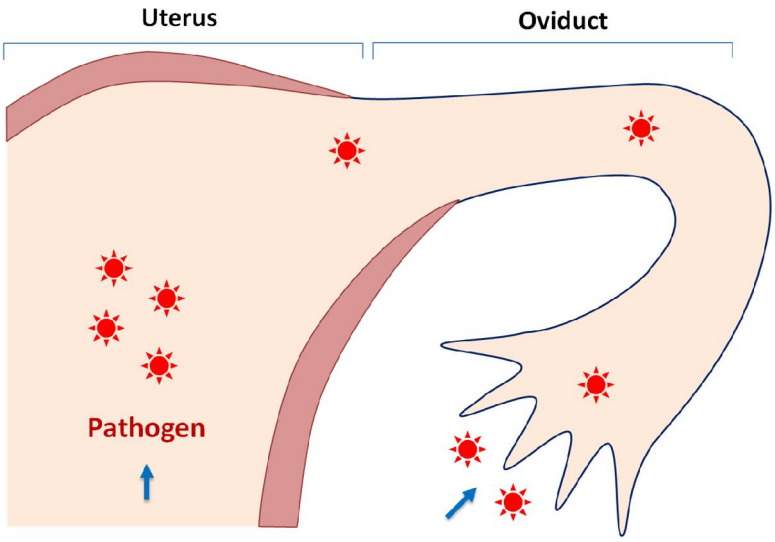

Fig. 1-2. Illustration showing the *pathogens* in the female reproductive tract. Arrow (↑) indicate possible entrances of microbial infection to the FRT.

2.2 Spermatozoa

During insemination, spermatozoa enter in the uterus (Fig.1-3), these are allogenic cells to the FRT since they are paternal in origin. It is reported that the deposition of semen in the FRT at estrus elicits an inflammatory response characterized by migration of neutrophils into the uterine lumen [6, 7]. These migrated neutrophils phagocyte a large number of spermatozoa in the uterus to remove the non-motile and dead spermatozoa, and clear the uterine environment for accepting the embryo. It has been demonstrated that the number of migrated neutrophils in the uteine lumen during estrus decreased 5-days later of insemination, suggesting that the embryo is not normally exposed to a leukocytic environment in the bovine uterus [8].

After insemination, only good quality spermatozoa enter into the oviduct to take part in the fertilization process. The polymorphonuclear neutrophils (PMNs) are found in the oviduct lumen during pre-ovulatory period in ewes and cows [9, 10]. Therefore, spermatozoa need to protect themselves from phagocytosis by PMNs to ensure fertilization in the oviduct. Our laboratory recently showed that physiological concentration of PGE2 (10^{-8}M) in the oviduct suppresses the sperm phagocytosis by PMNs *in vitro* [10]. Moreover, the binding of spermatozoa to oviduct epithelial cells

induces an anti-inflammatory response (*TGFB1*, *IL10* and *PTGES*), which may create a favorable environment for survival of spermatozoa in the bovine oviduct [11].

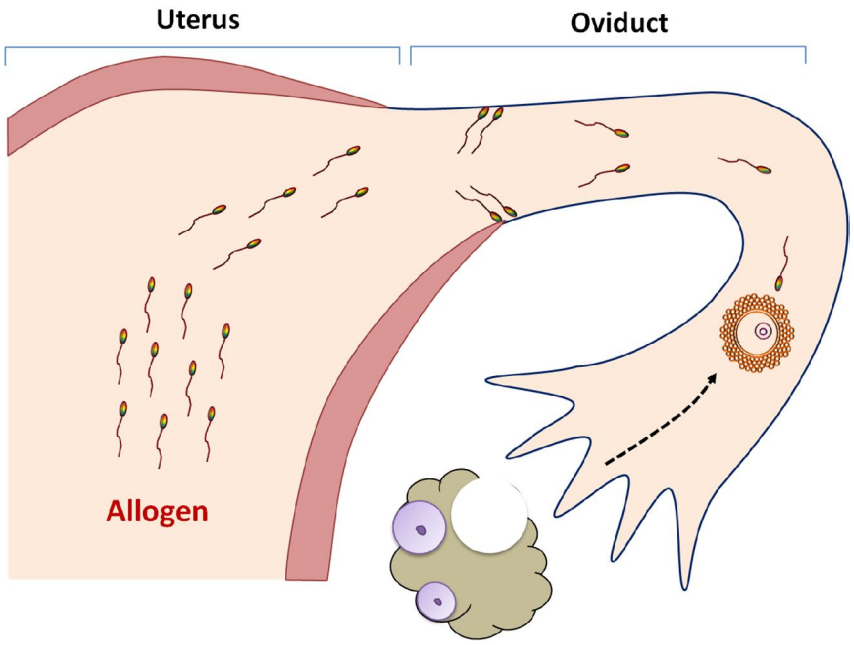

Fig. 1-3. Illustration showing the *allogenic* sperm in the female reproductive tract.

2.3 Embryo

After fertilization, embryo develops in the oviduct for first 3-4 days and moves into the uterus, where it establishes pregnancy (Fig. 1-4). The embryo expresses paternal antigen [12-14], thus, it could be considered as semi-allogen to the FRT. It is estimated that the 70-80% of total embryonic loss occur during the period from insemination to D16 of pregnancy in cattle [15]. Thus, it is possible that semi-allogenic embryo may suffer from maternal immune attack, which could be one of the main reasons for early embryo loss [16]. The immunologic interaction between a semi-allogenic embryo and the oviduct (D0-D4) is not yet properly studied in cattle. Moreover, little is known about the uterine immune response to the early embryo, particularly during the period from arrival of morula (D5) into the uterus until hatching of blastocysts (D9) in cattle.

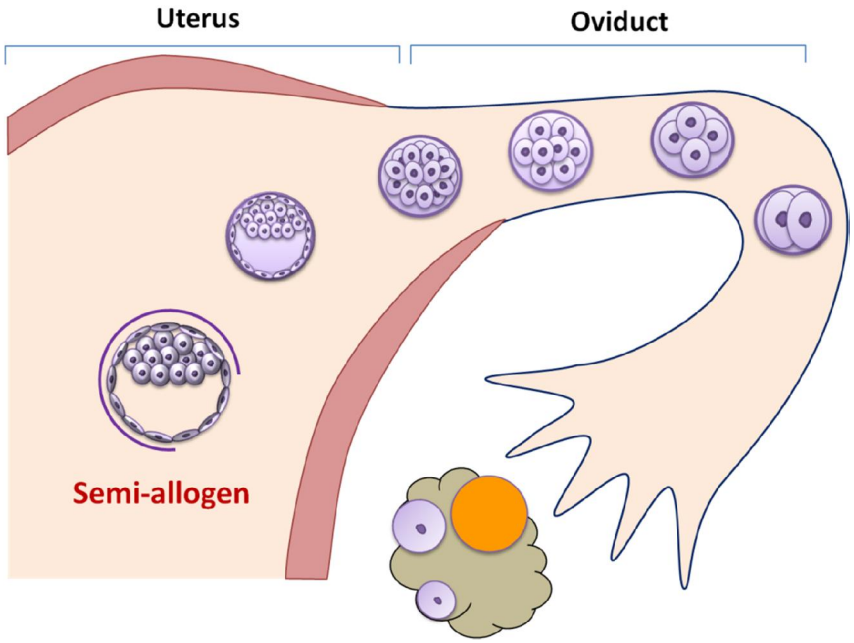

Fig. 1-4. Illustration showing the developing *semi-allogenic* embryo in the female reproductive tract.

3. Immune cells in the bovine FRT

The PMNs, innate immune cells, are found in the oviductal fluid of ewes and cows during physiological condition [9-10]. However, their functions in the oviduct lumen are still unknown. In the uterus, it is generally accepted that PMNs are the predominant phagocytic cells during follicular phase of the estrous cycle. At times other than estrus, the presence of PMNs in the uterine lumen is considered pathological [17].

Macrophage (MΦ)/ dendritic cells (DCs) and lymphocytes, adaptive immune cells, are found in the epithelium and stromal layer in the bovine oviduct [18, 19]. In the uterus, MΦ/ DCs are found only in the stromal region of bovine endometrium (Fig. 1-5). The CD8+ lymphocytes (cytotoxic T cell, Tc) are located exclusively in the luminal and glandular epithelium, as well as in the stroma immediately adjacent to the epithelium, while CD4+ lymphocytes (helper T cell, Th) are limited to stromal tissue [20]. B lymphocytes are rare in uninfected animals.

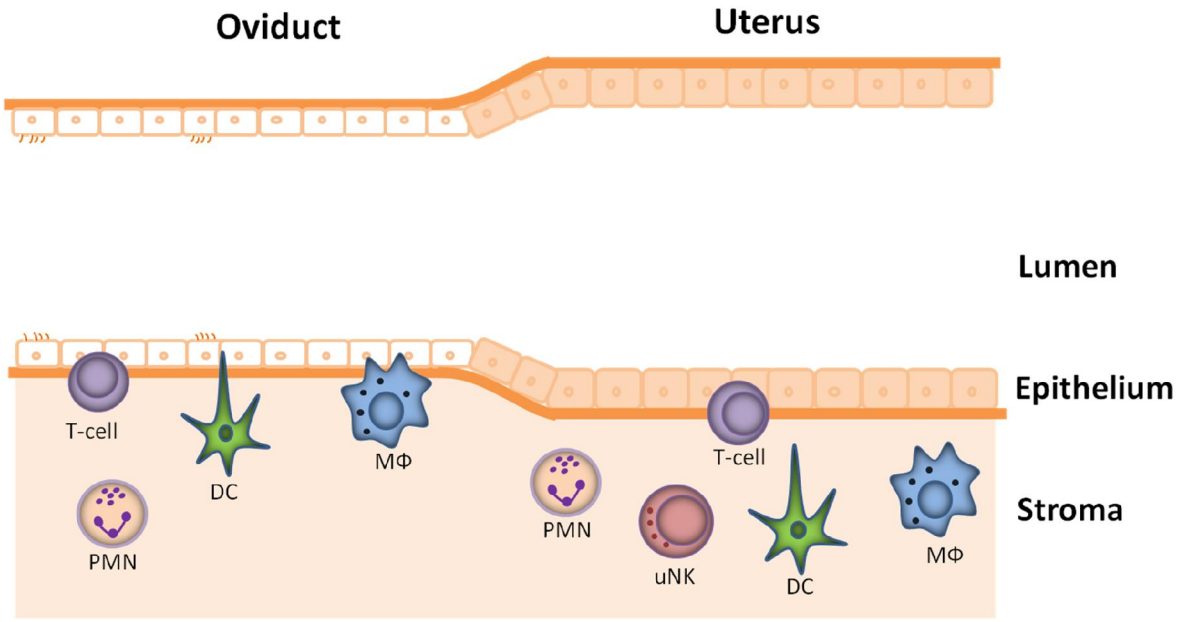

Fig. 1-5. Illustration showing the different types of immune cells in the FRT. T-cell = T-lymphocyte, DC = Dendritic cell, MΦ = macrophage, uNK = uterine natural killer cell, PMN = polymorphonuclear neutrophil; PMNs migrate into the lumen of the FRT during pre-ovulatory/estrus period in cows.

4. Local response of maternal immune cells during early pregnancy in the bovine

There are relatively few information available about the interaction between maternal immune cells and embryo particularly during the period of early pregnancy in cattle with compared to human and mice. Till date, there is no information on how the resident immune cells respond to a semi-allogenic embryo in the bovine oviduct. In the uterus, some recent studies investigated the interaction between early embryo and maternal immune cells during early pregnancy in cattle; however, results are inconsistent. It was reported that pregnancy had no effect on accumulation of CD4+ (T lymphocytes), CD14+ (macrophages) and CD21+ (B lymphocytes) cells in the endometrium on D16 of pregnancy in cattle [21]. In contrast, another study observed an initial expansion of monocyte (Mo), CD14+-cells (MΦ), and CD172a–CD11c+ n (DC) populations in the endometrial stroma on D13 of pregnancy in cattle [22], and suggested that these cells might play a role for pregnancy establishment. However, the same group found that there was no effect of pregnancy status on

lymphocyte populations (CD4+, CD8+, gamma delta T and FoxP3+) in the bovine endometrium from D5 to D16 of pregnancy; nevertheless, the endometrial gene expression revealed that Th1 cytokine (*IL1B*) was down-regulated, while Th2 cytokine (*IL10*) was up-regulated on D13-16 of pregnancy [23]. In a most recent study, a greater number of myeloid lineage cells (macrophages and DCs) with a tolerogenic phenotype (expressing *IDO1*, *CD163*, *SIRPA*) were observed in the endometrium on D17 of pregnancy in dairy heifers, which may play an important role for tolerance of a semi-allogenic embryo [24]. A decreased number of CD45+ (common marker for leukocytes) cells with increased *IDO* expression in the endometrium were also reported for protecting the semi-allogenic conceptus from maternal rejection on D18 of pregnancy in the bovine [25].

5. Response of peripheral immune cells during early pregnancy in the bovine

It is still mysterious that how the developing embryo from the reproductive tract influences the peripheral (systemic) immune response in the bovine. Interferon tau (IFNT) is an embryo-derived pregnancy recognition signal in ruminants, which is essential for maintenance of pregnancy by preventing luteal regression. It is also considered as an immune suppressive molecule that inhibits proliferation of lymphocytes *in vitro* [26] and, thus, may play an essential role for tolerance of embryo by maternal immune system. IFNT has been known to induce *ISGs* not only in the uterus [27], but also in the extra-uterine tissues such as corpus luteum [28] and peripheral blood leukocytes [29]. The activation of *ISGs* expression in peripheral blood immune cells during early pregnancy in cattle [29] beg a question that if this molecule is involved in peripheral immune tolerance for semi-allogenic embryo/fetus. It was observed that the *ISG15* and *OAS1* were stimulated in PBMCs on D8 of pregnancy in cows with up-regulation of *IL10*, a Th2 cytokine [29], suggesting that the peripheral blood leukocytes may recognize the presence of very early stage embryo and thus generate a Th2 immune response. A recent investigation observed an increase in the proportion of putative DC precursors (CD14+CD11+ cells) in the peripheral circulation of D17 pregnant heifers, although the significance of this finding remains unknown [24].

