EFFECTS OF NUTRITIONAL CONDITION OR ESTROUS CYCLE ON GMCSF AND MIF EXPRESSION IN BOVINE OVIDUCTS

栄養状態や性周期がウシ卵管での GMCSF や MIF の発現量に与える効果

The United Graduate School of Veterinary Science

Yamaguchi University

Asrafun NAHAR

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EFFECTS OF NUTRITIONAL CONDITION OR ESTROUS CYCLE ON GMCSF AND MIF EXPRESSION IN BOVINE OVIDUCTS

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ASRAFUN NAHAR

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Abstract

Obese heifers produce fewer excellent grade embryos than lean and normal heifers due to unknown mechanisms. In order to clarify mechanisms, this thesis focused 2 important proteins granulocyte macrophage colony-stimulating factor (GMCSF) and macrophage migration inhibitory factor (MIF) in bovine oviducts, because these recent discovered proteins have functions as promoter of fertilization and embryogenesis and as chemokine. Both GMCSF and MIF promote glucose transport by murine preimplantation embryos. Although glucose is an important energy source for preimplantation embryos, the presence of excess glucose is detrimental for the embryos during the early cleavage stages. Therefore, if the energy source is in excess in the maternal body, GMCSF or MIF expression in the oviduct may be downregulated to protect the embryo.

In Study 1, I hypothesized that GMCSF expression may be downregulated in the oviducts of obese cows compared to oviducts of normal cows. I collected ampullary or isthmic section of oviducts from lean [n = 5; body condition score (BCS) on a 5-point scale, 2.5], normal (n = 6; BCS, 3.0), and obese (n = 5; BCS, 4.0) Japanese Black cows and confirmed the expression of GMCSF mRNA and protein by real-time PCR, western blotting, and immunohistochemistry. I found that GMCSF mRNA and protein expression in the ampulla were lower ($P < 0.05$) in the obese group than in the normal group. Both mRNA and protein expression of GMCSF did not differ significantly in the isthmus among the 3 groups. Also the obese group showed weaker GMCSF immunoreactivities in the tunica mucosa of the ampulla than the normal and lean groups. Therefore, GMCSF may be a key molecular link between maternal nutritional status and early embryogenesis in the oviduct.

Oviduct may have another molecular link, because genetically GMCSF deficient female mice produce normal size litter. MIF may be such a molecule as promoter for sperm, ovum, and early embryos in bovine oviducts according to the maternal nutritional condition. Therefore, in Study 2, I evaluated whether MIF expression level downregulated in the oviducts of obese cows as compared to lean and normal cows. MIF mRNA and protein in ampullae or isthmuses were measured by real-time PCR or western blot. I found that MIF mRNA and protein expression were lower in both ampulla and isthmus parts of oviducts in the obese and lean groups than in the normal group ($P < 0.05$). Also immunohistochemistry revealed that the obese and lean groups had weaker MIF immunoreactivities in the tunica mucosa than the normal group. Therefore, MIF may be another key molecular link between maternal nutritional status and early embryogenesis in the oviduct.

In order to estimate importance of GMCSF or MIF in oviducts for early embryogenesis further, I evaluated a hypothesis that expression of GMCSF or MIF in oviducts are increased during post-ovulation phase when fertilized ovum are present in oviducts in Studies 3 and 4. I conducted a study to evaluate the GMCSF and MIF expression level during estrus (Day 0, $n = 5$), post-ovulation (Day 3, $n = 6$), and luteal (Days 9 to 12, $n = 5$) phases. The mRNA and protein expression of GMCSF or MIF in the ampullar and isthmic samples were measured by real-time PCR and western blot, respectively. GMCSF mRNA and protein expression levels were higher during estrus and post-ovulation phases than the luteal phase ($p < 0.05$). MIF mRNA and protein expression were higher in the post-ovulation phase than during the estrus and luteal phase ($P < 0.05$). Fluorescent immunohistochemistry confirmed that both GMCSF and MIF

mainly expressed in the tunica mucosa during all the evaluated phases. Therefore, the results of Studies 3 and 4 suggested the importance of GMCSF or MIF in oviducts for early embryogenesis also *in vivo*.

High blood insulin concentration was observed in the obese heifers produced fewer excellent-grade embryos than those observed in lean and normal heifers. Insulin control GMCSF and MIF expression in myocytes and adipocytes. Therefore, in Studies 5 and 6, I conducted a study to evaluate whether insulin control GMCSF or MIF expression in bovine oviduct epithelial cells (BOECs) in *in vitro*. In first, I confirmed expression of insulin receptor in the tunica mucosa by fluorescent immunohistochemistry. In next, I prepared epithelial cells of the tunica mucosa of ampullar and isthmic sections from Japanese Black heifers. Then, I cultured BOECs and confirmed expression of insulin receptor in the cultured BOECs. Then, I added insulin as final concentration of 0, 1, 20, or 5000 ng/mL in order to culture for 24 hours. Both mRNA and protein expression of GMCSF and MIF were measured by real-time PCR and western blot, respectively. Both mRNA and protein expression of GMCSF were higher in ampulla or isthmus BOECs treated with 20 ng/mL insulin than BOECs treated with 0, 1, or 5000 ng/mL ($P < 0.05$). Both mRNA and protein expression of MIF were higher in ampulla or isthmus BOECs treated with 20 ng/mL than BOECs treated with 0, and 5000 ng/mL ($P < 0.05$). Therefore, middle level insulin stimulate both GMCSF and MIF expression in oviducts whereas low and high level of insulin can stimulate only weakly, suggesting this may be the central mechanism to control both GMCSF and MIF expression in oviducts *in vivo*.

In conclusion, GMCSF and MIF expression is increased in bovine oviducts during the post-ovulation phase when embryo presents in oviduct, but GMCSF and MIF

expression is suppressed in the obese cows compared to the normal or lean cows probably because of high insulin concentration in blood.

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List of Abbreviations

ABI : Applied biosystems
ANOVA : Analysis of variance
BCS : Body condition score
BMI : Body mass index
BOECs : Bovine oviduct epithelial cells
BSA : Bovine serum albumin
cDNA : Complementary deoxyribonucleic acid
C : Celsius
C2orf29 : Chromosome 2 open reading frame 29
CCD : Charge-coupled device
CP : Crude protein
CO2 : Carbon dioxide
DAB : Diaminobenzidine
DAPI : 4',6-diamidino-2-phenylindole
DIC : Differential interference contrast
DM : Dry matter
DMEM : Dulbecco's modified eagle's medium
DNA : Deoxyribonucleic acid
ECL-Prime : Enhanced chemiluminescence-prime
EDTA : Ethylenediaminetetraacetic acid
FBS : Fetal bovine serum
GLUT1 : Glucose transporter 1
GMCSF : Granulocyte macrophage colony-stimulating factor
FSH : Follicle stimulating hormone
h : Hour(s)
HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP : Horseradish peroxidase
Hsph1 : Heat shock 105/110 kDa protein 1
IgG : Immunoglobulin G

IGF- 1 : Insulin like growth factor-1

InsR : Insulin receptor

IU : International Units

kg : Kilogram (s)

L : Liter (s)

LH : Luteinizing hormone

Ldlr : Low density lipoprotein receptor

Mcal : Megacalorie

ME : Metabolizable energy

MIF : Macrophage migration inhibitory factor

min : Minute (s)

mL : Mililiter (s)

mM : Millimolar

mRNA : Messenger ribonucleic acid

n : Number

NCBI : National Center for Biotechnology Information

NaCl : Sodium chloride

ng : Nanogram(s)

nM : Nanomolar

NEFA : Nonesterified fatty acids

PBS : Phosphate buffered saline

PCR : Polymerase chain reaction

PFA : Paraformaldehyde

PLSD : Protected least significant difference

Post-OV : Post-ovulation

PVDF : Polyvinylidene fluoride

RNA : Ribonucleic acid

SEM : Standard error of mean

SUZ12 : Suppressor of zeste 12

Slc2a1 : Solute carrier family 2, facilitated glucose transporter member 1

T-PER : Tissue protein extraction reagent

S	: Second
µL	: Microliter
µg	: Microgram
µm	: Micrometer

CHAPTER I

General Introduction

1.1. Background information

Obese women, particularly those with central obesity, have decreased pregnancy rates after insemination (Brewer and Balen, 2010; Norman, 2010). In previous field surveys, estimating the relationship between embryo production and body condition score (BCS), about 8% of all Holstein heifers were obese even in a free-stall barn with stanchions to avoid social status factors preventing some heifers from having enough feed (Kadokawa et al., 2008). Furthermore, the obese heifers produced fewer excellent-grade embryos than lean and normal heifers due to unknown mechanisms (Kadokawa et al., 2008).

The oviduct is the first site of maternal body contact with the early embryo. Oviduct plays important roles to accomplish gamete transport, fertilization and embryo development in a timely manner and to provide a healthy embryo to the uterus (Kolle et al., 2009; Besenfelder et al., 2012). For the early-stage embryo, the oviduct seems to supply any growth factor as well as nutrients via oviductal fluid (Hugentobler et al., 2010; Besenfelder et al., 2012).

There are strong lines of evidence to implicate granulocyte macrophage colony-stimulating factor (GMCSF) as a physiologically important promoter of early embryonic development, although this protein was originally identified as a product of activated T lymphocytes. GMCSF receptor mRNA is present in the fertilized oocyte and

at all subsequent stages of embryonic development (Robertson et al., 2001). GMCSF addition into culture medium improves the proportion of *in vitro* fertilized embryos developing to the blastocyst stage in cows, pigs, mice, and humans (de Moraes and Hansen, 1997; Sjoblom et al., 1999; Sjoblom et al., 2005; Neira et al., 2010; Kwak et al., 2012). Total cell numbers of blastocyst reduces in the $GMCSF^{-/-}$ mice in comparison with wild type control mice (Robertson et al., 2001). Treatment with GMCSF reduces the repetitive implantation failures in human (Würfel, 2015). Thus, GMCSF is very important for early stage embryo, and may be act as the molecular link between maternal nutritional status and early embryogenesis in the oviduct.

However, genetically GMCSF-deficient female mice produce litters of normal size (Hamilton et al., 2012) and suggest that oviduct may have another specific molecule to promote functions for spermatozoa, ovum and early-stage embryos. Macrophage migration inhibitory factor (MIF) may be such a molecule and is expressed in the ampullar and isthmic parts of the oviducts of mice and ewes (Suzuki et al., 1996; Lopes et al., 2011), as well as in ovary, testis and uterus (Meinhardt et al., 1998; Lopes et al., 2011; Paulesu et al., 2012). MIF at the optimal concentration promotes sperm capacitation, whereas low or excess MIF is inhibitory (Carli et al., 2007). Furthermore, MIF mRNA is expressed in ovulated oocytes, zygotes, two-cell embryos, eight-cell

embryos and blastocysts in mice (Suzuki et al., 1996). Important roles of MIF in establishing pregnancy have been reported in both upstream (ovary; Matsuura et al., 2002) and downstream (uterine; Vigano` et al., 2007; Bevilacqua et al., 2014) of the oviduct. Therefore, MIF may be another key molecular link between maternal nutritional status and early embryogenesis in the oviduct.

However, little is known about the level of expression of GMCSF or MIF at different stages of estrous cycle. The oviduct environment, where the embryos of most mammalian species spend their first 3 days, is known to affect the physiology and metabolism of the embryo (Leese, 1995). Therefore, the oviduct modifies its function at different phases of the estrous cycle in order to meet the demand of the growing embryos (Murray, 1995; Buhi et al., 2000). The oviduct function changes throughout the estrous cycle, and its optimum functions are regulated by the hormonal modifications in the oviduct (Wijayagunawardane et al., 1998; Buhi et al., 2000). Furthermore, estradiol is a very important factor in inducing the dramatic cyclic changes that occur in the epithelial lining and in the secretory status of the oviduct (Murray et al., 1995). GMCSF expression level is different in the uterine cells harvested at different times during the estrous cycle, and GMCSF expression peaks from the time of estrus to the time of implantation in mouse endometrium (Robertson and Seamark, 1992;

Robertson et al., 1996). Also estradiol enhances the production of GMCSF in human keratinocytes (Kanda and Watanabe, 2004). As for MIF, estradiol regulates the secretory pathway in the first-trimester during pregnancy in humans (Ietta et al., 2010). Additionally, estrogen decreases MIF production in the female rat colon (Houdeau et al., 2007). MIF expression is different in the human and mouse endometrium at different phases of estrous cycle (Suzuki et al., 1996; Kats et al., 2005). Therefore, GMCSF and MIF expression may also be cycle-dependent in bovine oviducts.

Nutrition is another important factor to control oviduct functions (Mburu et al., 1998; Novak et al., 2002), but details of the molecular mechanisms remain unclear. Insulin is a key metabolic hormone that plays a crucial role in regulating energy homeostasis in the body (Hu et al., 2016), especially controlling blood glucose level and various cell functions. During embryogenesis, glucose is an important energy source (Herrick et al., 2006; Sutton-McDowall et al., 2010). The optimum level of glucose is important in determining the maturation rate of bovine oocytes as well as their ability to grow after fertilization (Kim et al., 1993). Excessive glucose, on the other hand, is detrimental to the maturation of bovine oocytes and the development of early-stage embryos (Hashimoto et al., 2000; Kumar et al., 2012). GMCSF promotes glucose transport in pre-implantation embryos of mice *in vitro* (Robertson et al., 2001), as well

as in human sperm (Zambrano et al., 2001). Also MIF plays an important role in promoting glucose metabolism in muscle and adipose tissue (Benigni et al., 2000; Toso et al., 2008). Therefore, if energy sources are in excess in the maternal body, GMCSF or MIF gene expression in the oviduct may be downregulated in order to protect the embryo by decreasing glucose supply.

Therefore, the studies 1 and 2 evaluated the hypothesis that the obese cow's oviducts may express lower levels of GMCSF or MIF than normal and lean cows. The next studies 3 and 4 evaluated the hypothesis that the GMCSF and MIF expression is higher during the post-ovulation phase than estrus and luteal phases. High insulin concentration was observed in the obese heifers produced fewer excellent-grade embryos (Kadokawa et al., 2008). Thus, the studies 5 and 6 evaluated the hypothesis that insulin control GMCSF and MIF expression and the higher insulin level may decrease GMCSF or MIF expression level in the bovine oviduct epithelial cells (BOECs).

1.2. Objectives of the study

Based on the above background information, this thesis study was conducted with the following main objectives:

i. To determine the relationship between the body condition and the GMCSF expression in the oviducts of lean, normal and obese cows.
ii. To determine the relationship between the body condition and the MIF expression in the oviducts of lean, normal and obese cows.
iii. To determine the GMCSF expression level at the different phases of estrous cycle in the bovine oviducts.
iv. To determine the MIF expression level at the different phases of estrous cycle in the bovine oviducts.
v. To determine the effect of insulin on the GMCSF expression level in the bovine oviduct epithelial cells (BOECs)
vi. To determine the effect of insulin on the MIF expression level in the BOECs

1.3. Contents of dissertation

This thesis consists of nine chapters. Chapter I (General Introduction) deals with the background information and main objectives of the study. In Chapter II, we have reviewed the literatures directly or indirectly related to our study. Chapter III (Study 1) clarified the expression of GMCSF in oviduct ampullae of lean, normal, and obese cows. In Chapter IV (study 2), we examined the expression of MIF in the oviducts of lean, normal, and obese cows. In Chapter V (study 3), we examined the expression of GMCSF in bovine oviducts in the estrus, post-ovulation, and luteal phases. Chapter VI (study 4) evaluated the expression of MIF in bovine oviducts in the estrus, post-ovulation, and luteal phases. Chapter VII (study 5) and Chapter VIII (study 6) clarified the effect of insulin on the GMCSF and MIF expression level in the BOECs, respectively. Chapter IX discussed the main findings of this thesis study and their implications.

CHAPTER II

Review of Literature

2.1. Problems of obesity for reproduction and pregnancy outcomes

Obesity has been associated with reduced fertility in females, particularly among those with central adiposity (Robker et al., 2009; Wise et al., 2010). Obesity is a significant cause of anovulatory infertility, and the infertility rate may increase by 4% per body mass index (BMI) unit (Wise et al., 2010). Central adiposity increases the risk of polycystic ovary syndrome and contributes to anovulation through insulin resistance, hyperinsulinemia, or hyperandrogenemia (Joham et al., 2016). Pre-pregnancy obesity status has been shown to increase the risk for many poor maternal and fetal outcomes including preterm birth, macrosomia, shoulder dystocia, select birth defects, and stillbirth (Avci et al., 2015).

Obese heifers have been found to produce fewer excellent-grade embryos than lean and normal heifers. In previous field surveys, estimating the relationship between embryo production and BCS, about 8% of all Holstein heifers were obese even in a free-stall barn with stanchions to avoid social status factors preventing some heifers from having enough feed (Kadokawa et al., 2008). Furthermore, the obese heifers produced fewer excellent-grade embryos than did lean and normal heifers after superovulation due to unknown mechanisms (Kadokawa et al., 2008).

2.2. Importance of oviduct for early embryogenesis

Oviduct is the site where the maternal body first contacts with the early embryo. Oviducts are stage for a series of important events, e.g., gamete maturation, capacitation, sperm selection and early embryo development, and all of these must precisely initiate and complete (Kolle et al., 2009; Besenfelder et al., 2012). Ovum and sperm enter the oviduct from opposite ends and fuse to form an embryo. To meet all these demands, the oviduct consists of the infundibulum, ampulla and isthmus, which consist of longitudinal and circular aligned muscle layers, endothelial ciliated and non-ciliated cells. The isthmus is the functional sperm reservoir, and ampulla is the site for acrosome reaction in ruminants (Hunter, 2005).

Oviductal fluid secreted mainly by the non-ciliated epithelial cells, and oviductal fluid may provide growth factors as well as nutrients for the early embryo (Cox and Leese, 1997; Yaniz et al., 2000; Gray et al., 2001). Oviducts modulate its local mechanisms at the molecular level during the presence of gametes and embryos, which represent the first exchange of signals between the maternal environment and embryo (Holt and Fazeli, 2010; Kolle et al., 2010). The oviduct exhibits an extraordinary flexibility that is hormonally driven and exactly timed according to the embryonic stage (Abe and Hoshi, 2008; Nakahari et al., 2011), and the epithelium is the layer directly

contact with ovum, sperm and embryo. During early embryo development, the embryo needs support by the oviduct to establish pregnancy.

2.3. Declining of embryo development rate and role of oviduct factors

The high embryonic loss may be the result of insufficient communication between the embryo and the maternal environment (Wolf et al., 2003; Fazeli, 2008). Oviducts control its function in order to provide optimal environment for embryogenesis (Buhi, 2002). Oviducts produce a number of factors, and embryos express their receptors (Kane et al., 1997; Lee and Yeung, 2006). Several studies have identified embryotrophic factors in the oviducts and roles for embryos during preimplantation period (Kane et al., 1997; McCauley et al., 2003; Lee et al., 2006). The factors present in oviductal fluid promote embryo development and quality by protecting them against adverse impacts on mitochondrial DNA transcription and apoptosis induced by the culture environment *in vitro* (Lloyd et al., 2009).

Therefore, we need to improve our knowledge for the oviduct environment and factors secreted by the oviduct

2.4. Importance of GMCSF for fertility and embryogenesis

GMCSF, also known as colony stimulating factor 2, was first identified in mouse lung tissue-conditioned medium by its ability to stimulate proliferation of mouse

bone marrow cells *in vitro* and generate colonies of both granulocytes and macrophages (Burgess et al., 1977). GMCSF can be produced by a wide variety of tissue types, including fibroblasts, endothelial cells, T cells, macrophages, mesothelial cells, epithelial cells, and many types of tumor cells (Griffin et al., 1990a). The biological activities of GMCSF are exerted through binding to heteromeric cell-surface receptors that are expressed on monocytes, macrophages, granulocytes, lymphocytes, endothelial cells, and alveolar epithelial cells (Griffin et al., 1990b).

GMCSF may be act as a physiologically important regulator of early embryogenesis. The first important role of GMCSF in oviduct is as a promoter of fertility and embryogenesis. GMCSF promotes sperm viability and motility (Rodrı´guez-Gil et al., 2007). The addition of GMCSF in the *in vitro* fertilization process improves the yield of implantation competent blastocysts in the cow (de Moraes and Hansen, 1997) and human (Sjöblom et al., 1999). Another important role of GMCSF in the oviduct is as a chemokine. GMCSF is the chemotactic for neutrophils (Gomez-Cambronero et al., 2003) and promotes the proliferation and maturation of myeloid progenitors during the host defense and inflammatory reactions (Hamilton and Anderson, 2004; Hamilton, 2008). The defense activity of the oviduct is very important, because the uterus and oviduct receive pathogens that are present in sperm and seminal

plasma (Profet, 1993; Kelly et al., 1997). Treatment with GMCSF reduces the repetitive implantation failures or recurrent spontaneous abortions in human (Würfel, 2015).

2.5. Importance of MIF for fertility and embryogenesis

MIF is a pleiotropic protein, participating in inflammatory and immune responses. Its identification as a soluble factor affecting cells movement was built upon the pioneering work in cell migration by Rich and Lewis (1932) and George and Vaughan (1962). MIF was initially reported as a T-lymphocyte derived mediator capable of preventing the migration of target cells during the delayed-type hypersensitivity response (Bloom and Bennet, 1966; David, 1966). However, recent studies have evaluated the expression of MIF in reproductive organs, and MIF's roles in human and animal reproductive physiopathology (Suzuki et al., 1996; Vigano et al., 2007).

The first important role of MIF in the oviduct is as promoter for sperm, ovum, and embryos. The intraperitoneal injection of recombinant MIF the day after mating enhances the pregnancy rate in mice (Bondza et al., 2008). MIF at the optimal concentration promotes sperm capacitation *in vitro*, whereas low or excess levels of MIF are inhibitory (Carli et al., 2007). Another important role of MIF in the oviduct is as a cytokine. MIF is known to promote the host's responses to infection and

contributes to cell-mediated immunity (Weiser et al., 1991; Calandra et al., 1998; Bucala and Shachar, 2014). Immune responses of the oviduct are very important, because oviducts can get pathogens that are present in the sperm and seminal plasma (Profet, 1993; Kelly et al., 1997; Quale, 2002).

2.6. Role of estrogen for controlling GMCSF and MIF expression

During the estrus cycle, the oviductal epithelium releases various biomolecules to the lumen to promote embryo development. This secretory activity of the oviduct is regulated by steroid hormones and also modulated by gametes and embryos. Estradiol is a very important factor to induce the dramatic cyclic changes in the epithelial lining and the secretory status of the oviduct (Murray et al., 1995).

As for GMCSF, estradiol enhances GMCSF production in the human keratinocytes (Kanda and Watanabe, 2004). Also endometrial GMCSF expression is increased three times in ovariectomized ewes treated with estradiol than that of non-treated ovariectomized ewes (McGuire et al., 2002). GMCSF expression level is different in the uterine cells harvested at different times during the estrous cycle, and peaks from the time of estrus to the time of implantation in mouse endometrium (Robertson and Seamark, 1992; Robertson et al., 1996). Endometrial cells derived from ovariectomized mice produce 25-fold less GMCSF than did cells from estrous mice;

and pretreatement with estrogen increases GMCSF production in ovariectomized mice while combination with progesterone have a moderate inhibitory effect on GMCSF (Robertson et al., 1996).

As for MIF, estrogen decreases MIF production in colon of female rats (Houdeau et al., 2007), and estrogen decreases MIF expression to promote wound healing in humans and mice (Ashcroft et al., 2003; Hardman et al., 2005). In humans, the concentration of estradiol in the plasma is negatively correlated with the concentration of MIF in the plasma (Aloisi et al., 2005). Furthermore, estradiol downregulates the MIF secretory pathway in the human placenta (Ietta et al., 2010).

Receptors for estrogen are present in the mucosal cells of the human oviduct (Lam et al., 2005), and it is well known that estradiol secreted from the ovary is transferred from the ovarian vein to the oviduct artery via a counter-current transfer system (Hunter, 2005). Therefore, GMCSF or MIF expression in the oviduct may be controlled by the level of estrogen secreted from the ovaries, especially, high levels of estradiol during estrus may enhance GMCSF or suppress MIF expression in the bovine oviduct.

2.7. Importance of insulin for embryogenesis

Under physiological conditions, hormones transported by the blood reach the oviductal epithelium from the basolateral surface and regulate oviductal cell functions (Hunter, 2012). Insulin plays a key role in energy storage and cellular growth and differentiation (Cheatham and Kahn, 1995). It represents an essential component of many mammalian cell culture media and stimulates amino-acid transport (Kaye et al., 1986), protein synthesis (Harvey and Kaye, 1988). Insulin's effect is mediated via insulin receptors on cell surface (Harvey and Kaye 1990a). The level of insulin receptor of oviduct cells is regulated by steroid and peptide hormones in quail *in vitro* (Kato et al., 1990).

2.8. Relationship between insulin and GMCSF or MIF

Glucose homeostasis in the body depends upon the balance between glucose production and glucose utilization by the major insulin-dependent tissues, such as liver, adipose, and muscle, and by insulin-independent tissues, such as brain. Insulin stimulates glucose transport into peripheral tissues and inhibits hepatic gluconeogenesis (Cheatham and Kahn, 1995).

Glucose is an energy source for the embryo, but the presence of glucose at the concentrations found in maternal serum is detrimental for the embryos of several

species, including cows, during the early cleavage stages (Gutiérrez-Adán et al., 2001; Larson et al., 2001; Peippo et al., 2001). Recently, Bermejo-Alvarez et al. (2012) reported that maternal obesity causes the downregulation of both glucose transporter types 1 (Slc2a1) and low density lipoprotein receptor (Ldlr) in blastocysts recovered from diet-induced obese female mice. Insulin stimulates MIF expression in myocytes (Benigni et al., 2000) and adipocytes (Sakaue et al., 1999; Atsumi et al., 2007), and MIF inhibits glucose uptake by these cells. Insulin-resistant adipocytes show decreased MIF expression even in the presence of excess insulin (Sakaue et al., 1999). Therefore, insulin may be a molecular link between maternal nutritional condition and oviduct function.

