



HOKKAIDO UNIVERSITY

Title	Identification of the structural element of osteopontin for the restoration of the normal endometrial epidermal growth factor profile and fertility in repeat breeder dairy cows
Author(s)	谷田, 孝志
Degree Grantor	北海道大学
Degree Name	博士(獣医学)
Dissertation Number	甲第16468号
Issue Date	2025-03-25
DOI	https://doi.org/10.14943/doctoral.k16468
Doc URL	https://hdl.handle.net/2115/95483
Type	doctoral thesis
File Information	Takashi_Tanida.pdf



Identification of the structural element of osteopontin for
the restoration of the normal endometrial epidermal growth
factor profile and fertility in repeat breeder dairy cows

(乳用リピートブリーダー牛の
子宮内膜上皮成長因子発現と受胎性を回復させる
オステオポンチンの構造的最小単位の同定)

Takashi Tanida

Contents

Preface	1
 Chapter 1: The significance of posttranslational modifications of osteopontin for the restoration of the normal endometrial epidermal growth factor profile and fertility in repeat breeder dairy cows	5
Introduction	5
Materials and methods	6
Results	12
Discussion	13
Tables and figures	17
Summary	25
 Chapter 2: The element of primary structure of osteopontin for the restoration of the normal endometrial epidermal growth factor profile and fertility in repeat breeder dairy cows	26
Introduction	26
Materials and methods	27
Results	33
Discussion	35
Tables and figures	39
Summary	51
 Summary and conclusions	52
 Acknowledgements	55
 References	56
 Summary in Japanese	67

Abbreviations

AI: artificial insemination

ANOVA: analysis of variance

CIDR: controlled internal drug release devices

CL: corpus luteum

CO₂: carbon dioxide

COCs: cumulus-oocyte complexes

C-rOPN: C-terminal thrombin-cleaved fragment of recombinant osteopontin

DNA: deoxyribonucleic acid

DPBS: Dulbecco's phosphate-buffered saline

E. coli: *Escherichia coli*

E₂: estradiol-17 β

EB: estradiol benzoate

EGF: epidermal growth factor

ET: embryo transfer

GnRH: gonadotropin-releasing hormone

IL: interleukin

IVC: *in vitro* culture

IVF: *in vitro* fertilization

IVM: *in vitro* maturation

LC-MS/MS: liquid chromatography with tandem mass spectrometry

MS/MS: tandem mass spectrometry

N-rOPN: N-terminal thrombin-cleaved fragment of recombinant osteopontin

OPN: osteopontin

P₄: progesterone

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PGF_{2 α} : prostaglandin F_{2 α}

PMF: peptide mass fingerprinting

PVDF: polyvinylidene difluoride

rOPN: recombinant osteopontin

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SP: seminal plasma

TBS-T: Tris-buffered saline with Tween 20

Th-rOPN: thrombin-cleaved fragments of recombinant osteopontin

TNF α : tumor necrosis factor α

Notes

The contents of chapter I have been published in *Theriogenology*:

Tanida T, Tagami T, Sato H, Kyaw HM, Fujikawa T, Nagano M, Momozawa K, Yanagawa Y, Katagiri S (2024) Effects of recombinant osteopontin expressed in *Escherichia coli* on the recovery of the endometrial epidermal growth factor profile and fertility in repeat breeder dairy cows. *Theriogenology* **217** 159–168.

The contents of chapter II have been published in *Theriogenology*:

Tanida T, Tagami T, Yanagawa Y, Katagiri S (2025) Identification of an osteopontin structural element for the restoration of a normal endometrial epidermal growth factor (EGF) profile determined by the EGF concentration on day 3 of estrous cycle and pregnancy outcome in repeat breeder dairy cows. *Theriogenology* **231** 171–181.

Preface

The global demand of dairy products has been estimated to expand from 2024 to 2033 (OECD/FAO, 2024). Although a simple solution to produce high amount of milk may be the increase of the number of cows, this action also causes the competing to secure forage crops and an increase of the emissions of greenhouse gas such as methane (Montes *et al.*, 2013; Pérez-Marín and Quintela, 2023). Thus, another aspect is also needed to solve this issue of the expanded demand of milk, and the improvement of the efficiency of milk production in cows is one of the solutions. Selecting high-yielding cows genetically contributes to increase the milk production level in cows (Brito *et al.*, 2021), but reproduction is adversely affected by high yield, as a result of metabolic stress and feed imbalances and negative genetic relationships between yield and reproduction (Parkinson, 2019; Brito *et al.*, 2021). Reproductive performance has been known to strongly affect the efficiency of milk production in cows. In dairy cows, the level of milk yield in cows with high parity is higher than that in cows with low parity (Ray *et al.*, 1992). In addition, the level of milk production decreases as the days postpartum extends (Haile-Mariam and Goddard, 2008). Therefore, cows need to experience the parturition to increase and recover their milk production level. Reduced reproductive performance including the low fertility and subsequent prolonged calving interval, as well as other diseases such as mastitis (Sguizzato *et al.*, 2024) and infectious disease such as bovine leukemia (Norby *et al.*, 2016) and Johne's disease (Garvey, 2020), lead to the low efficiency of milk production in cows. Low productive efficiency of milk also causes the large economic loss and put a pressure on the farm management. Thus, improvement of the reproductive performance in cows has been needed for the sustainable dairy industry.

Cows failing to conceive after three or more inseminations without detectable abnormality in the estrous cycle, clinical signs and genital organs are generally defined as repeat breeder cows (Casida, 1961; Levine HD, 1999; Pérez-Marín and Quintela, 2023). The presence of repeat breeder cows is one of the major sources of reduced herd reproductive performance and, thus, causes reduced milk production and economic loss in dairy industry (Lafi *et al.*, 1992; Levine HD, 1999; Pérez-Marín and Quintela, 2023). Therefore, treatment for improving fertility in repeat breeder cows has been needed. The incidence of cows failing to conceive after three or more inseminations has been reported to be 16% in Japan (Katagiri *et al.*, 2019), 22% in the United States of America (Bartlett *et al.*, 1986) and 25% in Spain (Garcia-Ispuerto and López-Gatius, 2017). In Japan, approximately 50% of these cows had no detectable abnormality in the estrous cycle, clinical signs and genital organs and, thus, the incidence of true repeat breeder cows has been estimated to be 8% (Katagiri *et al.*, 2019).

The modest change in circulating ovarian steroid hormone concentrations (Erb *et al.*, 1976; Kimura *et al.*, 1987; Shelton *et al.*, 1990; Båge *et al.*, 2002) is one of the causes of low fertility in repeat

breeder cows. In repeat breeder cows, increase of plasma progesterone (P₄) concentrations after ovulation is slower and these concentrations show lower levels than fertile cows during the luteal phase (Kimura *et al.*, 1987; Shelton *et al.*, 1990; Båge *et al.*, 2002). Repeat breeder cows also show slower increase and lower levels of peak plasma estradiol (E₂) concentrations than fertile cows around the day of ovulation (Erb *et al.*, 1976). Although these changes are modest, they may be enhanced as an alternation of growth factors and cytokines in the endometrium since these factors are primarily regulated by E₂ and P₄ (Brigstock, 1991; Paria *et al.*, 2001; Katagiri and Moriyoshi, 2013). Epidermal growth factor (EGF) is one of those growth factors that is regulated by sex steroid hormones and plays an essential role in the regulation of uterine function (Brigstock, 1991; Nelson *et al.*, 1991; Johnson and Chatterjee, 1993a, b) and the embryo development (Paria and Dey, 1990; Brigstock, 1991). An alteration of the endometrial EGF profile has been linked to low fertility in repeat breeder cows (Katagiri and Takahashi, 2004a, 2006; Katagiri and Moriyoshi, 2013; Katagiri *et al.*, 2016). In fertile cows, endometrial EGF concentrations peak twice on days 2–4 and days 13–14 during the estrous cycle, while these peaks are lost in approximately 70% of repeat breeder Holstein cows (Katagiri and Takahashi, 2004a, 2006; Katagiri *et al.*, 2016). In most cases, the loss and recovery of the endometrial EGF peak on day 3 is accompanied by those on day 14 (Katagiri and Takahashi, 2006, 2008), thus making the endometrial EGF concentration on day 3 an indicator of the endometrial EGF profile (Katagiri, 2006; Katagiri *et al.*, 2013). In addition, cows recovered the cycle of the normal endometrial EGF profile early after the parturition ease to be pregnant within 150 days postpartum and cows which failed to recover the normal EGF profile in early postpartum period could be a source of repeat breeder cows (Ninpetch, 2022).

For cows with the alteration of the EGF profile, the administration of high dose of estradiol benzoate (EB) with an intravaginal progesterone-releasing device is effective to restore their normal endometrial EGF profile and fertility (Katagiri and Takahashi, 2008). However, there are two concerns to use this hormonal treatment. Firstly, the effectiveness of this treatment varies among herds (Katagiri *et al.*, 2019). Secondly, the use of estrogen products to food producing animals that include lactating cows has been prohibited in some countries and regions. Thus, an alternative treatment option is needed.

Seminal plasma (SP) infused into the uterus modulates the expression of growth factors and cytokines in the endometrium (Robertson, 2005) and improves fertility in mouse (Bromfield *et al.*, 2014) and pig (O’leary *et al.*, 2004) whose site of ejaculation is the uterus, whereas intrauterine SP infusion did not improve fertility in cows whose ejaculation site is the vagina (Odhiambo *et al.*, 2009; Ortiz *et al.*, 2019). However, SP infusion into the vagina has been reported to restore the normal endometrial EGF profile and fertility in repeat breeder dairy cows (Badrakh *et al.*, 2020a, b; Kyaw *et al.*, 2022). A

form of osteopontin (OPN) in SP showed the activity to normalize the endometrial EGF profile (Badrakh *et al.*, 2020b), and OPN purified from milk also showed this activity and improved fertility in repeat breeder dairy cows (Kyaw *et al.*, 2022). Therefore, as a treatment which does not require estrogen products, the treatment based on its OPN action are expected to be put to practical use for improving fertility in repeat breeder cows. However, as regards the application of OPN to fertility improvement technology at production sites, SP and raw milk may not be suitable as a source of OPN. SP is difficult to obtain in quantity and both SP (Givens, 2018) and raw milk (Vasavada, 1988; Herlekar *et al.*, 2013; Kuczewski *et al.*, 2021) may transmit infectious diseases. Thus, alternative sources of this protein without these disadvantages are needed. In addition, the mechanism by which OPN infused into the vagina restores the normal endometrial EGF profile and fertility remains unknown. Understanding this mechanism is important for the improvement of treatment to repeat breeder cows.