6. Objectives of the study

The molecular mechanism by which a semi-allogenic embryo escapes from attack by the maternal immune system remains largely unknown, particularly while the embryo stays in the oviduct (D0-D4) and during the period from arrival of morula into the uterus until the hatching of blastocyst (D5-D9). It is reported that a shift from Th1 (pro-inflammatory) to Th2 (anti-inflammatory) response is essential for successful pregnancy in human and mice [30, 31]; however, a situation remains unclear in cattle. The immunosuppressive and anti-proliferative activity of bovine IFNT has been demonstrated *in vitro*. *IFNT* is expressed in the bovine embryos as early as in the 8-16 cell stage and its protein can be detected in the trophoectoderm of non-hatched blastocyst on D7 [32-34]. Thus, the present study hypothesized that the early developing embryo may induce an anti-inflammatory response in the bovine oviduct and uterus, and a small amount of IFNT from very early stage of embryo could be involved, at least in part, for modulation of such local immune response in the bovine oviduct and uterus.

The general objective of the present study was to investigate the effect of early developing embryo on the immune-related gene expression of bovine oviduct epithelial cells (BOECs) and uterine epithelial cells (BUECs), and to examine the subsequent impact of *embryo-BOEC* and *embryo-BUEC* co-culture or embryo culture alone *via* conditioned media (CM) on gene expression of peripheral blood mononuclear cells (PBMCs) using an *in vitro* model (Fig. 1-6).

The specific objectives of the current study were:

In the oviduct:

i. To investigate the effect of early developing embryo (D0-D4) on gene expression of bovine oviduct epithelial cells (BOECs),
ii. To examine the impact of conditioned media from embryo-BOEC co-culture on gene expression of peripheral blood mononuclear cells (PBMCs), and
iii. To observe the impact of conditioned media from embryo culture alone on gene expression of PBMCs *in vitro*.

In the uterus:

i. To investigate the effect of early developing embryo (D5-D9) on gene expression of bovine uterine epithelial cells (BUECs),

ii. To examine the impact of conditioned media from embryo-BUEC co-culture on gene expression of peripheral blood mononuclear cells (PBMCs), and

iii. To observe the impact of conditioned media from embryo culture alone on gene expression of PBMCs *in vitro*.

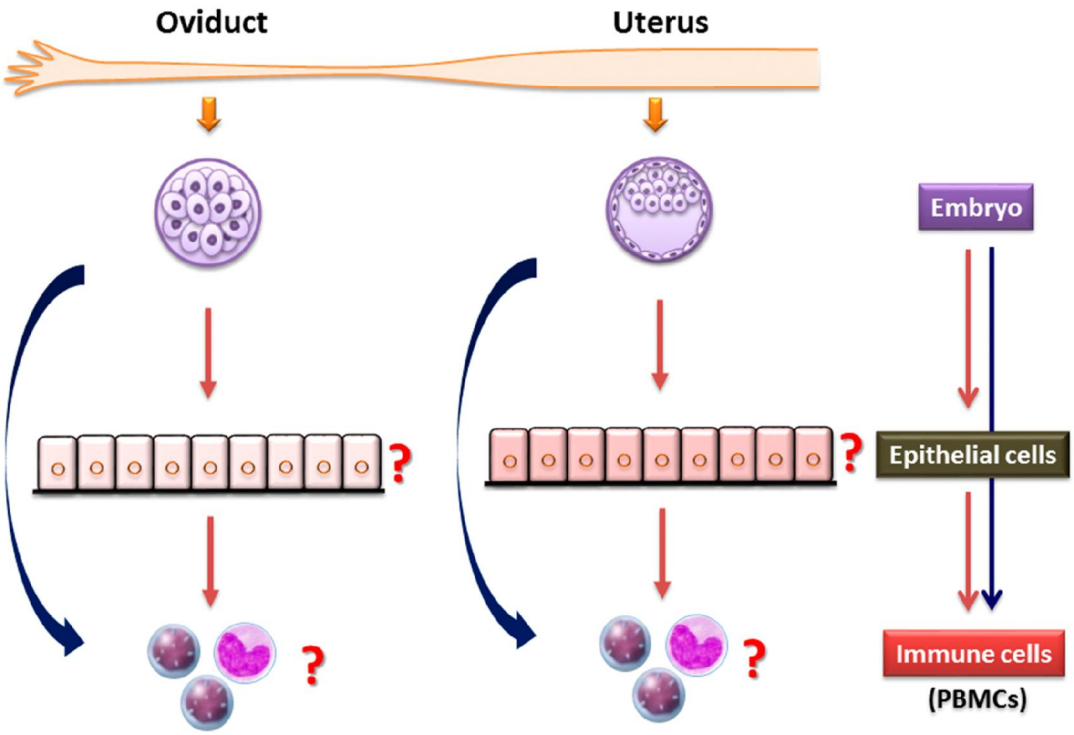

Fig. 1-6. Illustration showing the communication of the early embryo with the epithelial cells and immune cells in the bovine oviduct and uterus.

Chapter I

Bovine embryo and oviduct epithelial cells interact to induce anti-inflammatory response in immune cells *in vitro*

1. Introduction

The oviduct is a key organ responsible for final maturation of oocyte, transport of gametes, sperm capacitation, fertilization and early embryo development [35, 36]. The mucosal surface of the oviduct is often exposed to pathogen and endotoxin *via* uterus, peritoneal cavity and follicular fluid. Moreover, oviduct mucosa comes in contact with allogenic sperm and semi-allogenic embryo after insemination in the cow. Thus, the bovine oviduct should be equipped with an efficient and strictly-controlled immune system for accepting the allogenic sperm and semi-allogenic embryo, while providing protection against pathogen. Recently, we have shown that the oviduct possesses a unique immune system that generates a pro-inflammatory response (*NFkB*, *TNFA* and *IL1B*) against bacterial LPS [3, 37], while it induces an anti-inflammatory response (*PTGES*, *TGFB1* and *IL10*) to allogenic sperm *in vitro* [11].

After fertilization, newly formed embryo spends in the bovine oviduct for about 4 days and enters into the uterus approximately at the 16-cell to early morula stage [38]. Early cleavage-stage bovine embryo expresses MHC I transcript [14] and thus it could be considered as a foreign to the oviduct. Therefore, it is possible that the immunological crosstalk between the embryo and mother may start in the oviduct. It was observed in pigs that the presence of embryos in the oviduct was able to down-regulate immune gene expression in the uterus [39]. This study suggests the possibility that the embryo exerts a local effect in the oviduct that may extend to the uterus for tolerance of embryo. In a recent study, it has been demonstrated that endoscopic transfer of 50 zygotes induced changes in oviduct transcriptome between pregnant and cyclic heifers on D3 (D0 = estrus) including down-regulation of genes related to immune system, while single embryo did not induce changes in gene expressions, suggesting that a very local effect of embryo exists in the oviduct [40].

Interferon tau (IFNT) is an embryo-derived signal responsible for maternal recognition of pregnancy in ruminants [41, 42]. It is also considered as an immunosuppressive molecule that inhibits proliferation of lymphocytes and thus may play role for protection of semi-allogenic embryo/ fetus

from maternal immune attack [26]. IFNT has been shown to induce the expression of interferon-stimulated genes (*ISGs*: *ISG15*, *OAS1* and *MX2*) in the uterus [27] and corpus luteum [28] in cows. Moreover, *ISGs* were found activated in peripheral blood leukocytes during early pregnancy in cows [29, 43], suggesting that the local immune cells in the oviduct may also respond to IFNT. To the best of my knowledge, there is no information on the involvement of IFNT in embryo-maternal communication in the bovine oviduct. However, it is demonstrated that *IFNT* mRNA is expressed in the 8-16 cell *in vitro* produced bovine embryos [32, 34]. Therefore, it is possible that a small amount of IFNT from very early stage embryo could be involved in modulation of local immune response in the bovine oviduct.

The molecular mechanism by which a semi-allogenic embryo escapes from maternal immune attack in the oviduct remains largely unknown in cattle. The present study was, therefore, aimed to investigate the effect of early developing embryo on the immune-related gene expression in bovine oviduct epithelial cells (BOECs) using an *in vitro* model, and also to examine the impact of embryo-BOEC interaction or embryo alone *via* conditioned media (CM) on gene expression in peripheral blood mononuclear cells (PBMCs).

2. Materials and methods

2.1 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).

2.2 Experimental model

The present study used an *in vitro* model (Fig. 2-1) to investigate the communication of the developing embryo with oviduct epithelial cells and immune cells (PBMCs). First, BOECs were co-

cultured with IVF-derived zygotes (n=25-30) for D0-D4 (D0=IVF) that mimics the embryo development in the bovine oviduct *in vivo*. BOECs were cultured alone without embryo as control. The gene expression in BOECs and embryo was analyzed. Subsequently, PBMCs were cultured in conditioned media (CM) from embryo-BOEC co-culture or BOEC culture alone (control) and gene expression was evaluated. Next, zygotes were cultured alone without oviduct epithelial cells for 4 days, at which point CM were collected. At the same time, fresh media were incubated without embryos for 4 days. Further, PBMCs were cultured in conditioned media (CM) from embryo culture or in CM without embryo (control) for analysis of gene expression.

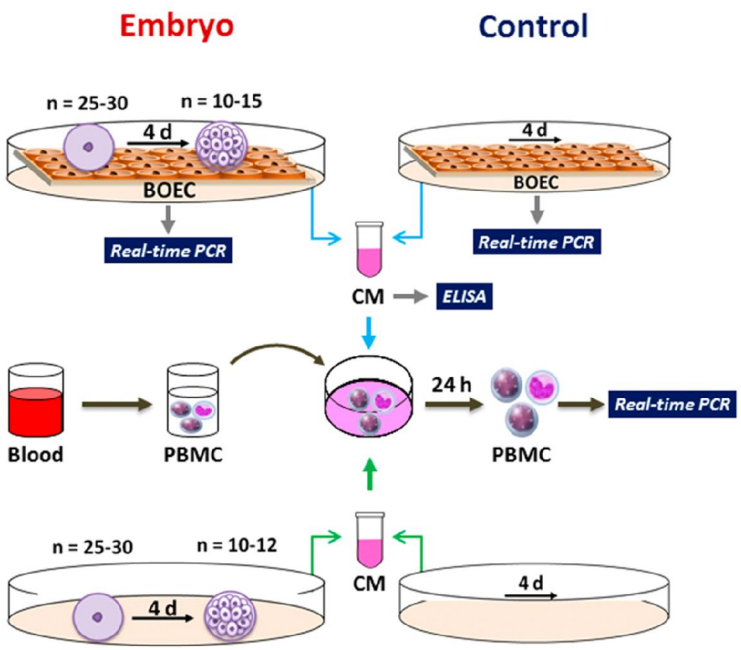

Fig. 2-1. Schematic representation of main experimental model. A BOCE monolayer was co-cultured with zygotes (n=25-30) or without (control) for D0-D4. At the end of co-culture, zygotes (n=10-15) developed into 16-cell to early morulae. The gene expression in BOECs and embryos was analyzed. PBMCs were cultured in conditioned media (CM) from embryo-BOEC co-culture or BOEC culture (control) for 24 h, and gene expression was evaluated. Next, zygotes (n=25-30) were cultured without epithelial cells for 4 days, when zygotes (n=10-12) developed into morulae. At the same time, fresh media without embryo was incubated for 4 days. PBMCs were cultured in CM from embryo culture or in CM without embryo (control) for 24 h, and gene expression was analyzed.

2.3 Culture of BOECs

Female reproductive tracts of cows were collected from a local slaughterhouse (Hokkaido Livestock Co., Doto Plant Tokachi Factory; Obihiro, Hokkaido, Japan). The phase of the estrous cycle was identified as previously reported [44], based on the appearance, weight, and colour of the corpus luteum, and the diameter of follicle. Only healthy oviducts ipsi-lateral to corpus luteum were selected. Oviducts were ligated from both ends, immersed in phosphate buffer saline without calcium or magnesium (PBS-/-) supplemented with 1% penicillin-streptomycin (Gibco, Grand Island, USA) and 1% amphotericin B (Gibco), and then transported to the laboratory. In the laboratory, oviducts were separated from the surrounding connective tissue, rapidly rinsed in 70% ethanol for disinfection, and rinsed three times with PBS-/-. Epithelial cells were isolated and cultured as previously described [45] with minor modifications. Briefly, epithelial cells from 3-4 oviducts were mechanically dislodged by gentle squeezing in a stripping motion with forceps. The cells were pooled and settled at the bottom of a tube, and washed with PBS-/- followed by separation medium consisting of DMEM/F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12; Gibco), 2.2% $NaHCO_3$ (Sigma-Aldrich, Steinheim, Germany), 1% penicillin-streptomycin, and 1% amphotericin B. Thereafter, the cells were obtained by centrifugation at 300 x*g* for 10 min at 10 °C. The resultant cell pellet was suspended in 10 ml separation medium, layered over 10 ml Percoll (Sigma-Aldrich), and centrifuged at 900 x*g* for 20 min at 4 °C. The epithelial cells were collected from the inter-phase between two layers and washed once with the separation medium, obtained by centrifugation at 300 x*g* for 10 min at 4 °C, and cultured in culture medium (DMEM/F12 supplemented with 2.2% $NaHCO_3$, 1% penicillin-streptomycin, 1% amphotericin B, and 10% fetal calf serum (FCS; Bio Whittaker, Walkersville, MD) in 4-well culture plate (Nalge Nunc International, Roskilde, Denmark) at 38.5 °C in 5% CO_2 and 95% air. The medium was renewed every 48 h until the growing BOECs reached to 80-90% confluence, at which point the cells were used for co-culture with the embryos. The cells in culture medium showed characteristic epithelial morphology (Fig. 2-2) that was confirmed by immunofluorescence staining of cytokeratin, a specific marker for epithelial cells.