CHAPTER III

(Study 1)

Expression of GMCSF in oviduct of lean, normal, and obese cows

Abstract

Obese heifers produce fewer excellent-grade embryos than lean and normal heifers due to unknown mechanisms. Oviducts synthesize GMCSF to promote embryogenesis, and GMCSF expression may be downregulated in the oviducts of obese cows. This study evaluated the relationship between the degree of obesity and GMCSF expression in the ampullary or isthmic section of oviducts utilizing lean (n = 5; BCS on a 5-point scale, 2.5), normal (n = 6; BCS, 3.0), and obese (n = 5; BCS, 4.0) Japanese Black cows. GMCSF mRNA and protein expression in the ampulla, measured by real-time PCR and western blotting, respectively, were lower ($P < 0.05$) in the obese group than in the normal group. Both mRNA and protein expression of GMCSF did not differ significantly in the isthmus among the 3 groups. The obese group showed weaker GMCSF immunoreactivities in the tunica mucosa, the primary site of GMCSF expression, of the ampulla than the normal and lean groups. In conclusion, unlike normal and lean cows, obese cows had suppressed GMCSF expression in the ampulla.

3.1. Introduction

Obese women, particularly those with central obesity, have decreased pregnancy rates after insemination (Brewer and Balen, 2010; Norman, 2010; Robker, 2008). In our previous field survey estimating the relationship between embryo production and body condition score (BCS), about 8% of all Holstein heifers were obese even in a free-stall barn with stanchions to avoid social status preventing inferior heifers from having enough feed (Kadokawa et al., 2008). Furthermore, these obese Holstein heifers produced fewer excellent-grade embryos after superovulation than did lean and normal heifers due to unknown mechanisms.

The oviduct is the first site of maternal body contact with the early embryo. In the oviduct, the ampullary section is an important site for the maturation of the oocyte, fertilization, and early embryogenesis. For the early-stage embryo, the oviduct supplies growth factors as well as nutrients via oviductal fluid (Besenfelder et al., 2012; Hugentobler et al., 2010).

There are strong lines of evidence to implicate GMCSF as a physiologically important regulator of early embryonic development. GMCSF receptor mRNA is present in the fertilized oocyte and at all subsequent stages of embryonic development (Robertson et al., 2001). GMCSF addition into culture medium improves the proportion of *in vitro* fertilized embryos developing to the blastocyst stage in cows, pigs, mice, and humans (de Moraes and Hansen, 1997; Kwak et al., 2012; Neira et al., 2010; Sjoblom et al., 1999, 2005). GMCSF also promotes glucose transport for human sperm (Zambrano et al., 2001) and murine preimplantation embryos (Robertson et al., 2001). Studies using *in situ* hybridization and immunohistochemistry reported that tunica mucosa in the ampullary and isthmic regions of the oviduct are the primary site of GMCSF expression

(de Moraes et al., 1999; Zhao and Chegini, 1994), with a greater intensity in the ampulla (de Moraes et al., 1999) and only a small amount of GMCSF in other sections of the oviducts (de Moraes et al., 1999; Zhao and Chegini, 1994). Thus, GMCSF may be the molecular linkage between maternal nutritional status and early embryogenesis in the oviduct.

Although glucose is an important energy source for preimplantation embryos, the presence of excess glucose is detrimental for the embryos during the early cleavage stages (Gutiérrez-Adán et al., 2001; Larson et al., 2001; Peippo et al., 2001). Therefore, if the energy source is in excess in the maternal body, GMCSF expression in the oviduct may be downregulated to protect the embryo. The present study aimed to evaluate the relationship between body condition and GMCSF expression in the oviducts of lean, normal, and obese cows.

3.2. Materials and methods

3.2.1. Animals and treatments

The experiments were performed in accordance with the Guiding Principles for the Care and Use of Experimental Animals in the Field of Physiological Sciences (Physiological Society of Japan) and approved by the Committee on Animal Experiments of the School of Veterinary Medicine, Yamaguchi University.

Multiparous Japanese Black cows (4 years old, calved 3 times, n = 16) were housed in a free-stall barn. Their calves were separated for weaning at 2 months after normal parturition. At the time of weaning, the cows' BCS on a 5-point scale (Ferguson et al., 1994) was 2.5, and mean body weight was 463 ± 2 kg (mean ± standard error of the mean [SEM]). The body weight required 11.2 Mcal of metabolizable energy (ME) as a maintenance level, according to the Japanese feeding standard (Agriculture, Forestry and Fisheries Research Council Secretariat, 2008). To create lean, normal, or obese cows, the cows were randomly allocated to 1 of the following 3 groups: (1) lean group (n = 5), which was fed 10.1 Mcal of ME (90% of the maintenance level) using Italian ryegrass hay (84.2% dry matter [DM]), 2.30 Mcal/kg DM of ME, 13.3% crude protein [CP]); (2) normal group (n = 6), which was fed 12.3 Mcal of ME (110% of the maintenance level) using the same Italian ryegrass hay plus concentrate (86.6% DM, 3.82 Mcal/kg DM of ME, 21.3% of CP); or (3) obese group (n = 5), which was fed 23.9 Mcal of ME (as the commonly used fattening level and as 213% of the maintenance level) using the same Italian ryegrass hay plus a greater amount of the same concentrate. After 6 months on these diets, the BCS of the lean group was 2.5 (body weight, 470 ± 2 kg); normal group, 3.0 (body weight, 501 ± 2 kg); and obese group, 4.0 (body weight, 563 ± 3 kg). Water and mineral blocks were provided ad libitum. Absence of disease,

including reproductive disease, was confirmed by daily observation or by weekly rectal palpation.

On the second day after ovulation induced by dinoprost (Pronalgon F, Pfizer, Tokyo, Japan), i.e., when oocytes are in the oviducts (el-Banna and Hafez, 1970), the cows were slaughtered. Oviducts on the ipsilateral side of ovulation were collected within 15 min of slaughter. The oviduct of Japanese Black cows is approximately 30 cm in length, and its anatomical structure is similar to those of other domestic animals (Hunter 2005). The length and diameter of the ampulla and isthmus are approximately 14 cm × 3 mm, and approximately 14 cm × 1.5 mm, respectively. We collected ampullar samples from areas at least 3 cm away from the fimbriated infundibulum as well as from the ampullary-isthmic junction. We collected the isthmus samples from areas also at least 3 cm away from the ampullary-isthmic junction as well as from the utero-tubal junction. The tissues surrounding the oviduct were removed carefully, and the oviducts were washed with phosphate-buffered saline (PBS). Half of the ampulla and half of the isthmus were frozen in liquid nitrogen and preserved at -80°C until RNA or protein extraction. The remaining halves of the ampulla and isthmus were stored in 4% paraformaldehyde (PFA) at 4°C for 12 h for immunohistochemistry studies.

3.2.2. RNA extraction, cDNA synthesis, and real-time PCR

DNA-free total RNA was extracted from the frozen ampulla and isthmus samples using an RNAiso Plus and the Ribonuclease-free Deoxyribonuclease Set (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. The RNA concentration and purity of each sample were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) at an acceptable 260/280 nm ratio of absorbance of 1.8–2.1. We synthesized cDNA from 2

μg RNA from each sample in 20-μL reactions with random hexamer primers using a high-capacity cDNA reverse transcription kit [Applied Biosystems (ABI), Foster City, CA, USA].

To prepare external standards as amplifying fragments of cDNA products containing target sequences for real-time PCR of GMCSF [National Center for Biotechnology Information (NCBI) reference sequences of bovine GMCSF are U22385.1] or 18S ribosomal RNA (18S rRNA; DQ222453.1), the PCR conditions were optimized by conventional PCR amplification using AmpliTaq Gold PCR Master Mix (ABI), 20 ng DNase-treated reverse-transcribed RNA, and primers. The presence of a single product was confirmed by electrophoresis on a 2% (w/v) agarose gel. The PCR-amplified products from the cDNA were purified using NucleoSpin Extract II columns (Takara Bio Inc.) to prepare external standards, as well as to verify the DNA sequence with a sequencer (ABI3130; ABI) using one of the PCR primers and the Dye Terminator v3.1 Cycle Sequencing Kit (ABI). The obtained sequences were used as query terms for homology searches in the DDBJ/GenBankTM/EBI Data Bank using the nucleotide basic local alignment search tool optimized for highly similar sequences (available on the NCBI website).

For real-time PCR, primers were designed by Primer Express Software v3.0 (ABI) based on the reference sequences. The forward and reverse primers of GMCSF were 5′-TGCAGGGCAGCCTCACTAG-3′ and 5′-TCGTAGTGGGTGGCCATCAT-3′, and the PCR-product size was 58 bp (258^{th}–315^{th} nucleotide of GMCSF). The primers of 18S rRNA were 5′-CCGCGGTTCTATTTTGTTGGT-3′ and 5′-CGGCCGCCCCTCTTAA-3′, and the PCR-product size was 57 bp (876^{th}–932^{nd} nucleotide of 18S rRNA protein). The mRNA expression levels of GMCSF or 18S

rRNA were measured in duplicate by real-time PCR analysis using 20 ng of cDNA, StepOne Real Time PCR System (ABI), and PowerSybr Green PCR Master Mix (ABI) with a 5-point relative standard curve and a non-template control. Series of 10-fold dilution standards were prepared using the purified, amplified fragments of DNA products. Because the real-time PCR system has only 48 wells, the ampulla and isthmus samples were analyzed separately. Temperature conditions for GMCSF were as follows: 95°C for 10 min for pre-denaturing and 45 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 20 s. Temperature conditions for 18S rRNA were as follows: 95°C for 10 min for pre-denaturing and 45 cycles of 95°C for 15 s and 60°C for 30 s. Melting curve analysis was performed for each amplicon for each annealing temperature with 95°C to ensure the absence of smaller non-specific products such as dimers. The concentrations of the PCR products were calculated by comparing the C_T values of the unknown samples to the standard curve using StepOne software ver. 2.1 (ABI). The expression of GMCSF was normalized by the expression of 18S rRNA in each sample.

3.2.3. Protein extraction and western blotting for GMCSF

The ampulla or isthmus samples were ground in liquid nitrogen and homogenized using the tissue protein extraction reagent (T-PER; Thermoscientific, Rockford, IL, USA) containing protease inhibitors (Halt protease inhibitor cocktail, Thermoscientific). Total protein content of each tissue homogenate was estimated using the bicinchoninic acid kit (Thermoscientific). The extracted samples (20 µg of total protein) were then loaded onto polyacrylamide gels with recombinant bovine GMCSF donated by Dr. Inumaru (National Institute of Animal Health, Japan; for details, Inumaru et al., 1997) as standards (12.5–100 ng per well) for estimating GMCSF concentration in the samples. Molecular weight markers ranging from 10 to 170 kDa (PageRuler prestained protein

ladder, 26616; Thermoscientific) were used to identify the bands for GMCSF. The proteins were electrophoresed through preformed sodium dodecyl sulfate polyacrylamide gels (Criterion TGX precast gel; Bio-Rad, Hercules, CA, USA). Because the gel had only 26 wells, the ampulla and isthmus samples were electrophoresed separately. The gels were run at 200 V for 30 min. The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes using the Transblot turbo transfer system (Bio-Rad). Immunoblotting was performed with anti-bovine GMCSF mouse monoclonal antibody (VMRD; Pullman, USA; 1:50,000 dilution) after treatment with blocking buffer containing 0.1% Tween 20 and 5% non-fat dry milk. The specificity of this antibody for western blotting and immunohistochemistry has been reported and utilized for bovine oviducts (de Moraes et al., 1999). Incubation with the antibody was done overnight at 4°C. Following washes with 10 mM Tris–HCl (pH 7.6) containing 150 mM NaCl and 0.1% Tween 20, horseradish peroxidase (HRP)-conjugated anti-mouse IgG (KPL Inc., Gaithersburg, MD, USA; 1:100,000 dilution) was added, and incubation was performed at 25°C for 1 h. Protein bands were visualized using an ECL-Prime chemiluminescence kit (GE Healthcare, Amersham, UK) and a charge-coupled device (CCD) imaging system (LAS-3000 Mini; Fujifilm, Tokyo, Japan). The concentrations of GMCSF protein in the samples were calculated by comparing the band strength of the unknown samples to the standard curve using the Multi Gauge software ver. 3.0 (Fujifilm). After removing the antibodies from the PVDF membrane using stripping solution (05364-55; Nacalai Tesque Inc., Kyoto, Japan), the membrane was blocked and incubated with anti-β-actin mouse monoclonal antibody (A2228; Sigma Aldrich, St. Louis, MO, USA; 1:50,000 dilution) overnight at 4°C. After washing, the membrane was incubated with the same HRP-conjugated anti-mouse IgG

(1:100,000 dilution) at 25°C for 1 h and visualized using an ECL-Prime chemiluminescence kit to capture images. The expression of GMCSF was normalized by the expression of β-actin in each sample.

3.2.4. Immunohistochemistry using anti-GMCSF antibody

After storage in 4% PFA at 4°C for 12 h, the tissue blocks were placed in 30% sucrose diluted in PBS until they were infiltrated with sucrose. The blocks were then frozen in an embedding medium (Tissue-Tek OCT compound; Sakura Finetechnical Co. Ltd., Tokyo, Japan) and maintained at -80°C. Next, the blocks were sectioned into 14-μm-thick cross sections using a cryostat (CM1900; Leica Microsystems Pty Ltd Wetzlar, Germany) and mounted on microscope slides (MAS coat Superfrost, Matsunami-Glass, Osaka, Japan). After treatment with 0.3% hydrogen peroxide in PBS for 10 min to inactivate endogenous peroxidase, followed by rinsing and treatment with 0.3% Triton X-100 in PBS for 15 min, the sections were incubated with 0.5 mL of PBS containing 10% normal goat serum for blocking for 1 h. The sections were then incubated overnight at 4°C in PBS containing 1 μg/mL of the same anti-GMCSF mouse monoclonal antibody and 0.5% normal goat serum as described above. After overnight incubation with the primary antibody, the sections were washed thoroughly and processed for 3,3′-diaminobenzidine (DAB) staining using a commercial kit (EnVision+ DualLink System-HRP, K4063;, DakoCytomation, Carpinteria, CA, USA). Briefly, sections were incubated with 1 drop of goat anti-mouse IgG conjugated to HRP-labeled polymer for 1 h, followed by washing and a final incubation with 1 mL of DAB chromogen substrate solution (LiquidDAB+ substrate chromogen system, K3467; DakoCytomation) for 20 min. The stained sections mounted on microscope slides were dehydrated in ethanol, cleared in xylene, and covered with a slip using a mountant

(DPX, 360294H; BDH Laboratory Supplies, Poole, UK) for microscopic observation (Eclipse E800; Nikon, Tokyo, Japan) and imaging with an attached CCD camera (Pixera600ES; Pixera Japan, Kawasaki, Japan) and its controller (Studio3.0.1; Pixera Japan). To verify the specificity of the signals, we included several negative control sections in which the primary antiserum had been omitted or pre-absorbed with 5 nM of the same recombinant GMCSF protein as described above or in which negative control rabbit IgG (Wako Pure Chemicals, Osaka, Japan) had been used instead of the primary antibody.

3.2.5. Statistical analysis

Data were analyzed using Statview version 5.0 for Windows (SAS Institute, Inc., Cary, NC, USA). The statistical significance of differences in the measured values were analyzed by one-factor analysis of variance (ANOVA) followed by post-hoc comparisons using Fisher's protected least significant difference (PLSD) test using a model consisting of variance from the effect of body condition (lean, normal, or obese) and the residual. The level of significance was set at $P < 0.05$. Data are expressed as mean ± SEM.

3.3. Results

3.3.1. GMCSF mRNA expression in bovine oviduct

ANOVA revealed a significant ($P < 0.05$) effect of body condition on the GMCSF mRNA expression in the ampulla samples. The obese group had lower ($P < 0.05$) expression than the normal group (**Fig. 3.1A**). There was no significant effect of body condition on the GMCSF mRNA expression in the isthmus samples (**Fig. 3.1B**).

3.3.2. GMCSF protein expression in bovine oviduct

Western blotting showed an immunoreactive protein band for all the ampulla and isthmus samples that migrated with an apparent molecular weight of 26.0 kDa (**Fig. 3.2A**). ANOVA revealed a significant ($P < 0.05$) effect of body condition on the GMCSF protein expression in the ampulla samples. The obese group had lower GMCSF expression than the normal group ($P < 0.05$; **Fig. 3.2B**). There was no significant effect of body condition on the GMCSF protein expression in the isthmus samples (**Fig. 3.2C**).

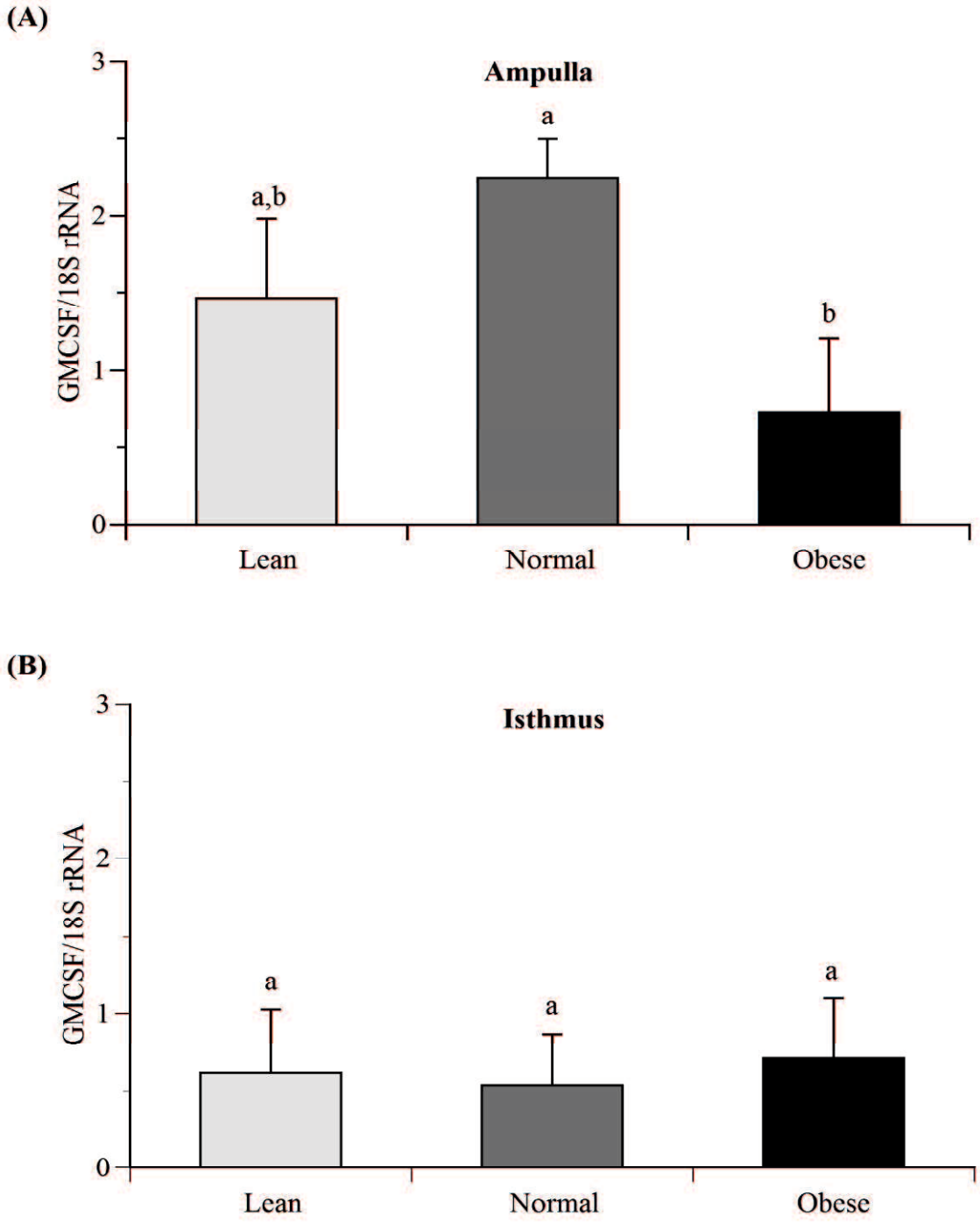

Fig. 3.1. Relative amount (mean ± SEM) of GMCSF mRNA expression analyzed by real-time PCR analysis normalized to 18S rRNA in the ampullary (A) and isthmus (B) sections of the oviducts of lean, normal, and obese cows. Letters (a *vs*. b) indicate significant ($P < 0.05$) differences compared to the normal cows.

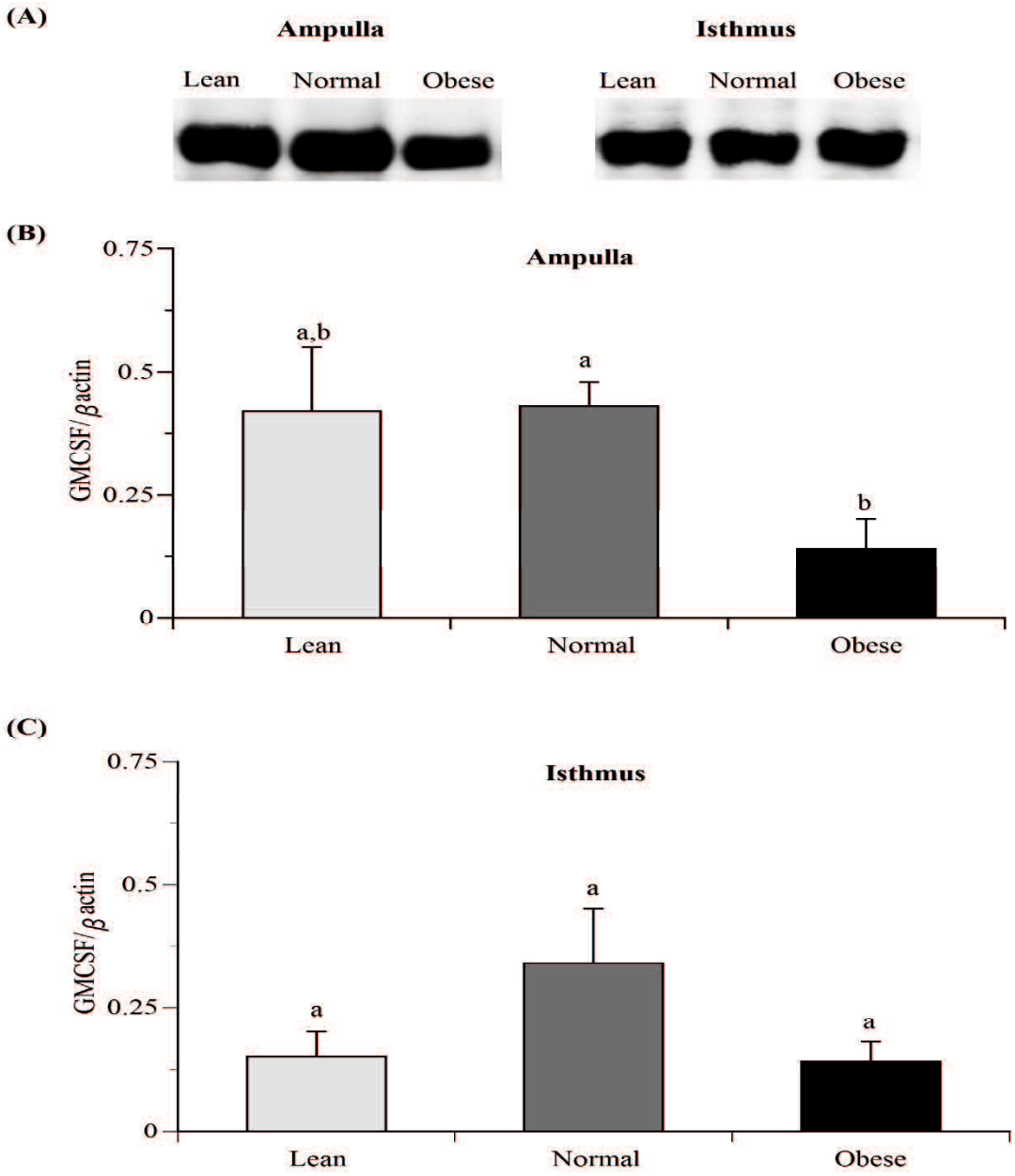

Fig. 3.2. Representative bands of GMCSF protein detected by western blotting with anti-GMCSF antibody in the ampullary and isthmic sections of oviducts of lean, normal, and obese cows (A), and the relationship of GMCSF protein expression normalized to β-actin in the ampullary (B) and the isthmic (C) sections of oviducts of the lean, normal, and obese cows. Letters (a *vs*. b) indicate significant ($P < 0.05$) differences compared to normal cows.

3.3.3. Immunohistochemistry

Strong GMCSF immunoreactivities were observed in the tunica mucosa of the ampulla in the normal and lean cows (**Fig. 3.3**). However, the GMCSF immunoreactivities were weak in the tunica mucosa of the ampulla of the obese cows.

The tunica mucosa of the isthmus also had moderate GMCSF immunoreactivities (**Fig. 3.4)**.