OPN is an acidic protein, consisting of a signal sequence and mature region of 16 and 262 amino acids in cattle, and also named as secreted phosphoprotein 1 (Denhardt and Guo, 1993; Icer and Gezmen-Karadag, 2018). This protein is secreted in various tissues (Sodek *et al.*, 2000) including the reproductive organs such as the ovary (Brunswig-Spickenheier and Mukhopadhyay, 2003; Poole *et al.*, 2013), oviduct (Gabler *et al.*, 2003), uterus (Johnson *et al.*, 1999; Garlow *et al.*, 2002; Joyce *et al.*, 2005), testis (Erikson *et al.*, 2007), and epididymis (Erikson *et al.*, 2007). OPN has multiple functions in the body, and this protein switches its function by the presence of posttranslational modifications and the binding to various receptors, i.e., integrins and CD44 (Lund *et al.*, 2009). OPN has roles in different physiological systems, including cancerous (Castello *et al.*, 2017), inflammatory (Lund *et al.*, 2009) and reproductive systems (Johnson *et al.*, 2003; Monaco *et al.*, 2009; Boccia *et al.*, 2013; Liu *et al.*, 2014; Kuwabara *et al.*, 2015). In the reproductive system, OPN has a role in sperm capacitation (Boccia *et al.*, 2013) and acrosome reaction (Monaco *et al.*, 2009), preimplantation embryo development (Monaco *et al.*, 2009; Liu *et al.*, 2014), conceptus attachment (Johnson *et al.*, 2003; Erikson *et al.*, 2009; Kim *et al.*, 2010), and corpus luteum (CL) formation (Kuwabara *et al.*, 2015).

One of the alternative sources of OPN to SP and raw milk may be the recombinant protein, which can be cost-effectively produced in *Escherichia coli* (*E. coli*) (Overton, 2014). However, such recombinant proteins do not undergo posttranslational modification, which is considered important for biological activity of various proteins (Sahdev *et al.*, 2008). For OPN, posttranslational modifications have shown to be crucial in the expression of its biological activity (Ashkar *et al.*, 2000; Weber *et al.*, 2002; Lund *et al.*, 2009; Minai-Tehrani *et al.*, 2013), but some functions of OPN do not depend on this modification (Ashkar *et al.*, 2000; Weber *et al.*, 2002; Erikson *et al.*, 2009; Kim *et al.*, 2010; Wing *et al.*, 2020; Wu *et al.*, 2022). In the function of OPN to restore the normal endometrial EGF profile and fertility

in repeat breeder cows, the significance of posttranslational modifications is unknown and should be examined.

OPN can be cleaved by thrombin, and this cleavage changes the biological activity of OPN (Senger *et al.*, 1996; Weber *et al.*, 2002; Yamamoto *et al.*, 2003; Lund *et al.*, 2009). Each cleaved fragment of OPN has different binding sites to integrins and CD44 (Lund *et al.*, 2009; Sørensen and Christensen, 2023). If the sites, or the structural element, which is responsible for the OPN action to restore the normal endometrial EGF profile and are identified, the recombinant protein moiety or the chemically synthesized peptide containing these sites may become a candidate of treatment agent to improve fertility. Furthermore, the clarification of the structural element may also help us to understand the mechanism by which this protein controls the uterine function via the vagina in cattle.

The objective of the present study was to identify the structural element of OPN for the restoration of the normal endometrial EGF profile and fertility in repeat breeder dairy cows. In chapter 1, the significance of posttranslational modifications of OPN for these reproductive functions was investigated by using the recombinant OPN expressed in *E. coli* that had no post-translational modification. In chapter 2, the element of the primary structure of this protein for the restoration of the normal endometrial EGF profile and fertility was determined by investigating the treatment effects of the fragments of rOPN cleaved by thrombin and the chemically synthesized peptides of integrin binding motif of OPN.

Chapter 1: The significance of post-translational modifications of osteopontin for the restoration of the normal endometrial epidermal growth factor profile and fertility in repeat breeder dairy cows

Introduction

Intravaginal infusion of OPN in SP and milk restores the normal endometrial EGF profile and fertility in repeat breeder dairy cows (Badrakh *et al.*, 2020b; Kyaw *et al.*, 2022). Nevertheless, SP and raw milk may not be suitable as raw materials to prepare OPN at production sites since SP is difficult to obtain in quantity and both SP (Givens, 2018) and raw milk (Vasavada, 1988; Herlekar *et al.*, 2013; Kuczewski *et al.*, 2021) may transmit the infectious diseases. One of the alternative sources of OPN without these disadvantages may be recombinant protein (Overton, 2014), which can be cost-effectively produced in *E. coli* (Overton, 2014). However, such recombinant proteins do not undergo posttranslational modification, which is considered important for biological activity of various proteins (Sahdev *et al.*, 2008).

For OPN, posttranslational modifications have shown to be crucial in the expression of its biological activity (Ashkar *et al.*, 2000; Weber *et al.*, 2002; Lund *et al.*, 2009; Minai-Tehrani *et al.*, 2013), but some functions of OPN do not depend on this modification (Ashkar *et al.*, 2000; Weber *et al.*, 2002; Erikson *et al.*, 2009; Kim *et al.*, 2010; Wing *et al.*, 2020; Wu *et al.*, 2022). OPN enhances the lung cancer cell growth depending on glycosylation in human (Minai-Tehrani *et al.*, 2013). OPN induces the expression of interleukin (IL) -12 (Ashkar *et al.*, 2000; Weber *et al.*, 2002) and tumor necrosis factor α (TNF α) (Weber *et al.*, 2002) in macrophage depending on phosphorylation, while it induces the chemotaxis of macrophages (Weber *et al.*, 2002) and suppresses the expression of IL-10 (Ashkar *et al.*, 2000) in a phosphorylation independent manner in mice. In sheep and pigs, recombinant rat OPN produced in *E. coli* without posttranslational modifications binds to integrins in uterine luminal epithelial cells (Erikson *et al.*, 2009), trophoctoderm cells (Erikson *et al.*, 2009; Kim *et al.*, 2010), endothelial progenitor cells (Wing *et al.*, 2020) and the placental tissue (Wu *et al.*, 2022), leading to promote trophoctoderm cell adhesion and migration (Erikson *et al.*, 2009; Kim *et al.*, 2010), endothelial progenitor cell incorporation into vasculature (Wing *et al.*, 2020), and ion transport across the placenta (Wu *et al.*, 2022).

Therefore, in the present study, we investigated the effect of full-length recombinant OPN (rOPN) expressed in *E. coli*, which has no posttranslational modification (Sahdev *et al.*, 2008), on the normalization of the endometrial EGF profile and the recovery of fertility in repeat breeder dairy cows. Fertility was evaluated with respect to the conception rate after artificial insemination (AI) and by the

survival and size of embryo transfer (ET) embryo recovered on day 14.

Materials and methods

rOPN preparation

A gene-encoding bovine OPN (Uniprot ID: P31096) was synthesized by GeneArt (Thermo Fischer Scientific, Waltham, MA, USA) with codon optimization for *E. coli* expression. The deoxyribonucleic acid (DNA) encoding was performed to predict the mature region of OPN (Leu17–Asn278) and amplified by polymerase chain reaction (PCR) using primers [5'-GCCGCGCGGCAGCC-ATATGCTGCCGGTTAAACCGACCAG-3' (sense; *NdeI*-site underlined) and 5'-CGAGTGCGGCCG-CAAGCTTTTAATTCACTTCACTGCTTG-3' (antisense; *HindIII*-site underlined)]. PCR was performed using KOD Plus Neo™ DNA polymerase (Toyobo, Osaka, Japan), and its product was purified with QIAEX II Gel Extraction kit (Qiagen, Hilden, Germany), and inserted into *NdeI*–*HindIII* sites of pET-28a (Merck Millipore, Darmstadt, Germany) using the seamless ligation cloning extract (Okegawa and Motohashi, 2015). The resultant plasmid vector was designated as SPP1/pET-28a.

The *E. coli* Rosetta (DE3) (Merck Millipore) cells harboring SPP1/pET-28a were cultured overnight on LB agar plates containing 50 µg/mL kanamycin and 30 µg/mL chloramphenicol at 37°C. A single colony was inoculated into 150 ml LB medium containing 50 µg/ml kanamycin and 30 µg/ml chloramphenicol, and to grow the cells, a seed culture was agitated overnight at 37°C and inoculated into 3000 mL LB medium containing 50 µg/mL kanamycin. When OD₆₀₀ of the culture medium reached to between 0.4 and 0.8, the culture medium was cooled in ice for 10 min, and to induce protein expression, 0.1 mM isopropyl β-D-thiogalactopyranoside was added. Then, the cells were cultured at 30°C for 20–22 h, harvested by centrifugation (10,000 × g at 25°C for 5 min), and frozen at –80°C until rOPN was purified according to a previous report (Tagami *et al.*, 2016). Briefly, the cells were resuspended in Buffer-A (20 mM sodium phosphate buffer, pH 7.0 and 0.3 M NaCl) at 4°C and disrupted by sonication. Cell-free lysate was obtained by centrifugation (6,000 × g at 4°C for 20 min) and applied to a Ni²⁺-charged Chelating Sepharose Fast Flow column (Cytiva, Marlborough, MA, USA), which was washed with 20 mM imidazole in Buffer-A. Then, rOPN was eluted with a 20–500 mM imidazole linear gradient in Buffer-A. Purified rOPN was dialyzed against the phosphate-buffered saline (PBS) (Takara Bio, Kusatsu, Japan) with cellulose membrane [cellulose tubing (30/32), Viskase Companies, Illinois, USA]. The purification and dialysis of rOPN were performed at 4°C. The dialyzed proteins containing rOPN and 1% (w/v) trehalose as a cryoprotectant was filtrated (pore size of the filter: 0.22 µm), and the solution containing 0.3 mg rOPN was aliquoted in 10 mL vials (NV-10, Nichiden Rika Glass, Kobe, Japan) and lyophilized. The lyophilized rOPN were stored at 4°C until infusion into repeat breeder cows.

Concentrations of rOPN in final preparation were calculated based on total protein concentrations and purities of rOPN. The total protein concentration was measured based on the absorbance at 280 nm ($E_{280\text{ nm}, 1\text{ cm}}^{1\%} = 10$) (Noble and Bailey, 2009), and the purity of rOPN calculated by the densitometry using the gel image of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by ImageJ (<https://imagej.net/ij/index.html>).