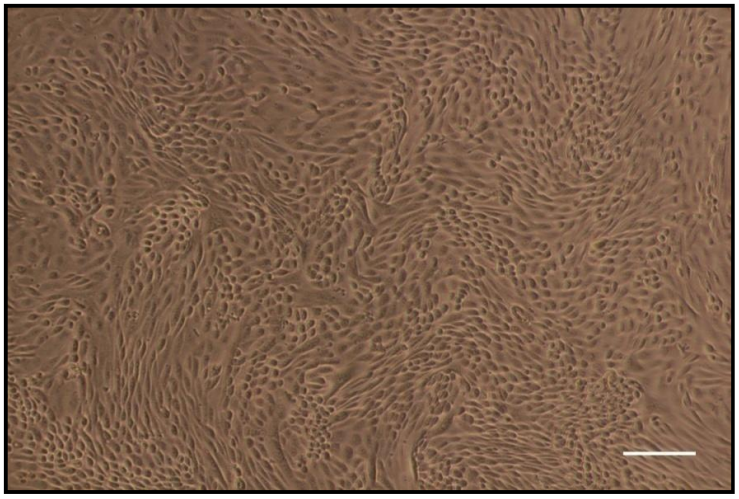

Fig. 2-2. The confluent BOEC monolayer used for co-culture with the embryos. Scale bar = 200 µm.

2.4 In vitro embryo production

2.4.1 Oocyte collection and in vitro maturation (IVM)

Bovine ovaries were collected from a local slaughterhouse (Hokkaido Livestock Co., Doto Plant Tokachi Factory; Obihiro, Hokkaido, Japan) and transported to the laboratory in physiological saline supplemented with 1% penicillin-streptomycin at 39 °C within 1–2 h of slaughter.

In the laboratory, follicular fluid was aspirated from individual visible antral follicles of 2–6 mm in diameter using 10 ml syringe attached to 18 G needle. The retrieved follicular fluid was transferred to 60 mm petridish (Thermo Fisher Scientific, Roskilde, Denmark) and examined for cumulus oocyte complexes (COCs) using a stereo-microscope (Leica M Stereomicroscope, Switzerland). Then, COCs were washed three times in oocyte collection medium (OCM; Research Institute for the Functional Peptides Co., Ltd., Yamagata, Japan) supplemented with 5% FCS in 35 mm petridish (Corning Inc., NY, USA). Only COCs with a homogenous cytoplasm and surrounded by at least three layers of compact cumulus cells 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 Co., Ltd.) supplemented with 10 µg/ml FSH (Folltropin-V, Bioniche Animal Health Inc., Belleville, Ontario, Canada) and 5% FCS in 4-well plates under mineral oil (Sigma-Aldrich) at 39 °C in 5% CO_2 with humidified air.

2.4.2 Sperm preparation and in vitro fertilization (IVF)

Two semen straws (0.5 ml) from the same bull (AG black 29) were thawed at 37 °C for 30 sec. The thawed semen was washed twice in fertilization medium IVF100 (Research Institute for the Functional Peptides Co., Ltd.) by centrifugation at 1200 rpm for 6 min at room temperature. Sperm pellet was diluted with IVF100 to prepare a final concentration of 5×10^6 /ml in the fertilization droplet.

In vitro fertilization was performed according to a previously described protocol [46] with minor modifications. Briefly, 50 µl droplets of fertilization medium IVF100 were prepared in 60 mm petridish under mineral oil. After maturation, COCs were washed twice in fertilization medium IVF100. Finally, 15 COCs and sperm (5×10^6 /ml) were transferred to a fertilization droplet for co-incubation for 6 h at 39 °C in 5% CO_2 in humidified air.

2.4.3 In vitro culture (IVC)

After 6 h of sperm-COCs co-incubation, presumptive zygotes (n=15) were denuded by repeated pipetting and transferred to 50 µl droplets of 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. At 12 h of IVC (18 h after IVF), presumptive zygotes were transferred onto a BOEC monolayer for co-culture.

For production of conditioned media (CM) from embryo culture without epithelial cells (Figure 2-1), IVC was performed using 25-30 zygotes in 400 µl KSOMaa medium supplemented with 5% FCS without mineral oil in 4-well plates for a further 4 day culture, at which point the medium was collected (denoted as CM of D0-D4 embryo culture).

2.5. Embryo-BOEC co-culture

The BOEC culture medium was completely replaced with 400 µl of KSOMaa with 5% FCS 6 h before adding the zygotes. The presumptive zygotes (n=25-30 per well), approximately 18 h after IVF, were transferred on a BOEC 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, BOECs were lysed with Trizol (Invitrogen, Carlsbad, USA) and stored at -80 °C until RNA extraction. Supernatants were stored at -80 °C for further use. The morulae, developed on the BOEC monolayer, were washed three times in PBS-/- and stored at -80 °C until RNA extraction. The experiment was repeated six times and 45.1 ± 2.9% (n=78/173) embryos developed into early morulae (≥16-32 cell) at the end of 4-day co-culture (Fig. 2-3 A, B).

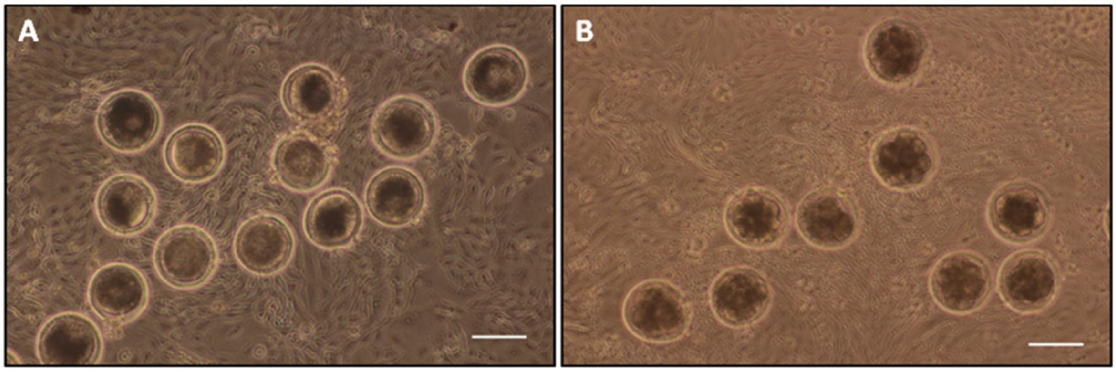

Fig. 2-3. (A) Zygotes on the BOEC monolayer at the start of 4 day co-culture, and **(B)** Morulae on the BOEC monolayer at the end of 4 day co-culture. Scale bar = 200 µm.

2.6. Isolation of PBMCs

PBMCs were isolated as previously described [29] with minor modifications. Blood collections were conducted at the Field Center of Animal Science and Agriculture of Obihiro University, and all experimental procedures complied with the guidelines for the care and use of agricultural animals at Obihiro University. Heparinized blood (20 ml) from a multiparous Holstein cow during luteal phase was collected and mixed with equal volume of PBS-/-, slowly layered over Ficoll-paque solution (Lymphoprep, Axis Shield, Oslo, Norway), and centrifuged at 1000 x*g* for 35 min at 10 °C. The buffy coat (white layer containing mononuclear cells) was collected as PBMCs, mixed with hemolysis buffer (NH_4Cl 155 mM, $KHCO_3$ 9.9 mM, EDTA 96.7µM) for 10 sec, and centrifuged at 500 x*g* for 10 min at 10 °C to remove red blood cells. After centrifugation, the cell pellet was washed twice by PBS-/-. The purity of PBMCs as evaluated by flow cytometry was > 98% and the viability was around 99% as assessed by Trypan blue staining.

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

PBMCs (5×10^6 cells) were cultured in a 48-well plate (Nalge Nunc International, Roskilde, Denmark) in 400 µl CM from embryo-BOEC co-culture or in CM of BOEC culture without embryo (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.

2.8 Culture of PBMCs in CM from D0-D4 embryo culture

PBMCs (5×10^6 cells) were cultured in a 48-well plate in 400 µl CM from D0-D4 embryo culture or in CM without embryo (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.

2.9 Immunocytochemistry

The purity of the epithelial cells was evaluated using immunofluorescence staining for specific marker (cytokeratin) of epithelial cells as previously described [47] with minor modifications. Briefly, oviduct epithelial cells were seeded on collagen-coated sterile coverslips in 6-well plates (Nalge Nunc International). Cells grown as a monolayer on a glass coverslip were examined after 6-7 days of culture. First, the cells were washed three times in PBS-/- containing 0.1% Tween-20 (PBS-T) and fixed with 4% paraformaldehyde (Wako Pure Chemicals Ltd., Osaka, Japan) for 10 min. Cells were exposed to 0.1% Triton-X 100 (Wako Pure Chemicals Ltd.) for 5 min for permeabilization. Then, the cells were incubated with 5% skim milk for 30 min at room temperature. After blocking, the cells were incubated with specific primary antibody (Rabbit monoclonal antibody to cytokeratin 8+18, Abcam, Tokyo, Japan; 1:200) or PBS-T as negative control for overnight at 4 °C. After incubation, the cells were incubated again with secondary antibody (Goat anti-Rabbit IgG Alexa Flour 546, Invitrogen; 1:200) for 60 min at room temperature. Finally, the coverslips were mounted with VECTASHIELD antifade mounting medium with DAPI (Vector Laboratories Inc., Canada) on slide

glasses. Fluorescence was observed using a fluorescence microscope (Nikon Microphot-SA, Tokyo, Japan). The purity of the cultured oviduct epithelial cells was > 98% (Fig. 2-4A, B).

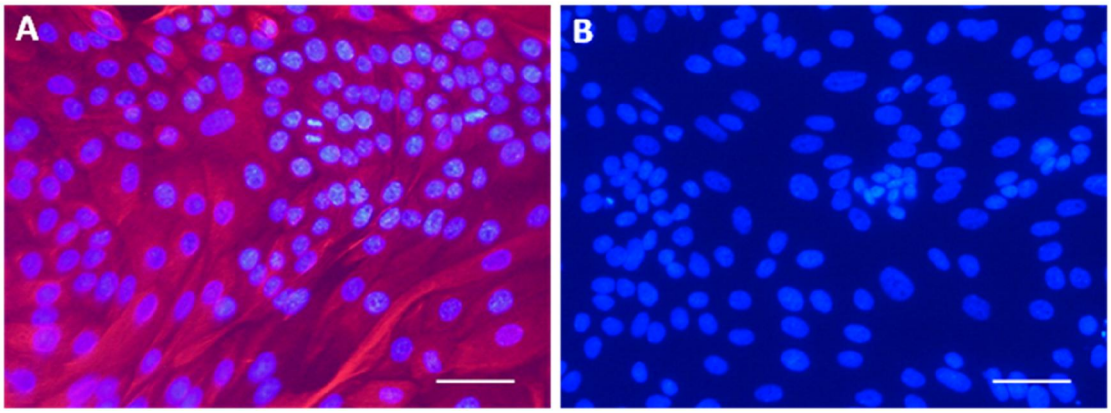

Fig. 2-4. (A) Immunofluorescence image of cultured BOECs with anti-cytokeratin antibody. Goat anti-rabbit IgG Alexa Flour 546 (red) was the secondary antibody, DAPI (blue) was used to visualize nuclei **(B)** Immunofluorescence image of cultured BOECs without anti-cytokeratin antibody as negative control. PBS-T was used instead of primary antibody.

2.10. RNA extraction and cDNA synthesis

Total RNA was extracted from BOECs and PBMCs using the Trizol reagent as a previously described protocol [48]. The RNA extraction from embryos was also performed according to the same protocol except addition of 1µl (25µg) glycogen (Roche Diagnostics GmbH, Mannheim, Germany) before precipitation with isopropanol. 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 production. A DNase treatment step was performed using RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA) to remove residual genomic DNA and other contaminations. The extracted RNA (1 µg in 7 µl) was incubated for 30 min at 37 °C with 1 unit of the 10× RQ1 RNase-free DNase reaction buffer and 2 µl of the 1 µg/µl RNase-free DNase. To terminate the reaction, 1 µl of the RQ1 DNase Stop solution (20 mM EDTA) was added to the sample, and the mixture was incubated for 10 min at 65 °C. First-strand cDNA synthesis was conducted

according to the commercial protocol described in the Super Script II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). The mixture was prepared using 1.5 µl of 50 ng/µl random primer (Invitrogen, Carlsbad, CA, USA), 1.5 µl of 10 mM PCR Nucleotide Mix (dNTP; Roche Diagnostics, Indianapolis, IN, USA), and 4 µl of H_2O to obtain a total volume of 18 µl per sample. This mixture was then incubated at 65 °C for 5 min in a thermal cycler (Bio-Rad, Munich, Germany). The samples were kept on ice while the second mixture, which consisted of 3 µl of 0.1M dithiothreitol (DTT, Invitrogen, Carlsbad, CA, USA), 1.5 µl of 40 units/µl RNasin Ribonuclease Inhibitor (Promega, Madison, WI, USA), and 6 µl of 5× First-Strand Buffer (Invitrogen, Carlsbad, CA, USA), was added to each tube. The samples were incubated for 2 min at 42 °C, and 0.2 µl of 200 units/µl SuperScript II Reverse Transcriptase was added to each tube. The thermal cycler was programmed at 25 °C for 10 min, 42 °C for 50 min, and then 70 °C for 15 min. The synthesized cDNA was stored at -30 °C.