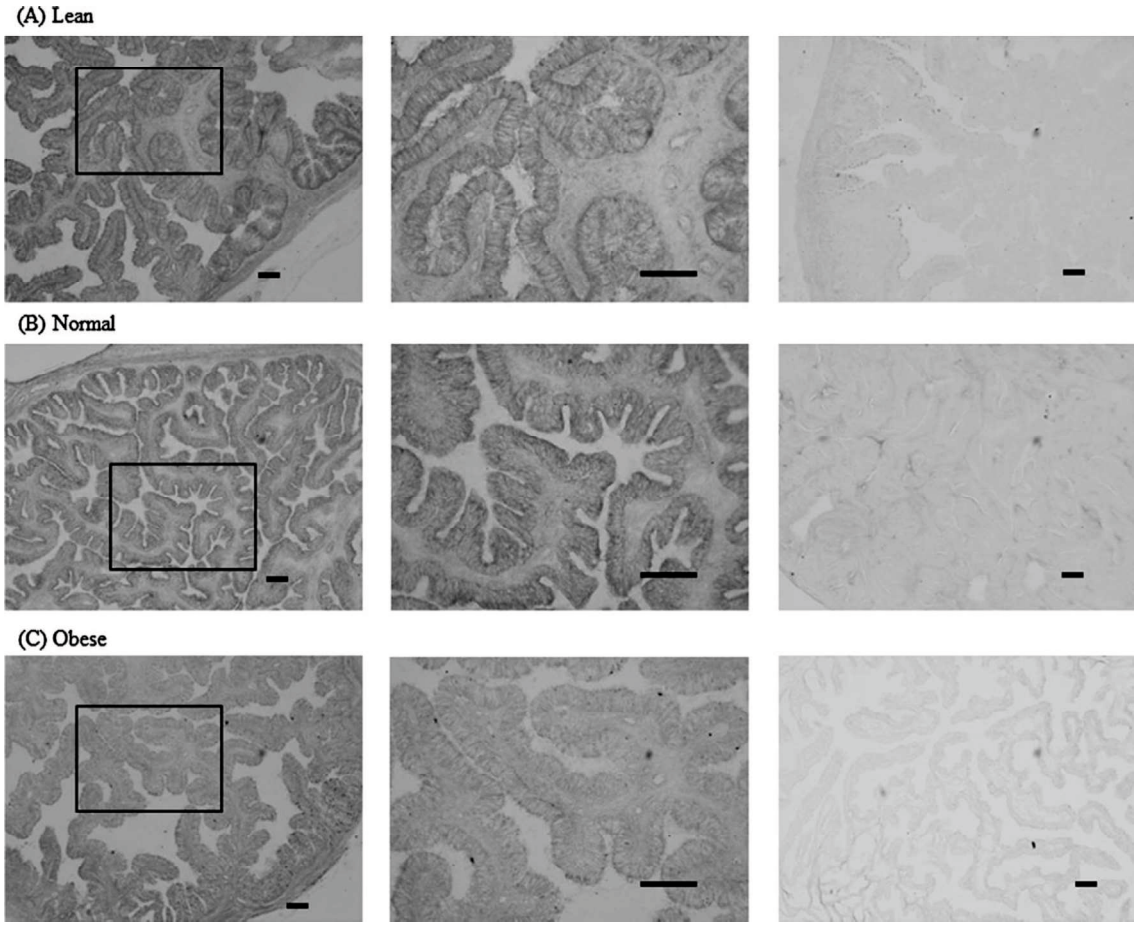

Fig. 3.3. Immunohistochemistry of ampullary sections stained with anti-GMCSF antibody of lean (A), normal (B), or obese cows (C). Left panels show stained regions as indicated by arrows at a lower magnification, and central panels show stained cells at a higher magnification. Right panels show negative controls using the same primary antibody pre-absorbed with 5 nM of the recombinant GMCSF protein. Scale bars are 100 μm.

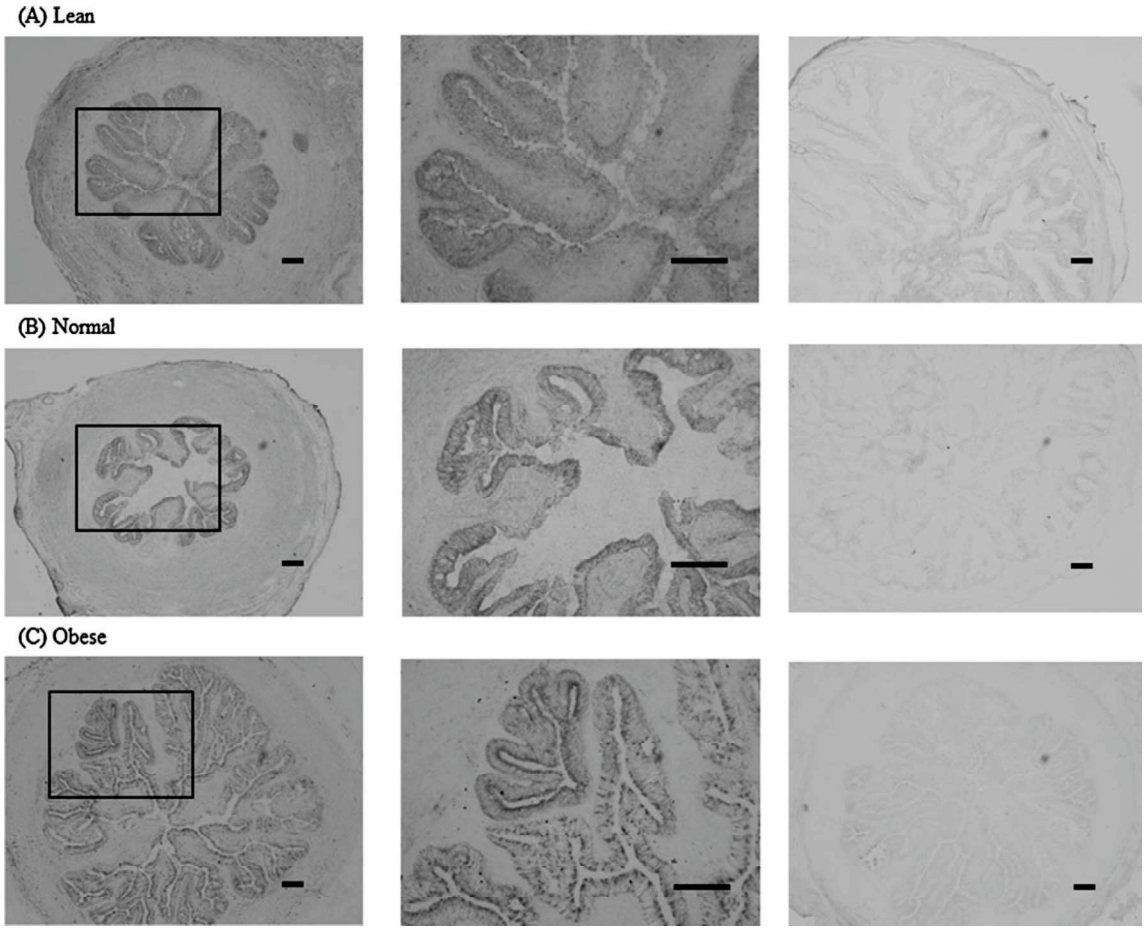

Fig. 3.4. Immunohistochemistry of isthmus sections stained with anti-GMCSF antibody of lean (A), normal (B), or obese cows (C). Left panels show stained regions as indicated by arrows at a lower magnification, and central panels show stained cells at a higher magnification. Right panels show negative controls using the same primary antibody pre-absorbed with 5 nM of the recombinant GMCSF protein. Scale bars are 100 μm.

3.4. Discussion

The mammalian oviduct plays a critical role in the establishment of a successful pregnancy (for a review, see Hunter, 1988). Fertilization, cleavage, and development to the 8- cell and 16-cell stage take place within the ampulla, and then, the embryo passes quickly through the isthmus and enters the uterus (el-Banna and Hafez, 1970). Early embryonic development depends on factors present in the oviductal fluid. We observed significantly lower expression of GMCSF in the ampulla of obese cows. In accordance with previous studies (de Moraes et al., 1999; Zhao and Chegini, 1994), the immunoreactive GMCSF was localized to the tunica mucosa in the ampullary and isthmic sections of the oviduct. Therefore, the suppressed GMCSF expression in the ampulla of obese cows suggested decreased expression in the tunica mucosa. These cells are most likely the main source of GMCSF to bind with the GMCSF receptors in early-stage embryos (Robertson et al., 2001). Therefore, in concordance with an earlier study (Kadokawa et al., 2008), our present data suggested that decreased GMCSF in the ampulla of obese females may be an important inhibiting factor for embryo development.

GMCSF promotes glucose transport for murine preimplantation embryos *in vitro* (Robertson et al., 2001), as well as for human sperm (Zambrano et al., 2001). Glucose is an energy source for the embryo, but the presence of glucose at the concentrations found in maternal serum is detrimental for the embryos of several species, including cows, during the early cleavage stages (Gutiérrez-Adán et al., 2001; Larson et al., 2001; Peippo et al., 2001). Recently, Bermejo-Alvarez et al. (2012) reported that maternal obesity causes the downregulation of both glucose transporter types 1 (Slc2a1) and low density lipoprotein receptor (Ldlr) in blastocysts recovered from diet-induced obese

female mice. However, such maternal obesity does not affect embryo development in this mouse model, probably by preventing excessive nutrient uptake because of Slc2a1 and Ldlr downregulation. Whether GMCSF has important roles in controlling the expression of Slc2a1 and Ldlr in embryo remains to be investigated.

GMCSF inhibits the cellular stress response and apoptosis pathways to facilitate mouse embryonic growth and survival (Chin et al., 2009). GMCSF-null mutation caused elevated expression of heat shock 105/110 kDa protein 1 (Hsph1) in *in vivo* developed mouse blastocysts (Chin et al., 2009). Therefore, in the oviduct of obese cows, the embryo may have increased cellular stress response and activated apoptosis pathways, at least of Hsph1. It is a matter of speculation that obese cows might have more damaged embryos in the summer season than normal cows. This hypothesis, however, warrants further studies.

Little is known about the mechanism of GMCSF expression control. Nonesterified fatty acids (NEFA) increase GMCSF secretion through protein kinase C activation in THP-1 macrophages (Bahramian et al., 2004). Because blood NEFA levels in Japanese Black cattle decrease during fattening (Shimada et al., 1987), NEFA may be associated with the suppressed expression of GMCSF in the oviduct. Other important nutritional factors might exist in the oviduct of cows. However, to the best of our knowledge, no studies on the effect of metabolic hormones on GMCSF expression in any tissue have been performed so far. Therefore, it is very important to clarify the underlying mechanisms of maternal nutritional condition affecting GMCSF expression in oviducts. Our study did not exclude the possibility that low expression of GMCSF might inhibit functions of the oviduct itself, or if any other incidental factor suppressed embryo development.

We cannot exclude the contribution of the uterus of obese females on differences in embryo development among lean, normal, and obese females. In addition, there are pronounced discrepancies in the litter size of genetically GMCSF-deficient female mice. For instance, Hamilton et al. (2012) reported normal litter size, while Robertson et al. (1999) reported decreased litter size. Therefore, in addition to GMCSF, other factors for differences in embryo development can be conceivable. As reviewed by Lumeng (2013), previous research on obesity and its associated diseases have increased the overlap between the complex molecular and cellular mechanisms that control metabolism and inflammation. MIF has emerged as an important regulator of inflammation, playing a central role in the control of both innate and antigen-specific immunity. MIF has oxidoreductase and tautomerase activities and important roles for embryonic development after gestation in humans (Viganò et al., 2007), and plasma MIF concentrations are elevated in obese women (Kim et al., 2011). Genetically MIF-deficient female mice are fertile, and their litter size is normal (Bozza et al., 1999). However, MIF might also play a significant role in embryonic development in cows, because interferon-tau stimulates MIF secretion from endometrial epithelial cells (Wang and Goff, 2003). A recent study in sheep reported that MIF is expressed in the reproductive duct as well as placenta (Lopes et al., 2011). Therefore, further studies are required to clarify mechanisms that alter embryo development in obese ruminants.

In conclusion, the obese cows had suppressed GMCSF expression in the ampulla unlike the normal and lean cows, however, there was no difference in GMCSF mRNA or protein expression in the isthmus among lean, normal, and obese cows.

CHAPTER IV

(Study 2)

Expression of MIF in oviduct of lean, normal, and obese cows

Abstract

Oviducts synthesize MIF to promote sperm capacitation and embryogenesis. This study aimed to test a hypothesis that the oviducts of obese cows may express MIF at a lower level than those of normal and lean cows. Ampullar or isthmic oviduct sections were collected from lean (n = 5; BCS on a 5-point scale, 2.5), normal (n = 6; BCS, 3.0), and obese (n = 5; BCS, 4.0) Japanese Black cows. MIF mRNA and protein were extracted from ampullae and isthmuses and their levels measured by real-time PCR or western blot. Immunohistochemistry was performed on frozen sections of ampullae and isthmuses by using antibodies to MIF. MIF mRNA and protein expression were lower in the obese and lean groups than in the normal group ($P < 0.05$). Immunohistochemistry revealed that the primary site of MIF expression in the ampulla and isthmus is the tunica mucosa. In addition, the obese and lean groups had weaker MIF immunoreactivity in the tunica mucosa than the normal group. In conclusion, the obese cows had suppressed MIF expression in the ampullae and isthmuses of oviducts, as we hypothesized, but unexpectedly, MIF expression was also lower in lean cows.

4.1. Introduction

In study 1, we found that GMCSF may be a key molecular signaling link between maternal nutritional status and early embryogenesis in the oviduct, and that GMCSF is expressed at lower levels in the ampullar part of the oviduct of obese cows than in normal and lean cows. This reduced expression may be useful for limiting the glucose supplied to pre-implantation embryos, because excess glucose is detrimental during the early cleavage stage (Hashimoto et al., 2000; Sutton-McDowall et al., 2010; Kumar et al., 2012). However, there may be another molecular link between maternal nutritional condition and embryo development in the oviduct, because genetically GMCSF-deficient female mice produce litter of normal size (Hamilton et al., 2012). MIF may be such a molecule, promoting functions for sperm, ovum, and early-stage embryos in the oviducts according to the maternal nutritional condition. MIF is expressed in the ampullar and isthmic parts of the oviducts of mice and ewes (Suzuki et al., 1996; Lopes et al., 2011), as well as ovary, testis, and uterus (Meinhardt et al., 1998; Lopes et al., 2011; Paulesu et al., 2012). Tunica mucosa, the interface layer between the oviduct and sperm, oocyte, and early-stage embryos, is the primary site of MIF expression in the oviduct (Suzuki et al., 1996). MIF at the optimal concentration promotes sperm capacitation, whereas low or excess MIF is inhibitory (Carli et al.,

2007). In mice, furthermore, MIF mRNA is expressed in ovulated oocytes, zygotes, 2-cell embryos, 8-cell embryos, and blastocysts (Suzuki et al., 1996). Important roles of MIF in establishing pregnancy have been reported both upstream (ovary; Matsuura et al., 2002) and downstream (uterine; Viganò et al., 2007; Bevilacqua et al., 2014) of the oviduct. Therefore, MIF synthesized by the oviduct may be important in early embryogenesis or oviduct functions, although little is known of the expression and role of the MIF receptors CD44, CD74, and CXCR4 (Leng et al., 2003; Schwartz et al., 2009; Rodriguez Hurtado et al., 2011) in the oviduct as well as in the oocyte, sperm, and early-stage embryo. Furthermore, MIF plays an important role in promoting glucose metabolism in muscle and adipose tissue (Benigni et al., 2000; Toso et al., 2008). If, therefore, energy sources are in excess in the maternal body, MIF gene expression in the oviducts may be downregulated to protect the embryos. Therefore, study 2 aimed to test the hypothesis that the oviducts of obese cows may express MIF at lower levels than normal and lean cows.

4.2. Materials and methods

4.2.1. Animals and treatments

Multiparous Japanese Black cows (age: 4 years, calved 3 times, n = 16) were housed in a free-stall barn. Their calves were separated for weaning two months after normal parturition. At the time of weaning, the cows had a BCS of 2.5 on a 5-point scale (Ferguson et al., 1994), and the mean body weight was 463 ± 2 kg (mean ± SEM). The maintenance of this body weight requires 11.2 Mcal of ME per day, according to the Japanese feeding standard (Agriculture, Forestry and Fisheries Research Council Secretariat, 2008). The cows were randomly allocated to one of the following three groups: (1) lean group (n = 5), fed 10.1 Mcal of ME (90% of the maintenance level) as Italian ryegrass hay (84.2% DM), 2.30 Mcal of ME/kg DM, 13.3% crude protein CP); (2) normal group (n = 6), fed 12.3 Mcal of ME (110% of the maintenance level) using the same Italian ryegrass hay plus concentrate (86.6% DM, 3.82 Mcal of ME per kg DM, 21.3% of CP); (3) obese group (n = 5), fed 23.9 Mcal of ME (the commonly used fattening level; 213% of the maintenance level) using the same Italian ryegrass hay plus extra concentrate. After 6 months on these diets, the BCS of the lean group was 2.5 (body weight, 470 ± 2 kg), that of the normal group was 3.0 (body weight, 501 ± 2 kg), and the obese group had a score of 4.0 (body weight, 563 ± 3 kg). Water and mineral

blocks were provided ad libitum. Absence of disease, including reproductive disease, was confirmed by daily observation and weekly rectal palpation.

On the second day after ovulation induced by dinoprost, the cows were slaughtered and the oviducts on the ipsilateral side of ovulation collected within the next 5 min. The tissues surrounding the oviduct were carefully removed and the oviducts washed with PBS. Half of the ampullae and half of the isthmuses were frozen in liquid nitrogen and preserved at −80°C until use for RNA or protein extraction. The remainder was stored in 4% PFA at 4°C for 12 h before being used for immunohistochemistry studies.

4.2.2. RNA extraction, cDNA synthesis, and real-time PCR

DNA-free total RNA was extracted from the frozen ampullar and isthmic samples using an RNAiso Plus and Ribonuclease-free Deoxyribonuclease Set (Takara Bio Inc.) according to the manufacturer's protocol. The concentration and purity of each RNA sample were evaluated to ensure the A260/A280 nm ratio was in the acceptable range of 1.8–2.1. Electrophoresis of total RNA followed by staining with ethidium bromide was performed to verify the mRNA quality of all samples, and the 28S:18S ratios were 2:1.

We synthesized cDNA from 2 μg RNA from each sample in 20-μL reactions with random hexamer primers using a high-capacity cDNA reverse transcription kit (ABI).

To prepare external standards for amplified fragments of cDNA products containing target sequences for real-time PCR of *MIF* (NM_001033608.1), or two housekeeping genes, *C2orf29* (XM_002691150.2) and *SUZ12* (NM_001205587.1), the PCR conditions were optimized by conventional PCR amplification using AmpliTaq Gold PCR Master Mix (ABI), 20 ng DNase-treated reverse-transcribed RNA, and primers. The primers were designed by Primer Express Software v3.0 (ABI) based on the reference sequences. The forward primer for MIF standard was 5′-GCGGTCACGTAGCTCAGGTT-3′ (12th–31st nucleotide of mRNA, on 1st Exon), and the reverse primer for MIF standard was 5′-ACACCGTTTATTGCTCCTTCCA-3′ (541st –562th nucleotide of mRNA, on 3rd Exon), then, and the size of *MIF* PCR-product for standard was 551 bp. Two housekeeping genes, *C2orf29* and *SUZ12*, were used to normalize the real-time PCR results, because they are identified by both geNorm and Normfinder programs as most stable and reliable housekeeping genes in bovine endometrium and corpus luteum (Walker et al., 2009; Rekawiecki et al., 2012). The forward and reverse primers for C2orf29 standard were 5′-AAGTTTTTTCTTTCCCAGCTCATG-3′ (666th–688th nucleotide of mRNA, on 2nd

Exon) and 5′-CAGGAAGTTTGGCTGGAGTGA-3′ (1207th–1227th nucleotide of mRNA, on 5th Exon) and the size of C2orf29 PCR-product for standard was 562 bp. The forward and reverse primers for SUZ12 standard were 5′-GGAAGAGACTGCCTCCATTTGA-3′ (1019th–1040th nucleotide of mRNA, on 10th Exon) and 5′-CCCTGAGACACCATCTGTTTCC-3′ (2166th–2187th nucleotide of mRNA, on 16th Exon), and the size of SUZ12 PCR-product for standard was 1169 bp. All primers used in the present study were produced by a commercial service (Fasmac Co. Ltd, Tokyo, Japan). The presence of a single product was confirmed by electrophoresis on a 2% (w/v) agarose gel. The PCR-amplified products from the cDNA were purified using NucleoSpin Extract II columns (Takara Bio Inc.) to prepare external standards, as well as to verify the DNA sequence with a sequencer (ABI3130; ABI) using one of the PCR primers and the Dye Terminator v3.1 Cycle Sequencing Kit (ABI). The obtained sequences were used as query terms for homology searches in the DDBJ/GenBankTM/EBI Data Bank using the basic nucleotide local alignment search tool optimized for highly similar sequences (available on the NCBI website).

In addition, for real-time PCR, primers were designed by Primer Express Software v3.0 based on the reference sequences. The forward and reverse primers for MIF were 5′-GCGCCTGCGCATTAGC-3′ (355th–370th nucleotide of mRNA, on 2nd Exon) and

5′-CGCGTTCATGTCGCAGA-3′ (393th–409th nucleotide of mRNA, on 3rd exon), and the size of MIF real-time PCR product was 55 bp. The sequences of primers for C2orf29 and SUZ12 were the same as that reported in the previous paper (Rekawiecki et al., 2012). The primers of C2orf29 were 5′-TCAGTGGACCAAAGCCACCTA-3′ (928th–948th nucleotide of mRNA, on 3rd Exon) and 5′-CTCCACACCGGTGCTGTTCT-3′ (1077th–1097th nucleotide of mRNA, on 4th Exon), and the PCR-product size was 170 bp. The primers of SUZ12 were 5′-CATCCAAAAGGTGCTAGGATAGATG-3′ (1441th–1465th nucleotide of mRNA, on 13th Exon) and 5′-TTGGCCTGCACACAAGAATG-3′ (1581th–1600th nucleotide of mRNA, on 14th Exon), and the PCR product size was 160 bp.

The expression levels of MIF, C2orf29, and SUZ12 were measured in duplicate by real-time PCR analyses using 20 ng of cDNA, StepOne Real Time PCR System (ABI), and Power SYBR Green PCR Master Mix (ABI) with a 5-point relative standard curve and a non-template control. Series of 10-fold dilution standards were prepared using the purified amplified fragments of DNA products. Because the real-time PCR system has only 48 wells, the ampullar and isthmic samples were analyzed separately. Temperature conditions for MIF were as follows: 95°C for 10 min for pre-denaturing, 5 cycles of 95°C for 15 s, 66°C for 30 s, and 40 cycles of 95°C for 15 s, and 60°C for 60 s.

Temperature conditions for C2orf29 and SUZ12 were as follows: 95°C for 10 min for pre-denaturation and 40 cycles of 95°C for 20 s and 60°C for 30 s. Melting curve analyses were performed at 95°C for each amplicon for each annealing temperature to ensure the absence of smaller non-specific products such as dimers. The concentrations of the PCR products were calculated by comparing the CT values of the unknown samples with the standard curve by using StepOne software ver. 2.1 (ABI). The expression of *MIF* gene was normalized to the geometric means of *C2orf29* and *SUZ12* expression; thus, the MIF amount was divided by the geometric mean of C2orf29 and SUZ12 in each sample.

4.2.3. Protein extraction and western blotting for MIF

The ampullar or isthmic samples were ground in liquid nitrogen and homogenized using T-PER containing Halt protease inhibitor cocktail. Total protein content of each tissue homogenate was estimated using a bicinchoninic acid kit. The extracted samples (30 μg of total protein) were then loaded onto polyacrylamide gels with recombinant human MIF as standards (12.5–100 ng per well; CYT-575, ProSpec-Tany TechnoGene Ltd., Rehovot, Israel) for estimating MIF concentration in the samples. Molecular weight markers ranging from 10 to 170 kDa were used to identify MIF bands. The proteins were electrophoresed through preformed sodium dodecyl sulfate

polyacrylamide gels. The gels were run at 200 V for 30 min. The proteins were then transferred to PVDF membranes. Immunoblotting was performed with anti-human MIF mouse monoclonal antibody (Clone 12302, MAB289, R&D systems, Inc., Minneapolis, MN, USA; 1:25,000 dilution) after treatment with blocking buffer containing 0.1% Tween 20 and 5% non-fat dried milk. Bovine MIF has 92% homology with the antigen, human MIF, and the specificity of anti-human MIF monoclonal antibody on bovine placentomal tissue has been assessed previously by western blot (Paulesu et al., 2012), and it has been used for western blot, immunoprecipitation, and immunohistochemistry on bovine tissues (Wadgaonkar et al., 2005; Paulesu et al., 2012). Incubation with the antibody was carried out overnight at 4°C. After washing with 10 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and 0.1% Tween 20, HRP-conjugated anti-mouse IgG (1:50,000 dilution) was added, and incubation was performed at 25°C for 1 h. Protein bands were visualized using an ECL-Prime chemiluminescence kit. The concentrations of MIF protein in the samples were calculated by comparing the band strength of the unknown samples to the standard curve. After removing the antibodies from the PVDF membrane with stripping solution, the membrane was blocked and incubated with the same mouse anti-β-actin monoclonal antibody for overnight at 4°C. After washing, the membrane was incubated with the same HRP-conjugated anti-mouse IgG (1:100,000

dilution) at 25°C for 1 h and the bands visualized using an ECL-Prime chemiluminescence kit. The expression of MIF was normalized to the expression of β-actin in each sample.

4.2.4. Immunohistochemistry using anti-MIF antibody

After storage in 4% PFA at 4°C for 12 h, the tissue blocks were placed in 30% sucrose that was diluted with PBS until they were infiltrated with sucrose. The blocks were then frozen in Tissue-Tek OCT compound and maintained at −80°C. Next, the blocks were sectioned into 14-μm-thick cross sections using the cryostat and mounted on microscope slides. The sections were treated with 0.3% hydrogen peroxide in PBS for 10 min to inactivate endogenous peroxidase, followed by rinsing and treatment with 0.3% Triton X-100 in PBS for 15 min. They were then blocked by incubating for 1 h at room temperature with 0.5 mL PBS containing 10% normal goat serum. The sections were then incubated overnight at 4°C in PBS containing 1 μg/mL of the same anti-MIF mouse monoclonal antibody and 0.5% normal goat serum, as described above. After overnight incubation with the primary antibody, the sections were washed thoroughly and processed for 3, DAB staining. Sections were incubated with 1 drop of goat anti-mouse IgG conjugated to HRP-labeled polymer for 1 h at room temperature, followed by washing and a final incubation with 1 mL of LiquidDAB+ substrate

chromogen system for 20 min. The stained sections mounted on microscope slides were dehydrated in ethanol, cleared in xylene, and covered for microscopic observation and imaging. To verify the specificity of the signals, a bovine uterine section was used as the positive control (Paulesu et al., 2012). We included several negative control sections in which the primary antiserum had been omitted or pre-absorbed with 5 nM of the recombinant MIF protein described above, or in which negative control mouse IgG (Biocare Medical, LLC, Concord, CA, USA) had been used instead of the primary antibody.

4.2.5. Statistical analysis

The statistical significance of differences in the measured values was assessed by one-factor ANOVA followed by post-hoc comparisons with Fisher's PLSD test using a model consisting of variance from the effect of body condition (lean, normal, or obese) and the residual.

4.3. Results

4.3.1. MIF mRNA in the bovine oviduct

ANOVA revealed an effect ($P < 0.05$) of body condition on the amount of MIF mRNA in both the ampullar samples (**Fig. 4.1A**) and the isthmic samples (**Fig. 4.1B**) from bovine oviducts. The obese and lean groups had lower ($P < 0.05$) MIF mRNA expression in both parts of the oviducts than the normal group.