The identity of rOPN was confirmed by western blotting according to previous methods (Kyaw *et al.*, 2022), with slight modification. Briefly, in the western blotting, the purified rOPN was subjected to SDS-PAGE, and the proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Immun-Blot PVDF Membrane, Bio-Rad Laboratories, Berkeley, California, USA). The membrane was blocked by incubating it in PBS with 5% bovine serum albumin at room temperature for 1 h. The membrane was washed with Tris-buffered saline with Tween 20 (TBS-T) (pH 7.5) and then incubated in the TBS-T with the anti-OPN polyclonal antibody (LB-4225, Cosmo bio, Cambridge, UK) at a 1:1,000 dilution for 3 h at room temperature. The unbound primary antibody was washed out using TBS-T, and the membrane was incubated in the TBS-T with the secondary antibody (anti-IgG-horseradish peroxidase; #4050-05, SouthernBiotech, Birmingham, AL, USA) at a 1:4,000 dilution for 2 h at room temperature. The unbound secondary antibody was washed using TBS-T, and the peroxidase was detected using 50 mM Tris (pH7.0) dissolved 3,3'-diaminobenzidine tablet (Fujifilm Wako Pure Chemical Industries, Osaka, Japan).

SP preparation

SP was prepared as reported previously (Badrakh *et al.*, 2020a). Briefly, semen was collected from seven fertile Holstein bulls using an artificial vagina at a commercial AI center (Genetics Hokkaido, Tokachi Shimizu, Hokkaido, Japan). Two ejaculates were collected on the same day twice a week. SP was separated from the semen by centrifugation ($1000 \times g$ for 10 min), stored at -20°C , and transported to the laboratory. Frozen SP was thawed and centrifuged ($5,000 \times g$ at 4°C for 20 min). The resulting supernatant from SP collected from seven bulls were pooled and used as the SP sample, which was aliquoted in 0.5 mL and stored at -30°C until infusion into repeat breeder cows.

***In vitro* embryo production and freezing for direct transfer**

In the present study, ovaries were obtained from Japanese black or F1 crossbred (Japanese black $\times$ Holstein) cattle slaughtered at a local abattoir located within 1 h from the laboratory and transported to the laboratory within 4 h after collection. All media (BO-IVM, BO-IVF, BO-SemenPrep, and BO-IVC) used in these experiments were purchased from IVF Bioscience (Cornwall, UK).

Cumulus-oocyte complexes (COCs) were aspirated from follicles (2–8 mm in diameter) of slaughterhouse-derived ovaries were submitted to *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) as described previously (Chelenga *et al.*, 2020). For IVM, COCs were washed twice in TCM199 (Invitrogen, Grand Island, NY, USA) supplemented with 0.1% polyvinyl alcohol, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM sodium bicarbonate, and 50 µg/mL gentamicin sulfate (isolation medium, pH 7.4 at 37°C), as described elsewhere (Harada *et al.*, 1997), and once in BO-IVM media before culture in 50 µL BO-IVM droplets (approximately 10 COCs/50 µL droplets) covered with paraffin oil at 39°C for 20–24 h in 5% carbon dioxide (CO₂) in humidified air. After IVM, COCs were subjected to IVF and IVC. Commercial frozen semen (Genetics Hokkaido) derived from a Holstein bull was used for IVF. The sperms were washed twice by centrifugation (300 × g for 5 min) in BO-SemenPrep media, and 2 × 10⁶ cells/mL sperm was coincubated with oocytes in 500 µL of BO-IVF medium (day 0) in a 4-well dish without an oil overlay at 39°C for 18–24 h in 5% CO₂ humidified air for IVF. After IVF, inseminated oocytes were denuded of the surrounding cumulus cells and excess spermatozoa by vortexing. For IVC, presumptive zygotes were washed three times in BO-IVC media and then cultured in a 30 µL BO-IVC droplet (approximately 10–25 zygotes/droplet) with an overlay of paraffin oil at 39°C for 144–150 h in 5% oxygen, 5% CO₂, and 90% nitrogen. An inverted microscope was used to examine the development of the embryos at days 6 and 7. Grade 1 blastocysts according to the International Embryo Technology Society classifications (Robertson and Nelson, 2010) were then frozen in 1.5 M ethylene glycol in Dulbecco's PBS (DPBS) supplemented with 0.1 M sucrose and 20% calf serum (freezing medium) in a program freezer (ET-1, Fujihira, Tokyo). The embryos were directly transferred into the freezing medium and then loaded into a 0.25 mL straw to equilibrate for 15–20 min. The straws were then placed in a methanol bath of a programmable freezer at –7°C for 1 min, seeded at –7°C, maintained at –7°C for 9 min, cooled to –30°C at the rate of 0.3°C/min, and plunged into liquid nitrogen (Dochi *et al.*, 2008).

Animals

All animal experiments were performed in accordance with the Japanese Act on Welfare and Management of Animals and approved by the Institutional Animal Care and Use Committee of Hokkaido University (Approval No. 16-0071 and 19-0030). In the present study, 269 lactating Holstein cows were used and housed in sixteen commercial farms in Hokkaido, Japan. They were multiparous lactating cows (> 9500 kg of 305-day fat-corrected milk) and between two and six in parity. We excluded cows with problems at calving and during the postpartum period requiring three or more veterinary visits.

Repeat breeder cows (n = 251) were selected by local veterinarians according to the criteria

for failure to conceive after three or more AIs without a detectable abnormality during an estrous cycle, clinical signs and genital organs by rectum palpation, vaginal examination, and ultrasonography (Badrakh *et al.*, 2020a; Kyaw *et al.*, 2022). The absence of any abnormality in the uterine morphology was confirmed by transrectal ultrasonography (Mee *et al.*, 2009) and the uterine cytology by cytobrush (Dubuc *et al.*, 2010) before the enrollment in the study.

Fertile controls (n = 18) were cows 120 days postpartum on the day of ET and between two and six in parity before the first service after parturition. They were also confirmed to have no detectable abnormality in the estrous cycle, clinical signs and genital organs by rectum palpation, vaginal examination, ultrasonography, and uterine cytology before the enrollment of the study. They became pregnant within two inseminations after the study period.

Synchronization of estrus and ovulation

Estrus was synchronized by giving a single administration of prostaglandin F_{2α} (PGF_{2α}) (20 or 25 mg of dinoprost tromethamine im; Pronalgon F, Zoetis Japan, Tokyo, Japan) or an Selectsynch protocol (Larson *et al.*, 2006) in cows. In the Selectsynch protocol, gonadotropin-releasing hormone (GnRH) (100 µg fertirelin acetate im; Conceral, MSD Animal Health, Tokyo, Japan) was treated between day 7 and day 9 of the estrous cycle, and PGF_{2α} (25 mg of dinoprost tromethamine im) was administrated 7 days after the GnRH treatment. All cows synchronized by a single administration of PGF_{2α} and the Selectsynch protocol showed estrus within 5 and 4 days after PGF_{2α} treatment, respectively, detected either by observation twice daily after the PGF_{2α} treatment or by using an automated activity monitor. The day of estrus was defined as day 0.

Ovulation was synchronized by an Ovsynch protocol (Wiltbank and Pursley, 2014), in which the cows were treated with the first GnRH (100 µg of fertirelin acetate im) followed by PGF_{2α} (25 mg dinoprost tromethamine im) 7 days later and the second GnRH (100 µg fertirelin acetate im) 55 ± 2 h after PGF_{2α} treatment. The next day of the second GnRH treatment was considered as day 0.

Endometrial tissue collection, EGF assay, and the analysis of the endometrial EGF profile

Endometrial tissues were collected for the EGF assay as described previously (Katagiri and Takahashi, 2004a). Tissues were weighed and frozen in liquid nitrogen within 10 min and then stored at -20°C until the EGF assay.

Endometrium tissue samples were processed, and the tissue extracts were used for the EGF assay (Katagiri and Takahashi, 2006; Badrakh *et al.*, 2020a). An antihuman EGF mouse monoclonal antibody (MAB636, R & D Systems, Minneapolis, MN, USA) and antihuman EGF rabbit antiserum

(#5022-100, Biogenesis, Poole, UK) were used as the solid-phase antibody and detection antibody, respectively. The assay system was verified for the measurement of bovine EGF using recombinant bovine EGF (Badrakh *et al.*, 2020a). Assay sensitivity was 10 pg/well. The intra and interassay coefficients of variation at 50 pg/well were 4.2% and 6.8%, respectively.

In the present study, endometrial EGF concentrations were measured on day 3, and a normal endometrial EGF profile was defined by concentrations within the normal range for day 3 (4.70–13.50 ng/g tissue weight) (Katagiri and Takahashi, 2006; Katagiri *et al.*, 2016).

AI and ET

AI was performed with commercial frozen-thawed Holstein semen by professional AI technicians within 4–12 h after estrus detection. For ET, commercial, *in vivo* derived, frozen-thawed Japanese black embryos or *in vitro* derived, frozen-thawed embryos were transferred on day 7 into the uterine horn ipsilateral to the side of the ovaries with a CL by transcervical method using a flexible transfer catheter (Mo–NO.4, Misawa Medical Industry, Kasama, Japan).

Infusion of samples into the vagina

All samples were prepared in 10 mL PBS, loaded into a 10 mL syringe, and infused into the vagina as previously described (Badrakh *et al.*, 2020a; Kyaw *et al.*, 2022). Briefly, the tip of a disposable plastic catheter for AI, which was attached to the syringe loaded with an infusion sample, was introduced into the vagina and guided deep into the vagina adjacent to the external orifice of the cervix. The plunger was pushed to deposit the samples.

Embryo collection and measurement of embryo length

Transferred embryos were collected on day 14 (i.e., 7 days after ET) via the balloon catheter either 18 Fr, two eyes (#01209300, Nipro, Osaka, Japan) or 20 Fr, two eyes (#0120752520, Nipro) flushing the uterus nonsurgically with a commercial medium for embryo collection (Embryotech, Zenoaq, Fukushima, Japan). Side holes of the balloon catheter were enlarged by removing the rubber between two side holes next to each other. Embryo length was determined as the length of the long axis of collected embryos, or twice the length of fragmented filamentous embryos with embryonic disc that were recovered.