2.11. Real-time PCR

The primers of target genes used in the present study are listed in Table 2-1. Quantitative real-time PCR 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 run with an initial activation step (15 min at 9 °C), followed by 40 cycles of PCR (15 sec denaturation at 95 °C, 30 sec annealing at 55–58 °C, and 20 sec extension at 72 °C). Melting curve was evaluated at the end of the run to observe the specificity of the amplification. The calculated cycle threshold (Ct) values were normalized using *B-actin* as the internal standard using the Delta-Delta comparative threshold method [49] to quantify the fold change between the samples.

Table 2-1. 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	
IFNT	Forward	GCCCTGGTGCTGGTCAGCTA	AF238612
	Reverse	CATCTTAGTCAGCGAGAGTC	

2.12. ELISA for determination of IFNT concentration in conditioned media

A commercially available ELISA kit (Clould-Clone Corpo., Texas, USA) was used for determination of IFNT concentration in conditioned media obtained from embryo-BOEC co-culture following the manufacturer's instructions. The optical density (OD) value was detected using ELISA microplate reader (Labsystem Multiskan MS 352, Labsystems, Finland) at 450 nm wavelengths. The standard curve used for this assay was 7.8-500 pg/mL.

2.13 Statistical analysis

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

3. Results

3.1 The effect of the developing embryos on gene expression in BOECs

The developing embryos did not induce expression of interferon (IFN)-stimulated genes (*ISGs*; *ISG15*, *OAS1* and *MX2*), a key factor for IFNT-signaling (*STAT1*), and type-1 IFN receptors (*IFNAR1* and *IFNAR2*) in BOECs. However, embryos significantly stimulated *PTGES* ($P<0.05$) and suppressed *NFkB2* and *NFkBIA* ($P<0.01$) in BOECs. Moreover, there was no significant ($P>0.05$) change was observed in the expression of pro- (*TNFA* and *IL1B*) and anti- (*TGFB1* and *IL10*) inflammatory cytokines in BOECs in the presence of the embryos (Fig. 2-5).

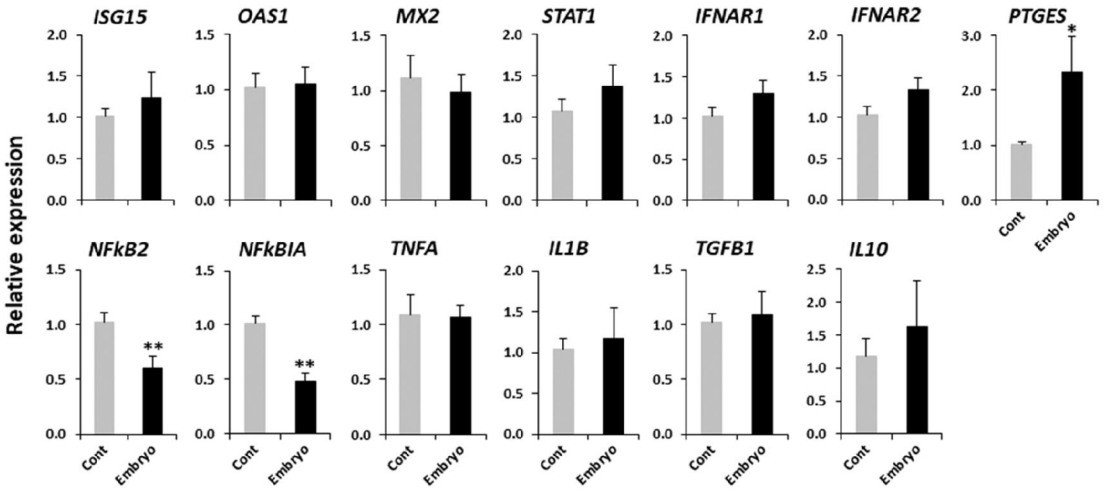

Fig. 2-5. The effect of the developing embryos on gene expression in BOECs. Relative mRNA expression of IFN-stimulated genes (*ISG15*, *OAS1* and *MX2*), a key factor for IFN-signaling (*STAT1*), type-1 IFN receptors (*IFNAR1* and *IFNAR2*), enzyme involved in prostaglandin E synthesis (*PTGES)*, key factors for inflammatory response (*NFKB2* and *NFKBIA*), pro-inflammatory cytokines (*TNFA* and *IL1B*) and anti-inflammatory cytokines (*TGFB1* and *IL10*). Data are presented as mean ± SEM of six experiments. Asterisks denote statistical difference: * $P<0.05$, ** $P<0.01$, when compared to the control.

3.2. The effect of CM from embryo-BOEC co-culture on gene expression in PBMCs

The CM from embryo-BOEC co-culture clearly stimulated *ISGs* and *STAT1* in PBMCs (P<0.05). Moreover, CM from embryo-BOEC co-culture significantly increased *TGFB1* and *PTGES*, but suppressed *IL17* in PBMCs (P<0.05) (Fig. 2-6).

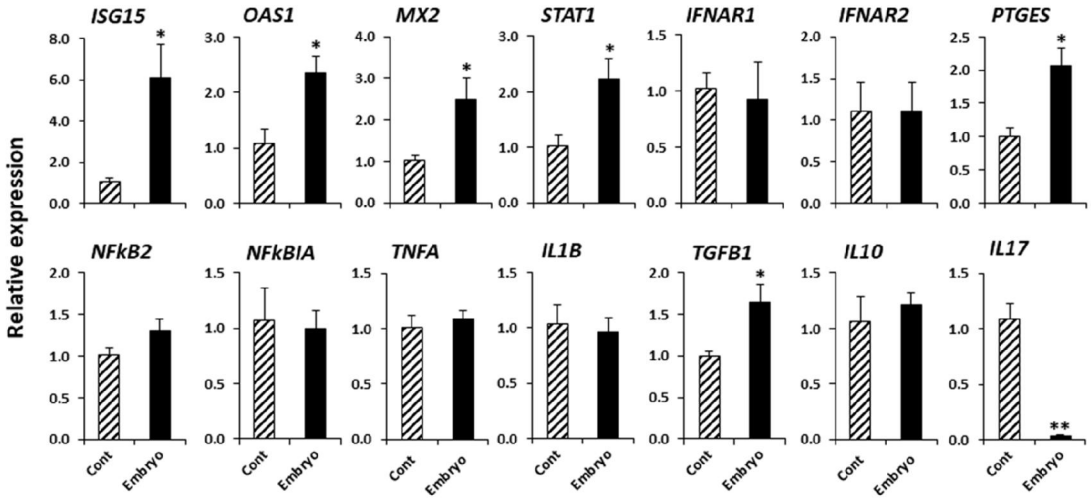

Fig. 2-6. The effect of conditioned media from embryo-BOEC co-culture on gene expression in PBMCs. Relative mRNA expression of IFN-stimulated genes (*ISG15*, *OAS1* and *MX2*), a key factor for IFN-signaling (*STAT1*), type-1 IFN receptors (*IFNAR1* and *IFNAR2*), an enzyme involved in prostaglandin E synthesis (*PTGES)*, key factors for inflammatory response (*NFKB2* and *NFKBIA*), pro-inflammatory cytokines (*TNFA* and *IL1B*), anti-inflammatory cytokines (*TGFB1* and *IL10*) and Th17 cytokine (*IL17*). Data are presented as mean ± SEM of six experiments. Asterisks denote statistical difference: * P<0.05, ** P<0.01, when compared to the control.

3.3. The effect of CM from D0-D4 embryo culture on gene expression in PBMCs

The CM from embryo culture did not affect *ISGs* or other immune-related genes in PBMCs (Fig. 2-7).

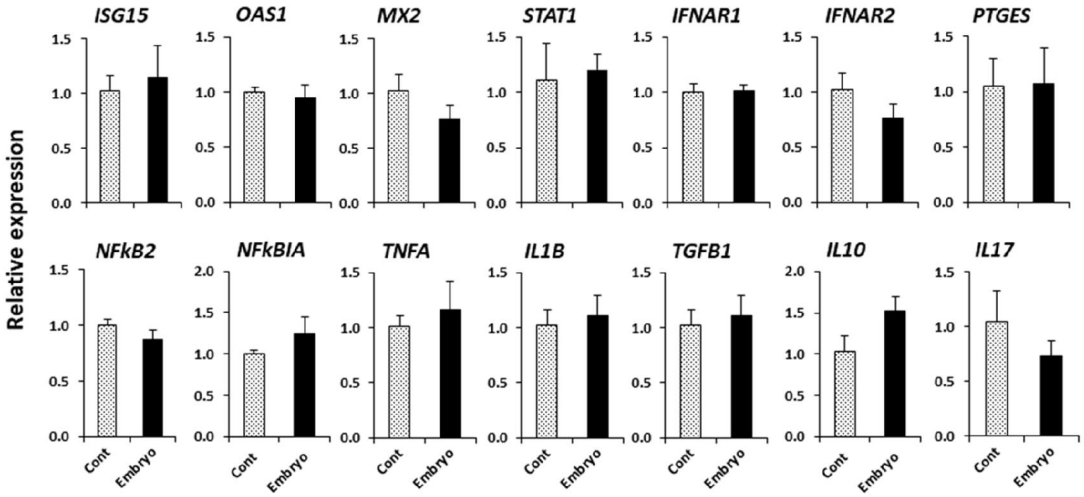

Fig. 2-7. The effect of conditioned media from D0-D4 embryo culture on gene expression in PBMCs. Relative mRNA expression of IFN-stimulated genes (*ISG15*, *OAS1* and *MX2*), key factor for IFN-signal (*STAT1*), type-1 IFN receptors (*IFNAR1* and *IFNAR2*), an enzyme involved in prostaglandin E synthesis (*PTGES)*, key factors for inflammatory response (*NFKB2* and *NFKBIA*), pro-inflammatory cytokines (*TNFA* and *IL1B*), anti-inflammatory cytokines (*TGFB1* and *IL10*) and Th17 cytokine (*IL17*). Data are presented as mean ± SEM of three experiments.

3.4 Expression of type-1 IFN receptors (IFNAR1 and IFNAR2) in BOECs and PBMCs

The gene expressions of type-1 IFN receptors were compared between the BOECs and PBMCs. PBMCs expressed significantly (P<0.01) higher *IFNAR1* and *IFNAR2* with compared to BOECs (Fig. 2-8).

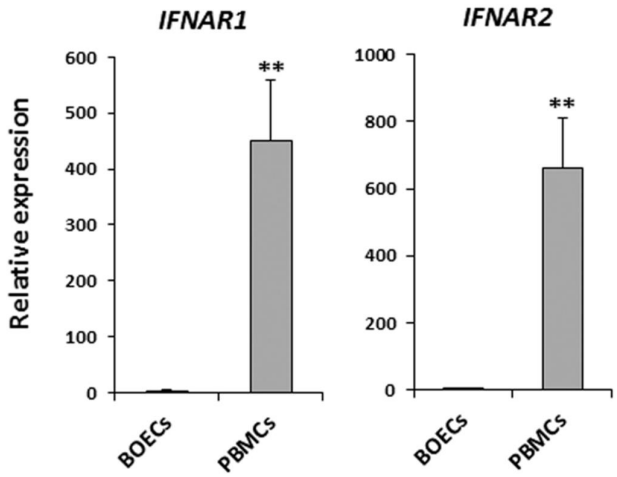

Fig. 2-8. Relative mRNA expression of *IFNAR1* and *IFNAR2* in BOECs and PBMCs. Data are presented as mean ± SEM of six experiments. Asterisks denote statistical difference: ** P<0.01, when compared to the BOECs.

3.5 Gene expression (PTGES and IFNT) in the bovine embryos

The gene expression in morulae, developed on the BOEC monolayer, was compared with that of blastocysts (positive control). Both *PTGES* and *IFNT* were expressed in the 16-32 cell morula stage embryos developed on the BOEC monolayer. Blastocysts expressed about 8-fold higher *IFNT* than morulae ($P<0.01$) (Fig. 2-9).

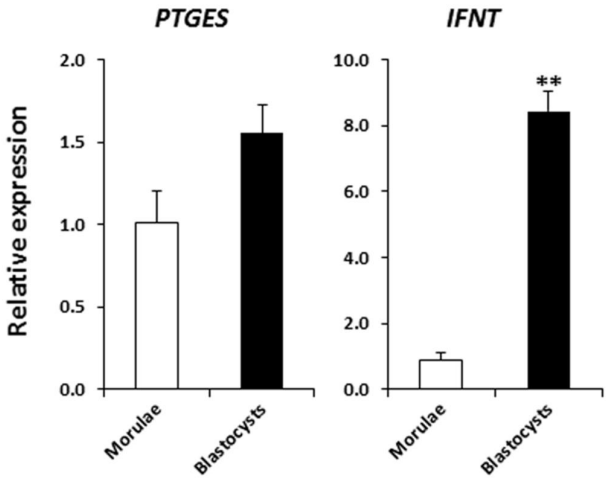

Fig. 2-9. Relative mRNA expression of *PTGES* and *IFNT* in the bovine morulae at the end of co-culture. Data are presented as mean ± SEM of six experiments. Asterisks denote statistical difference: ** $P<0.01$, when compared to the morulae.

3.6. IFNT concentration in conditioned media

It was not possible to determine IFNT concentration in the conditioned media from embryo-BOEC co-culture by ELISA.