4.3.2. MIF protein in the bovine oviduct

Western blotting showed an immunoreactive protein band that migrated with an apparent molecular weight of 12.5 kDa in all the ampullar (**Fig. 4. 2A**) and isthmic samples (**Fig. 4. 2B**). ANOVA revealed an effect of body condition on the amount of MIF protein in both kinds of samples: the obese and lean groups had less MIF in their ampullae ($P < 0.05$; **Fig. 4. 2C**) and isthmuses ($P < 0.05$; **Fig. 4. 2D**) than the normal group.

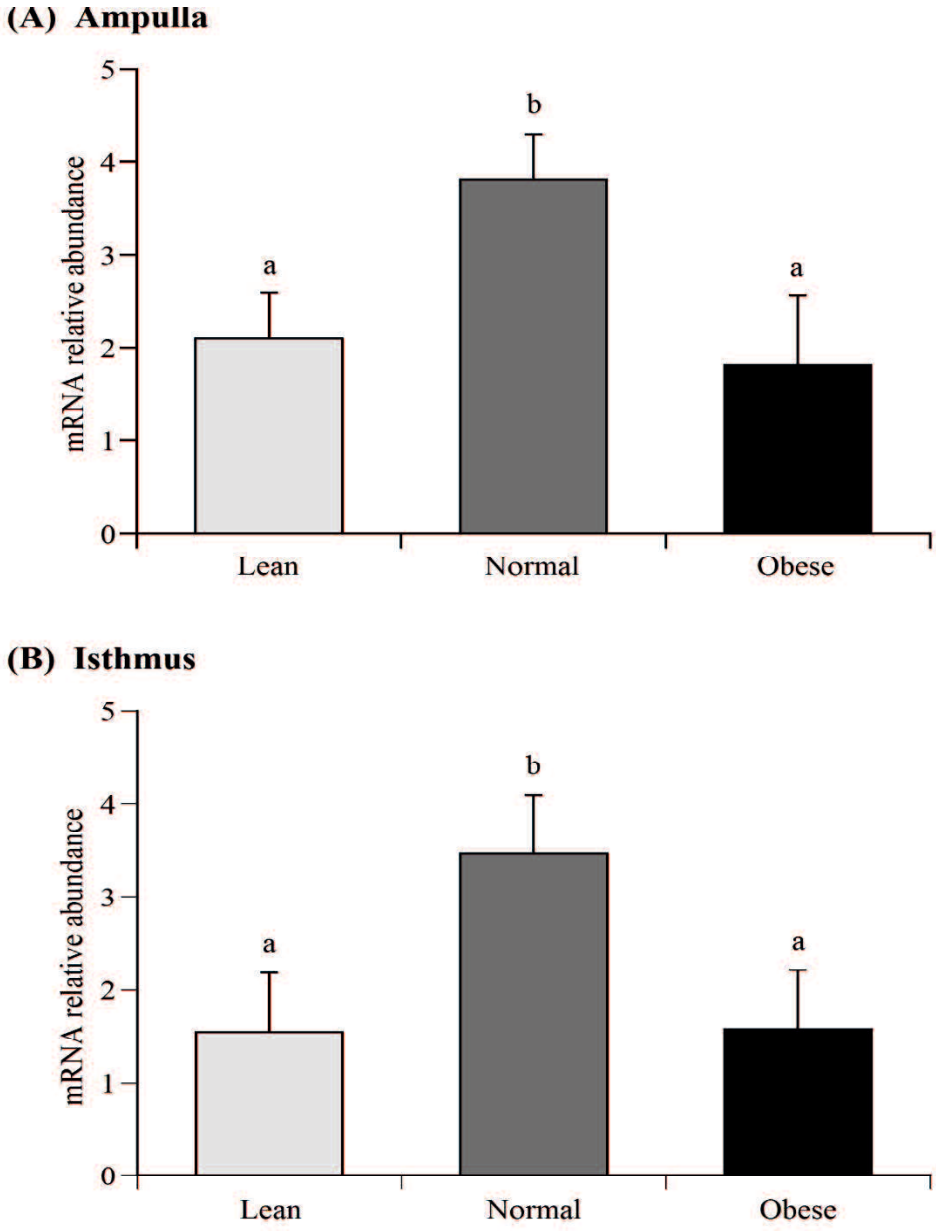

Fig. 4.1. Relative MIF mRNA expression (mean ± SEM) determined by real-time PCR analysis in ampullar (A) and isthmic (B) sections of oviducts from lean, normal, and obese cows. Results are normalized to geometric mean of two housekeeping genes, namely, *C2orf29* and *SUZ12* expression. Letters (a *vs*. b) indicate differences ($P < 0.05$) compared with normal cows.

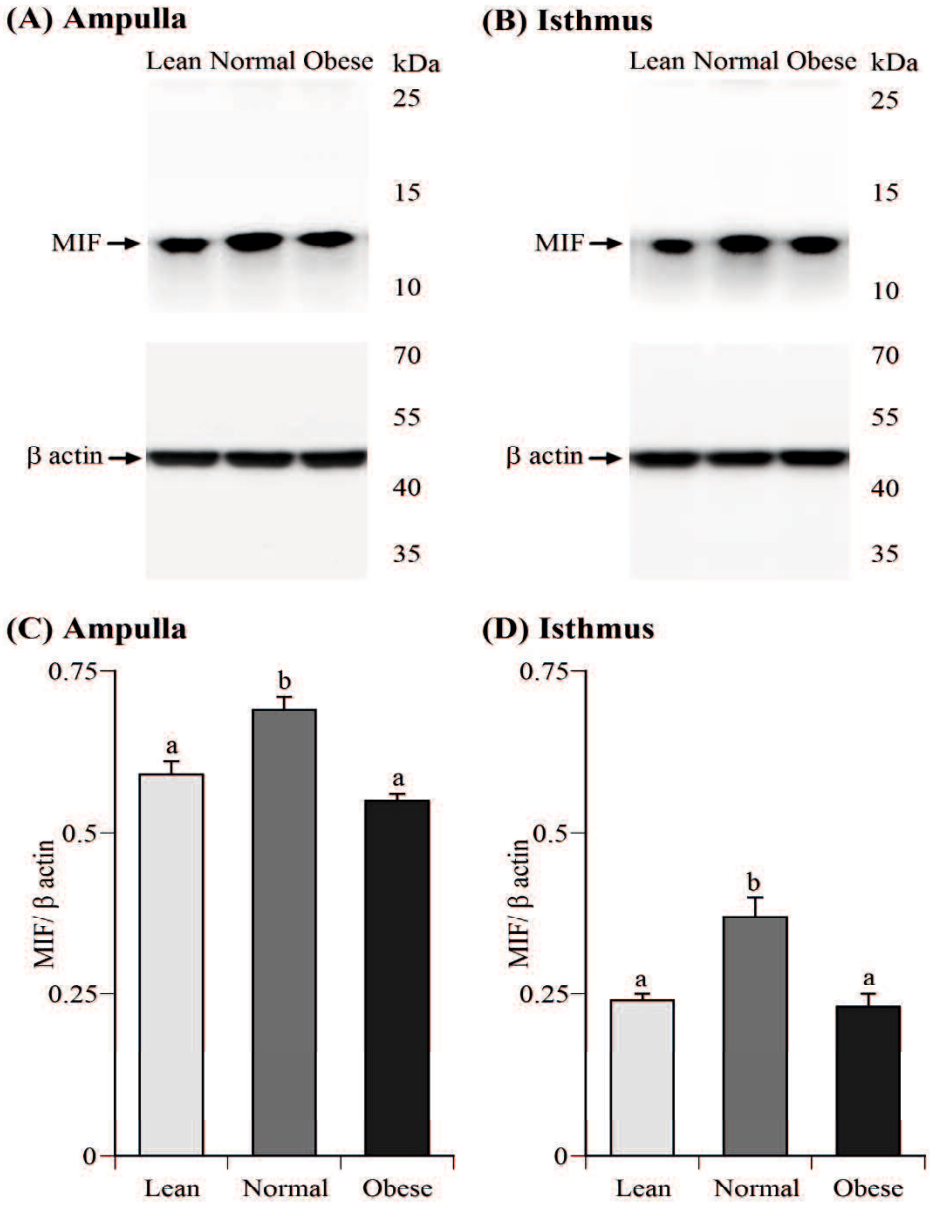

Fig. 4.2. Representative MIF and β-actin protein bands detected by western blotting with anti-MIF or anti-β-actin antibody in ampullar (A) and isthmic (B) sections of oviducts from lean, normal, and obese cows (the molecular weight marker proteins are shown on the right side of each image), and the relationship of MIF protein expression normalized to β-actin in ampullar (C) and isthmic (D) sections of oviducts from lean, normal, and obese cows. Letters (a *vs*. b) indicate differences ($P < 0.05$) compared with normal cows.

4.3.3. Immunohistochemistry

The results showed that normal cows had higher MIF immunoreactivity than lean and obese cows in the tunica mucosa of both ampulla (**Fig. 4. 3**) and isthmus (**Fig. 4.4**). Intense staining was observed in the epithelium of tunica mucosa, particularly in the apical portion of cells, while a comparatively weak staining was observed in the stroma.

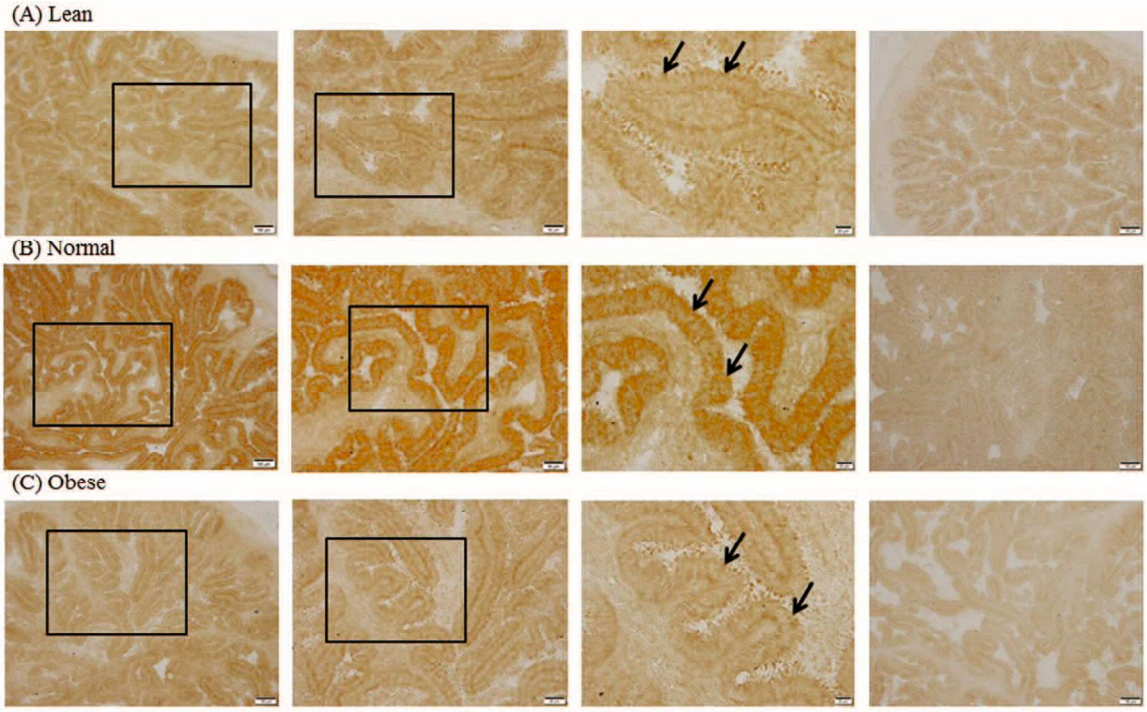

Fig. 4.3. Immunohistochemistry of ampullar sections from lean (A), normal (B), or obese cows (C) stained with anti-MIF antibody. Left panels show stained regions at a lower magnification (Scale bar is 100 μm). The central two panels show stained cells from the rectangular area of its left panel at a higher magnification (Scale bars are 50 μm or 20 μm). Arrows indicate the immunoreactivity found in the tunica mucosa. Right panels show negative controls using the same primary antibody pre-absorbed with 5 nM of recombinant MIF protein (Scale bar is 100 μm).

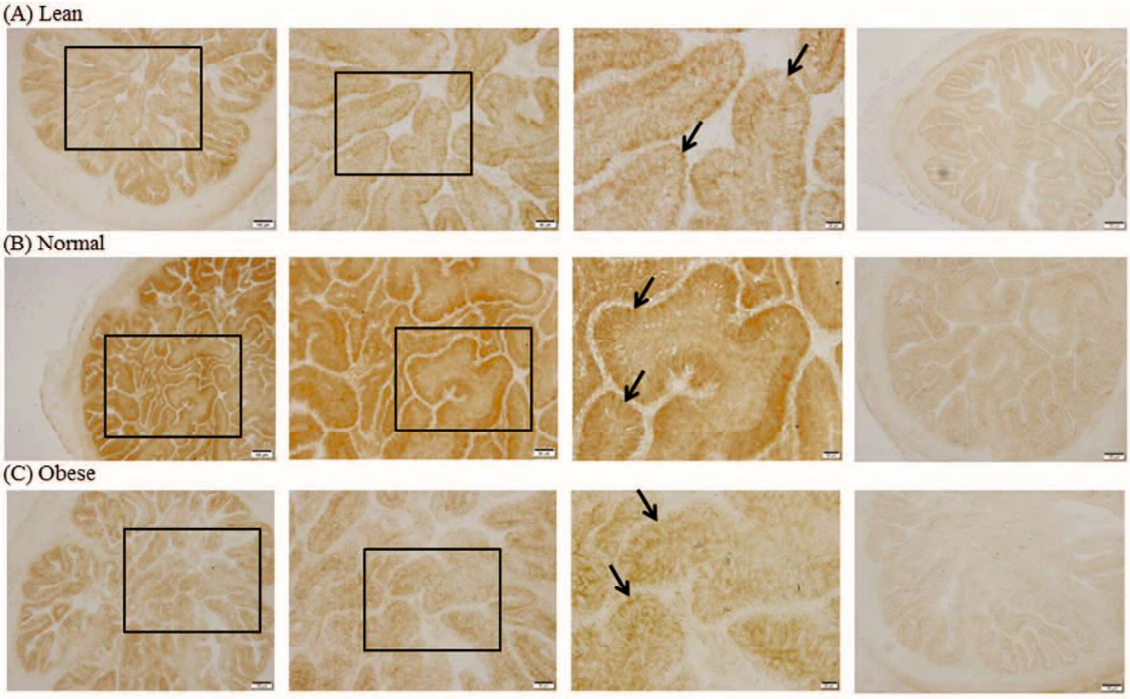

Fig. 4.4. Immunohistochemistry of isthmic sections from lean (A), normal (B), or obese (C) cows stained with anti-MIF antibody. Left panels show stained regions at a lower magnification (Scale bar is 100 μm). The central two panels show stained cells from the rectangular area of its left panel at a higher magnification (Scale bars are 50 μm or 20 μm). Arrows indicate the immunoreactivity found in the tunica mucosa. Right panels show negative controls using the same primary antibody pre-absorbed with 5 nM of recombinant MIF protein (Scale bar is 100 μm).

4.4. Discussion

The present study aimed to test the hypothesis that the oviducts of obese cows express lower levels of MIF than those of normal and lean cows. The data we obtained reveal that MIF expression is lower in the ampulla and isthmus of obese cows, as predicted, but unexpectedly, was also lower in lean cows. We therefore need to consider the possible role of lower MIF expression in obese and lean cows after discussing its importance in oviducts.

In agreement with a previous study (Suzuki et al., 1996), immunoreactive MIF was localized to the tunica mucosa of the ampulla and isthmus in mice. The tunica mucosa is the layer producing oviductal fluid for gamete transport and maturation, fertilization, and early embryo development in various animals including bovine (Leese et al., 2001; Cebrian-Serrano et al., 2013). MIF has been detected in the oviducts of amphibians, sheep, and mice, and strong expression has been observed in the epithelial cells of tunica mucosa (Suzuki et al., 1996; Jantra et al., 2011; Lopes et al., 2011). This is the first report on the expression of MIF in bovine oviducts, furthermore, our results show that MIF is strongly expressed in bovine epithelial cells. The suppressed levels of MIF in the ampullae and isthmuses of obese and lean cows therefore suggest that MIF gene expression is decreased in the tunica mucosa. MIF mRNA is expressed in ovulated oocytes, zygotes, 2-cell embryos, 8-cell embryos, and blastocysts of mice (Suzuki et al.,

1996). At the appropriate concentration, MIF promotes human sperm capacitation in vitro, but not at higher or lower levels (Carli et al., 2007). MIF plays important roles in human endometrium during gestation and it promotes embryonic growth (Arcuri et al., 2001; Akoum et al., 2005). In mice, intraperitoneal injection of recombinant MIF the day after mating increases the pregnancy rate (Bondza et al., 2008). Although little is known of the role of MIF in the oviduct, our findings suggested that MIF synthesized by the oviduct might play important roles in fertilization and early embryo development.

MIF promotes glucose metabolism (Benigni et al., 2000; Toso et al., 2008). During embryogenesis, glucose is an important energy source (Downs et al., 1998; Zheng et al., 2001; Herrick et al., 2006; Sutton-McDowall et al., 2010). However, very little is known about regulation of glucose in bovine oviduct. In buffalo, glucose concentration in the oviduct fluid is known to increase during the pre-ovulatory phase and to decrease dramatically after ovulation (Vecchio et al., 2010), which is followed by early embryo development in the oviduct. The optimum level of glucose is important in determining the maturation rate of bovine oocytes as well as their ability to grow after fertilization (Kim et al., 1993). Excessive glucose, on the other hand, is detrimental to the maturation of bovine oocytes and the development of early stage embryos (Hashimoto et al., 2000; Larson et al., 2001; Kumar et al., 2012). In addition, a

high-energy diet has deleterious effect on the early embryo development in the oviducts of ewes (Lozano et al., 2003; Kakar et al., 2005). This study shows that MIF mRNA and protein expression in the oviducts of obese cows is lower than that in normal cows. If energy sources are in excess in the maternal body, *MIF* gene expression in the oviduct may be downregulated to protect the embryo.

Cells targeted by insulin, including myocytes (Benigni et al., 2000) and adipocytes (Sakaue et al., 1999; Atsumi et al., 2007), produce MIF in order to regulate glucose uptake and glycolysis. Therefore, the low glucose and insulin levels might be the reason for the low MIF expression in lean cows. However, insulin-resistant adipocytes show decreased MIF expression even in the presence of excess insulin and glucose (Sakaue et al., 1999). Obesity-associated insulin resistance is well known also in ruminants (McCann et al., 1986; Bergman et al., 1989), and peripheral tissues of obese ruminants do not respond to insulin. Obesity-associated insulin resistance, therefore, may be the reason for the low MIF expression in obese cows. Further studies are required to test the hypothesis that oviducts in lean and obese cows express lower MIF in muscle layers, wherein glucose uptake and glycolysis are downregulated to decrease sperm transport and inhibit pregnancy when energy is in short supply or in excess.

Peritoneal macrophages phagocytize normal sperm in humans (Oren-Benaroya et al., 2007). Furthermore, in humans, peritoneal macrophages migrate specifically to the oviducts. Infertile patients with endometriosis had more peritoneal macrophages than did fertile normal women or infertile women with distal or proximal tubal obstruction (Haney et al., 1983). Therefore, further studies are required to evaluate the hypothesis that decreassed MIF expression increases macrophages to contribute to infertility in obese and lean animals.

In conclusion, obese cows had suppressed MIF expression in the ampulla and isthmus of the oviduct, as we had hypothesized, but unexpectedly, MIF expression was also lower in lean cows.

CHAPTER V

(Study 3)

Expression of GMCSF in bovine oviducts during estrus, post-ovulation, and luteal phases

Abstract

GMCSF is an important promoter of fertilization and early embryogenesis in *in vitro*. Therefore, *in vivo*, GMCSF expression in bovine oviducts may be higher when fertilization and early embryogenesis occur. This study tested this hypothesis by comparing GMCSF expression levels between estrus (day 0), post-ovulation (day 3), and luteal phases. Both ampullar and isthmic samples were collected from Japanese Black heifers during estrus (n = 5), post-ovulation (day 3; n = 6), and luteal (days 9 to 12; n = 5) phases. GMCSF mRNA and protein were extracted from the ampullar and isthmic samples, and their levels were measured by real-time PCR and western blot, respectively. Fluorescent immunohistochemistry was performed on frozen ampullar and isthmic sections using antibodies against GMCSF. GMCSF mRNA and protein expression levels were higher during estrus and post-ovulation phases relative to those observed during the luteal phase ($p < 0.05$). Fluorescent immunohistochemistry confirmed that during all evaluated estrous-cycle phases, the primary site of GMCSF expression in the ampulla and isthmus was the tunica mucosa. Our results revealed that the bovine ampulla and isthmus showed higher GMCSF-expression levels during the estrus phase as compared to the luteal phase. Furthermore, higher GMCSF expression levels were also observed during the post-ovulation phase. Therefore, GMCSF seems as an important factor for fertilization and early embryogenesis also *in vivo*.

5.1. Introduction

The addition of GMCSF to culture medium improves the proportion of embryos from cows (de Moraes and Hansen, 1997; Hansen et al., 2014), humans (Sjo¨blom et al., 1999), and pigs (Kwak et. al., 2012) growing to the blastocyst stage and increases post-transfer embryonic survival in mice (Sjo¨blom et al., 2005; Loureiro et al., 2009). GMCSF improves inner cell mass of the trophectoderm of bovine embryos, enabling their survival in a pluripotent state (Dobbs et al., 2013). Therefore, GMCSF is an important promoter of fertilization and early embryogenesis during *in vitro*. Also, *in vivo*, GMCSF expression in bovine oviducts may be higher when fertilization and early embryogenesis occur. To test whether GMCSF expression in bovine oviducts is higher when fertilization and early embryogenesis occur, we compared GMCSF expression levels during estrus (day 0), post-ovulation (day 3), and luteal phases.

5.2. Materials and methods

5.2.1. Animals and treatments

Post-pubertal Japanese Black heifers (30-month-old) were housed in a free-stall barn. Their daily diet included Italian ryegrass hay (84.2% DM, 2.30 Mcal ME/kg DM, and 13.3% CP) and concentrate (86.6% DM, 3.82 Mcal ME/kg DM, and 21.3% CP), and they were given 23.9 Mcal of ME in average. Water and mineral blocks were provided *ad libitum*. The absence of diseases, including reproductive disease, was confirmed by daily observation and weekly rectal palpation. The heifers received two intramuscular injections of dinoprost to control their estrus stages. The heifers were sacrificed on day 0 (estrus; $n = 5$), day 3 (post-ovulation; $n = 6$), or day 9 to 12 (luteal; $n = 5$) after ovulation, and the oviducts on the side ipsilateral to ovulation were collected within 5 min. The tissues surrounding the oviduct were carefully removed, and the oviducts were washed with PBS on ice. Half of the ampullae and half of the isthmuses were frozen in liquid nitrogen and preserved at −80°C until use for RNA or protein extraction. The remaining samples were stored in 4% PFA at 4°C for 12 h before being used for immunohistochemistry studies.

5.2.2. RNA extraction, cDNA synthesis, and real-time PCR

Total RNA was extracted from the frozen ampullar and isthmic samples, using RNAiso Plus, and subsequently treated with ribonuclease-free deoxyribonuclease. The concentration and purity of each RNA sample were evaluated to ensure the A_{260}/A_{280} ratio was in the acceptable range of 1.8 to 2.1. Electrophoresis of total RNA, followed by staining with ethidium bromide, was performed to verify the mRNA quality of all samples, and the 28S:18S ratios were 2:1. We synthesized cDNA from 2 μg RNA from each sample in 20-μL reactions, using random hexamer primers and a high-capacity cDNA reverse-transcription kit.

The expression levels of *GMCSF* and two housekeeping genes, *C2orf29* and *SUZ12*, were measured in duplicate by real-time PCR. Details of the real-time PCRs were described in the previous chapters. The expression of the *GMCSF* gene was normalized to the geometric means of *C2orf29* and *SUZ12* expression.

5.2.3. Protein extraction and western blotting for GMCSF

The ampullar or isthmic samples were ground in liquid nitrogen and homogenized using T-PER containing protease inhibitor. The total protein content of each tissue homogenate was estimated using the bicinchoninic acid kit. The extracted samples (20 μg of total protein) were then loaded onto polyacrylamide gels with a

recombinant bovine GMCSF used as a standard (12.5–100 ng/well) for estimating GMCSF concentration in the samples. Details of both sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting using the same primary antibodies and the same anti-β-actin mouse monoclonal antibody were described in the previous chapters. GMCSF expression was normalized to the expression of β-actin in each sample.

5.2.4. Fluorescent immunohistochemistry and confocal microscopic observation

After storage in 4% PFA at 4°C for 12 h, the tissue blocks were placed in 30% sucrose, which was diluted with PBS until the blocks were infiltrated with sucrose. The blocks were then frozen in Tissue-Tek OCT compound and maintained at −80°C. Next, the blocks were sectioned into 14-μm thick cross-sections using the cryostat and mounted on microscope slides. Briefly, the sections were treated with 0.3% Triton X-100 in PBS for 10 min and blocked with 0.5 mL PBS containing 10% normal goat serum for 1 h. The sections were then incubated overnight at 4°C in PBS containing 1 μg/mL of the same anti-GMCSF mouse monoclonal antibody and 0.5% normal goat serum. After overnight treatment with the primary antibody, the sections were washed thoroughly and incubated with a cocktail of fluorochrome-conjugated secondary antibody (4 μg/mL Alexa Fluor 546 goat anti-mouse IgG; Invitrogen, Carlsbad, CA,

USA) and 1 μg/mL of 4′,6′- diamino-2-phenylindole (DAPI; Wako Pure Chemicals) for 2 h at room temperature. The stained sections were then observed by confocal microscopy (LSM710; Carl Zeiss, Göttingen, Germany). To verify the specificity of the signals, we included several negative-control sections in which the primary antiserum had been omitted or pre-absorbed with 5 nM of the recombinant GMCSF protein described above, or the negative-control mouse IgG had been used instead of the primary antibody.

5.2.5. Statistical analysis

The statistical significance of the differences between the measured values was assessed by one-factor ANOVA, followed by post-hoc comparisons with Fisher's PLSD test using a model consisting of variance from the effect of the estrous-cycle phase (estrus, post-ovulation, or luteal) and the residual.