Study design

Study 1-1: The effect of rOPN on the restoration of the normal endometrial EGF profile and

fertility

After the screening the repeat breeder cows for any detectable abnormality in the uterine morphology and cytology, estrus or ovulation was synchronized by a single administration of PGF_{2α} or the Ovsynch protocol. The endometrial EGF concentration was determined on day 3. The cows with an altered EGF profile were used for the study (Fig. 1-1A). Repeat breeder cows were synchronized for estrus according to the Selectsynch protocol and infused with 10 mL PBS (negative control, n = 74), 0.5 mL frozen-thawed SP (positive control, n = 37), or 0.3 mg rOPN (n = 105) into the vagina within 4–12 h after estrus detection, corresponding to the timing of AI. To examine the effects of the treatments on the endometrial EGF profile, the endometrial EGF concentration was measured on day 3 for the second time. Some cows infused with PBS (n = 58), SP (n = 20), or rOPN (n = 83) were subjected to AI immediately before the samples were infused. Pregnancy was confirmed by transrectal ultrasonography between days 30 and 35 after AI.

Study 1-2: The effect of the rOPN on the embryo survival and elongation

After the screening for any detectable abnormality in the uterine morphology and cytology in repeat breeder cows and fertile control cows, the endometrial EGF concentration was measured on day 3 on the same schedule as in study 1-1 (Fig. 1-1B). Repeat breeder cows with an altered EGF profile (n = 35) and fertile controls with normal EGF profile (n = 18) were synchronized for estrus and infused with rOPN or PBS, as in study 1-1. Repeat breeder cows were infused with PBS (n = 18, PBS group) or rOPN (n = 17, rOPN group), while fertile controls were infused with PBS (n = 18, fertile control group). To examine the endometrial EGF profile, the endometrial EGF concentration was measured on day 3 for the second time. On day 7, the cows were transferred either *in vivo* derived embryos or *in vitro* derived embryos (two embryos in each cow: PBS group, n = 4; rOPN group, n = 5; fertile control group, n = 6; three embryos in each cow: PBS group, n = 14; rOPN group, n = 12; fertile control group, n = 12). In total, 50, 46, and 48 embryos were transferred in the PBS, rOPN, and fertile control group, respectively. On day 14, the embryos were recovered, and the embryo lengths were measured.

Data analyses

Fisher's exact test with the Benjamini-Hochberg method was performed to compare the restoration rates of the normal endometrial EGF profile, conception rates, the proportion of cows from which embryos were collected, and embryo recovery rates between groups. The Steel–Dwass test was used to compare endometrial EGF concentrations between groups. The Wilcoxon signed-rank test was conducted to compare endometrial EGF concentrations before and after treatment. Embryo lengths were

denoted by the sinh-arcsinh normal distribution, and the transformed data were evaluated by a two-way analysis of variance (ANOVA) with groups and embryo types as factors. Tukey's test was used to compare embryo lengths transformed into the sinh-arcsinh normal distribution between groups. The Pearson's rank correlation coefficient was used to determine the correlation between the endometrial EGF concentration and the embryo length transformed into the sinh-arcsinh normal distribution.

Data were analyzed using the computer software JMP Pro 16 (SAS Institute Inc., Tokyo, Japan). The P-values in Fisher's exact test were adjusted for multiple comparisons using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). P values less than 0.05 were considered for statistical significance.

Results

Preparation of rOPN

The predicted mature OPN, where a predicted signal peptide was truncated, was produced in *E. coli* as N-terminally His6-tagged recombinant protein. By western blotting, OPN was confirmed as the band with the apparent molecular mass of approximately 45.0×10^3 on the SDS-PAGE gel (Fig. 1-2). In eight batches of rOPN preparations, the bands of rOPN accounted for $49.0\% \pm 11.8\%$ (range: from 34.6% to 65.3%) of the total protein content in the final preparation. The yields of rOPN were 1.9 ± 1.0 mg/L of culture.

Study 1-1: The effect of rOPN on the restoration of the endometrial EGF profile and fertility

Endometrial EGF concentrations before treatment were comparable in all groups ($P > 0.1$) (Fig. 1-3). After treatment, endometrial EGF concentrations in the rOPN and SP groups were comparable but higher than those in the PBS group ($P < 0.05$). The restoration rates of the normal endometrial EGF profile of the rOPN and SP groups were comparable (58.1% vs. 62.2%) but higher than that of the PBS group (29.7%) ($P < 0.05$, Table 1-1).

The conception rates of inseminated cows with normalized endometrial EGF profile after treatment with rOPN and PBS infusion were higher than those of inseminated cows with unnormalized endometrial EGF profile ($P < 0.05$, Table 1-1), while the conception rate of cows after SP infusion was not different between cows with normalized and unnormalized endometrial EGF profile. The overall conception rate of the rOPN group was higher than that of the PBS group (47.0% vs. 28.1%) ($P < 0.05$). The conception rate of the SP group was intermediate between PBS and rOPN groups.

Study 1-2: The effect of the rOPN on the embryo survival and elongation

The proportion of cows with a normal endometrial EGF profile after treatment of PBS and rOPN groups were 44.4% (8/18) and 58.8% (10/17), respectively. The fertile control group has the highest proportion of cows with a normal endometrial EGF profile after treatment (94.4%, 17/18), which was higher than those of the PBS (44.4%) and rOPN groups (58.8%) ($P < 0.05$). One cow in the fertile control group had an altered endometrial EGF profile after treatment (endometrial EGF concentration: 4.39 ng/g tissue). Endometrial EGF concentrations after treatment of the PBS and rOPN groups were similar but lower than those of the fertile control group ($P < 0.05$, Fig. 1-4).

The number of filamentous embryos that lost one of the distal ends and the number of distal end fragments without embryo discs were equal. Therefore, the number of embryo discs represented the number of embryos. The proportion of cows from which embryos were collected and embryo recovery rates were similar between *in vitro* and *in vivo* derived embryos within groups (Table 1-2, $P > 0.1$). Embryo recovery rates of the rOPN and fertile control groups were comparable (58.7% vs. 58.3%) and higher than that of the PBS group (32.0%) ($P < 0.05$). The proportion of cows from which embryos were collected and embryo recovery rate in cows with a normal endometrial EGF profile after treatment were higher than those in cows with an altered endometrial EGF profile after treatment ($P < 0.05$, Table 1-3). In most of cows from which embryos were collected, multiple embryos were recovered.

The two-way ANOVA revealed the effect of treatments but not embryo types, without correlation ($P > 0.1$). Embryo lengths of all recovered embryos and the largest embryos in each cow from the fertile control and rOPN groups were comparable and greater than those from the PBS group ($P < 0.05$, Table 1-2). Endometrial EGF concentrations was not correlated to the embryo lengths of all recovered embryos ($r = -0.01$) or the largest embryos in each cow ($r = -0.09$) (Fig. 1-5, $P > 0.1$).

Discussion

In the present study, rOPN affected the normalization of the endometrial EGF profile and recovery of fertility in repeat breeder cows with an altered EGF profile. Despite the role of posttranslational modifications in some activities of OPN (Ashkar *et al.*, 2000; Weber *et al.*, 2002; Minai-Tehrani *et al.*, 2013), these modifications were not essential and the peptide or protein moiety without the modifications can restore the normal endometrial EGF profile and fertility in repeat breeder cows. In study 1-1, rOPN normalized the endometrial EGF profile to a similar extent as SP in repeat breeder cows. Furthermore, the effect of normalizing the endometrial EGF profile by rOPN (58.1%) was similar to that of milk OPN (56.1%) (Kyaw *et al.*, 2022). The present results also showed that the normalization of the endometrial EGF profile after rOPN infusion resulted in the recovery of fertility in

repeat breeder cows. The conception rate of cows infused with rOPN (47.0%) was similar to those that underwent hormone therapy (40.0%) (Katagiri and Takahashi, 2008), SP (44.4%) (Badrakh *et al.*, 2020a) and mOPN (43.5%) (Kyaw *et al.*, 2022). However, further study including a larger number of animals is needed to confirm the effect of rOPN on fertility.

It remains unknown how OPN infused into the vagina restores the normal endometrial EGF profile. However, it is unlikely that SP (i.e., OPN) ejaculated into the vagina reaches the uterus (Fernandez-Fuertes, 2023) because of the low volume of semen ejaculated from bull (around 5 ml) (Murphy *et al.*, 2018), the length of the cervix (Perkins *et al.*, 1954) and the high viscosity of cervical mucus (Suarez and Pacey, 2006) although sperm have the potential to act as a vehicle of SP factors into the uterus (Chu *et al.*, 1996; Fernandez-Fuertes, 2023). Accordingly, our previous report showed that SP restored the normal endometrial EGF profile and fertility by infusion into the vagina, but not into the uterus, in repeat breeder cows (Badrakh *et al.*, 2020a). Furthermore, intrauterine infusion of SP at the time of AI had no effect on pregnancy rates in cows (Odhiambo *et al.*, 2009; Ortiz *et al.*, 2019). These reports indicate that the site of action of SP and OPN to restore the normal endometrial EGF profile and fertility in repeat breeder cows is the vagina.

Hypothetically, OPN activates the immune cells in the vagina, causing uterus-targeted or systemic changes by the activation of immune cells in the adjacent lymph nodes (Kyaw *et al.*, 2022). These activated immune cells in the vagina and lymph nodes may recirculate and infiltrate into the uterus, causing the normalization of the endometrial EGF profile in repeat breeder cows. OPN causes proinflammatory response in the immune cells and activate them, including macrophages, dendritic cells, and lymphocytes (Lund *et al.*, 2009). Proinflammatory cytokines, such as IL-6 (Campanile *et al.*, 2021) and TNF α (Gori *et al.*, 2011), play roles in the regulation of endometrial functions. In proinflammatory cytokines, TNF α has been hypothesized to be involved in the normalization of the EGF profile by OPN infusion (Kyaw *et al.*, 2022) since EGF synthesis is primary regulated by estrogen in the endometrium (Huet-Hudson *et al.*, 1990; Brigstock, 1991) and TNF α activates estrogen receptor signaling pathways in endometrial epithelial cells (Gori *et al.*, 2011). The role of immune cells and the proinflammatory cytokines (e.g., IL-6 and TNF α) in normalizing the endometrial EGF profile after intravaginal OPN infusion is needed to be investigated. In addition, the active site of OPN to normalize the endometrial EGF profile and receptive mechanism of OPN in the vagina also need to be studied to understand the mechanism of this function of OPN.