4. Discussion

The present study provides *in vitro* evidence that an immunological crosstalk indeed exists between the developing embryo and bovine oviduct epithelial cells. Importantly, the subsequent interaction between the embryo and the oviduct epithelial cells *via* conditioned media induced a clear anti-inflammatory response in the immune cells (PBMCs). The findings from the present study

suggest a pivotal role of BOECs to enhance IFNT secretion from the developing embryo in the bovine oviduct, and such a low amount of IFNT is likely to be involved for modulation of gene expression in the immune cells (PBMCs).

In the present study, developing embryos on D0-D4 did not induce *ISGs* in BOECs. However, gene expression in the embryos from the current study and from others [32, 34] shows that the early bovine embryos (16-cell to early morula stage) express *IFNT* mRNA. Therefore, these findings suggest that the early bovine embryo starts to secrete IFNT in the oviduct; however, such a small amount of IFNT is not sufficient to induce *ISGs* expression in BOECs. On the contrary, another study reported that the early bovine embryo can induce *ISGs* expression in BOECs during 8 days *in vitro* co-culture, where embryos were developed until blastocyst stage [50]. Due to very long period for co-culture, the result obtained in that study was not oviduct-specific but very probably *via* IFNT from the blastocyst that is normally developing *in utero*.

The result of the present study reveals that D0-D4 embryos clearly induced *PTGES* expression in BOECs. Moreover, early morula stage (16-32 cells) embryos at the end of 4 days embryo-BOEC co-culture expressed *PTGES*. The PGE2 was detected in *in vitro* produced bovine embryo by immunofluorescence technique, while intensity of PGE2 gradually increased from 2-cell to blastocyst [51]. Therefore, it is likely that both the embryo and oviduct epithelial cells contribute to generate a PGE2 rich environment in the bovine oviduct. PGE2 is known as an immunosuppressive molecule that can inhibit proliferation of lymphocytes in culture at a concentration of 10^{-8}M [52]. Our laboratory recently demonstrated that sperm stimulates PGE2 biosynthesis from oviduct epithelial cells that acts as a main regulator for induction of anti-inflammatory response in the bovine oviduct *in vitro*[11]. Moreover, previous study from our laboratory showed that PGE2 plays a major role in suppressing sperm phagocytosis by polymorphonuclear neutrophils (PMNs) in bovine oviduct in a dose-dependent manner [10]. Therefore, in the current study, embryo-stimulated PGE2 biosynthesis from BOECs seems to be involved in suppression of local immunity in the oviduct.

The developing embryos on D0-D4 induced a slight anti-inflammatory response in BOECs, *via* suppression of *NFkB2* and *NFkBIA*, without influencing pro- (*TNFA* and *IL1B*) and anti (*TGFB1* and *IL10*) - inflammatory cytokines expression. Similar result was also observed by Maillo et al. [40] who demonstrated that 50 embryos in the oviduct down-regulated *NFkB2* transcript in cattle. The mechanism by which the embryo suppresses NFkB system in the oviduct requires further investigation. It is possible that the early bovine embryo may release unknown molecule(s) that prevent nuclear translocation of NFkB and thus suppress the activation of NFkB/NFkBIA signaling pathway in the oviduct.

In the present study, although the mechanism by which PGE2 synthesis is stimulated in BOECs by the embryos is unknown, the conditioned media (CM) from embryo-BOEC co-culture significantly increased the expression of *PTGES* and *TGFB1* in PBMCs. It is well known that TGFβ synergistically works with PGE2 to induce the differentiation of naïve T cell (Th0) to regulatory T cell for immune suppression and tolerance [53]. On the other hand, TGFβ also enhances the differentiation of Th0 to Th17 cell, which expresses *IL17*, in the presence of pro-inflammatory cytokines, such as IL1 or IL6 [54, 55]. In the current study, the expression level of *IL17* was significantly suppressed in PBMCs by CM from embryo-BOEC co-culture. Therefore, embryo induced *TGFB1* in PBMCs and PGE2 synthesis from BOECs both may synergistically play an important role for induction of an anti-inflammatory and immune tolerance conditions in the bovine oviduct.

Surprisingly, CM from embryo-BOEC co-culture induced *ISGs* and *STAT1* in PBMCs suggesting that this CM contains IFNT, although embryos did not stimulate *ISGs* in BOECs. The possible reason is that PBMCs are much more responsive to IFNT as they expressed relatively higher type-1 IFN receptors with compared to BOCEs. Interestingly, CM of D0-D4 embryo culture alone did not stimulate *ISGs* and also did not influence other immune-related genes in PBMCs, suggesting that this CM did not contain IFNT or amount of IFNT was less than that of CM of embryo-BOEC co-

culture. These results suggest that the BOECs might enhance IFNT secretion from the developing embryos during co-culture period, and such a small amount of IFNT might be involved for regulation of gene expression of immune cells (PBMCs). Further study, such as neutralization of IFNT in CM from embryo-BOEC co-culture using an anti-IFNT antibody, is currently on-going to specify the role of IFNT for regulation of immune cells gene expression.

Taken together, findings of this study support the hypothesis that the bovine embryo starts to secrete IFNT in the oviduct (D0-D4) and induces an anti-inflammatory response in epithelial cells, without stimulation of *ISGs*. The embryo-BOECs interaction induces an anti-inflammatory response in immune cells (PBMCs) with activation of *ISGs*, while the developing embryo alone ‘‘cannot’’ regulate gene expression of the immune cells. A very small amount of IFNT is likely to be involved in modulation of such “local” immune response in the bovine oviduct.

Chapter II

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

1. Introduction

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 [56, 57]. 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 [38, 58]. 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 [58, 59]. 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) [22-24, 27, 60]. 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 arrival of the morula into the uterus (D5) until the hatching of the blastocyst (D9). It was observed that nuclear factor kappa B (NFkB), a central mediator of inflammatory and immune responses, was down-regulated in cattle uterus on D8 of pregnancy, which may confer tolerance to the semi-allogenic embryo in the uterus [61]. 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 [41, 42]. 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 [26]. Furthermore, the interferon-stimulated genes (*ISGs*) were activated in peripheral blood leukocytes during early pregnancy in cows [29, 43], suggesting that the resident immune cells in the FRT may also respond to IFNT. A very recent study demonstrated the involvement of IFNT in embryo-maternal

communication on D7 of pregnancy in cattle [62]. Moreover, IFNT mRNA is expressed and its protein can be detected in the trophectoderm of non-hatched blastocysts on D7 [32, 33]. 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 BUECs and immune cell gene expression.

2. Materials and methods

2.1 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).

2.2 Experimental model

An *in vitro* model (Fig. 3-1) was used to investigate the communication of the developing 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.

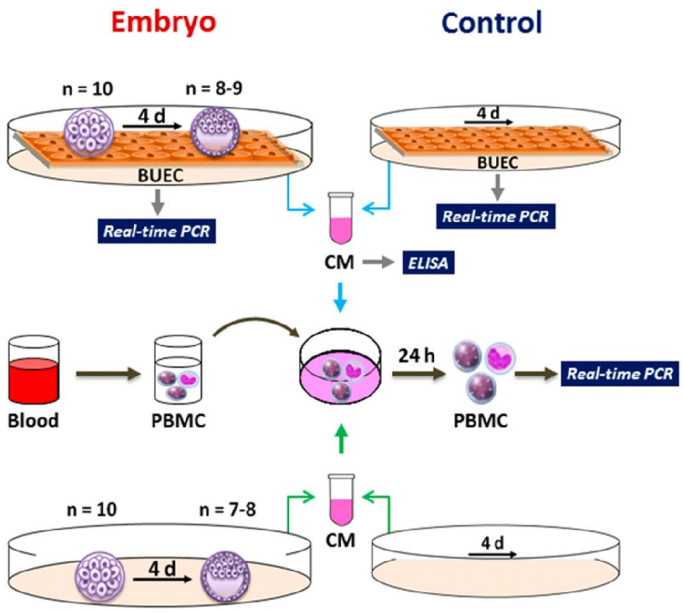

Fig. 3-1. Schematic representation of the experimental model. A BUEC monolayer was co-cultured with morulae (n=10) or without (control) for 4 days. At the end of co-culture, morulae (n=8-9) were developed into blastocysts. Gene expression was analyzed in BUECs. Specific ELISAs for PGE2 and IFNT were 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.

2.3 Culture of BUECs

The reproductive tracts of female cows at luteal phase (D5-D7) were collected from a local slaughterhouse (Hokkaido Livestock Co., 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 [44]. The uterine horn, ipsilateral to the corpus luteum, was used for isolation and culture of epithelial cells according to a previously described method [63], with minor modifications. In the laboratory, uterine horn was separated from surrounding connective tissue and washed three times in physiological saline. A polyvinyl catheter was inserted through the tip of horn and fixed with ligation. The other end near the uterine body was ligated in order to retain the enzyme solution for solubilizing the epithelial cells as described below. The lumen of uterine horn was washed three times with 30–50 ml of Hank's balanced salt solution (HBSS) supplemented with 1% penicillin-streptomycin, 1% amphotericin B and 0.1% BSA (Sigma-Aldrich, Steinheim, Germany). Twenty milliliters of enzyme solution (sterile HBSS containing 0.05% collagenase I (Sigma-Aldrich), 0.005% deoxyribonuclease I (Sigma-Aldrich), and 0.1% BSA) was then infused into the uterine lumen through the catheter. Epithelial cells were isolated by incubation at 38.5 °C for 60 min with gentle shaking. The cell suspension was filtered through sterile nylon mesh (100 μm) to remove undissociated tissue fragments. The filtrate was washed by centrifugation (180 x*g* for 10 min at 4 °C) with Tris-buffered ammonium chloride (pH 7.5) to remove hematocytes followed by three times wash with DMEM/F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12; Gibco) supplemented with 1% penicillin-streptomycin, 1% amphotericin B and 0.1% BSA. After the washes, the cells were counted with a hemocytometer. The cell viability was higher than 95% as assessed by 0.5% trypan blue dye exclusion. The final pellet of the epithelial cells was resuspended in culture medium containing DMEM/F12 supplemented with 2.2% $NaHCO_3$ (Sigma-Aldrich), 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 [5]. The cells in culture medium showed characteristic

epithelial morphology (Fig. 3-2) that was confirmed by immunofluorescence staining of cytokeratin, a specific marker for epithelial cells.

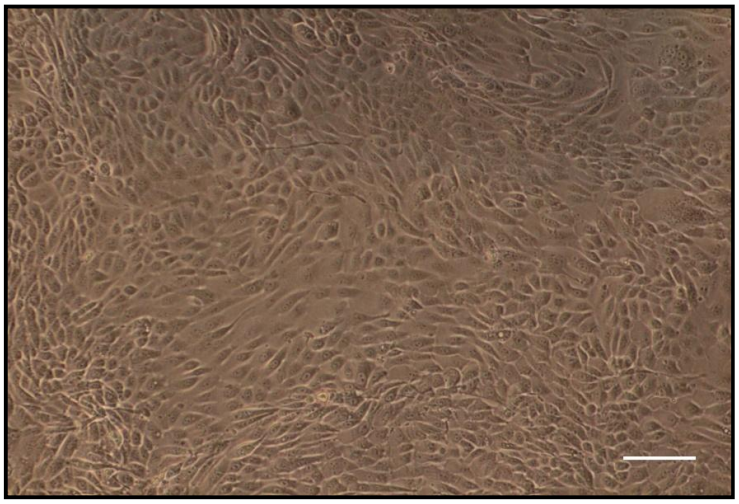

Fig. 3-2. The confluent BUEC monolayer at which point cells were given a second passage. Scale bar = 200 μm.

2.4 In vitro embryo production

2.4.1 Oocyte collection and in vitro maturation (IVM)

Bovine ovaries were collected from a local slaughterhouse (Hokkaido Livestock Co., Doto Plant Tokachi Factory; Obihiro, Hokkaido, Japan) and transported to the laboratory in physiological saline supplemented with 1% penicillin-streptomycin at 39 °C within 1–2 h of slaughter.

In the laboratory, follicular fluid was aspirated from individual visible antral follicles of 2–6 mm in diameter using 10 ml syringe attached to 18 G needle. The retrieved follicular fluid was transferred to 60 mm petridish (Thermo Fisher Scientific, Roskilde, Denmark) and examined for cumulus oocyte complexes (COCs) using a stereo-microscope (Leica M Stereomicroscope, Switzerland). Then, COCs were washed three times in oocyte collection medium (OCM; Research Institute for the Functional Peptides Co., Ltd., Yamagata, Japan) supplemented with 5% FCS in 35 mm petridish (Corning Inc., NY, USA). Only COCs with a homogenous cytoplasm and surrounded by at least three layers of compact cumulus cells 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 Co., Ltd.) supplemented with 10 µg/ml FSH (Folltropin-V, Bioniche Animal Health Inc., Belleville, Ontario, Canada) and 5% FCS in 4-well plates under mineral oil (Sigma-Aldrich) at 39 °C in 5% CO_2 with humidified air.

2.4.2 Sperm preparation and in vitro fertilization (IVF)

Two semen straws (0.5 ml) from the same bull (AG black 29) were thawed at 37 ℃ for 30 sec. The thawed semen was washed twice in fertilization medium IVF100 (Research Institute for the Functional Peptides Co., Ltd.) by centrifugation at 1200 rpm for 6 min at room temperature. Sperm pellet was diluted with IVF100 to prepare a final concentration of 5×10^6 /ml in the fertilization droplet.

In vitro fertilization was performed according to a previously described protocol [46] with minor modifications. Briefly, 50 µl droplets of fertilization medium IVF100 were prepared in 60 mm petridish under mineral oil. After maturation, COCs were washed twice in fertilization medium IVF100. Finally, 15 COCs and sperm (5×10^6 /ml) were transferred to a fertilization droplet for co-incubation for 6 h at 39 °C in 5% CO_2 in humidified air.