5.3. Results

The ANOVA revealed an effect ($p < 0.05$) of the estrous-cycle phase on the amount of GMCSF mRNA in both the ampullar (**Fig. 5.1A**) and isthmic samples (**Fig. 5.1B**) of bovine oviduct. Both the estrus and post-ovulation phases showed higher ($p < 0.05$) GMCSF mRNA levels in both parts of the oviduct relative to those observed in the luteal phase. There was no significant difference observed between the estrus and post-ovulation phases ($p > 0.1$).

Western blots showed an immunoreactive protein band with an apparent molecular weight of 26 kDa in all of the ampullar (**Fig. 5. 2A**) and isthmic samples (**Fig. 5. 2B**) from bovine oviducts. The ANOVA revealed an effect of the estrous-cycle phase on the amount of GMCSF protein in both samples, with the ampullar ($p < 0.05$; **Fig. 5. 2C**) and isthmic ($p < 0.05$; **Fig. 5. 2D**) samples showing higher GMCSF expression levels during the estrus and post-ovulation phases as compared to the luteal phase. There was no significant difference between the estrus and post-ovulation phases.

Furthermore, immunohistochemistry results revealed that GMCSF-positive cells were present in the tunica mucosa of both the ampullar (**Fig. 5.3**) and isthmic (**Fig. 5.4**) samples, while a comparatively weak staining was observed in the stroma.

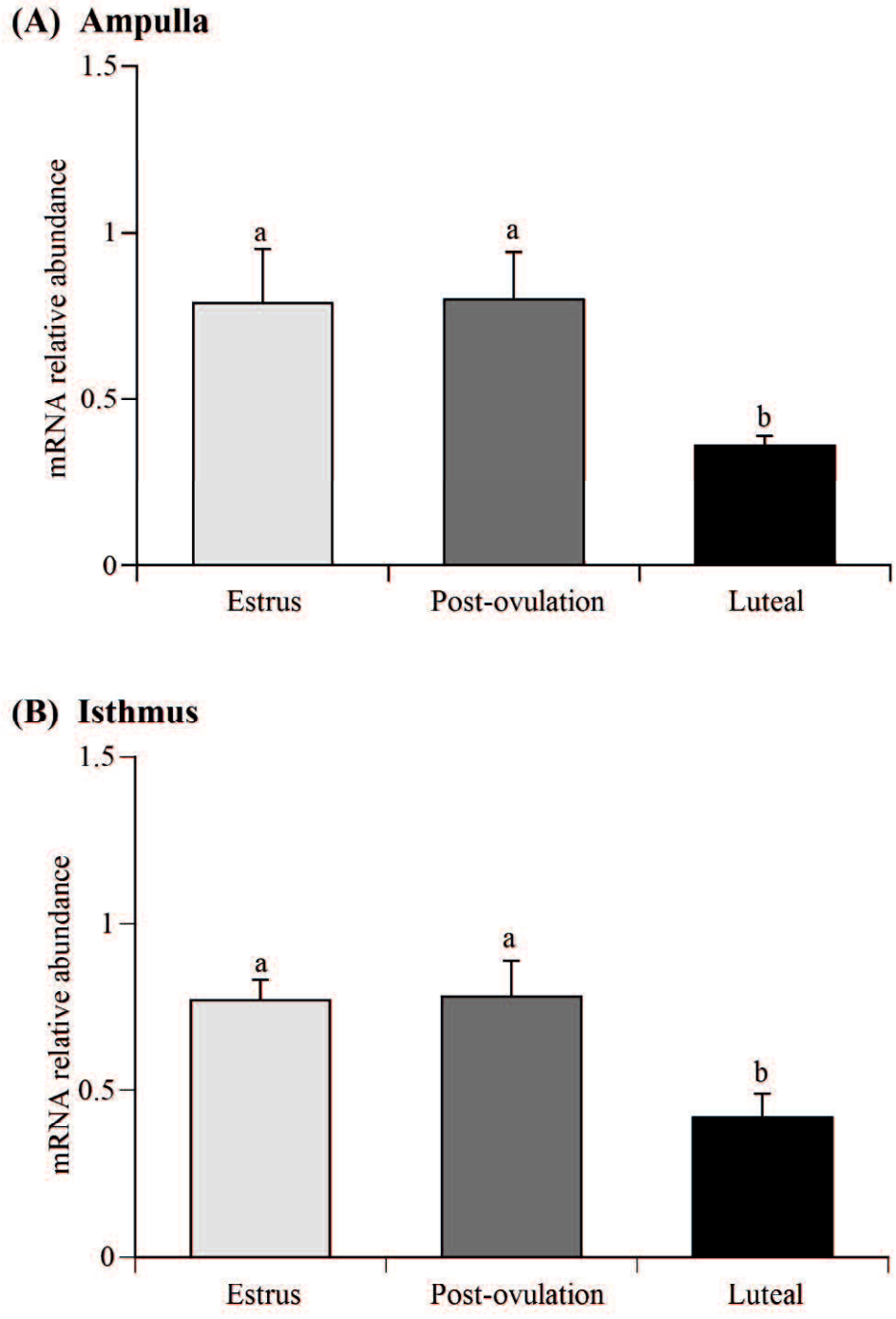

Fig. 5.1. Relative GMCSF mRNA expression (mean ± SEM) as determined by real-time PCR analysis in (A) ampullar and (B) isthmic sections of bovine oviduct during estrus, post-ovulation, and luteal phases. Data are normalized to the geometric mean of the expression of two housekeeping genes (*C2orf29* and *SUZ12*). Letters (a vs. b) indicate differences ($p < 0.05$) between phases

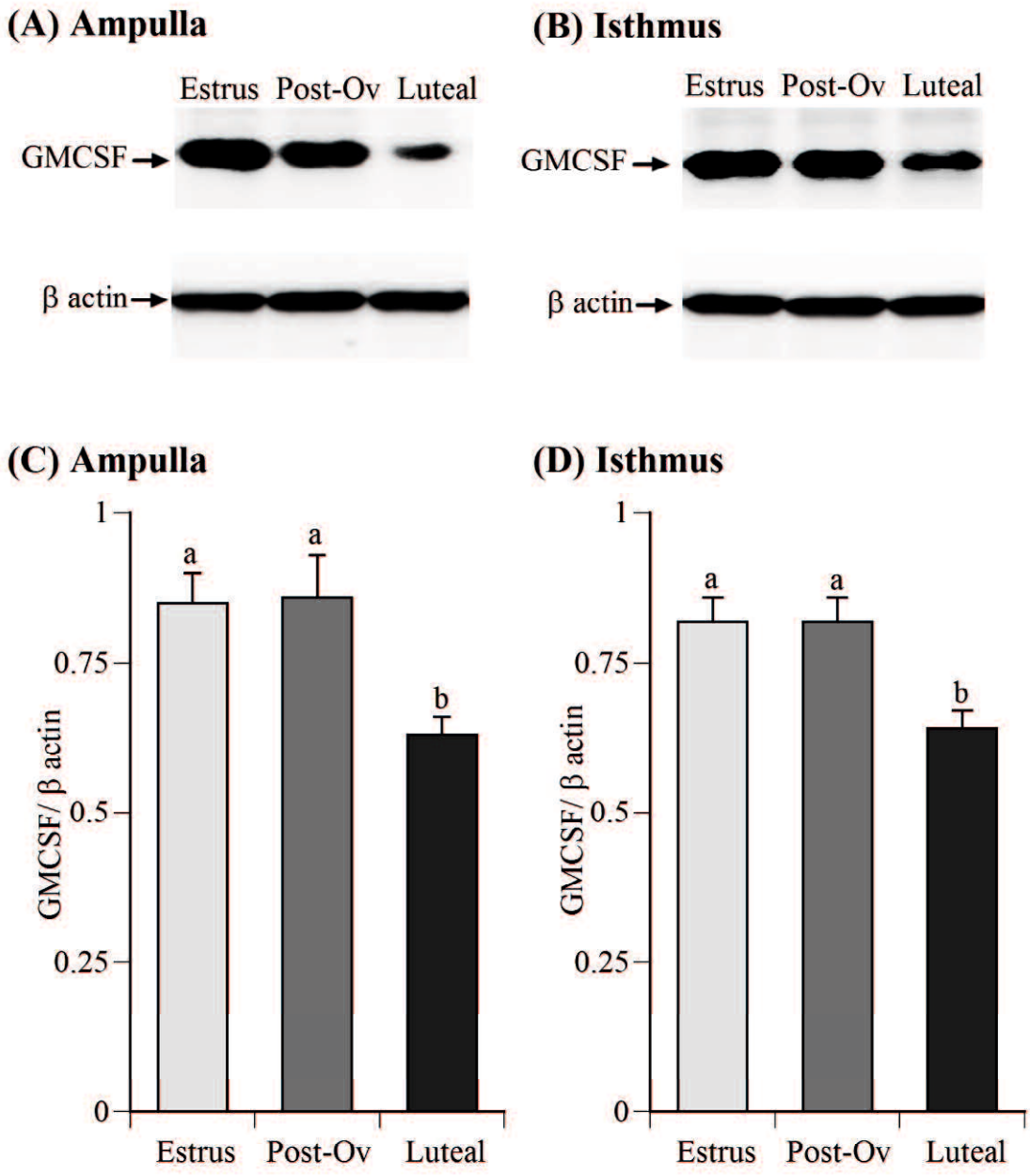

Fig. 5.2. Representative GMCSF and β-actin protein bands detected by western blotting with anti-GMCSF or anti-β-actin antibodies in (A) ampullar and (B) isthmic sections of bovine oviducts during the estrus, post-ovulation, and luteal phases. Levels of GMCSF-protein expression normalized to β-actin expression in (C) ampullar and (D) isthmic sections of bovine oviducts obtained during the estrus, post-ovulation (Post-OV), and luteal phases. Letters (a vs. b) indicate differences ($p < 0.05$) between phases.

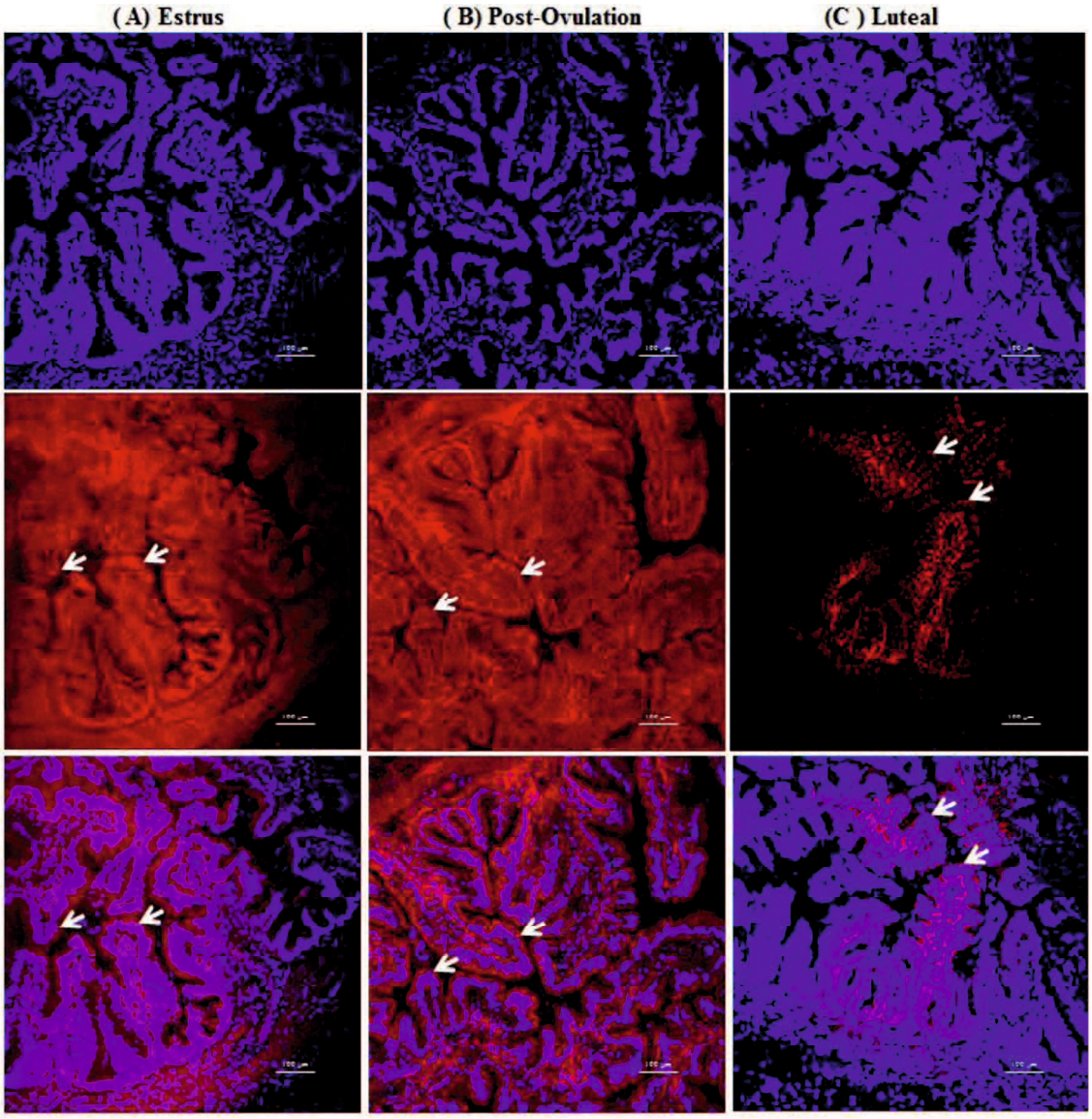

Fig. 5.3. Immunohistochemistry confirmed the presence of GMCSF-positive cells (red) in the ampullar sections of the bovine oviduct during the (A) estrus, (B) post-ovulation, or (C) luteal phases. The blue color denotes DAPI staining, which was used to show the location of the nucleus of the DNA. Arrows indicate GMCSF-positive cells in the tunica mucosa. Scale bar is 100 µm.

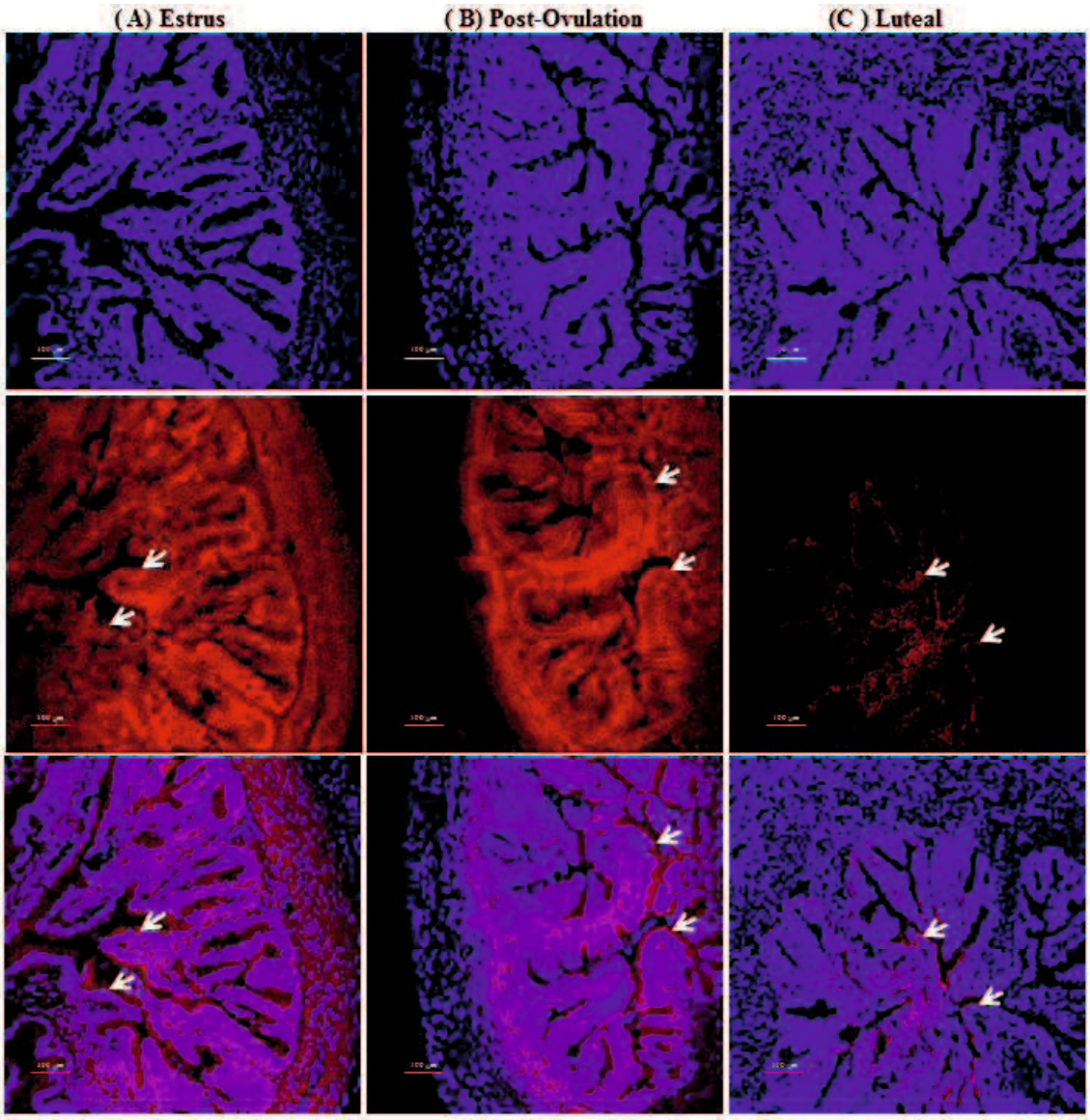

Fig. 5.4. Immunohistochemistry confirmed the presence of GMCSF-positive cells (red) in the isthmic sections of the bovine oviduct during the (A) estrus, (B) post-ovulation, or (C) luteal phases. The blue color denotes DAPI staining. Arrows indicate GMCSF-positive cells in the tunica mucosa. Scale bar is 100 µm.

5.4. Discussion

Our results supported the hypothesis that the bovine ampulla and isthmus exhibit higher GMCSF expression levels during the estrus and post-ovulation phases as compared to that during the luteal phase. Here, we observed the GMCSF expression in the tunica mucosa of the bovine oviduct, which was in agreement with a previous report (de Moraes et al., 1999). As previously mentioned, addition of GMCSF to the *in vitro* fertilization process improved the yield of implantation-competent blastocysts in the cow (de Moraes and Hansen, 1997; Loureiro et al., 2009), human (Sjöblom et al., 1999) and pig (Cui et al., 2004; Kwak et al., 2012). Additionally, GMCSF promotes sperm viability and motility (Rodrı´guez-Gil et al., 2007). Furthermore, both mouse and human embryos synthesize GMCSF receptor from the time of fertilization through all subsequent stages of blastocyst development (Robertson et al., 2001; Sjoblom et al., 2002). The isthmus is the functional sperm reservoir, and the ampulla is the site for acrosome reactions in ruminants (Hunter, 2005). Therefore, our observations of higher GMCSF-expression levels in the ampulla and isthmus of the bovine oviduct during estrus and post-ovulation phases suggested that GMCSF may play important roles in fertilization and early-stage embryo development also *in vivo*.

A second potential major GMCSF function in the bovine oviduct may be as a chemokine. GMCSF enhances neutrophil chemotaxis in the human reproductive tract (Shen et al., 2004). The immune-defense activity of the oviduct is very important, because the uterus and oviduct receive pathogens that are present in sperm and seminal plasma (Profet, 1993; Kelly et al., 1997). Moreover, pathogens and endotoxins may enter into the oviduct via the cervix/uterus, peritoneal cavity, and follicular fluid (Mårdh et al., 1981; Hoof 2007; Tang et al., 2013). For example, *Neisseria gonorrhoeae* bacteria may ascend from the endocervix into the uterus and oviducts and induce salpingitis, a major cause of infertility (Westrom and Wolner-Hanssen, 1993). Therefore, the higher GMCSF-expression levels observed in the bovine oviduct during the estrus and post-ovulation phases may be essential to induce immune responses to pathogens during fertilization and early embryogenesis.

The most probable factor controlling GMCSF expression in bovine oviducts is estradiol, for the following reasons. First, estradiol is important for induction of dramatic cyclic changes in the epithelial lining and the secretory status of the oviduct (Murray et al., 1995). Second, estradiol increases GMCSF production in human keratinocytes (Kanda and Watanabe, 2004) and the endometria of ewes and mice (Robertson et al., 1996; McGuire et al., 2002). Estrogen receptors are present in the

mucosal cells of the human oviduct (Lam et al., 2005), and estradiol secreted from the ovary is transferred from the ovarian vein to the oviduct artery via a counter-current transfer system (Hunter, 2005). Therefore, GMCSF expression in the oviduct may be controlled by the level of estrogen secreted from the ovaries, and high levels of estradiol secreted during estrus may enhance GMCSF expression in the bovine oviduct.

This study also revealed high GMCSF-expression levels during the post-ovulation period after the estrus phase, when estradiol concentrations are not high in bovine oviduct and oviductal fluid (Wijayagunawardane et al., 1998; Lamy et al., 2016). Lamy et al. (2016) recently reported that estrone levels in bovine oviductal fluid were moderate during the estrus phase, highest at the post-ovulation phase, and lowest during the luteal phase. Therefore, GMCSF expression in the oviduct may also be controlled by estrone levels.

One previous study evaluating changes in GMCSF expression in bovine oviducts showed similarities in immunoreactive GMCSF-expression levels in the oviducts of Holstein or crossbred beef cows at days 0, 7, and 14 of the estrous cycle (de Moraes et al., 1999). Our results indicated that GMCSF-expression levels were significantly higher during the estrus and post-ovulation phases (day 3) as compared to the luteal phase. One possible explanation for these contradictory results may be the

difference in animals. Specifically, differences between heifers or multiparous cows suggests the importance of further studies to clarify the mechanism of GMCSF expression in the bovine oviduct, given that heifers generally exhibit better fertility relative to cows.

In conclusion, bovine ampulla and isthmus showed higher GMCSF-expression levels during the estrus and post-ovulation phases as compared to that during the luteal phase, indicating that GMCSF is important for fertilization and early embryogenesis also *in vivo*.

CHAPTER VI

(Study 4)

Expression of MIF in bovine oviducts during estrus, post-ovulation, and luteal phases

Abstract

The hypothesis that MIF in the bovine oviduct is very important for early embryogenesis has not been well-substantiated. This study was designed to test the hypothesis that bovine oviducts express higher levels of MIF during the post-ovulation phase. Both ampullar and isthmic samples were collected from Japanese Black heifers during estrus (Day 0, n = 5), post-ovulation (Day 3, n = 6), and the luteal (Days 9 to 12, n = 5) phase. MIF mRNA and protein were extracted from the ampullar and isthmic samples and their levels measured by the real-time PCR and western blot, respectively. Fluorescent immunohistochemistry was performed on frozen ampullar and isthmic sections by using antibodies against MIF. MIF mRNA and protein expression were higher in the post-ovulation phase than they were during estrus and the luteal phase ($P < 0.05$). Fluorescent immunohistochemistry confirmed that in all of the estrous cycle phases evaluated, the primary site of MIF expression in the ampulla and isthmus was the tunica mucosa. In conclusion, the bovine ampulla and isthmus showed higher MIF expression in the post-ovulation phase. Further studies are needed to clarify the role of MIF in bovine oviducts.

6.1. Introduction

The oviduct is the first region where the maternal body makes contact with the early embryo and may play an essential role in embryogenesis. The classical view has been that the ampulla of the bovine oviduct is an important site for gamete maturation, fertilization, and development to the 8-cell and 16-cell stage embryo (El-Banna and Hafez, 1970). However, recent studies have reported that the site of fertilization is actually in the vicinity of the ampullary-isthmic junction (Croxatto, 2002; Hunter, 2005). Therefore, it is likely that the embryo spends most of its time in the isthmus prior to entering into uterus, and not in the ampulla. In support of this theory, Maillo et al. (2015) found that most of the non-fertilized oocytes/embryos were located in the isthmus on day 3 after estrus. The oviduct is known to provide growth factors and nutrients via oviductal fluid to promote embryogenesis (Hugentobler et al., 2010; Besenfelder et al., 2012), and the oviduct environment, where the embryos of most mammalian species spend their first 3 days, is known to affect the physiology and metabolism of the embryo (Leese, 1995). The oviduct modifies its activity at different phases of the estrous cycle in order to meet the requirements of the developing embryos (Murray, 1995; Tadokoro et al., 1995; Buhi et al., 2000; Gabler et al., 2001). MIF is an important molecule in the oviduct (Suzuki et al., 1996). Therefore, higher levels of MIF

in the oviduct during post-ovulation may have any important role in creating the optimal environment for embryogenesis. However, the hypothesis that MIF in the bovine oviduct is very important for early embryogenesis has not been well-substantiated.

As mentioned above, the microenvironment of the oviduct changes during the estrous cycle, where optimal functions are controlled by hormonal changes (Wijayagunawardane et al., 1998; Buhi et al., 2000; Buhi, 2002). Estradiol in particular is a very important factor in inducing the dramatic cyclic changes that occur in the epithelial lining as well as in the secretory status of the oviduct (Murray et al., 1995). Estradiol regulates the MIF secretory pathway in the first-trimester during pregnancy in humans (Ietta et al., 2010). MIF expression is also cycle-dependent in the mouse ovary, human and mouse endometrium, and bovine corpus luteum (Suzuki et al,. 1996; Bove et al., 2000; Kats et al., 2005; Lopes et al., 2011). Additionally, estrogen decreases MIF production in the female rat colon (Houdeau et al., 2007). Therefore, MIF expression may also be cycle-dependent in the bovine oviducts.

This study aimed to test the hypothesis that MIF may be more highly expressed in the bovine oviduct during the post-ovulation phase.

6.2. Materials and Methods

6.2.1. Samples

Bovine oviducts samples were collected as written in the chapter V.

6.2.2. RNA extraction, cDNA synthesis, and real-time PCR

We extracted total RNA, and synthesized cDNA from 2 μg RNA from each sample as written in the chapter V.

The expression levels of *MIF*, *C2orf29*, and *SUZ12* were measured in duplicate by real-time PCR. Details of the real-time PCRs were described in the chapter IV. The expression of the *MIF* gene was normalized to the geometric means of *C2orf29* and *SUZ12* expression.

6.2.3. Protein extraction and western blotting for MIF

We extracted total protein from each sample as written in the chapter V. The extracted samples (30 μg of total protein) were used for western blotting using the same protocol written in the chapter IV. The expression of MIF was normalized to the expression of β-actin in each sample.