Although the normalization of the endometrial EGF profile in repeat breeder cows increases embryo survival or promotes embryo development, thereby recovering the conception rate (Katagiri and Takahashi, 2006; Katagiri and Moriyoshi, 2013), this hypothesis had not been tested. The proportion of

cows from which embryos were collected and embryo recovery rate by day 14 of pregnancy reflects the embryo survival in the uterus (Lonergan *et al.*, 2007; Sánchez *et al.*, 2019; Mateo-Otero *et al.*, 2020; O’Callaghan *et al.*, 2021). In the present study, the normalization of the endometrial EGF profile after OPN infusion increased the embryo survival to day 14 in the uterus in repeat breeder cows. Since EGF controls the endometrium function (Brigstock, 1991; Nelson *et al.*, 1991; Johnson and Chatterjee, 1993a, b), EGF secreted by the endometrium on days 2–4 would regulate the uterine environment to suit embryo development in cattle (Katagiri and Takahashi, 2004a). Furthermore, the recovery of the endometrial EGF peak on day 3 of the estrous cycle is accompanied by that on day 14 in most cases (Katagiri and Takahashi, 2008). Bovine elongating embryos (days 13–16 of pregnancy) express EGF receptors, but not ligand (Kliem *et al.*, 1998), thus EGF secreted by the endometrium on days 13–14 may act on the embryo and stimulate embryo development (Kliem *et al.*, 1998; Katagiri and Takahashi, 2004a).

Alternatively, we could not find the relationship between embryo size and the endometrial EGF concentration on day 3. Endometrial EGF concentrations on days 3 and 14 have been correlated to embryo length on day 16 of pregnancy (Katagiri and Takahashi, 2004b). Thus, embryo elongation might become apparent after the exposure of the EGF peak on days 13–14. The elongation of a bovine embryo begins between days 12 and 14 (Lonergan and Forde, 2014), and mean values of embryo length indicated logarithmic growth between day 10 and 16 (Betteridge *et al.*, 1980). To confirm that the normalization of the endometrial EGF profile may promote embryo elongation in repeat breeder cows, further investigation should evaluate embryo lengths later than day 14 (i.e., day 16).

Interestingly, the present results suggest that in addition to the normalization of the endometrial EGF profile in repeat breeder cows, OPN infusion into the vagina might enhance embryo development in the uterus by an unknown mechanism independent of the endometrial EGF concentration on day 3. The embryo recovery rates were comparable between the rOPN group and the fertile control group despite lower proportion of cows with a normal endometrial EGF profile and endometrial EGF concentrations on day 3 after treatment of rOPN group than those of the fertile control group. Furthermore, although the embryo length was not correlated to the endometrial EGF concentration on day 3 after treatment, embryo lengths on day 14 were comparable between the rOPN group and the fertile control group but higher than those of the PBS group. Although OPN has been shown to directly promote adhesion, migration and cytoskeletal remodeling of trophectoderm cells that is essential for elongation and attachment of trophectoderm cells to uterine luminal epithelium for implantation in sheep (Kim *et al.*, 2010) and pigs (Erikson *et al.*, 2009), as discussed above, it seems unlikely that rOPN infused into the vagina directly enhance the development of transferred embryos in the uterus in repeat breeder cows. OPN infusion into the vagina may help in controlling the endometrial function by

activating immune cells in the vagina and adjacent lymph nodes (Kyaw *et al.*, 2022), which in repeat breeder cows, may regulate the uterine environment via a pathway independently on the endometrial EGF concentration on day 3. Furthermore, the formation of CL has been described as an inflammation involving various immune cells (Fair, 2015; Alrabiah *et al.*, 2021). In cattle, P₄ secreted from CL enhances embryo elongation in the uterus by regulating the secretion of histotrophic substances and growth factors from the endometrium (Clemente *et al.*, 2009; Forde *et al.*, 2011, 2012; Spencer *et al.*, 2016). Thus, immune cells activated by OPN infusion into the vagina might support CL formation and enhance progesterone secretion from a CL, thus independently promoting embryo development in the uterus with endometrial EGF concentration on day 3.

In conclusion, the present results indicate that rOPN infused into the vagina restores the normal endometrial EGF profile and fertility in repeat breeder cows without posttranslational modifications. The normalization of the endometrial EGF profile by OPN infusion improves embryo survival in the uterus. However, whether the endometrial EGF concentration on day 3 is directly correlated to the embryo size on day 14 remains unclear. Further study must confirm the relationship between the normalization of the endometrial EGF profile and embryo elongation.

Tables and figures

Table 1-1. The effect of recombinant osteopontin (rOPN) on the endometrial epidermal growth factor (EGF) profile and fertility in repeat breeder cows.

Groups (n)	No. (%) of cows with a normal EGF profile after treatment	Endometrial EGF profile after treatment	No. of inseminated cows	No. (%) of conceived cows
PBS (74)	22 (29.7) ^A	Normalized	19	9 (47.4) ^C
		Unnormalized	39	7 (17.9) ^D
		Total	58	16 (27.6) ^A
SP (37)	23 (62.2) ^B	Normalized	9	6 (66.7)
		Unnormalized	11	3 (27.3)
		Total	20	9 (45.0) ^{AB}
rOPN (105)	61 (58.1) ^B	Normalized	47	32 (68.1) ^C
		Unnormalized	36	7 (19.4) ^D
		Total	83	39 (47.0) ^B
Total (216)	106 (49.1)	Normalized	75	47 (62.7) ^C
		Unnormalized	86	17 (19.8) ^D
		Total	161	64 (39.8)

SP: Seminal plasma.

A, B Restoration rates of the normal endometrial EGF profile and conception rates with different letters in the same column differ significantly ($P < 0.05$).

C, D Conception rates with different letters between different endometrial EGF profiles within the same group differ significantly ($P < 0.05$).

Table 1-2. The effect of recombinant osteopontin (rOPN) on the embryo survival and elongation

Groups	Embryo type	No. of cows	No. (%) of cows from which embryos were collected	No. of transferred embryos*	No. (%) of recovered embryos	Embryo lengths of all recovered embryos (mm)	Embryo lengths of the largest embryos in each cow (mm)
PBS	In vitro	10	4 (40.0)	26	9 (34.6)	9.6 $\pm$ 11.4	17.5 $\pm$ 13.5
	In vivo	8	3 (37.5)	24	7 (29.2)	14.8 $\pm$ 17.0	16.5 $\pm$ 21.5
	Total	18	7 (38.9)	50	16 (32.0) ^A	11.9 $\pm$ 13.8 ^A	17.1 $\pm$ 15.7 ^A
rOPN	In vitro	10	7 (70.0)	25	16 (64.0)	31.6 $\pm$ 25.9	46.7 $\pm$ 26.9
	In vivo	7	4 (57.1)	21	11 (52.4)	27.1 $\pm$ 27.1	57.0 $\pm$ 16.4
	Total	17	11 (64.7)	46	27 (58.7) ^B	29.7 $\pm$ 26.0 ^B	50.4 $\pm$ 23.3 ^B
Fertile control	In vitro	10	7 (70.0)	24	13 (54.2)	53.3 $\pm$ 26.8	70.3 $\pm$ 18.2
	In vivo	8	6 (75.0)	24	15 (62.5)	30.9 $\pm$ 23.8	48.4 $\pm$ 22.4
	Total	18	13 (72.2)	48	28 (58.3) ^B	41.3 $\pm$ 27.2 ^B	60.1 $\pm$ 22.4 ^B

Embryo lengths are described as means $\pm$ standard deviations.

* Two or three embryos were transferred into the uterus in each cow.

A, B Embryo lengths and embryo recovery rates with different letters in the same column differ significantly ($P < 0.05$).

Table 1-3. The relationship between the endometrial epidermal growth factor (EGF) profile after treatment and the embryo survival

Endometrial EGF profile after treatment	Groups	No. of cows	No. (%) of cows from which embryos were collected	No. of transferred embryos*	No. (%) of recovered embryos	No. of cows transferred three embryos and recovered indicated number of embryos			No. of cows transferred two embryos and recovered indicated number of embryos	
						Three	Two	One	Two	One
Normal	PBS	8	6 (75.0) ^A	23	14 (60.9) ^A	2	3	0	1	0
	rOPN	10	9 (90.0) ^A	27	21 (77.8) ^A	4	2	0	2	1
	Fertile control	17	12 (70.6)	46	27 (58.7)	3	4	1	4	0
	Total	35	27 (77.1) ^A	96	62 (64.6) ^A	9	9	1	7	1
Altered	PBS	10	1 (10.0) ^B	27	1 (3.7) ^B	0	0	0	0	1
	rOPN	7	2 (28.6) ^B	19	6 (31.6) ^B	2	0	0	0	0
	Fertile control	1	1 (100.0)	2	1 (50.0)	0	0	0	0	1
	Total	18	4 (22.2) ^B	48	8 (16.7) ^B	2	0	0	0	2

* Three or two embryos were transferred into the uterus per cows.

A, B The proportions of cows from which embryos were collected and embryo recovery rates with different letters within the same group in the same column differ significantly ($P < 0.05$).

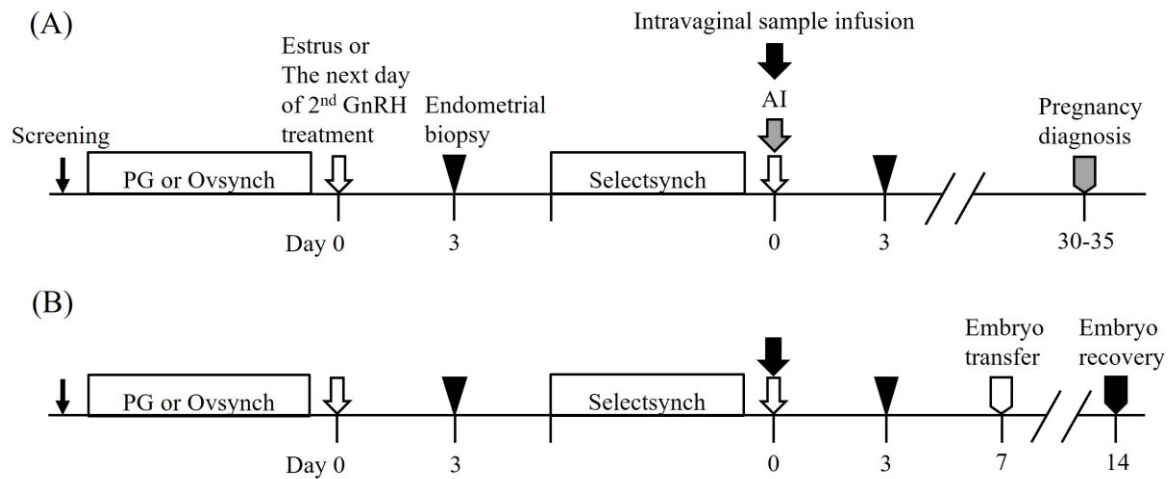


Fig. 1-1. The outline of activities during study 1-1 (A) and study 1-2 (B).