2.4.3 In vitro culture (IVC)

After 6 h of sperm-COCs co-incubation, presumptive zygotes (n=15) were denuded by repeated pipetting and transferred to 50 µl droplets of 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. At 96 h of IVC (102 h after IVF), morulae were transferred onto a BUEC monolayer for co-culture.

For production of conditioned media (CM) from embryo culture without uterine epithelial cells (Fig. 3-1), 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).

2.5 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% (n=48/60) morulae developed into blastocysts, of which 35.0 ± 2.2% were hatched at the end of the 4-day co-culture (Fig 3-3.A-D).

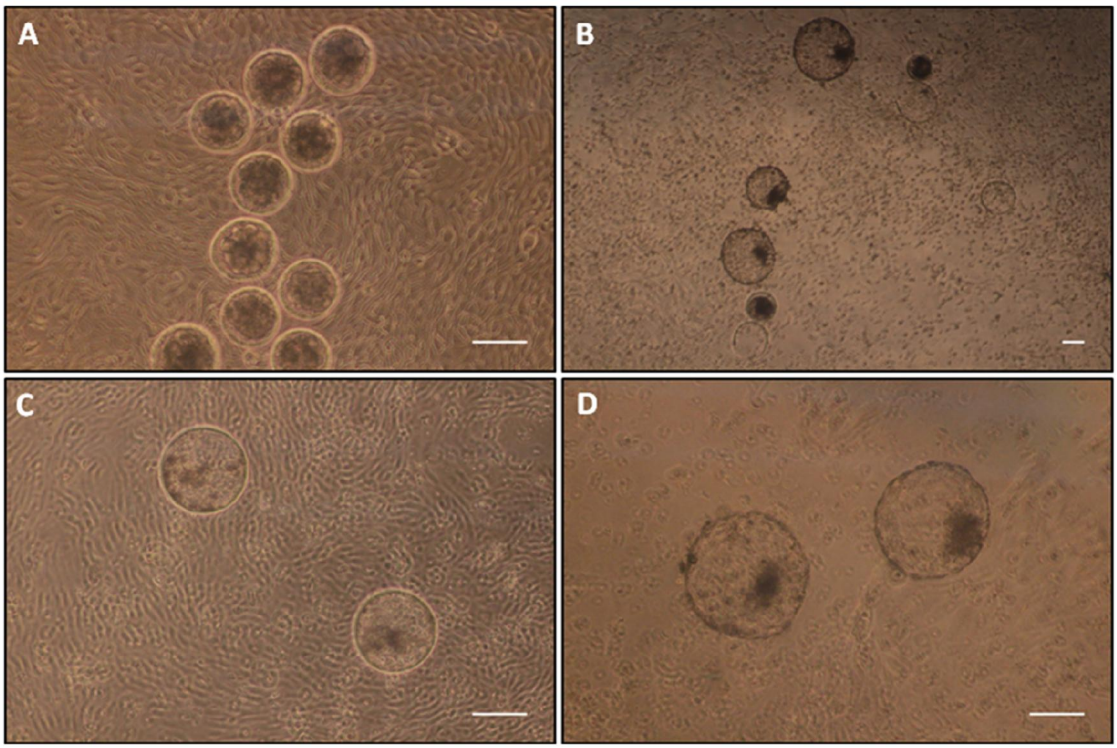

Fig. 3-3. (A) Morulae on the BUEC monolayer at the start of 4 day co-culture, **(B)** Different types of blastocysts on the BUEC monolayer at the end of 4 day co-culture, **(C)** Non-hatched blastocysts on the BUEC monolayer, and **(D)** Hatched blastocysts on the BUEC monolayer. Scale bar = 200 µm.

2.6 Isolation of PBMCs

PBMCs were isolated as previously described [29] 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 x*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 x*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%.

2.7 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.

2.8. Culture of PBMCs in CM from D5-D9 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.

2.9 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.

2.10 Immunocytochemistry

The purity of the epithelial cells was evaluated using immunofluorescence staining for specific marker (cytokeratin) of epithelial cells as previously described [47] with minor modifications. Briefly, uterine epithelial cells were seeded on collagen-coated sterile coverslips in 6-well plates (Nalge Nunc International). Cells grown as a monolayer on a glass coverslip were examined after 6-7 days of culture. First, the cells were washed three times in PBS-/- containing 0.1% Tween-20 (PBS-T) and fixed with 4% paraformaldehyde (Wako Pure Chemicals Ltd., Osaka, Japan) for 10 min. Cells were exposed to 0.1% Triton-X 100 (Wako Pure Chemicals Ltd.) for 5 min for permeabilization. Then, the cells were incubated with 5% skim milk for 30 min at room temperature. After blocking, the cells were incubated with specific primary antibody (Rabbit monoclonal antibody to cytokeratin 8+18, ab53280, Abcam, Tokyo, Japan; 1:200) or PBS-T as negative control for overnight at 4 °C. After incubation, the cells were incubated again with secondary antibody (Goat anti-Rabbit IgG Alexa Flour 546, Invitrogen; 1:200) for 60 min at room temperature. Finally, the coverslips were mounted with VECTASHIELD antifade mounting medium with DAPI (Vector Laboratories Inc., Canada) on slide glasses. Fluorescence was observed using a fluorescence microscope (Nikon Microphot-SA, Tokyo, Japan). The purity of the cultured uterine epithelial cells was > 98% (Fig. 3-4. A, B).

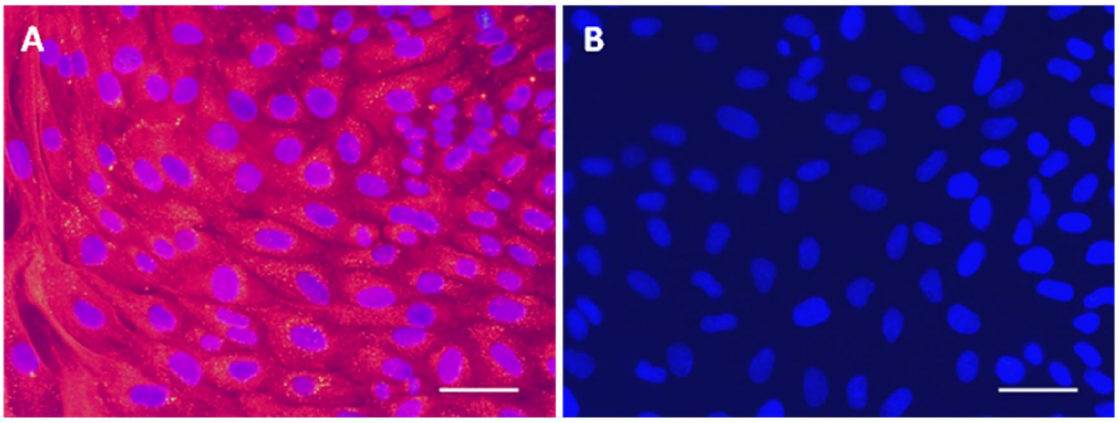

Fig. 3-4. (A) Immunofluorescence image of cultured BUECs with anti-cytokeratin antibody. Goat anti-rabbit IgG Alexa Flour 546 (red) was the secondary antibody, DAPI (blue) was used to visualize nuclei **(B)** Immunofluorescence image of cultured BUECs without anti-cytokeratin antibody as negative control. Scale bar = 100 µm.

2.11 RNA extraction and cDNA synthesis

Total RNA was extracted from BUECs and PBMCs using the Trizol reagent as previously described [48]. 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 [11]. Briefly, a DNase treatment step was performed using RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA) to remove residual genomic DNA and other contaminations. The extracted RNA (1 µg in 7 µl) was incubated for 30 min at 37 °C with 1 unit of the 10× RQ1 RNase-free DNase reaction buffer and 2 µl of the 1 µg/µl RNase-free DNase. To terminate the reaction, 1 µl of the RQ1 DNase Stop solution (20 mM EDTA) was added to the sample, and the mixture was incubated for 10 min at 65 °C. First-strand cDNA synthesis was conducted according to the commercial protocol described in the Super Script II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). The mixture was prepared using 1.5 µl of 50 ng/µl random primer (Invitrogen, Carlsbad, CA, USA), 1.5 µl of 10 mM PCR Nucleotide Mix (dNTP; Roche Diagnostics, Indianapolis, IN, USA), and 4 µl of H_2O

to obtain a total volume of 18 µl per sample. This mixture was then incubated at 65 °C for 5 min in a thermal cycler (Bio-Rad, Munich, Germany). The samples were kept on ice while the second mixture, which consisted of 3 µl of 0.1M dithiothreitol (DTT, Invitrogen, Carlsbad, CA, USA), 1.5 µl of 40 units/µl RNasin Ribonuclease Inhibitor (Promega, Madison, WI, USA), and 6 µl of 5× First-Strand Buffer (Invitrogen, Carlsbad, CA, USA), was added to each tube. The samples were incubated for 2 min at 42 °C, and 0.2 µl of 200 units/µl SuperScript II Reverse Transcriptase was added to each tube. The thermal cycler was programmed at 25 °C for 10 min, 42 °C for 50 min, and then 70 °C for 15 min. The synthesized cDNA was stored at -30 °C.

2.12 Real-time PCR

Quantitative real-time PCR of target genes (Table 3-1) 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 run with an initial activation step (15 min at 9 °C), followed by 40 cycles of PCR (15 sec denaturation at 95 °C, 30 sec annealing at 55–58 °C, and 20 sec extension at 72 °C). Melting curve was evaluated at the end of the run to observe the specificity of the amplification. The calculated cycle threshold (Ct) values were normalized using *B-actin* as the internal standard by applying the Delta-Delta comparative threshold method [49] to quantify the fold change between samples.

Table 3-1. 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	

2.13 ELISA for determination of PGE2 and IFNT concentration in conditioned media

The specific ELISA kits were used for determination of PGE2 (R&D systems, Minneapolis, MN, USA) and IFNT (Cloud-Clone Corp., 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.

2.14 Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed using SPSS software, version 14.0 (SPSS Inc., 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.

3. Results

3.1 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. 3-5). It was not possible to determine IFNT concentration in conditioned media from embryo-BUEC co-culture by ELISA.

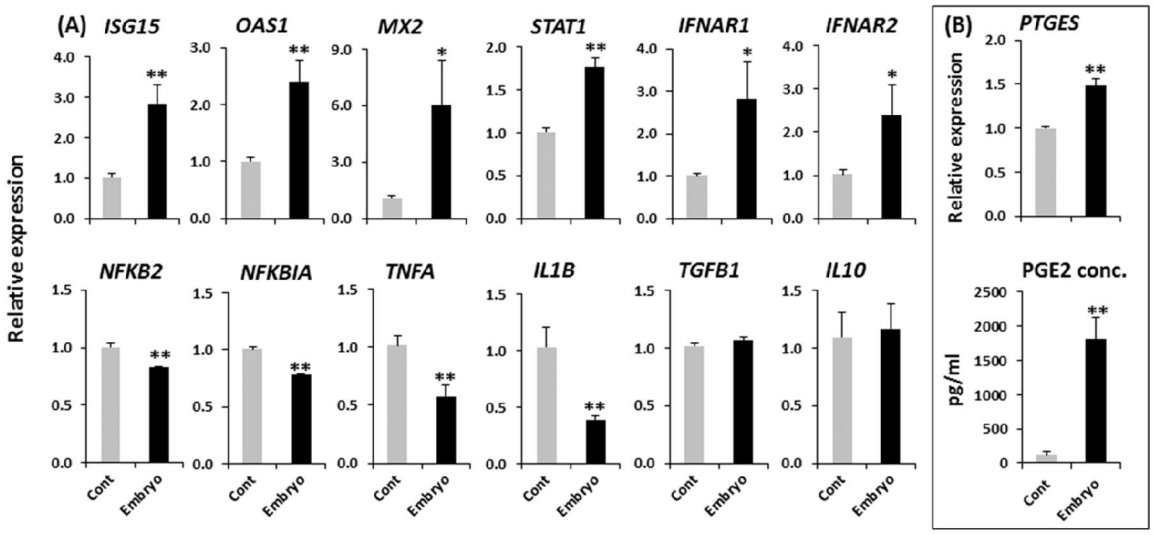

Fig. 3-5. The effect of developing embryos on gene expression and PGE2 secretion in BUECs. **(A)** Relative mRNA expression of IFN-stimulated genes (*ISG15*, *OAS1* and *MX2*), a key factor for IFN-signaling (*STAT1*), type-1 IFN receptors (*IFNAR1* and *IFNAR2*), key factors for the inflammatory response (*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.

3.2 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.3-6).