6.2.4. Fluorescent immunohistochemistry and confocal microscopic observation

We prepared tissue samples and stained for fluorescent immunohistochemistry using the same protocol written in the chapter IV.

6.2.5. Statistical analysis

The statistical significance of the differences in the measured values was assessed by one-factor ANOVA followed by post-hoc comparisons with Fisher's PLSD test using a model consisting of variance from the effect of the phase of the estrous cycle (estrus, post-ovulation, or luteal) and the residual.

6.3. Results

6.3.1. MIF mRNA in the bovine oviduct

The ANOVA revealed an effect of estrous cycle phase on the quantity of *MIF* mRNA in both the ampullar (**Fig. 6.1A**) and isthmic samples (**Fig. 6.1B**) from bovine oviducts ($P < 0.05$). MIF mRNA expression was observed in all three phases of the estrous cycle; however, the post-ovulation phase showed higher MIF mRNA levels in both parts of the oviduct than the estrus and luteal phase ($P < 0.05$).

6.3.2. MIF protein in the bovine oviduct

Western blots showed an immunoreactive protein band with an apparent molecular weight of 12.5 kDa in all ampullar (**Fig. 6. 2A**) and isthmic samples (**Fig. 6. 2B**) from bovine oviducts. The ANOVA revealed an effect of estrous cycle phase on the quantity of MIF protein from both types of tissue, with the ampullar ($P < 0.05$; **Fig. 6. 2C)** and isthmic ($P < 0.05$; **Fig. 6. 2D**) samples showing higher MIF expression levels during the post-ovulation phase than during estrus and the luteal phase.

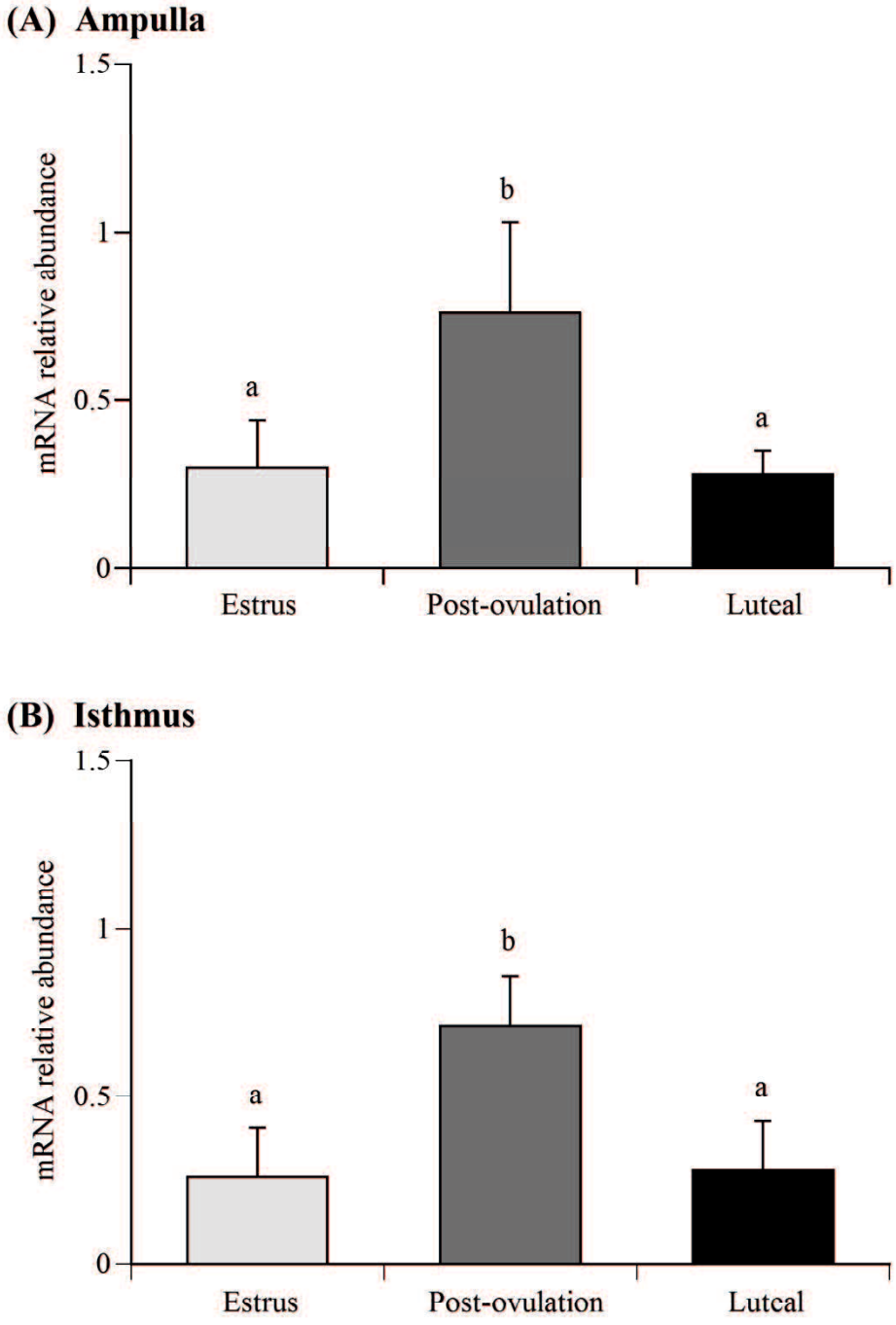

Fig 6.1. Relative MIF mRNA expression (mean ± SEM), as determined by real-time PCR analysis of ampullar (A) and isthmic (B) sections of bovine oviduct during estrus (n=5), post-ovulation (n=6), and the luteal (n=5) phase. Data are normalized to the geometric mean of the expression of two housekeeping genes, namely, *C2orf29* and *SUZ12*. Letters (a *vs*. b) indicate differences ($P < 0.05$) among the phases.

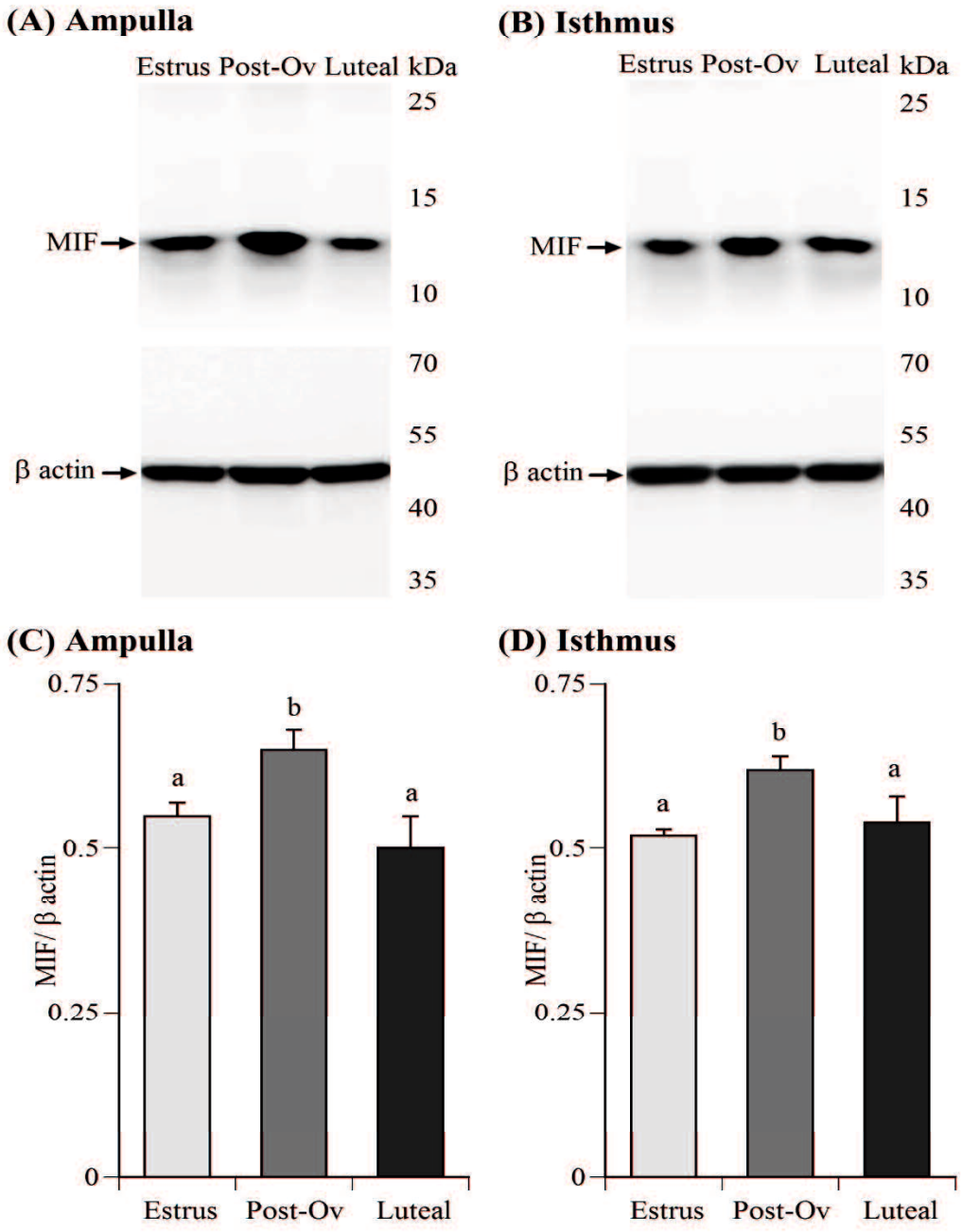

Fig 6.2. Representative MIF and β-actin protein bands detected by western blotting. (A-B) Detection with anti-MIF or anti-β-actin antibodies in ampullar (A) and isthmic (B) sections of bovine oviduct during estrus (n=5), post-ovulation (n=6), and the luteal (n=5) phase. (C-D) Relationship of MIF protein expression normalized to β-actin in ampullar (C) and isthmic (D) sections of bovine oviduct obtained during estrus, post-ovulation (Post-OV), and the luteal phase. Letters (a *vs*. b) indicate differences ($P < 0.05$) among the phases.

6.3.3. Fluorescent immunohistochemistry

MIF-positive cells were observed in the tunica mucosa of both ampullar (**Fig. 6.3**) and isthmic (**Fig. 6.4**) samples.

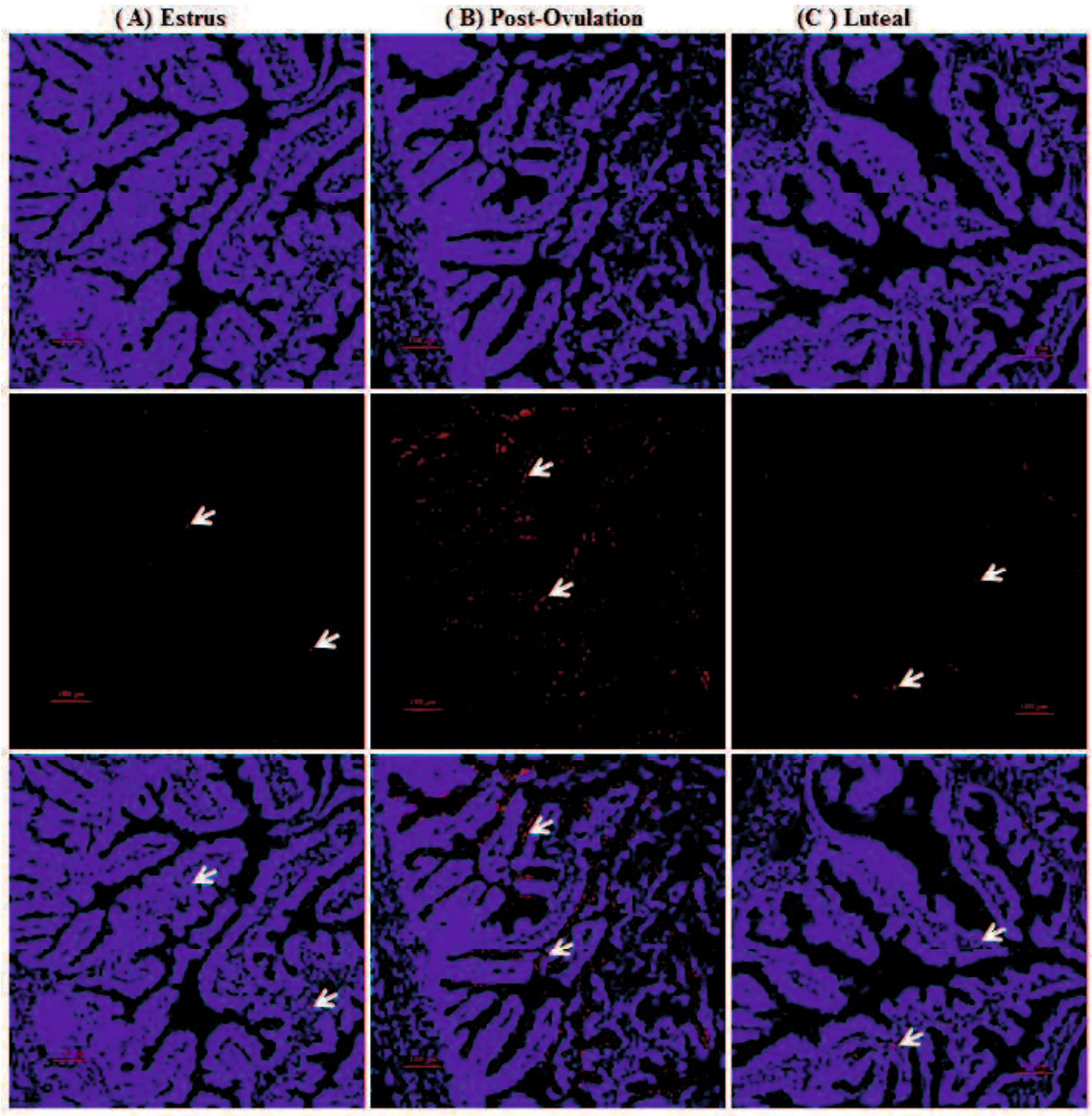

Fig 6.3. Fluorescent immunohistochemistry confirmed the presence of MIF-positive cells (red) in ampullar sections of the bovine oviduct during estrus (A), post-ovulation (B), and the luteal (C) phase. The blue color is DAPI. Arrows indicate the positive cells in the tunica mucosa. Scale bar is 100 µm.

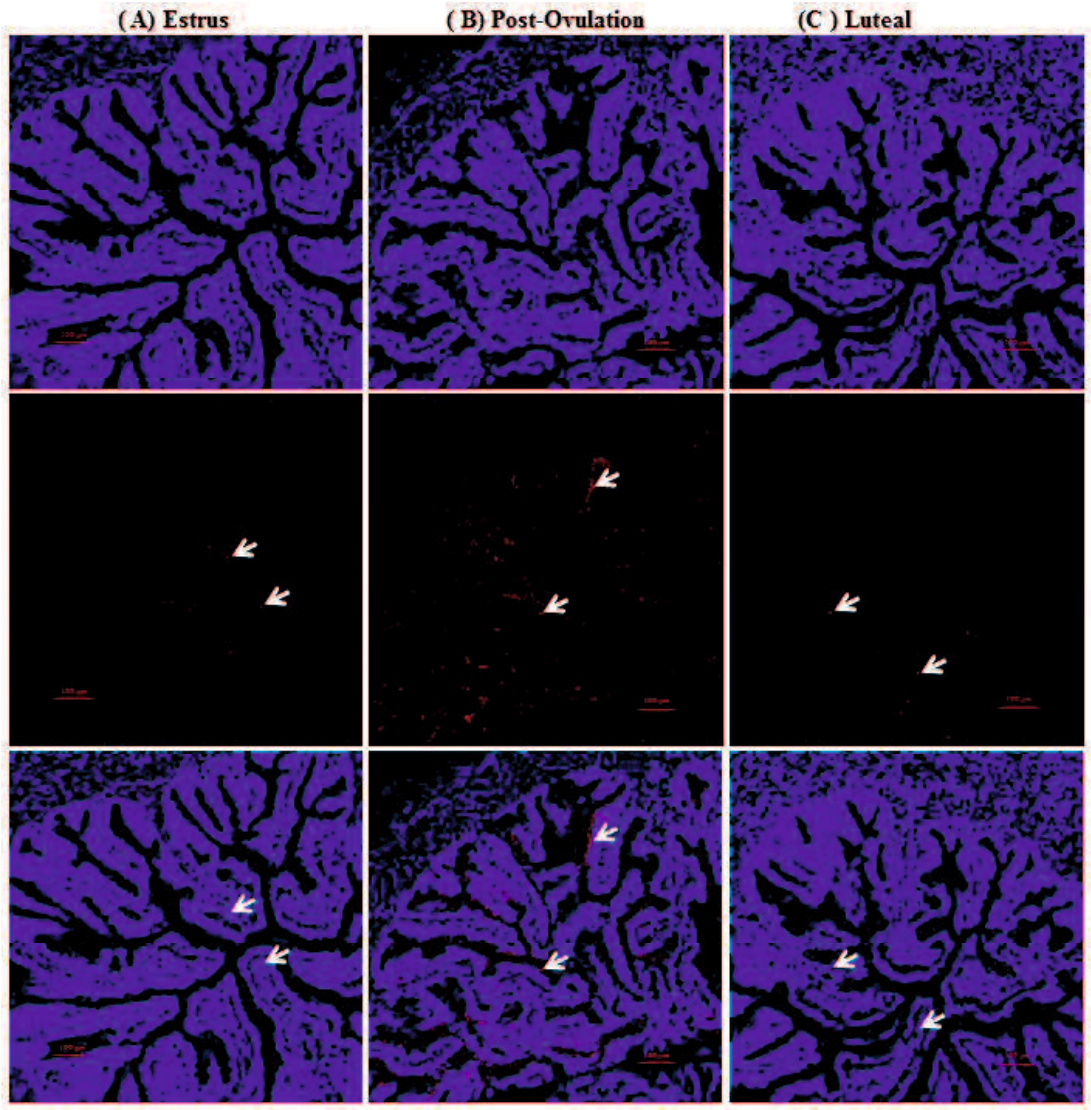

Fig 6.4. Fluorescent immunohistochemistry confirmed the presence of MIF-positive cells (red) in isthmic sections of the bovine oviduct during estrus (A), post-ovulation, (B), and the luteal (C) phase. The blue color is DAPI. Arrows indicate the positive cells in the tunica mucosa. Scale bar is 100 µm.

6.4. Discussion

MIF in the bovine oviduct may be very important for early embryogenesis, but this hypothesis does not have substantial support. The present study revealed that MIF mRNA and protein expression in the oviduct was higher during the post-ovulation phase than during estrus and luteal phase.

The first major potential function of MIF in the oviduct may be as a growth factor for sperm, oocytes, and embryos. In agreement with a previous study in mice (Suzuki et al., 1996), the present study confirmed the expression of MIF in the tunica mucosa of the bovine oviduct. As mentioned previously, the intraperitoneal injection of recombinant MIF the day after mating enhances the pregnancy rate in mice (Bondza et al., 2008). MIF at the optimal concentration promotes sperm capacitation in vitro, whereas low or excess levels of MIF are inhibitory (Carli et al., 2007). Furthermore, the key role of MIF in establishing pregnancy has been previously reported in the ovary, uterus, placenta, and corpus luteum (Suzuki et al., 1996; Bove et al., 2000; Kats et al., 2005; Faria et al., 2010; Lopes et al., 2011; Paulesu et al., 2012). Additionally, decreased MIF levels in maternal serum are known to cause first-trimester miscarriages in humans (Yamada et al., 2003). Nevertheless, further studies are required to clarify any role of the higher MIF levels observed in the bovine isthmus for embryogenesis during the post-ovulation phase.

A second potential major function of MIF in the oviduct may be as a cytokine. For instance, MIF is known to promote the host's responses to infection and contributes to cell-mediated immunity (Weiser et al., 1991; Calandra et al., 1998; Bucala and Shachar, 2014). Immune responses of the oviduct are vital as the uterus and oviduct are subject to pathogens that are present in the sperm and seminal plasma (Profet, 1993; Kelly et al., 1997; Quale, 2002). Moreover, pathogens and endotoxins may enter into the oviduct via the cervix/uterus, peritoneal cavity, and follicular fluid (Mårdh et al., 1981; Herath et al., 2007; Hoof, 2007; Tang et al., 2013). For example, *Chlamydia trachomatis* infections move upwards from the vagina or cervix to the oviduct, damaging the oviduct and inducing infertility in humans, guinea pigs, and mice (de Jonge et al., 2011; Donati et al., 2015; Hafner, 2015). Research shows that the oviducts of amphibians also have immunologic defenses against pathogens (Jantra et al., 2011). A study in humans demonstrated that elevated MIF levels reduced infection in placental explants, whereas the lack of MIF upregulation resulted in a higher susceptibility to infection (de Oliveira Gomes et al., 2011). Although these studies support a defensive role for MIF, further studies are needed to clarify the immunological role of increased MIF expression observed in the bovine oviduct during the post-ovulation phase.

The site of fertilization is near the ampullary-isthmic junction (Croxatto, 2002;

Hunter, 2005), and embryos are predominantly observed in the isthmus at 3 days post-estrus (Maillo et al., 2015). The present study revealed that MIF is expressed in both the ampulla and the isthmus. In the fallopian tubes, the ampulla has alternating propulsive forces towards and away from the uterus at the mid-follicular phase; however, ipsilateral transport to the ovary increases with increasing follicular diameter at the time of ovulation (Wildt et al., 1998). Furthermore, it has been observed that pregnancy rates after intercourse are higher in those women who demonstrate ipsilateral transport, as opposed to those who fail to show lateralization (Wildt et al., 1998). MIF induces vasoconstriction of the pulmonary artery in rats (Zhang et al., 2012). Therefore, further studies are required to clarify any relationship between MIF and force within the bovine oviduct.

Previous studies have revealed that MIF appears to be hormonally regulated. For instance, estrogen decreases MIF production in the colon of female rats (Houdeau et al., 2007), and has also been shown to decrease MIF expression to promote wound healing in humans and mice (Ashcroft et al., 2003; Hardman et al., 2005). In humans, the concentration of estradiol in the plasma is negatively correlated with the concentration of MIF in the plasma (Aloisi et al., 2005). Furthermore, estradiol has been shown to downregulate the MIF secretory pathway in the human placenta (Ietta et al., 2010).

Receptors for estrogen are present in the mucosal cells of the human oviduct (Lam et al., 2005), and it is well known that estradiol secreted from the ovary is transferred from the ovarian vein to the oviduct artery via a counter-current transfer system (Hunter, 2005). Therefore, MIF expression in the oviduct may be controlled by the level of estrogen secreted from the ovaries, where high levels of estradiol during estrus may downregulate MIF expression in the bovine oviduct.

Interestingly, the tunica mucosa of the porcine oviduct expresses luteinizing hormone (LH) receptors (Gawronska et al., 1999), while MIF expression in granulosa cells is increased by human chorionic gonadotropin (Wada et al., 1999). On day 3 after ovulation, when blood concentrations of estradiol and progesterone are low, pulsatile LH secretion is activated in heifers (Kadokawa and Yamada, 1999). Therefore, LH may be another factor that controls MIF expression in the bovine oviduct.

In a recent study by Maillo et al. (2015), RNA sequencing revealed 278 differentially expressed genes (from a total of 24,557 listed genes) in the ipsilateral oviduct with multiple embryos transferred by endoscopy on day 1.5 post-estrus. Although MIF was listed among the 24,557 genes expressed in the isthmus on day 3, its expression did not change significantly by the endoscopical transfer of multiple embryos into the oviduct. Schmaltz-Panneau et al., (2014) utilized microarray and an *in*

vitro co-culture system of bovine oviduct epithelial cells with early bovine embryos to show that the expression of 11 genes was increased by the presence of embryos and had a major impact on the antiviral and immune response. However, MIF was not listed among the 11 genes. Our experimental design did not use artificial insemination or endoscopical transfer of zygotes into the oviduct. Therefore, further studies should explore whether MIF is an important protein in oviducts.

The current study found a significant effect of the estrous cycle phase on MIF mRNA and protein expression in both ampullae and isthmuses. However, we also found large individual differences in each group, suggesting other factors may control MIF expression in oviducts. For example, reported factors controlling MIF expression in muscle and adipose tissues include glucose uptake, glycolysis, insulin, and insulin resistance (Atsumi et al., 2007; Benigni et al., 2000; Toso et al., 2008; Sakaue et al., 1999). Plasma MIF concentrations may increase with age, parity, and undefined factors in healthy Japanese Black cows (Koizumi et al., 2016). Therefore, factors not measured in this study may explain the large individual differences.

In conclusion, bovine ampullae and isthmuses showed the highest levels of MIF expression during the post-ovulation phase. Further studies should be conducted to explore and clarify the role(s) of MIF in bovine oviducts.

CHAPTER VII

(Study 5)

Effect of insulin on GMCSF expression in BOECs

Abstract

This study aimed to evaluate the effect of insulin on GMCSF expression level in BOECs. In first, we confirmed expression of insulin receptor in the tunica mucosa of both ampulla and isthmus parts of oviduct utilizing fluorescent immunohistochemistry. In next, both ampullar and isthmic BOECs from Japanese Black heifers were prepared, cultured, and passage. Then, BOECs were cultured until 70% confluency, and insulin (final concentrations were 0, 1, 20, and 5000 ng/mL) were added to the medium for culture for 24 hours. GMCSF mRNA and protein were measured by real-time PCR and western blot, respectively. Fluorescent immunohistochemistry was performed on BOECs from either ampullar or isthmic sections using one of antibody against cytokeratin, vimentin, insulin receptor, or GMCSF. Fluorescent immunohistochemistry showed that all of the cultured cells were cytokeratin-positive and vimentin-negative, thus, all of the cultured cells were verified as epithelial cells. Also the cultured BOECs expressed both insulin receptor and GMCSF. GMCSF mRNA and protein expression levels were higher in BOECs treated with 20 ng/mL insulin than BOECs treated with 0, 1 or 5000 ng/mL insulin ($p < 0.05$). In conclusion, moderate level of insulin stimulated GMCSF expression in the cultured BOECs, whereas low or excess insulin had no

stimulation, suggesting insulin may regulate GMCSF expression level in bovine oviduct also *in vivo*.

7.1. Introduction

High blood insulin concentration was observed in the obese heifers produced fewer excellent-grade embryos than those observed in lean and normal heifers (Kadokawa et al., 2008). Therefore, the present study aimed to investigate (1) the expression of insulin receptor in oviduct tissues and cultured BOECs, and (2) the effect of insulin on GMCSF expression level in cultured BOECs.