(A) Repeat breeder cows, which failed to conceive after three or more artificial inseminations (AI) without a detectable abnormality in the estrous cycle, clinical signs and genital organs, were subjected to the examination of uterine ultrasonography and cytology (Screening). The cows showing no abnormality at screening were synchronized estrus or ovulation, and the endometrial epidermal growth factor (EGF) concentration was determined on day 3 [day 0 = the day of estrus or the next day of the second gonadotropin-releasing hormone (GnRH) treatment of the Ovsynch program] and cows with an altered EGF profile were used for the further study. Cows were synchronized estrus and infused with samples into the vagina 4 to 12 h after estrus detection. The endometrial EGF concentration was determined on day 3 for the second time. Some cows were subjected to AI immediately before the infusion with samples. Pregnancy was diagnosed between day 30 and 35 after AI.

(B) Repeat breeder cows and fertile control cows were subjected to the screening and the endometrial EGF concentration was determined on day 3 same as study 1-1. Repeat breeder cows with an altered EGF profile and fertile control cows with a normal EGF profile were synchronized estrus and infused with samples into the vagina 4 to 12 h after estrus detection. The endometrial EGF concentration was determined on day 3 for the second time. Thereafter, cows were transferred embryos on day 7. Embryos were recovered on day 14 and embryo lengths were measured.

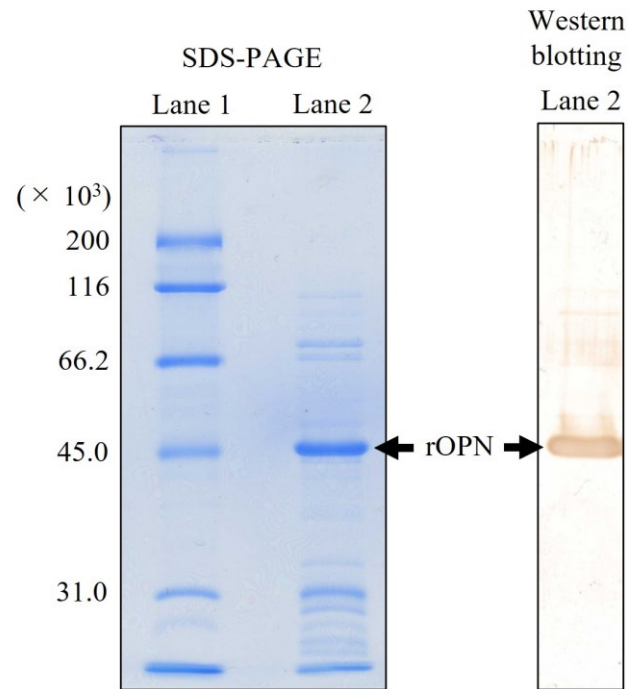


Fig. 1-2. Verification of recombinant osteopontin (rOPN) by Western blotting.

The band with the apparent molecular mass of approximately 45.0×10^3 on the SDS-PAGE gel (lane 2) was identified as OPN by Western blotting (2.4 μg of total protein per lane). Lane 1: molecular mass marker, lane 2: final preparation.

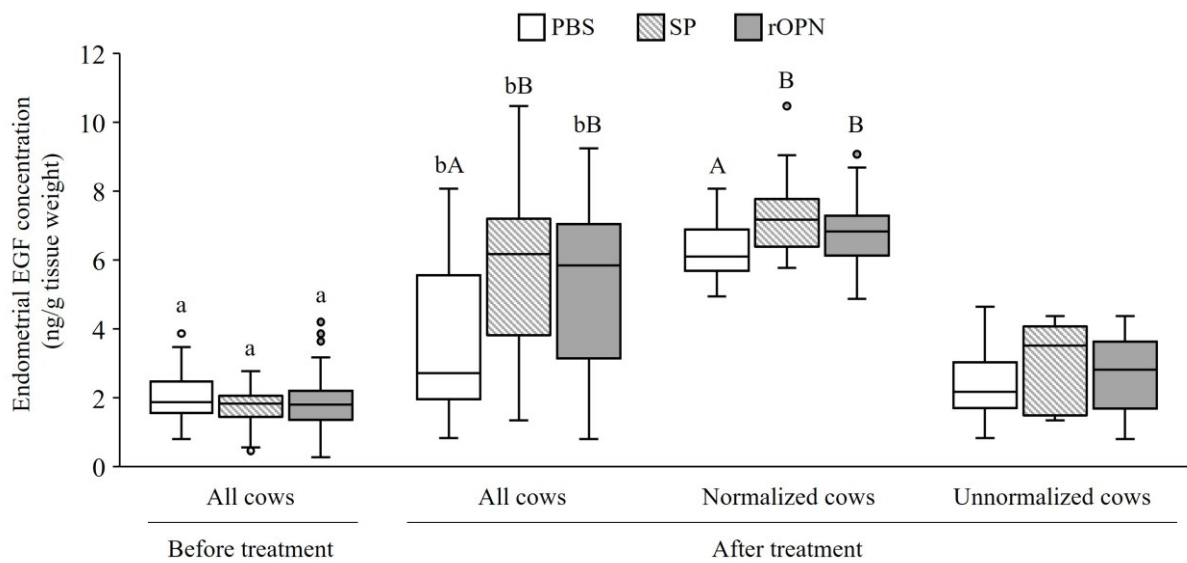


Fig. 1-3. The effect of recombinant osteopontin (rOPN) on endometrial epidermal growth factor (EGF) concentrations in repeat breeder dairy cows in study 1-1.

Repeat breeder cows with an altered EGF profile were infused with PBS (n = 74), seminal plasma (SP, n = 37) or rOPN (n = 105) into the vagina at the estrus. Endometrial EGF profiles of 22, 23 and 61 cows were normalized in PBS, SP and rOPN groups, respectively. Endometrial EGF profiles of 52, 14 and 44 cows were unnormalized in PBS, SP and rOPN groups, respectively. a, b Values with different letters between before and after treatment within the same group differ significantly ($P < 0.05$). A, B Values with different letters between groups within all cows before treatment or all, normalized or unnormalized cows after treatment differ significantly ($P < 0.05$).

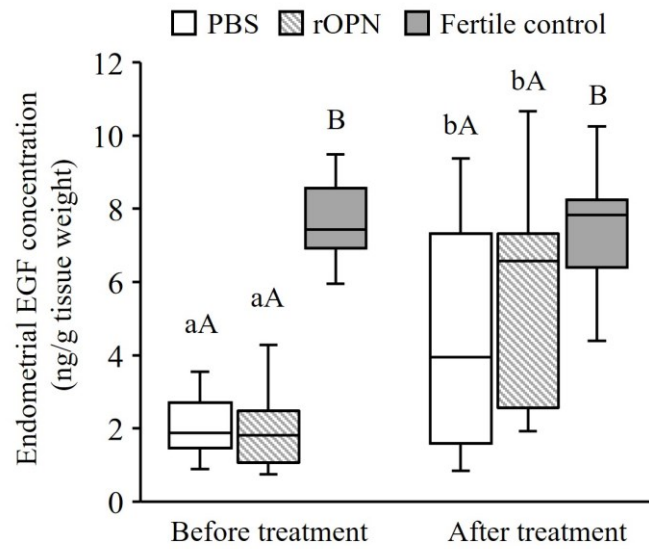


Fig. 1-4. The effect of recombinant osteopontin (rOPN) on endometrial epidermal growth factor (EGF) concentrations in repeat breeder dairy cows in study 1-2. Repeat breeder cows with an altered EGF profile were infused with PBS (PBS group, n = 18) or rOPN (rOPN group, n = 17) into the vagina at the estrus. Fertile cows with a normal EGF profile (Fertile control group, n = 18) were infused with PBS into the vagina at the estrus. a, b Values with different letters between before and after treatment within the same group differ significantly ($P < 0.05$). A, B Values with different letters between groups within cows before or after treatment differ significantly ($P < 0.05$).

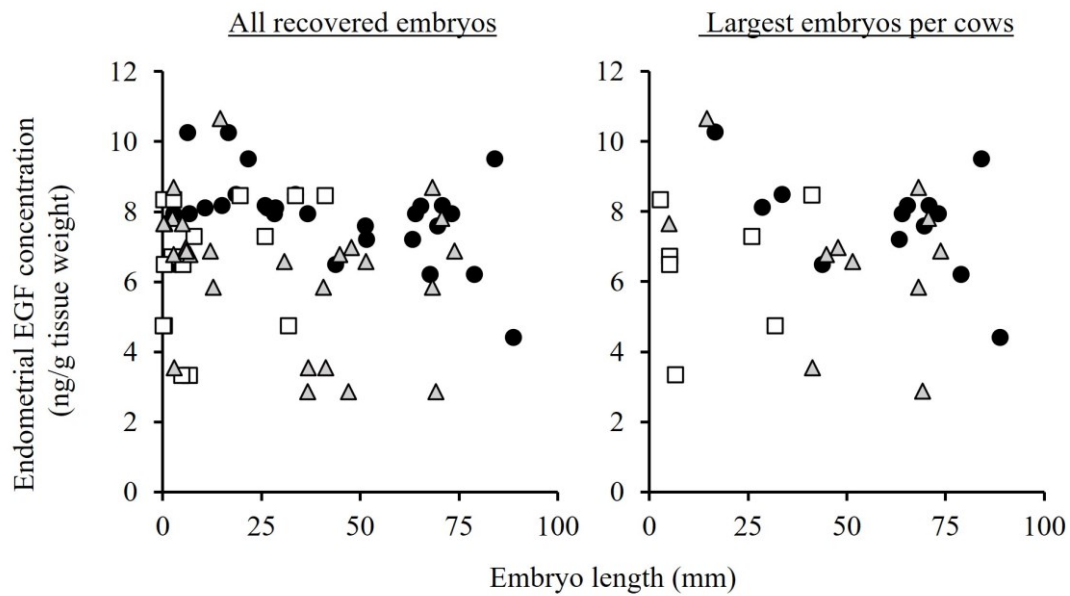


Fig.1-5. The relationship between the endometrial epidermal growth factor (EGF) concentration and the embryo length. Embryos were transferred to repeat breeder cows infused with PBS (white squares) or recombinant OPN (gray triangles) or to fertile cows infused with PBS (black circles). There were no correlations between the endometrial EGF concentration and the embryo length of all recovered embryos ($r = -0.01$) or that of the largest embryos per cows ($r = -0.09$), respectively ($P > 0.1$. The correlation was evaluated between the data of endometrial EGF concentrations and that of embryo lengths transformed into the sinh-arcsinh normal distribution).