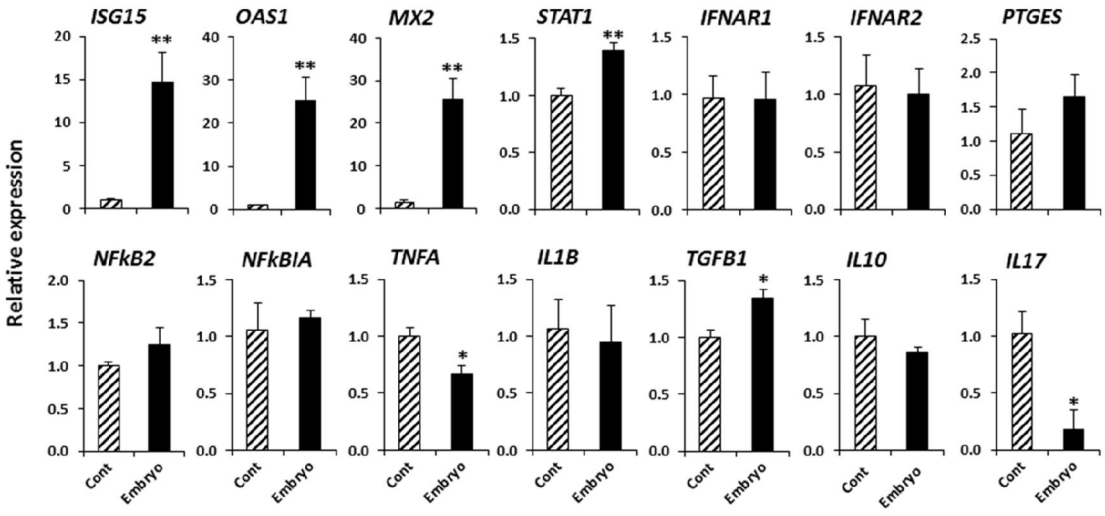

Fig. 3-6. The effect of conditioned media from embryo-BUEC co-culture on gene expression in PBMCs. CM from BUEC culture without embryos served as the control. Relative mRNA expression of IFN-stimulated genes (*ISG15*, *OAS1* and *MX2*), a key factor for IFN-signaling (*STAT1*), type-1 IFN receptors (*IFNAR1* 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.

3.3 The effect of CM from D5-D9 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.3-7).

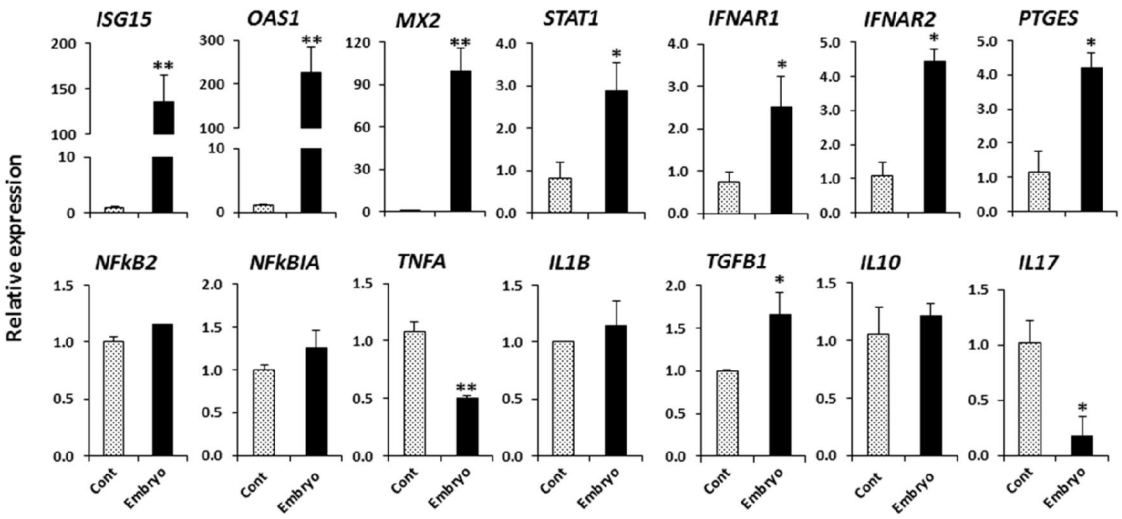

Fig. 3-7. The effect of conditioned media from D5-D9 embryo culture on gene expression in PBMCs. CM without embryos served as the control. Relative mRNA expression of IFN-stimulated genes (*ISG15*, *OAS1* and *MX2*), a key factor for IFN-signaling (*STAT1*), type-1 IFN receptors (*IFNAR1* and *IFNAR2*), 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.

3.4 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. 3-8A). 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. 3-8B).

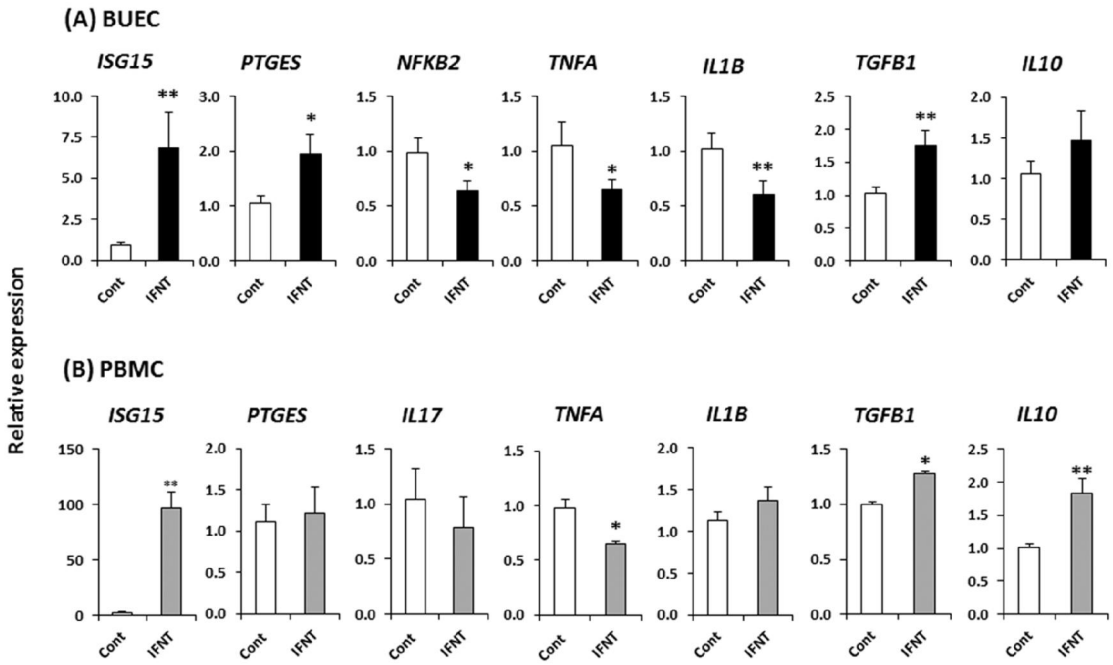

Fig. 3-8. The effect of IFNT (100 pg/ml) on gene expression in BUECs and PBMCs. **(A)** Relative mRNA expressions of *ISG15*, *PTGES*, *NFkB2*, pro-inflammatory cytokines (*TNFA* and *IL1B*) and anti-inflammatory cytokines (*TGFB1* and *IL10*) in BUECs, and **(B)** Relative mRNA expressions of *ISG15*, *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.

4. 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, a simplified co-culture model was used 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. These 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 to these 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 [27]. 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. A very recent study identified the IFN-signaling 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[62]. The present study applied 10 embryos onto the BUEC monolayer, which might have amplified the embryo-derived IFNT signal and thus the *ISGs* expression in BUECs. The findings of the current study suggest that a “very local” communication exists between the early developing embryo and endometrium during the first four days of embryo development in the uterus (D5-D9) in cattle. In fact, it was not possible to determine IFNT concentration in the conditioned media from embryo-BUEC co-culture. This may be due to presence of 5% FCS in the media used 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 [64], and this strongly supports finding of the present study.

PTGES expression and PGE2 secretion were stimulated in BUECs by early developing embryos. This 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 [65, 66]. 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 [52]. 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 [67]. 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 [68]. 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 [69, 70], 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 [30], while Th1 dominance is observed in pregnancy failure [31]. 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 [53]. 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 [54, 55]. 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, it is also possible 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, BUECs were stimulated 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, findings of the present study 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 [23], while the embryo is also known to induce expression of *ISGs* in the endometrium in pregnant cows [27]. 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 [71, 72].

Taken together, findings of the present study support the hypothesis that the bovine 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 the bovine.

General Discussion and Conclusion

The mucosal epithelium plays important roles in regulation of the local immunity of the female reproductive tract. It recognizes the pathological and physiological stimulus present in the lumen of the FRT and send off the appropriate signal to the underlying immune cells. Thus, the immune cells become either recruited and activated or suppressed. The epithelium actually sets up the entire environment of the FRT to initiate an inflammatory response or confer tolerance to a specific stimulus. The recognition system of pathogen or endotoxin in the FRT is well known. Pathogens or bacterial LPS/ lipopeptides are recognized by the TLRs of the bovine FRT epithelium [3-5]; however, it is still unknown by which the FRT epithelium can recognize the gametes or embryo.

An integration in the functions of immune system, endocrine system, and reproductive system is crucial for an acceptance of a semi-allogenic embryo/fetus in the female reproductive tract. The mechanism by which an embryo escapes from attack by the maternal immune system is a complex process, which is accompanied by combined action of variety of factors from developing embryo including IFNT and PGE2, and those from maternal ovary, oviduct and uterus such as P4, cytokines, chemokines, prostaglandins, and growth factors etc. The present study used an *in vitro* model to study the immunological interactions between the early developing embryo and oviduct/ uterine epithelial cells (BOECs/BUECs) in the bovine. Subsequently, the impact of embryo-BOEC and embryo-BUEC interactions or the effect of embryo alone *via* conditioned media on gene expression of immune cells (PBMCs) was investigated.

The embryo comes in intimate contact with oviduct and uterine epithelial cells and, therefore, the *in vitro* co-culture of early embryo with BOECs and BUECs could provide a valuable tool to investigate the molecular mechanisms and regulatory pathways involved in embryo-maternal interaction in cattle. One could argue that the present study used multiple embryos (25-30 zygotes or 10 morulae) for co-culture that does not happen under physiological condition in cattle. In the current study, multiple embryos were applied in order to amplify the signals from the embryos so that changes in gene expression in BOECs and BUECs can reach up to the detectable level. One good

example is that, in superovulation and embryo transfer program, multiple embryos are present in the oviduct and uterus until D7 of pregnancy in cattle without producing any abnormal response in maternal tract. Therefore, it is likely that the multiple embryos did not exhibit any abnormal response in BOECs and BUECs in the present study. It should be noted that multiple embryos are also used by others to study embryo-maternal interactions in cattle both *in vitro* and *in vivo* conditions [40, 50, 61].

The early cleavage-stage embryo expresses MHC I transcript and thus it could be considered as foreign to the bovine oviduct. Therefore, in chapter I, the present study hypothesized that the early developing embryo may induce an anti-inflammatory response in the bovine oviduct for immunological acceptance. Results of the present study reveal that the oviduct epithelium somehow recognizes the early developing embryo and generates an anti-inflammatory response to the embryo *via* suppression of NFkB system and up-regulation of PGE2 synthesis. The mechanism by which the embryo is recognized by epithelial cells in the bovine oviduct is not known. Further, the present study found that the embryo-BOEC interaction *via* CM modulates gene expression of immune cells towards anti-inflammatory response with stimulation of *ISGs*, even though embryo did not induce *ISGs* in epithelial cells. These findings suggest that the developing embryo in the oviduct (D0-D4) starts to secrete a very small amount of IFNT, which is recognized by the immune cells but not by the epithelial cells. The possible reason is that the PBMCs are more responsive to small IFNT-signaling with compared to oviduct epithelial cells.

The embryo alone *via* CM did not influence *ISGs* in PBMCs, although CM from embryo-BOEC co-culture clearly stimulated *ISGs* in PBMCs, suggesting that CM of embryo culture alone did not contain IFNT or amount was less than that of CM of embryo-BOEC co-culture. These results clearly indicate a pivotal role of BOECs to enhance IFNT secretion from the developing embryo in the bovine oviduct. The changes in other immune-related gene expressions in the PBMCs were also not observed by CM from embryo culture alone, suggesting that a possible involvement of IFNT for regulation of gene expression of immune cells in the bovine oviduct.

It is well known that the bovine blastocyst expresses paternal antigen of MHC molecule I and thus it can be considered as foreign to the uterus, immediately after its arrival from the oviduct following insemination or after an embryo transfer on D7 in cows. The IFNT has been detected in the bovine morulae and blastocysts which have anti-proliferative, anti-luteolytic and immunosuppressive properties. Thus, in chapter II, the present study hypothesized that the developing bovine embryo, in the first four days in the uterus (D5-D9), may induce an anti-inflammatory response in the epithelial cells and also in immune cells. The IFNT from early developing embryo during the same period may be involved for induction of this local anti-inflammatory response in the uterus. The results revealed that morula to blastocyst embryo secretes a considerable amount of IFNT, which is recognized by the uterine epithelial cells, and epithelial cells generate a clear anti-inflammatory response to the developing embryo. Also, embryo alone without a help of uterine epithelial cells regulates gene expression of immune cells towards anti-inflammatory action, with the activation of *ISGs*. Finally, the current study examined the direct effect of IFNT on BUECs and PBMCs and found that IFNT induced a similar response in gene expression of BUECs and PBMCs as with embryo. These findings clearly indicate the involvement of IFNT, as one of the intermediators from embryo, for modulation of local immune response in the uterus during very early stage of pregnancy in cows.

In conclusion, findings of the present study support the hypothesis (Fig. 4-1) that developing embryo in the bovine oviduct (D0-D4) starts to secrete a small amount of IFNT and induces a slight anti-inflammatory response in epithelial cells, without *ISGs* stimulation. The interaction between the embryo and epithelial cells stimulates an anti-inflammatory response in immune cells with activation of *ISGs*, while embryo alone ‘‘cannot’’ regulate gene expression of immune cells. A very small amount of IFNT might be involved in modulation of such ‘‘local’’ immune response the bovine oviduct. On the other hand, developing embryo, in the first four days in the bovine uterus (D5-D9), induces relatively a strong anti-inflammatory response in uterine epithelial cells with *ISGs* stimulation. Also, the developing embryo during same period of development can ‘‘directly’’ modulate gene expression of immune cells towards an anti-inflammatory action without a help of

uterine epithelial cells. The IFNT is likely to act as one of the intermediators from early embryo for modulation of ‘‘local’’ immune response in the bovine uterus.