7.2. Materials and methods

7.2.1. Preparation and culture of BOECs

We established culture system of BOECs as following. Oviducts were obtained from Japanese Black heifers and put into tube containing 25 mM HEPES buffer (pH 7.2) containing 10 mM glucose with 100 IU/mL penicillin (Nacalai Tesque, Inc., Kyoto, Japan) and 100 μg/mL streptomycin (Nacalai Tesque), and the tube were transported in 37ºC hot water in thermos from the local slaughterhouse to the laboratory. The ampulla and isthmic part of oviduct were separated from surrounding tissues in room temperature. The luminal wall of each ampulla or isthmus part was washed five times with 1 ml of sterile DMEM/F12 medium (12400-024, Thermo Fisher) containing 0.1% (wt/vol) Bovine serum albumin (BSA; Wako Pure Chemicals), 100 IU/ml penicillin and 100μg/ml streptomycin. Then ampulla or isthmus was filled with 1mg/ml collagenase (Wako Pure Chemicals) dissolved in DMEM/F12 medium containing 0.1% BSA, 100 IU/ml penicillin and 100μg/ml streptomycin to incubate at 38 ºC for 1 hr. After 1 h, each ampulla or isthmus was squeezed one into a 15ml-tube respectively in order to pool epithelial cells of each heifer (volume were 1 ml of isthmus cells or 1.5 ml of ampulla cells). The cell suspension was diluted in 3ml of DMEM/F12, and then the

dissociated epithelial cells were filtered (200 μm; Filcon N cup, 2-7209-06, Asone, Osaka, Japan) to remove undissociated tissue fragments. The filtrates were washed by centrifugation (180 g for 10 min at 20ºC) with 10 ml tris-buffered ammonium chloride (pH 7.5) to remove hemocytes. Then, the cells were washed twice with 10ml DMEM/F12 supplemented with 0.1% (w/v) BSA, 100 IU/ml penicillin, and 100μg/mL streptomycin. After the washing, the cells were counted using cell-counter (Model TC20, Bio-Rad). Cell viability was >95% as assessed by 0.5% trypan blue dye exclusion. The final pellets of the epithelial cells were resuspended in about 3ml of DMEM/Ham's F-12 with 10% (v/v) fetal bovine serum (FBS; Gibco), 20 mg/ml gentamicin (Sigma–Aldrich), and 2 μg/ml amphotericin B (Gibco). Then, 3 ml of cell suspension (at a density of 0.33×10^5 cells/ml; total was 10^5 per ampulla or isthmus) were seeded into one well of 6-well culture dishes (Thermo Scientific) and cultured at 38.5ºC in a humidified atmosphere of 5% CO_2 and 95% air. Because the epithelial cells attached 24–48 h after plating, the medium in the epithelial cell culture was replaced 48 h after seeding. The medium was changed further every 2 days after until cell growth reached 70 to 80% confluency, usually on 6 days after the beginning of culture.

For passage, the cells were treated with trypsin as following. The cells in the culture plates were washed with 3 ml PBS twice. After washing, cells were treated with

0.6ml of 0.25% trypsin and 0.02 % EDTA in PBS for about 3 min to remove cells from the plate. The obtained cells were washed twice with 10 ml DMEM/F12 containing 10% FBS, 100 IU/ml penicillin, and 100μg/mL streptomycin. Then, the cells were re-suspended in 6 to 8 ml of DMEM/F12 containing 10% FBS, 100 IU/ml penicillin, and 100μg/mL streptomycin (at a density of 1.0×10^5 cells/ml; total was 6 to 8×10^5 per ampulla or isthmus).

7.2.2. Fluorescent immunohistochemistry

We evaluated expression of insulin receptor in bovine oviduct tissues obtained from Japanese Black heifers (n = 5, 26 months of age) utilizing fluorescent immunocytochemistry by incubation with an anti-insulin receptor antibody (1:1000; Thermo Scientific) for 2 h at room temperature, and incubation with 4μg/mL Alexa Fluor 546 goat anti-mouse IgG and 1μg/mL DAPI for 2 h at room temperature.

Oviducts as ipsilateral side to the corpus luteum were obtained from Japanese Black heifers (*n* = 5, 26 months of age, at luteal phase) for cell culture. The BOECs were prepared and cultured as the protocol written previously, and then treated with trypsin for passage. The 150 μl cell suspensions (at a density of 1.0×10^5 cells/ml) per lane was cultured using a microscopy chamber (μ-Slide VI 0.4, Ibidi, Planegg, Germany) for 3 days. Cells attached to the bottom of the microscopy chamber were

treated with 4% PFA in PBS for 3 min. Also CellCover (ANACYTE Laboratories UG, Kuhreder, Hamburg) was used for fixation cells instead of 4% PFA for 3 min to verify the location of insulin receptor on cell-surface of BOECs. After washing, the cells were treated with Triton X-100 for 3 min. Then the cells were treated with PBS containing 10% normal goat serum for blocking for 30 min, and they were incubated with either anti-cytokeratin antibody (1:600; Sigma–Aldrich, St. Louis, USA), anti-vimentin antibody (1:1000; Sigma–Aldrich), the same anti-GMCSF mouse monoclonal antibody used in chapter III (1:1000), or the same anti-insulin receptor antibody used for the tissue in this chapter (1:1000; Thermo Scientific) for 2 h at room temperature. Epithelial cells are cytokeratin-positive and vimentin- negative (Yamamoto et al., 2014). Thus, fluorescent immunohistochemistry with either anti-cytokeratin mouse monoclonal antibody or anti-vimentin mouse monoclonal antibody were used in order to confirm the purity of epithelial cells among the cultured cells. After the primary antibody reaction, cells were incubated with 4μg/mL Alexa Fluor 546 goat anti-mouse IgG and 1μg/mL DAPI for 2 h at room temperature. The stained cells were then observed by confocal microscopy and fluorescence microscopic images and DIC images were obtained as single plane. To verify the specificity of the signals, several negative controls were included in which the primary antiserum had been omitted or pre-absorbed with 5 nM

antigen peptide or in which the normal mouse IgG had been used instead of the primary antibody.

7.2.3. Culture to evaluate effect of insulin on GMCSF expression in the BOECs

Oviducts as ipsilateral side to the corpus luteum were obtained from Japanese Black heifers (n = 5, 26 months of age, at luteal phase) for cell culture. The BOECs were prepared and cultured as the protocol written previously, then treated with trypsin for passage. Then, 1.5 ml of cell suspension (at a density of 1.0×10^5 cells/ml) were seeded into 12-well culture plates to culture at 38.5ºC in 5% CO2 and 95% air. The medium was changed every 48 h until the BOEC monolayer reached 70 to 80% confluency (9 or 10 days after starting the culture; 3 or 4 days after passage).

In order to evaluate the effect of insulin on GMCSF expression, medium was replaced by insulin solution (final concentration of insulin were 0, 1, 20, 5000 ng/mL) dissolved in DMEM/Ham's F-12 containing 10% FBS, 100 IU/mL penicillin, and 100μg/mL streptomycin to culture for 24 h. After 24 h of culture, medium was removed and remaining cells were washed with PBS 3 times, then, RNA or protein were extracted from the cells.

7.2.4. RNA extraction, cDNA synthesis, and real-time PCR

Total RNA was extracted from the each well containing cultured ampullar and isthmic BOECs, using 300μl of RNAiso Plus according to the manufacturer's protocol but with a slight modification, namely, the addition of 3 μL of high molecular weight acrylamide polymer solution (Ethachinmate, Nippon Gene, Tokyo, Japan) as a carrier solution for isopropyl alcohol precipitation of RNA. The concentration and purity of each RNA sample were evaluated using the A260/A280 ratio. The cDNA was synthesized from 2 μg of RNA from each sample in 20-μL reactions with random hexamer primers, by using a ReverTra Ace qPCR RT Master Mix (Toyobo, Tokyo, Japan).

The expression levels of *GMCSF*, *C2orf29*, and *SUZ12* were measured by real-time PCRs. The expression of the *GMCSF* gene was normalized to the geometric means of *C2orf29* and *SUZ12* expression.

7.2.5. Protein extraction for western blotting

Total protein was extracted from the each well containing cultured ampullar and isthmic BOECs using 200μl of Tper containing protease inhibitor. The same western blotting method mentioned in the chapter III was used to measure GMCSF protein expression.

7.2.6. Statistical analysis

The statistical significance of the differences between the measured values was assessed by one-factor ANOVA followed by Fisher's PLSD test using a model consisting of variance from the effect of different insulin concentration and the residual.

7.2. Results

Immunohistochemistry confirmed the expression of insulin receptor in the ampullar (**Fig. 7.1A**) and isthmic **(Fig. 7. 1B**) tissues of bovine oviduct, especially strong expression was observed in tunica mucosa.

The purity of the epithelial cells was verified by the presence of cytokeratin (**Fig. 7.2A and Fig. 7.3A**) and absence of vimentin (**Fig. 7.2B and Fig. 7.3B**) in all of the cells prepared from ampullar or isthmic sections of bovine oviduct, respectively.

Immunohistochemistry after fixing by 4% PFA showed that all of the cultured BOECs prepared from ampulla (**Fig. 7.4A**) or isthmus **(Fig. 7.4B**) expressed insulin receptor in the cytoplasm. Also immunohistochemistry after fixing by CellCover showed all of the cultured BOECs prepared from ampulla (**Fig. 7.5A**) or isthmus **(Fig. 7.5B**) expressed insulin receptor on the cell-surface.

Immunohistochemistry showed that all of the cultured BOECs prepared from ampulla (**Fig. 7. 6A**) or isthmus **(Fig. 7. 6B**) expressed GMCSF.

ANOVA revealed an effect ($p < 0.05$) of the different insulin concentration on the GMCSF mRNA and protein expression in BOECs from the ampullar (**Fig. 7.7A** and **Fig. 7.8A**) and isthmic **(Fig. 7.7B** and **7.8B**) sections of bovine oviducts, respectively. BOECs treated with 20 ng/mL insulin showed higher ($p < 0.05$) GMCSF mRNA and

protein expression in both parts of the oviduct than the BOECs treated with 0, 1 and 5000ng/mL insulin.

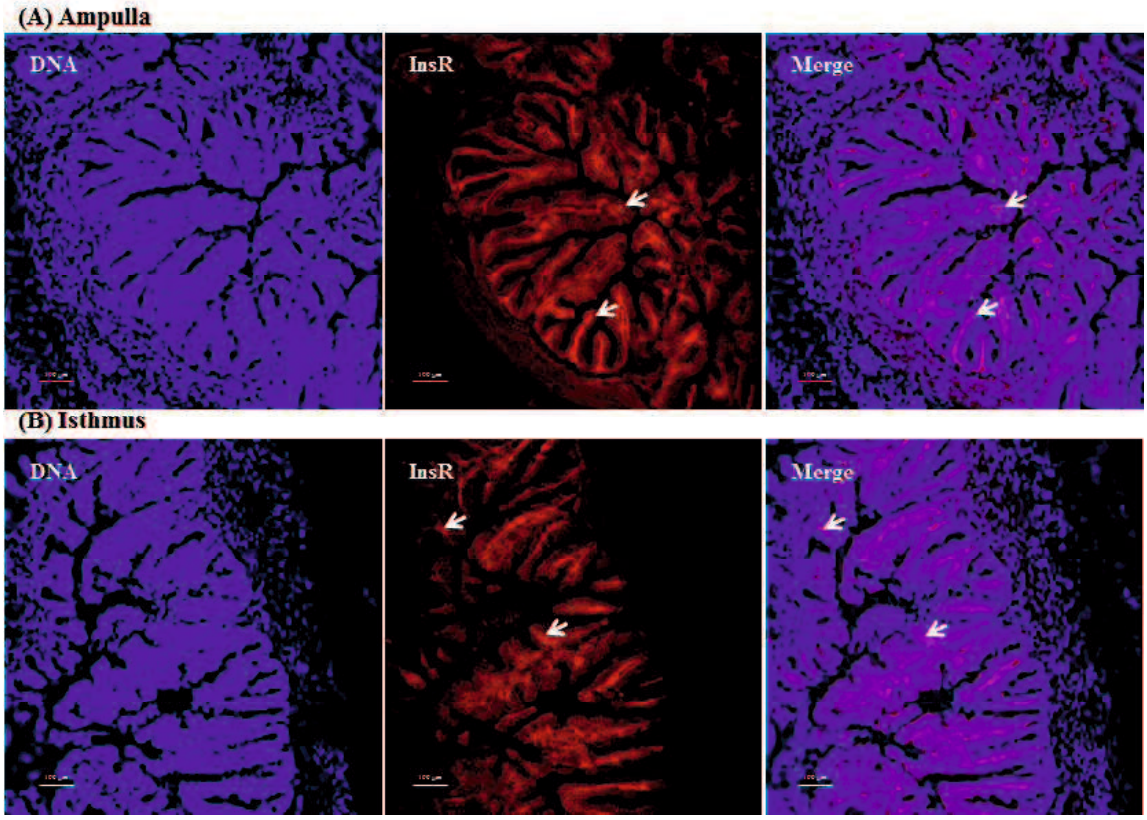

Fig. 7.1. Immunohistochemistry confirmed that the ampulla (**A**) and isthmus **(B)** expressed insulin receptor (InsR). The blue color in left pictures denotes DAPI staining, which was used to show the location of the DNA in nucleus. The red color in center pictures denotes insulin receptor staining. The right pictures show the merge. Arrows indicate insulin receptor positive cells in the tunica mucosa. Scale bar is 100 µm.

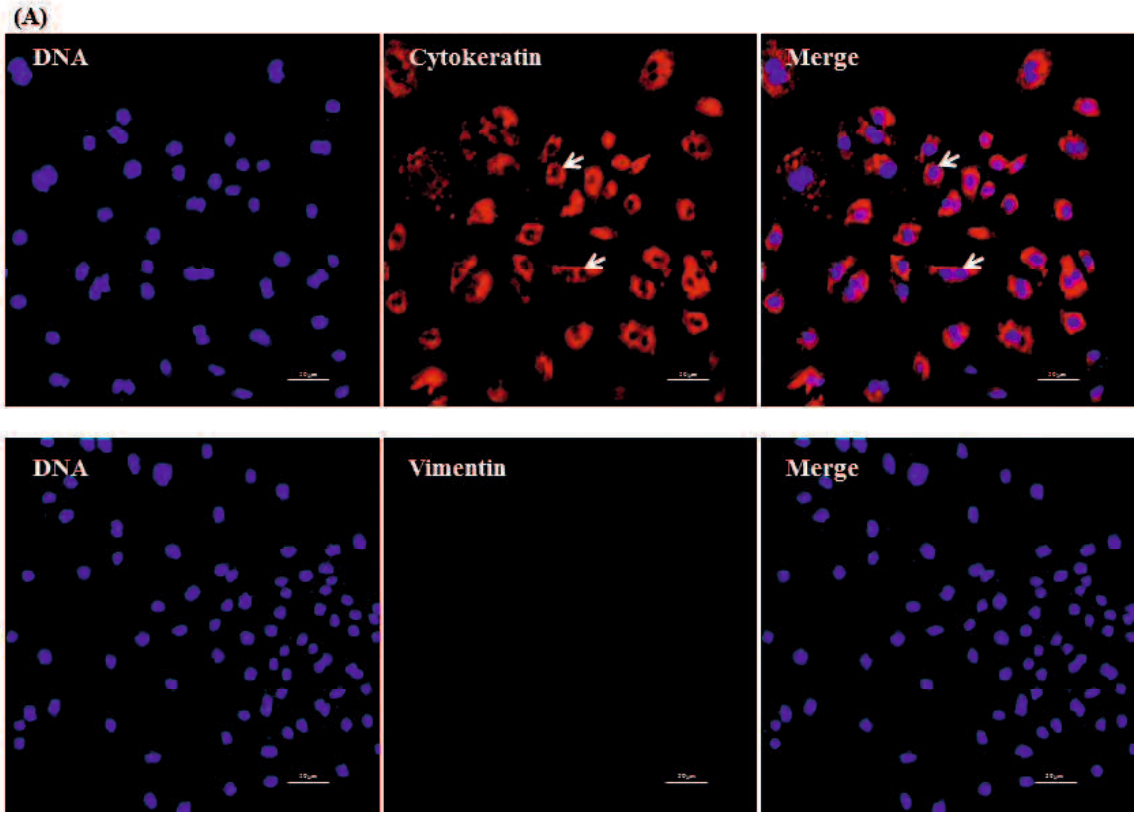

Fig. 7.2. Immunohistochemistry confirmed that all of the cultured cells prepared from ampulla were cytokeratin-positive (A) and vimentin-negative (B). The blue color in left pictures denotes DAPI staining. The red color in center pictures denotes cytokeratin or vimentin staining. The right pictures show the merge. Arrows indicate cytokeratin positive cells. Scale bar is 50 μm.

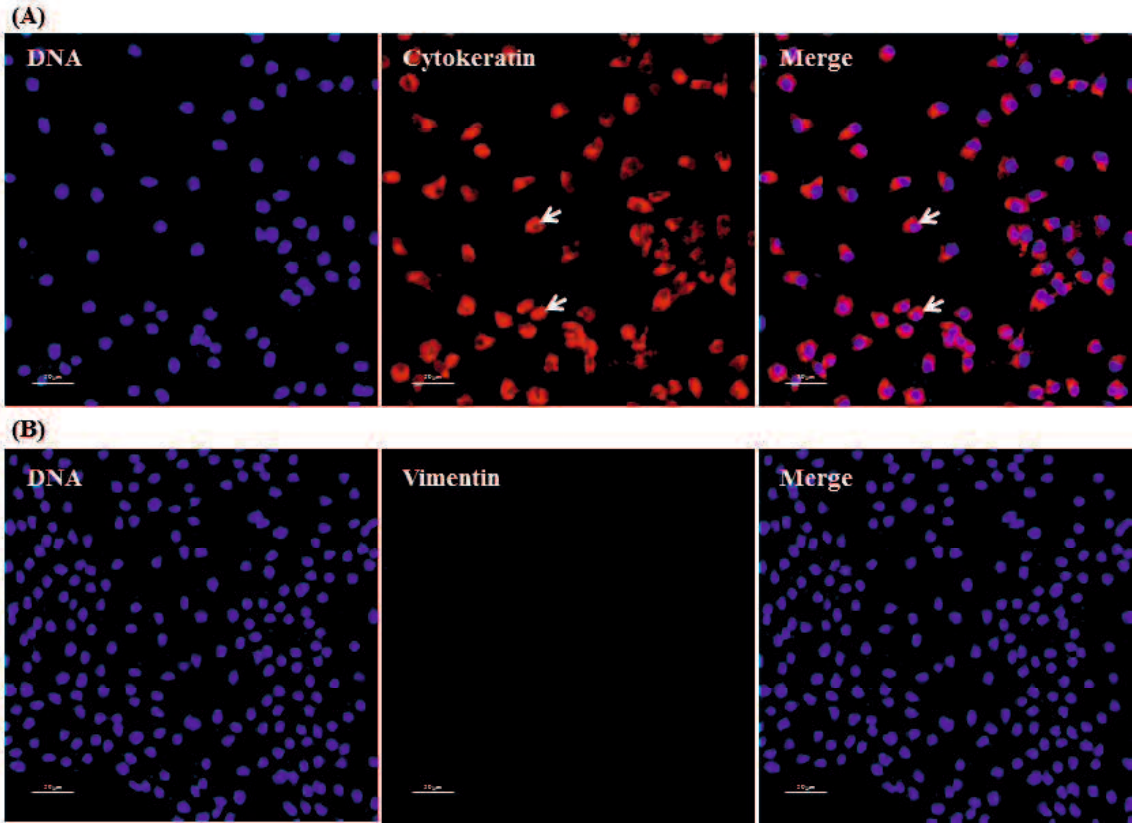

Fig. 7.3. Immunohistochemistry confirmed that all of the cultured cells prepared from isthmus were cytokeratin-positive (A) and vimentin-negative (B). The blue color in left pictures denotes DAPI staining. The red color in center pictures denotes cytokeratin or vimentin staining. The right pictures show the merge. Arrows indicate cytokeratin positive cells. Scale bar is 50 μm.

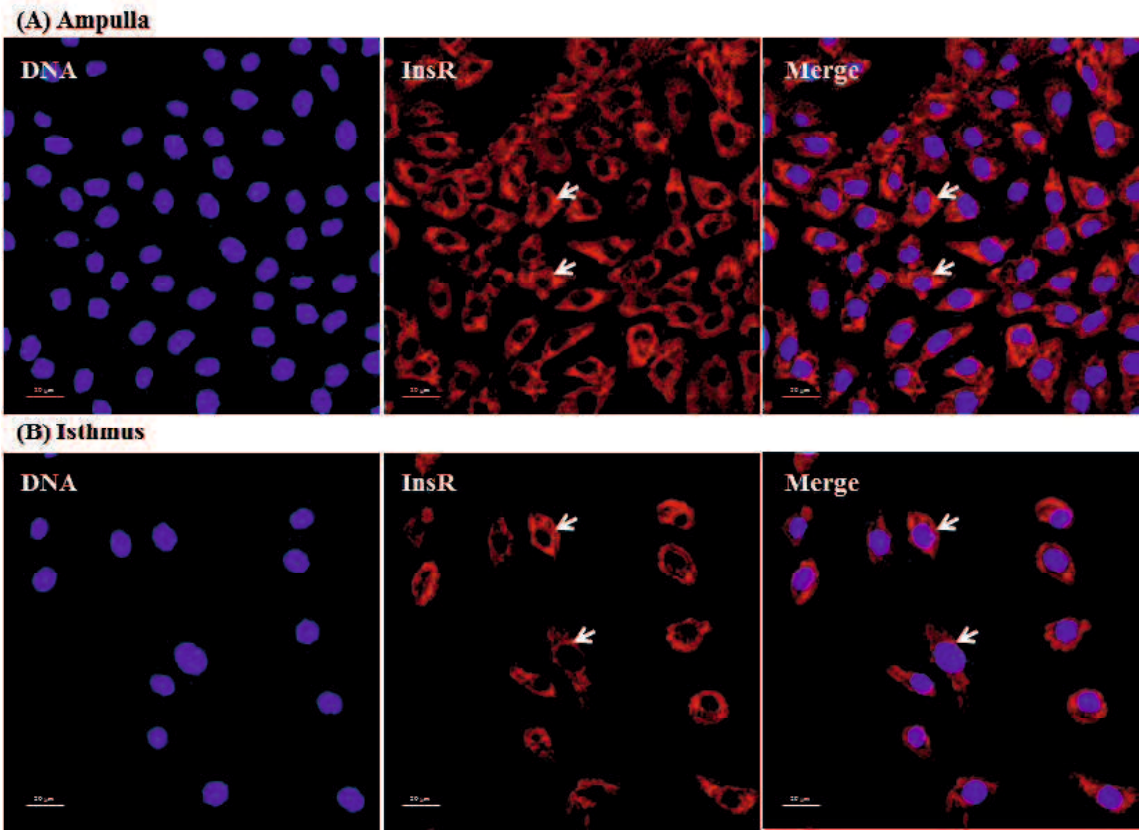

Fig. 7.4. Immunohistochemistry confirmed that all of the cultured cells (fixed by 4% PFA) prepared from ampulla (A) or isthmus (B) expressed insulin receptor (InsR). The blue color in left pictures denotes DAPI staining. The red color in center pictures denotes insulin receptor staining. The right pictures show the merge. Arrows indicate insulin receptor positive cells. Scale bar is 20 μm.

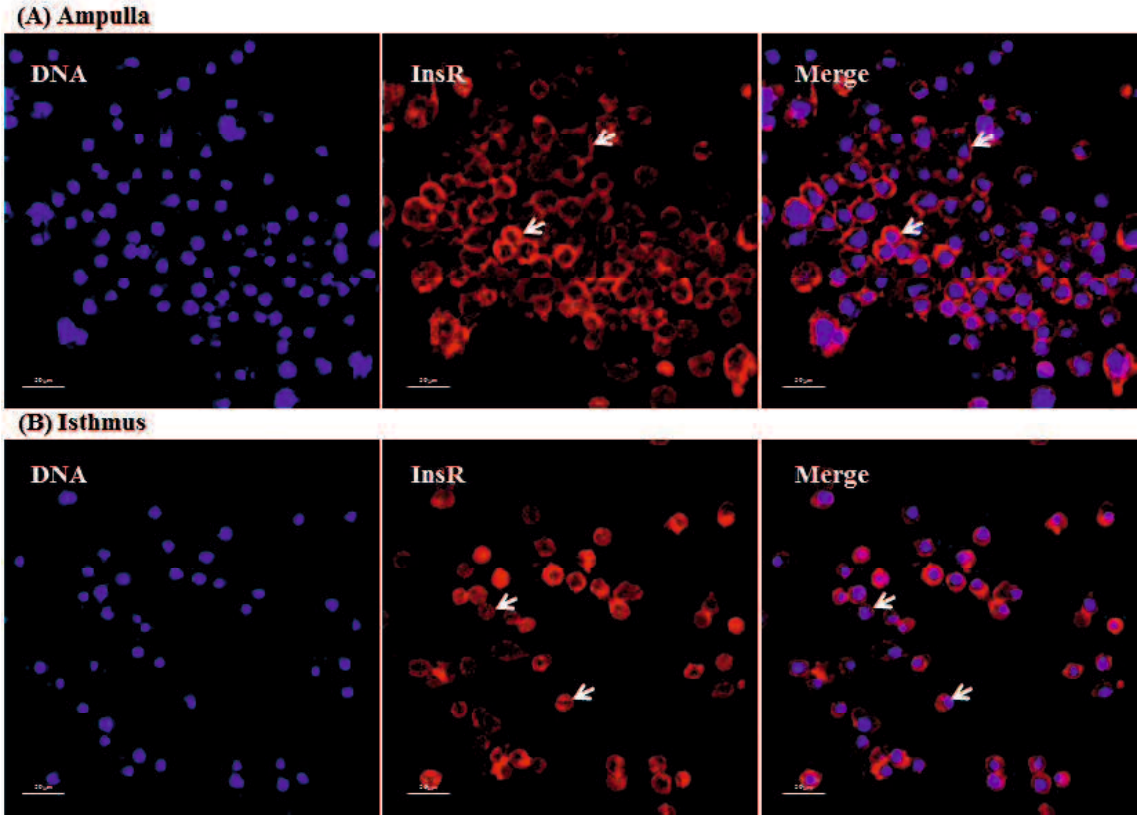

Fig. 7.5. Immunohistochemistry confirmed all of the cultured cells (fixed by CellCover) prepared from ampulla (A) or isthmus (B) expressed insulin receptor (InsR). The blue color in left pictures denotes DAPI staining. The red color in center pictures denotes insulin receptor staining. The right pictures show the merge. Arrows indicate insulin receptor positive cells. Scale bar is 50 μm.