Summary

Endometrial EGF shows a cyclic change with two peaks on days 2–4 and days 13–14 of the estrous cycle in fertile cows. In repeat breeder cows, loss of the peaks has been associated with reduced fertility. By infusing SP and OPN derived from SP and milk into the vagina, their EGF profile and fertility are restored. However, SP is difficult to obtain, and both SP and OPN derived from SP and milk can transmit infectious diseases. While OPN can be sourced from recombinant protein without this risk, recombinant OPN expressed in *E. coli* (rOPN) should be examined for its effects on the EGF profile, since it does not undergo posttranslational modification, which is important for its biological activity. Thus, in chapter 1, the effect of rOPN on the restoration of the normal endometrial EGF profile and fertility in repeat breeder cows was investigated. In study 1-1, PBS, SP (0.5 ml), and rOPN (0.3 mg) were infused into the vagina at estrus (day 0) in 74, 37, and 105 repeat breeder Holstein cows with an altered EGF profile, respectively. Endometrial EGF concentrations were measured on day 3. Some cows (n = 58, 20, and 83, respectively) were inseminated immediately before the infusion and then diagnosed for pregnancy between days 30 and 35. The restoration rate of the normal endometrial EGF profile and conception rate in rOPN group (58.1% and 47.0%, respectively) were not significantly different from those in SP group (62.2% and 45.0%, respectively) but higher than those in PBS group (29.7% and 28.1%, respectively) ($P < 0.05$). In study 1-2, repeat breeder cows with an altered EGF profile were infused with PBS (n = 18) and rOPN (n = 17), while fertile controls with a normal EGF profile (n = 18) were infused with PBS on day 0. Then, endometrial EGF concentrations were measured on day 3. Two or three embryos were transferred into cows on day 7 and then recovered on day 14. Embryo recovery rates of the rOPN and fertile groups were comparable (58.7% vs. 58.3%) but higher than that of the PBS group (32.0%) ($P < 0.05$). The embryo recovery rate of cows with normalized EGF profile was higher than that of cows with unnormalized EGF profile (64.4% vs. 16.7%) ($P < 0.05$). The embryo sizes of cows in the rOPN and fertile groups were comparable but larger than those in the PBS group ($P < 0.05$). However, the embryo size was not correlated to the corresponding endometrial EGF concentrations. In conclusion, rOPN without posttranslational modifications restored the normal endometrial EGF profile and fertility in repeat breeder cows. Improved fertility by normalization of the EGF profile could be attributed partly to the increased embryo viability up to day 14.

Chapter 2: The element of primary structure of osteopontin for the restoration of the normal endometrial epidermal growth factor profile and fertility in repeat breeder dairy cows

Introduction

An infusion of OPN into the vagina normalized the endometrial EGF profile and restored fertility in repeat breeder dairy cows (Badrakh *et al.*, 2020b; Kyaw *et al.*, 2022). Posttranslational modifications such as phosphorylation and glycosylation have been shown to be crucial for the expression of some biological activities of OPN (Ashkar *et al.*, 2000; Weber *et al.*, 2002; Lund *et al.*, 2009; Minai-Tehrani *et al.*, 2013). However, the results of chapter 1 showed that the intravaginal infusion of rOPN, which has no posttranslational modifications (Sahdev *et al.*, 2008), restored the normal endometrial EGF profile and fertility in repeat breeder dairy cows. These results indicate that posttranslational modifications were not essential, and the primary structure of OPN may play an important role in this action of OPN in the uterus.

Thrombin cleavage has also been demonstrated to be crucial for the regulation of OPN bioactivity (Senger *et al.*, 1996; Weber *et al.*, 2002; Yamamoto *et al.*, 2003; Lund *et al.*, 2009). Thrombin-cleaved OPN stimulates the migration of endothelial cells more than native OPN in humans (Senger *et al.*, 1996). Thrombin-cleaved OPN, but not full-length OPN, induces the cell migration of splenic monocytes obtained from arthritic mice (Yamamoto *et al.*, 2003). The N-terminal fragment of thrombin-cleaved OPN induces haptotaxis and cell spreading of macrophages, whereas the C-terminal fragment induces the chemotaxis of these cells in mice (Weber *et al.*, 2002).

Bovine OPN possesses one conserved thrombin-cleavage site (between Arg163 and Ser164), and OPN fragments produced by thrombin treatment bind to different receptor types, such as CD44 and integrins (Sørensen *et al.*, 1995; Lund *et al.*, 2009). The C-terminal fragment of thrombin-cleaved murine OPN binds to CD44, although its binding site is unknown (Weber *et al.*, 2002; Lund *et al.*, 2009). The Thr121–Val140 motif of murine OPN, which corresponds to Val127–Ala148 of bovine OPN, also binds to CD44 (Lin and Yang-Yen, 2001). However, in bovine OPN, there are two integrin binding motifs, including the RGD (Arg152–Asp154) and SVAYGLK (Ser155–Lys161) motifs, in the N-terminal fragment of thrombin-cleaved OPN (Sørensen *et al.*, 1995; Lund *et al.*, 2009; Christensen and Sørensen, 2016). RGD motifs are often identified as cell attachment sites for various adhesive proteins, such as fibronectin, fibrinogen, and OPN (Mezu-Ndubuisi and Maheshwari, 2021). The SVAYGLK motif next to the RGD motif in bovine OPN corresponds to the SVVYGLR and SLAYGLR motifs in human and rodent OPNs, respectively (Christensen and Sørensen, 2016; Sørensen and Christensen, 2023). The SVVYGLR motif in human OPN mediates interactions with integrins after thrombin

cleavage (Yokosaki *et al.*, 1999; Barry *et al.*, 2000). The target receptors of the OPN RGD motif are the $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha 5\beta 1$, and $\alpha 8\beta 1$ integrins, whereas the target receptors of the SVAYGLK motif are the $\alpha 4\beta 1$, $\alpha 4\beta 7$, and $\alpha 9\beta 1$ integrins (Lund *et al.*, 2009; Sørensen and Christensen, 2023). In the case of human OPN, the ELVTDFPTDLPAT (Glu131–Thr143) motif, which corresponds to the bovine OPN EVDFPTDIPTI (Glu126–Ile136) motif, is also involved in $\alpha 4\beta 1$ integrin binding (Bayless and Davis, 2001).

In chapter 2, I aimed to determine the element of primary structure of OPN to restore the normal endometrial EGF profile and fertility in repeat breeder dairy cows. I examined the effect of OPN fragments generated by thrombin treatment and chemically synthesized peptides possessing integrin binding OPN motifs (RGD and SVAYGLK motifs) on the restoration of the normal endometrial EGF profile and fertility in repeat breeder dairy cows.

Materials and methods

Preparation of rOPN

rOPN was expressed in *E. coli* as an N-terminally His6-tagged recombinant protein of predicted mature OPN (Leu17–Asn278), purified by Ni^{2+} affinity column chromatography, dissolved in PBS with 1% (w/v) trehalose as a cryoprotectant and filtered, according to a method described in chapter 1. In the seven batches of rOPN preparations, the purity of the rOPN was estimated to be $46.3 \pm 6.0\%$ (range: 35.9–55.5%) in the final preparation. The solutions containing 16 nmol rOPN, calculated based on the amino-acid analysis (study 2-1) (Luo *et al.*, 2011), or 9.5 nmol rOPN, calculated based on the total protein concentration, purity and the rOPN molecular weight as described in chapter 1 (study 2-2), were aliquoted into 10 mL glass vials and lyophilized. The lyophilized rOPN was stored at 4°C until infusion into repeat breeder cows.

The results of chapter 1 found that 0.3 mg rOPN, calculated based on the total protein concentration and purity, was effective dose for the treatment for restoration of the normal endometrial EGF profile. The dose of the same amount of rOPN was calculated in different methods. In study 2-1, this dose was equivalent to 16 nmol calculated based on the amino-acid analysis while, in study2-2, this dose was equivalent to 9.5 nmol rOPN, calculated based on the total protein concentration, purity and the rOPN molecular weight.

Preparation of thrombin-cleaved rOPN

The purified rOPN (11–18 nmol/mL, calculated based on the amino-acid analysis) in PBS was treated with thrombin (2.5 IU/mL; #27084601, Cytiva, Marlborough, MA, USA) at room

temperature (20–25°C) for 16 h. After adding trehalose to the reaction mixture containing the thrombin-cleaved fragments (Th-rOPN) to achieve a final concentration of 1%, the solution was filtered (pore size: 0.22 µm). The solution containing the Th-rOPN that was produced by 16 nmol of rOPN was aliquoted into 10 mL glass vials, lyophilized, and stored at 4°C until infusion into repeat breeder cows.

Separation, verification, and preparation of the N- and C-terminal fragments of Th-rOPN

The Th-rOPN fragments were separated using benzamidine column chromatography, which is usually used to remove serine proteases, including thrombin. The Th-rOPN solution containing thrombin dissolved in PBS with 0.3 M NaCl was added to a Benzamidine Sepharose 4 Fast Flow column (Cytiva), and the unbound fraction was collected. The bound fraction was eluted with 20 mM *p*-aminobenzamidine (#01937-91, Nacalai Tesque, Kyoto, Japan).

N-terminal (N-rOPN) and C-terminal (C-rOPN) Th-rOPN fragments in either the bound or unbound fraction separated by benzamidine column chromatography were verified by peptide-mass fingerprinting (PMF) analysis (Perkins *et al.*, 1999). Each protein band that was cut from the SDS-PAGE gel was washed four times with 50% methanol and sonication on ice, gradually dehydrated with 50 and 100% acetonitrile, and swollen with 50 mM ammonium bicarbonate. Dehydration with 100% acetonitrile and swelling with 50 mM ammonium bicarbonate were repeated. The gel pieces were then dehydrated with 50% acetonitrile containing 50 mM ammonium bicarbonate and 100% acetonitrile. The dehydrated gels were dried in a vacuum. The dried gels were swollen in a trypsin solution (25 mg/mL dissolved in 50 mM ammonium bicarbonate) and incubated at 37°C for 19 h. Tryptic peptides were extracted three times with 45% acetonitrile containing 0.1% trifluoroacetic acid and sonication on ice. The extracts were dried in a vacuum and dissolved in 0.1% formic acid containing 5% acetonitrile. The resultant peptides were analyzed on the nanoflow liquid chromatography with tandem mass spectrometry (LC-MS/MS) system (LTQ-OrbitrapXL, ThermoFisher Scientific, Waltham, MA, USA). The tandem mass spectrometry (MS/MS) values detected were evaluated using a Mascot MS/MS ion search (<https://www.matrixscience.com/>).