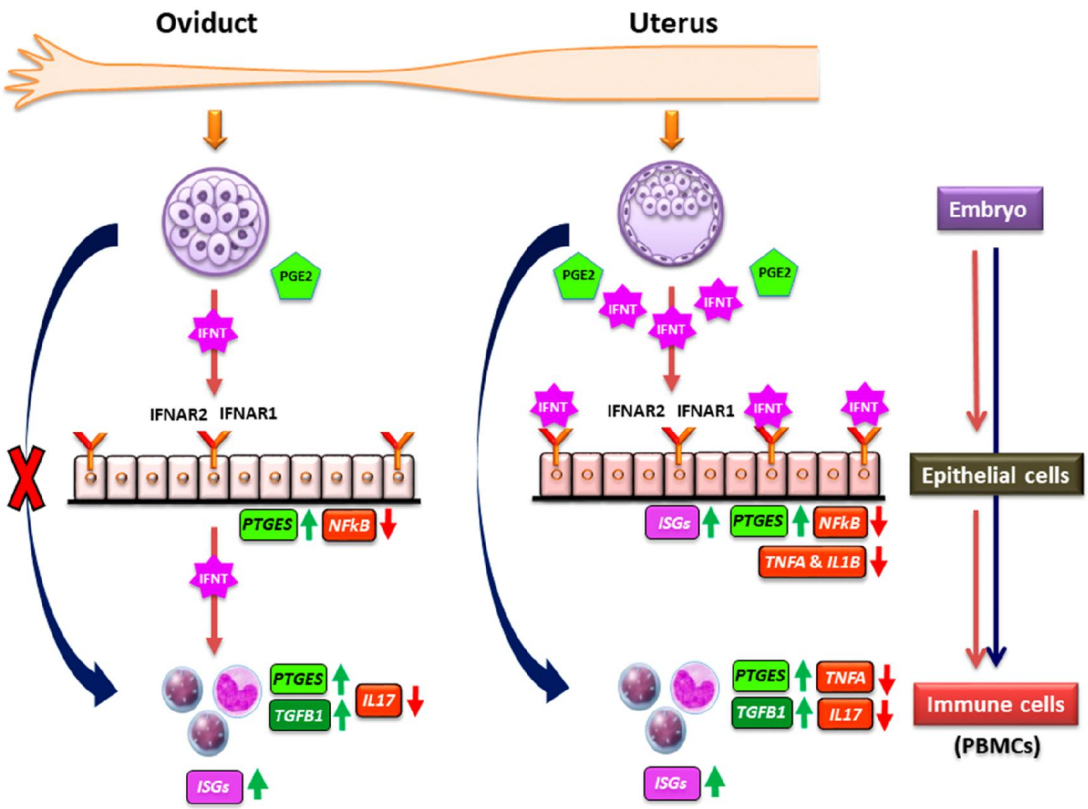

Fig. 4-1. Hypothetical representation of communication of the early developing embryo with the epithelial cells and immune cells (PBMCs) in the bovine oviduct and uterus.

Summary

The mucosal immune system of the female reproductive tract (FRT) plays dual roles by accepting allogenic spermatozoa and semi-allogenic embryo, whilst it provides protection against pathogens. Following fertilization, the bovine embryo stays in the oviduct for about 4 days and enters into the uterus at approximately the 16-cell to early morula stage. In the uterus, the embryo develops to blastocyst by D7 and hatches from the zona pellucida between D9 and D10 of pregnancy. Recent investigations suggest that the embryo-maternal communication starts in the bovine oviduct (D0-D4) and uterus immediately after arrival of morula until hatching of blastocyst (D5-D9). The early developing embryo expresses paternal antigen, and thus it could be considered as foreign to the bovine oviduct and uterus. However, the semi-allogenic embryo somehow escapes from attack by maternal immune system and establishes pregnancy. The molecular mechanism involved in acceptance of semi-allogenic embryo in the bovine oviduct and uterus, particularly at very early stage of pregnancy, remains mostly unknown. Interferon tau (IFNT) is an embryo-derived pregnancy recognition signal in cattle that inhibits the luteal regression and thereby maintains pregnancy. In addition, it has anti-proliferative and immunosuppressive properties. *IFNT* mRNA is expressed in 8-16-cell bovine embryo and its protein can be detected in late morula and non-hatched blastocyst on D7. Therefore, it is possible that a small amount of IFNT may regulate local immune response in the bovine oviduct and uterus at very early stage of pregnancy in cattle. All together, the present study aimed to investigate the effect of early developing embryo on the immune-related gene expression in bovine oviduct epithelial cells (BOECs) and uterine epithelial cells (BUECs) *in vitro*. Further, the current study examined the effect of embryo-BOECs and embryo-BUECs interaction, or embryo alone *via* conditioned media (CM) on gene expression in peripheral blood mononuclear cells (PBMCs).

In chapter I, BOECs were co-cultured with zygotes (n=25-30) and without zygotes (control) for D0-D4 (D0=IVF), and gene expression in BOECs and developing embryos was analyzed. ELISA was performed to determine IFNT concentration in CM from embryo-BOEC co-culture. Subsequently, PBMCs were cultured in CM from embryo-BOEC co-culture or BOEC culture (control), and gene

expression was evaluated. Next, zygotes (n=25-30) were cultured alone without BOECs for D0-D4. At the same time, fresh medium was also incubated for D0-D4. PBMCs were cultured in CM from embryo culture or in CM without embryos (control), and gene expression was evaluated. In BOECs, the developing embryos did not induce interferon (IFN)-stimulated genes (*ISGs*: *ISG15*, *OAS1* and *MX2*), a key factor for IFN-signaling (*STAT1*), type-1 IFN receptors (*IFNAR1* and *IFNAR2*), but stimulated *PTGES* (an enzyme for PGE2 synthesis) and suppressed *NFkB2* and *NFkBIA* (key factors for inflammatory and immune response). Interestingly, in PBMCs, CM from embryo-BOEC co-culture stimulated *ISGs*, *STAT1*, *PTGES* and *TGFB1* (Th2 cytokine), but suppressed *IL17* (Th17 cytokine). In contrast, CM from D0-D4 embryo culture alone did not influence *ISGs* and other immune-related genes expression in PBMCs. *IFNT* and *PTGES* were expressed in the 16-32 cell embryos developed on BOEC monolayer at the end of culture. It was not possible to determine IFNT concentration in CM of embryo-BOEC co-culture.

In chapter II, BUECs were co-cultured with morulae (n=10) and without morulae (control) for D5-D9 (D0=IVF), and gene expression in BUECs was analyzed. ELISA was performed to determine PGE2 and IFNT concentration in CM from embryo-BUEC co-culture. Further, PBMCs were cultured in CM from embryo-BUEC co-culture or BUEC culture (control), and gene expression was evaluated. Next, morulae (n=10) were cultured alone without BUECs for D5-D9. Simultaneously, fresh medium was also incubated for D5-D9. PBMCs were cultured in CM from embryo culture or in CM without embryos (control), and gene expression was analyzed. Finally, BUECs and PBMCs were treated with IFNT (100 pg/ml) for analysis of gene expression. In BUECs, the developing embryos induced *ISGs*, *STAT1*, *IFNAR1* and *IFNAR2*, with suppression of *NFkB2*, *NFkBIA* and pro-inflammatory cytokines (*TNFA* and *IL1B*). The embryos also stimulated *PTGES* in BUECs. In accordance with gene expression, the developing embryos stimulated PGE2 secretion by 20-fold from BUECs with compared to control; however, it was not possible to determine IFNT concentration in CM of embryo-BUEC co-culture. In PBMCs, both CM from embryo-BUEC co-culture and embryo culture alone induced *ISGs*, *STAT1* and *TGFB1*, while suppressing *TNFA* and *IL17*. Similar to the embryo, IFNT at

100 pg/ml suppressed *NFkB2*, *TNFA* and *IL1B* in BUECs, and also stimulated *TGFB1* and suppressed *TNFA* in PBMCs.

Taken together, the results suggest that the early cleavage-stage embryo starts to secrete IFNT in the bovine oviduct, which is recognized by the immune cells. The developing embryo induces an anti-inflammatory response in the oviduct epithelial cells without stimulation of *ISGs*; however, the embryo alone cannot regulate gene expression of immune cells. An interaction between the developing embryo and BOECs modulates gene expression of immune cells (PBMCs) towards an anti-inflammatory action with activation of *ISGs*. On the other hand, the 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 with activation of *ISGs*. In addition, the developing embryo during this period of development is capable of regulating gene expression of the immune cells towards suppression in the bovine uterus. A small amount of IFNT form the early developing embryo is likely to be involved in modulation of such "local" immune response in the bovine oviduct and uterus.

Finally, the present study has provided basic information for understanding the molecular crosstalk between the early developing embryo and the immune cells in the bovine oviduct and uterus. The findings of this study could be useful to make further plan, such as intra-uterine infusion of small amount of IFNT at the time of embryo transfer on D7, for ensuring a favorable immune-environment in the uterus to facilitate acceptance of a semi-allogenic embryo by the maternal immune system of cow. This could be one of the possible ways for improvement of the fertility and productivity in dairy cows. It should also be mentioned that the pathogenic infections (diseases) and physiological stress for example heat stress and any other stress inside the herd may disrupt the physiological immune response of the FRT to the developing embryo during very early stages of pregnancy in cows. Thus, appropriate hygiene should be strictly followed in the herd to maximize the fertility and production of the cows.

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要　旨

雌性生殖器内側の粘膜免疫系は、異質遺伝子型である精子や半異質遺伝子型である受精卵を受け入れる一方で、病原体に対しては防御するという、両方の役割を持つ。卵管での受精後、ウシ初期胚は発育しながら卵管内に４日間程度留まり、５日目に 16 細胞期胚あるいは桑実胚として子宮内に入る。初期胚は７日目に胚盤胞となり９日目以降に透明帯から脱出する。これらの初期胚は父側の抗原を持つことが知られているが、卵管と子宮内では母体側の免疫システムの攻撃は受けずに寛容され、受胎に至る。ウシにおいて上述の受精後の７日間の初期胚と卵管・子宮との免疫調節に関わるクロストークについてはほとんど解っていない。本研究では、ウシ初期胚と卵管あるいは子宮上皮細胞の共培養系を用いて、発育する初期胚がそれぞれの上皮細胞の免疫応答に関わる遺伝子発現に及ぼす影響を調べることを目的とした。さらに、共培養した培養上清で単離した血中単核球（PBMC）を培養して、その遺伝子発現への影響も調べた。

第１章では、ウシ卵管上皮細胞（BOEC）の初代培養系で、体外受精直後のウシ受精卵を４日間共培養して 16 細胞期−桑実胚に発生させ、BOEC 遺伝子発現を real-time PCR で調べた。その結果、４日目までの初期胚の存在は PGE2 合成酵素（*PTGES*）遺伝子発現を刺激し、一方で、炎症反応の調節因子である *NFkB2* 遺伝子を抑制した。インターフェロンτ（IFNT）は胚盤胞以降の栄養膜細胞から分泌され、子宮内の母体側の妊娠認識に関わるが、IFNT で刺激される遺伝子群発現に変化はなかった。ところが、初期胚と BOEC を共培養した培養上清で PBMC を培養したところ、IFNT で刺激される遺伝子（*ISG15, OAS1, MX2*）および IFNT シグナル遺伝子（*STAT1*）、さらには、寛容に働く Th2 サイトカイン（*TGFB1*）遺伝子が刺激され、炎症性サイトカインである *IL17* 遺伝子を抑制していた。以上の結果から、16-32 細胞期の初期胚は既に微量の IFNT を卵管内で分泌し始めており、PBMC はそれを察知して寛容に誘導される可能性が示唆された。

第２章では、体外受精で得たウシ受精卵を桑実胚まで体外発生させ、それらを子宮上皮細胞（BUEC）の単層培養系で４日間共培養し、胚盤胞まで発生させた。その後、BUEC 遺伝子発現を real-time PCR で調べた。その結果、５日−９日目までの初期胚の存在は *PTGES* と IFNT で刺激される遺伝子（*ISG15, OAS1, MX2*）および IFNT シグナル遺伝子（*STAT1*）、IFNT 受容体遺伝子（*IFNAR1, IFNAR2*）遺伝子が刺激されていた。一方で、炎症反応の調節因子である *NFkB2*、炎症性サイトカインである *TNFA* と *IL1B* 遺伝子を抑制していた。さらに、５日−９日目の初期胚の共培養上清は、PBMC の遺伝子発現を、IFNT で刺激される遺伝子群、寛容に作用する *TGFB1* 遺伝子を強く刺激し、一方で、炎症性の *TNFA* と *IL17* 遺伝子を抑制していた。重要なことに、低濃度のウシ組み換え IFNT（100 pg/ml）は、BUEC と PBMC の双方において、上述のような初期胚と同様

の遺伝子変化を誘導した。以上の結果から、初期胚が卵管から子宮に移動した最初の４日間の時期でも、子宮は既に初期胚を認識し免疫細胞ともに寛容型に変化すること、初期胚からの IFNT はそのクロストークの重要な因子の１つであることが示唆された。

以上の一連の知見から、ウシ卵管と子宮の上皮細胞は、受精後の初期胚を認識するシステムを備えており、基本的には初期胚を寛容する局所免疫環境を整えることが伺われた。さらに、初期胚から分泌される微量の IFNT が、その重要なコミュニケーション因子の１つであることが初めて示された。したがって、本研究の知見は、ウシの超初期妊娠に関わる卵管・子宮が備える初期胚の認識システムの一部を明らかにし、高泌乳牛の健康と繁殖の基礎概念の構築に大きく貢献するものと考えられた。