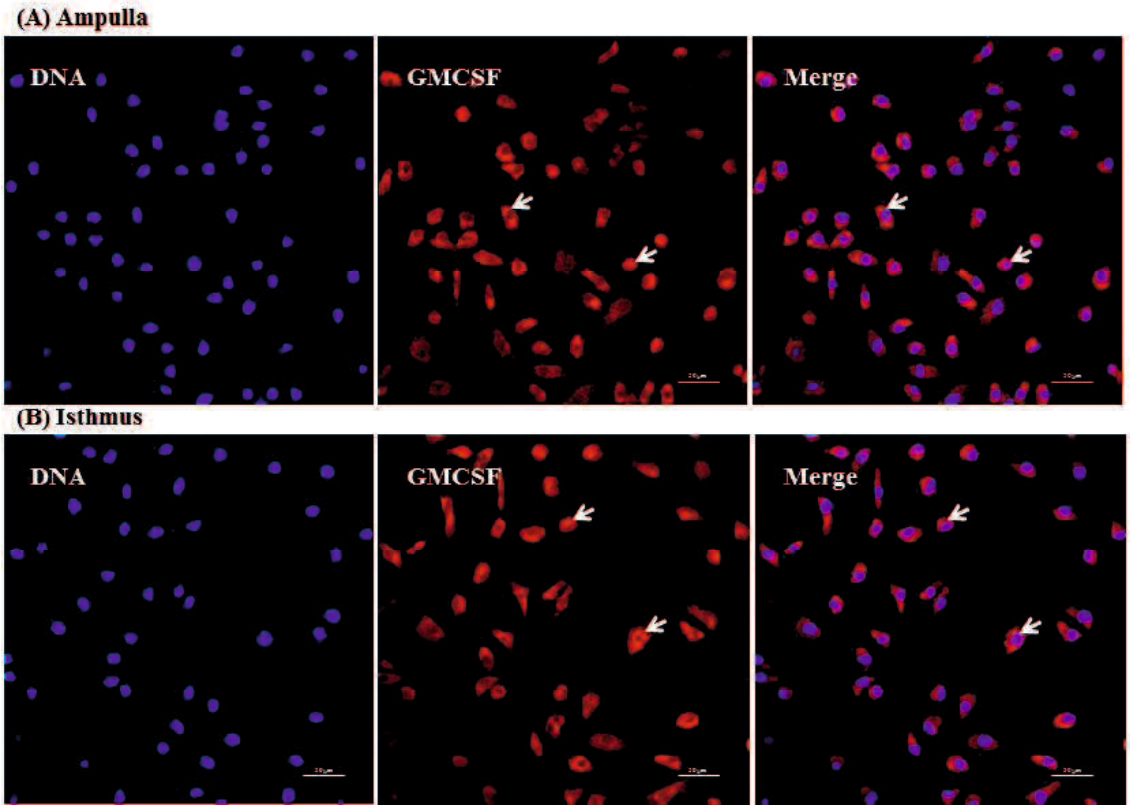

Fig. 7.6. Immunohistochemistry confirmed all of the cultured cells prepared from ampulla (A) or isthmus (B) expressed GMCSF. The blue color in left pictures denotes DAPI staining. The red color in center pictures denotes GMCSF staining. The right pictures show the merge. Arrows indicate GMCSF positive cells. Scale bar is 50 μm.

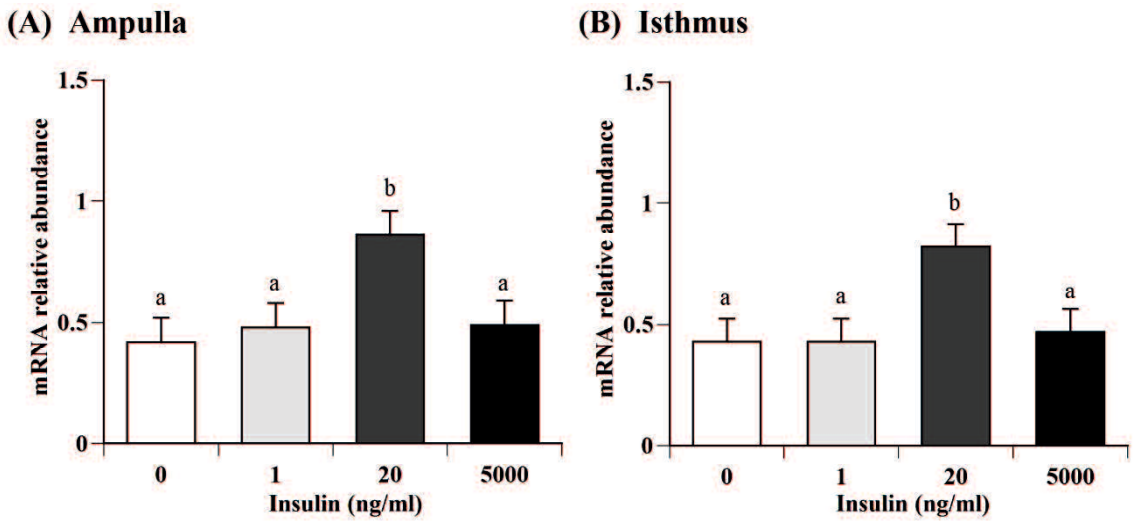

Fig. 7.7. The effect of insulin on the relative GMCSF mRNA expression (mean ± SEM) in BOECs prepared from (A) ampulla and (B) isthmus. Data are normalized to the geometric mean of the expression of two housekeeping genes (*C2orf29* and *SUZ12*). Letters (a vs. b) indicate differences ($p < 0.05$) between treatments.

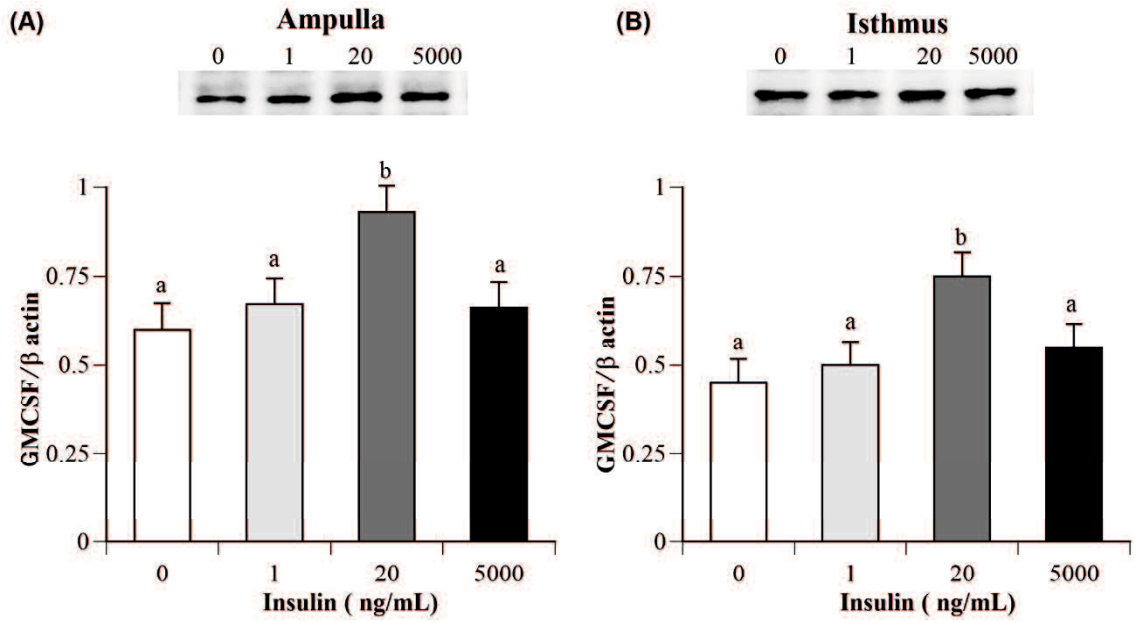

Fig. 7.8. The effect of insulin on the GMCSF protein expression (mean ± SEM) in BOECs prepared from (A) ampulla and (B) isthmus. Data are normalized to the expression of β-actin genes. Letters (a vs. b) indicate differences ($p < 0.05$) between treatments.

7.3. Discussion

The present study revealed that both tunica mucosa in tissue and cultured BOECs expressed insulin receptor as well as GMCSF. Furthermore, insulin, especially at the middle level of concentration, stimulated GMCSF expression. Insulin is the most important hormone in the regulation of blood glucose concentrations. The obese heifers which produced fewer excellent-grade embryos than lean and normal heifers had high insulin concentration in blood (Kadokawa et al., 2008). Therefore, these results suggested that appropriate concentration of insulin stimulates GMCSF expression, however, excess insulin have no stimulation for GMCSF expression, in tunica mucosa via insulin receptor.

This study used the same concentration of insulin used in the previous study for cultured BOECs (Palma-Vera et al., 2014), but we need to note that the lowest concentration approximated the physiological level present in bovine plasma [1 ng/mL; (Sternbauer and Luthman, 2002)].

We conclude that BOECs treated with moderate insulin increased, whereas excess insulin have no stimulation for GMCSF expression level, suggesting that insulin regulates GMCSF expression level in tunica mucosa of bovine oviduct *in vivo*.

CHAPTER VIII

(Study 6)

Effect of insulin on MIF expression in BOECs

Abstract

Insulin stimulated GMCSF expression in the cultured BOECs in chapter 7. Therefore, this study evaluated the effect of insulin on MIF expression level in cultured BOECs. Both ampullar and isthmic epithelial cells were collected from the oviducts of Japanese Black heifers. After preparation and passage, BOECs were cultured until 70% confluency, and then insulin (final concentrations were 0, 1, 20, and 5000 ng/mL) were added to the medium for culture for 24 hours. MIF mRNA and protein were measured by real-time PCR and western blot, respectively. Fluorescent immunohistochemistry was performed on BOECs from either ampullar or isthmic sections using antibodies against MIF. Fluorescent immunohistochemistry showed that all of the cultured cells were MIF-positive. The insulin, especially at middle level, stimulated MIF expression in the cultured BOECs. Therefore, these results suggested that insulin may regulate MIF expression in bovine oviduct *in vivo*.

8.1. Introduction

Higher blood insulin concentration was observed in the obese heifers produced fewer excellent-grade embryos than those observed in lean and normal heifers (Kadokawa et al., 2008). Insulin stimulated GMCSF expression in the cultured BOECs in chapter VII. Therefore, this study evaluated the effect of insulin on MIF expression level in cultured BOECs.

8.2. Materials and methods

8.2.1. Cultuer of BOECs and fluoresecent immunohistochemistry for MIF

Oviducts as ipsilateral side to the corpus luteum were obtained from Japanese Black heifers (n = 5, 26 months of age, at luteal phase) for cell culture. The BOECs were prepared and cultured as the protocol written previously, then treated with trypsin for passage.

The 150 μl cell suspension (at a density of 1.0×10^5 cells/ml) per lane was cultured using the μ-Slide VI 0.4 for 3 days for fluoresecent immunohistochemistry using the same protocol written in the chapter VII and anti-MIF anitibody (1:1000).

Also 1.5 ml of cell suspension (at a density of 1.0×10^5 cells/ml) were seeded into 12-well culture plates to culture at 38.5ºC in 5% CO2 and 95% air. The medium was changed every 48 h until the BOEC monolayer reached 70 to 80% confluency (9 or 10 days after starting the culture; 3 or 4 day after the passage).

8.2.2. RNA extraction, cDNA synthesis, and real-time PCR

Total RNA was extracted from the BOECs of ampullar and isthmic using the same method written in the chapter VII. The expression levels of *MIF*, *C2orf29*, and *SUZ12* were measured by real-time PCRs. The expression of the *MIF* gene was normalized to the geometric means of *C2orf29* and *SUZ12* expression.

8.2.3. Protein extraction for western blotting

Total protein was extracted from the each well containing cultured ampullar and isthmic BOECs using T-PER containing protease inhibitor. The same western blotting method used in the chapter III was used to measure MIF protein expression.

8.2.4. Statistical analysis

The statistical significance of the differences between the measured values was assessed by one-factor ANOVA followed by post-hoc comparisons with Fisher's PLSD test using a model consisting of variance from the effect of different insulin concentration and the residual.

8.3. Results

Immunohistochemistry results revealed that all of the BOECs were MIF positive prepared from both ampulla (**Fig. 8. 1A**) and isthmus **(Fig. 8. 2B**).

ANOVA revealed an effect ($p < 0.05$) of the different insulin concentration on the MIF mRNA and protein expression in BOECs prepared from the ampulla (**Fig. 8.2A and 8.3A**) or isthmus **(Fig. 8.2B and 8.3B**), respectively. The BOECs treated with 20 ng/mL insulin showed higher ($P < 0.05$) MIF mRNA and protein expression in both parts of the oviduct than BOECs treated with 0 or 5000 ng/mL insulin.

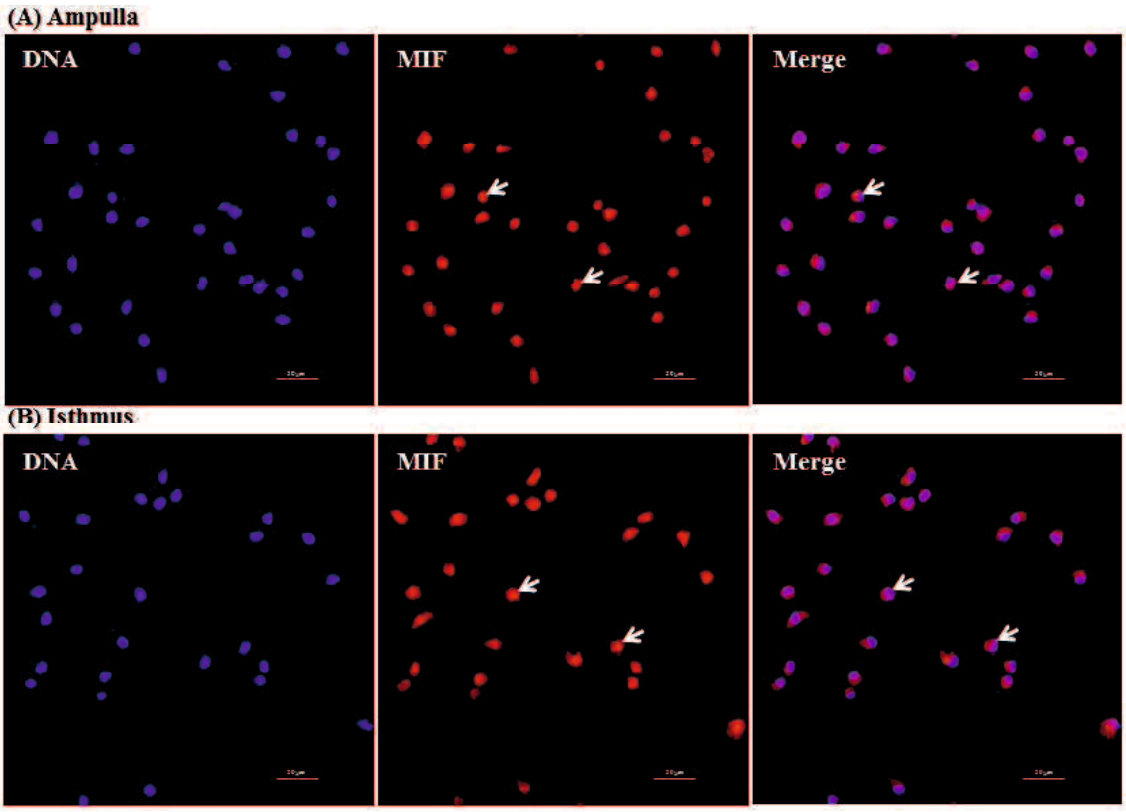

Fig. 8.1. Immunohistochemistry confirmed that all of the cultured cells prepared from ampulla (A) or isthmus (B) expressed MIF. The blue color in left pictures denotes DAPI staining. The red color in center pictures denotes insulin receptor staining. The right pictures show the merge. Arrows indicate MIF positive cells. Scale bar is 50 μm.

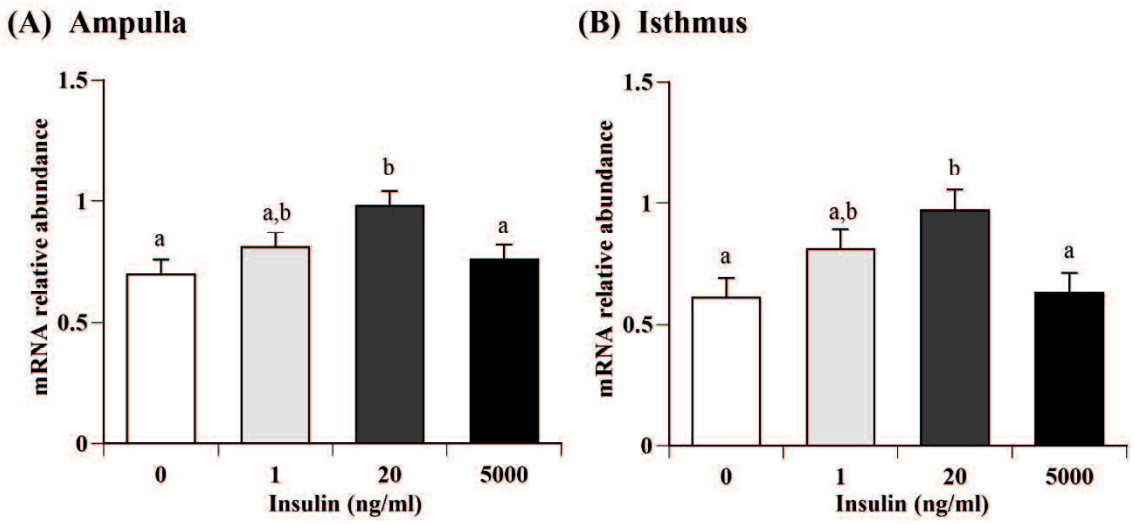

Fig. 8.2. The effect of insulin on the relative MIF mRNA expression (mean ± SEM) in BOECs prepared from (A) ampulla and (B) isthmus. Data are normalized to the geometric mean of the expression of two housekeeping genes (*C2orf29* and *SUZ12*). Letters (a vs. b) indicate differences ($P < 0.05$) between treatments.

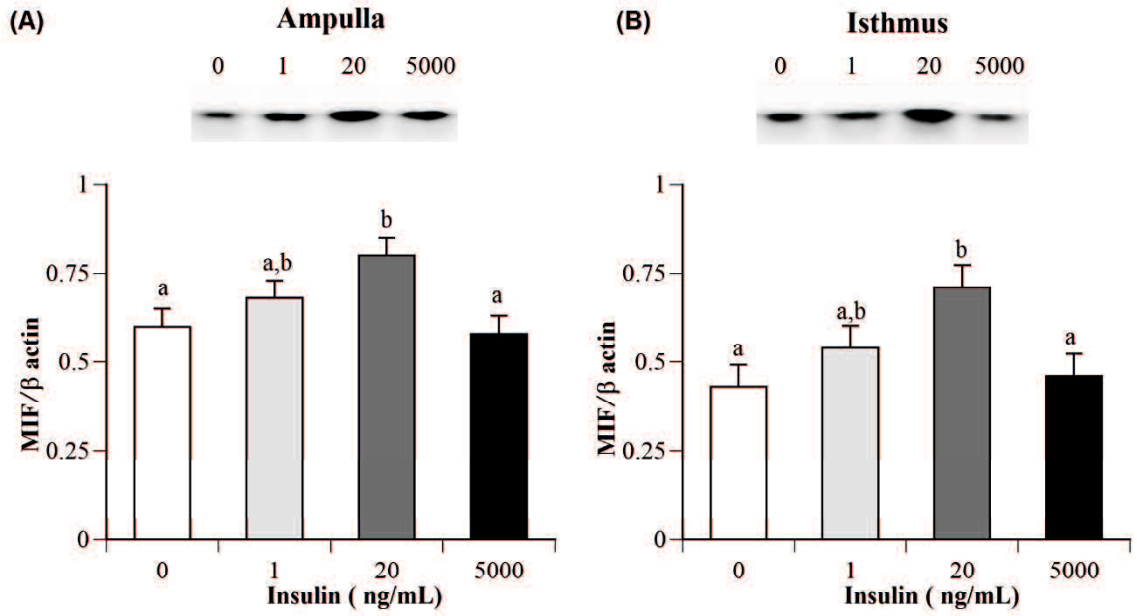

Fig. 8.3. The effect of insulin on MIF protein expression (mean ± SEM) in BOECs prepared from (A) ampulla and (B) isthmus. Data are normalized to the expression of β-actin gene. Letters (a vs. b) indicate differences ($P < 0.05$) between treatments.

8.4. Discussion

This study clarified that insulin stimulate MIF expression in the cultured BOECs. Cells targeted by insulin, including myocytes (Benigni et al., 2000) and adipocytes (Sakaue et al., 1999; Atsumi et al., 2007), produce MIF in order to regulate glucose uptake and glycolysis. Therefore, the present data suggested that insulin control MIF expression in oviduct to regulate glucose uptake and glycolysis by early embryo.

The highest concentration of insulin showed the weaker stimulation of MIF expression in BOECs. Insulin-resistant adipocytes have decreased MIF expression even in the presence of excess insulin *in vivo* (Sakaue et al., 1999). Thus, the high insulin dose may induce insulin resistance in BOECs.

We conclude that insulin, especially at the middle concentration, stimulate MIF expression in the cultured BOECs, suggesting insulin's role to regulate MIF expression level in bovine oviduct *in vivo*.

CHAPTER IX

General Discussion and Conclusion

9.1. General Discussion

In the study of Chapter III, we observed lower expression of GMCSF in the ampullae of obese cows. In accordance with previous studies (Zhao and Chegini, 1994; de Moraes et al., 1999), the immunoreactive GMCSF was localized to the tunica mucosa in the ampullae and isthmuses. The suppressed GMCSF expression in the ampullae of obese cows suggested decreased expression in the tunica mucosa. The tunica mucosa is the layer producing oviductal fluid for gamete transport and maturation, fertilization, and early embryo development in various animals including bovine (Leese et al., 2001; Cebrian-Serrano et al., 2013). The GMCSF positive cells are most likely the main source of GMCSF to bind with the GMCSF receptors in early-stage embryos (Robertson et al., 2001). Therefore, our present data suggested that GMCSF may be a key molecular link between maternal nutritional status and early embryogenesis in the oviduct, and decreased GMCSF in the ampulla of obese females might be an important inhibiting factor for embryogenesis.

Oviduct may have another molecular link, because genetically GMCSF deficient female mice produce normal size litter (Hamilton et al., 2012). MIF may be such a molecule, promoting functions for sperm, ovum, and early-stage embryos in the oviducts according to the maternal nutritional condition. In Chapter IV, we conducted another experiment and the data revealed that MIF expression was lower in the ampulla and isthmus of obese cows, as predicted, but unexpectedly, was also lower in lean cows. Immunohistochemistry showed that MIF was also localized in the tunica mucosa of the ampulla and isthmus of oviduct. The suppressed levels of MIF in the ampullae and isthmuses of obese and lean cows therefore suggested that MIF gene expression is

decreased in the tunica mucosa and may be one reason for decreased embryo development in obese heifers (Kadokawa et al., 2008).

As previously mentioned, addition of GMCSF to the *in vitro* fertilization process improve the yield of implantation-competent blastocysts in the cow (de Moraes and Hansen, 1997), human (Sjöblom et al, 1999), and pig (Kwak et al., 2012). GMCSF promotes sperm viability and motility (Rodrı´guez-Gil et al., 2007). Also MIF at the optimal concentration promotes sperm capacitation *in vitro*, whereas low or excess levels of MIF are inhibitory (Carli et al., 2007). Decreased MIF levels in maternal serum are known to cause first-trimester miscarriages in humans (Yamada et al., 2003). Therefore, it is important to verify the expression level of GMCSF or MIF in the bovine oviducts during post-ovulation phase (Day 3) when fertilized ovum is present. Our results in Chapter V revealed that the bovine ampullae and isthmuses expressed higher GMCSF expression during the estrus and post-ovulation phases as compared to that during the luteal phase. In Chapter VI, our data revealed that MIF mRNA and protein expression in the bovine oviduct was higher during the post-ovulation phase than during estrus and the luteal phase. Therefore, the higher GMCSF or MIF levels in the ampulla and isthmus during the post-ovulation phase suggested that GMCSF and MIF play important roles in fertilization and early-stage embryo development also *in vivo*.

To clarify the mechanisms, we conducted other experiments using insulin in the cultured BOECs in Chapter VII and VIII. In first, we confirmed the expression of insulin receptor in the tunica mucosa by fluorescent immunohistochemistry. Our results showed that moderate insulin supplementation (20 ng/mL) increased, whereas excess insulin (5000 ng/mL) had no stimulation for GMCSF and MIF expressions in the

cultured BOECs. Our results suggested that insulin may regulate GMCSF and MIF expression in bovine oviduct *in vivo*. Therefore, the increased insulin in obese cows may suppress expression of GMCSF and MIF that may be the reason of suppressed fertility and embryogenesis in oviducts.

We could not deny the possibility that other metabolic hormones may control GMCSF and MIF expression. For example, IGF1 stimulates and leptin inhibits estradiol secretion from bovine granulosa cells (Armstrong et al., 2003; Scaramuzzi et al., 2010). Also this thesis study could not deny the possibility that IGF1 and IGF1 receptor may be more important than insulin and insulin receptor, and insulin used in the chapters VII and VIII may be activated IGF1 receptor, not insulin receptor, to increase GMCSF and MIF expression in the cultured BOECs. However, to the date, it was impossible to obtain neither insulin receptor-specific antagonist nor IGF1 receptor-specific antagonist. We could obtain only antagonist for both insulin and IGF1 receptors, BMS754807. Also cytoplasmic pathways to control GMCSF or MIF expression were remained to be clarified.

9.2. Conclusion

This thesis studies have clarified that GMCSF and MIF expression is increased in bovine oviducts during the post-ovulation phase when embryo presents in oviduct, but GMCSF and MIF expression is suppressed in the obese cows compared to the normal or lean cows probably because of high insulin concentration in blood.

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