N-terminal amino-acid sequence analysis of C-rOPN was determined by Edman degradation (Ikeuchi *et al.*, 1989; Mizuno *et al.*, 1999). The C-rOPN was separated by SDS-PAGE and transferred to a PVDF membrane (Immun-Blot PVDF Membrane, Bio-Rad Laboratories., Berkeley, CA, USA) using a semi-dry transfer system. The composition of the transfer buffer was 30 mM Tris, 17 mM boric acid, 0.055% SDS, and 20% methanol. The transferred proteins were stained with 0.1% Coomassie Brilliant Blue R-250 and destained with 50% methanol. A piece of the membrane was cut out and subjected to a protein sequencer (PPSQ-53A, Shimadzu, Kyoto, Japan).

The N-rOPN and C-rOPN that were purified by the Benzamidine Sepharose 4 Fast Flow column were dissolved in PBS with 1% trehalose, filtered (pore size: 0.22 μ m), and the solution containing 16 nmol N-rOPN or C-rOPN was aliquoted into 10 ml glass vials and lyophilized. Concentrations of N-rOPN and C-rOPN in the final preparations were calculated based on the amino-acid analysis. The lyophilized N-rOPN and C-rOPN were stored at 4°C until infusion into repeat breeder cows.

Preparation of peptides

Four peptides, GRGDSVAYGLK (peptide 1), GRGDS (peptide 2), SVAYGLK (peptide 3), and GRGESVAYGLK (peptide 4), were synthesized by Peptide Institute (Osaka, Japan). The amino-acid sequences of peptide 1, peptide 2, and peptide 3 corresponded to Gly151–Lys161, Gly151–Ser155, and Ser155–Lys161 of bovine OPN, respectively (Fig. 2-1). Peptide 4 shared the same sequence as peptide 1; however, aspartic acid (D) was replaced with glutamic acid (E). Peptides 1, 3, and 4 were synthesized in the form of a trifluoroacetate salt with >99.0% purity, and peptide 2 was prepared in the form of an acetate salt with >99.0% purity. The lyophilized peptides were dissolved in sterilized ultrapure water and filtered (pore size: 0.22 μ m). The concentration of each peptide was determined via amino-acid analysis. Peptide samples containing 32, 320, or 1,600 nmol were aliquoted into 10 mL glass vials and lyophilized. The lyophilized peptides were stored at 4°C until infusion into repeat breeder cows.

Determination of the concentrations of proteins and peptides

The concentrations of rOPN in the final preparation were calculated based on the amino-acid analysis (Luo *et al.*, 2011) in study 2-1 and the total protein concentration and the purity and molecular weight of rOPN as described in chapter 1 in study 2-2. The concentrations of the other recombinant proteins or synthesized peptides in the final preparations were calculated based on the amino-acid analysis. For amino-acid analysis, the samples were hydrolyzed in 6 N HCl for 24 h at 110°C, and the amounts of resultant amino acids were measured using an L-8900 Amino-Acid Analyzer (Hitachi High-Tech, Tokyo, Japan). The concentrations of the proteins and peptides were calculated by dividing the amount of each amino acid by the number of amino acids that the protein or peptide was composed of and averaging the values.

The total protein concentration and the purity of rOPN were calculated according to the method described in chapter 1. The molarity of rOPN in the final preparation was calculated by multiplying the total protein concentration and purity and dividing by the theoretical molecular weight

of rOPN (31535.40).

Animals

All animal experiments were performed in accordance with the Japanese Act on Welfare and Management of Animals. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Hokkaido University (Approval No. 19-0030). The present study was conducted from October 2021 to May 2023. Holstein lactating Repeat breeder cows were housed in nine commercial farms, each of which keeps 400–1500 Holstein lactating multiparous cows, in Hokkaido, Japan. The repeat breeder cows were multiparous lactating cows (> 9,500 kg of 305-day fat-corrected milk) and between two and six in parity. Cows with problems at calving and during the postpartum period requiring three or more veterinary visits were excluded from this study.

Repeat breeder cows were recruited by local veterinarians according to the criteria for failure to conceive after three or more AI without detectable abnormalities in their estrus cycle and genital organs by rectum palpation, vaginal examination, and ultrasonography or clinical signs (Badrakh *et al.*, 2020a; Kyaw *et al.*, 2022). The prevalence of repeat breeder cows in four farms which provided more than 90% of repeat breeder cows used in this study were between 13.0 and 18.2%. The absence of any abnormality in the uterine morphology by transrectal ultrasonography (Mee *et al.*, 2009) and uterine cytology by cytobrush (Dubuc *et al.*, 2010) were also confirmed before enrollment of study. Repeat breeder cows without abnormalities in the uterine morphology and cytology was subjected to the single examination of the endometrial EGF concentration on day 3 of the estrous cycle. Those showing an altered EGF profile (between 8.6 and 12.0% in cows kept in farms) were used in the present study.

Synchronization of ovulation

Ovulation was synchronized using an Ovsynch protocol, Ovsynch protocol with E₂ treatment, or Ovsynch protocol with P₄ supplementation (Wiltbank and Pursley, 2014). In the Ovsynch protocol, cows were first treated with GnRH (100 µg of fertirelin acetate im; Conceral, MSD Animal Health, Tokyo, Japan), followed by PGF_{2α} (25 mg of dinoprost tromethamine im; Pronalgon F, Zoetis Japan, Tokyo, Japan) 7 days later, and a second treatment of GnRH (100 µg of fertirelin acetate im) approximately 55 ± 2 h after the PGF_{2α} treatment. In the Ovsynch protocol with E₂ treatment, EB (1 mg im; Estradiol injection, Kyoritsu seiyaku, Tokyo, Japan) was administered one day after the PGF_{2α} treatment in addition to the Ovsynch protocol. In the Ovsynch protocol with P₄ supplementation, controlled internal drug release devices (CIDR) containing 1.9 g of progesterone (CIDR 1900; Zoetis Japan) were inserted on the day of the first GnRH treatment in addition to the Ovsynch protocol, and

the CIDR was removed on the day of the PGF_{2α} treatment. The Ovsynch protocol and the Ovsynch protocol with E₂ treatment were performed on days 5–10 or 18–20 of the estrous cycle. The Ovsynch protocol with P₄ supplementation was performed on days 13–17 of the estrous cycle. The day following the second GnRH treatment was considered day 0.

Endometrial tissue collection, EGF assay, and analysis of the endometrial EGF profile

Endometrial tissues were collected and processed, and EGF was extracted from the tissue homogenate as described in chapter 1. The EGF concentrations in the tissue extracts were also determined according to the method in chapter 1.

In the present study, the endometrial EGF profile was considered to be normal when the endometrial EGF concentration was within the normal range for day 3 (4.70–13.50 ng/g tissue weight), while the EGF profile was judged as altered when the EGF concentration was lower than 4.70 ng/g tissue weight (Katagiri and Takahashi, 2006; Katagiri *et al.*, 2016).

AI and infusion of samples into the vagina

AI was performed using commercial frozen-thawed Holstein semen by professional AI technicians within 16–20 h after the second GnRH treatment of the Ovsynch protocols for ovulation synchronization. All OPN treatment samples were dissolved in 10 mL of PBS, loaded into a 10 mL syringe, and infused into the vagina immediately after AI, as described in chapter 1.

Study design

Study 2-1

The effect of Th-rOPN, N-rOPN, and C-rOPN treatment on the restoration of the normal endometrial EGF profile was evaluated. Repeat breeder cows with an altered endometrial EGF profile (n = 65) were used for this study (Fig. 2-2). Repeat breeder cows were synchronized for ovulation with either the Ovsynch protocol, Ovsynch protocol with E₂ treatment, or Ovsynch protocol with P₄ supplementation and underwent AI. Cows were randomly assigned to four groups with 16 nmol of rOPN (n = 18), N-rOPN (n = 20), and C-rOPN (n = 13), as well as Th-rOPN produced by 16 nmol of rOPN (n = 14) that was infused into the vagina immediately after AI. The endometrial EGF concentration was determined on day 3 for the second time. Pregnancy was diagnosed by transrectal ultrasonography on days 30–35 after AI.

Study 2-2

The effects of OPN peptides on the restoration of the normal endometrial EGF profile and fertility were examined using 431 repeat breeder cows. The timeline of the study was the same as that of study 2-1. Repeat breeder cows with an altered endometrial EGF profile were synchronized for ovulation. The cows were then infused with treatment samples into the vagina immediately after AI. study 2-2 was conducted in two trials. In trial 1, one of the following samples was randomly infused into the vagina in repeat breeder cows to examine the effects of those samples on the normalization of the endometrial EGF profile: PBS (negative control; n = 45), rOPN (positive control, 9.5 nmol; n = 83), peptide 1 (32, 320, and 1,600 nmol; n = 25 each), peptide 2 (320 and 1,600 nmol; n = 20 each), or peptide 3 (320 and 1,600 nmol; n = 25 and 23, respectively). In trial 2, additional repeat breeder cows were infused into the vagina with PBS (n = 30), rOPN (n = 28), peptide 1 (320 and 1,600 nmol; n = 25 each), or peptide 3 (1,600 nmol; n = 32) to investigate the effect on fertility with an increased number of animals.

Study 2-3

The effect of peptide 4 on the restoration of the normal endometrial EGF profile was examined to clarify the role of the RGD motif. The timeline of the study was the same as that of study 2-1. Repeat breeder cows with an altered endometrial EGF profile were synchronized for ovulation by using the Ovsynch protocol and infused with PBS (negative control; n = 24), 320 nmol peptide 1 (positive control; n = 78), or peptide 4 (320 and 1,600 nmol; n = 50 and 26, respectively) into the vagina immediately after AI.

Data analyses

Endometrial EGF concentrations before and after treatment were compared using Wilcoxon signed-rank test. Endometrial EGF concentrations of the peptide 1, peptide 2, and peptide 3 groups in trials 1 and 2 of study 2-2 were compared to those of the negative (PBS) and positive (rOPN) control groups using Steel test. Endometrial EGF concentrations between the negative and positive control groups in trials 1 and 2 of study 2-2 were compared using Wilcoxon rank sum test. Endometrial EGF concentrations between groups in study 2-1, the total of trials 1 and 2 of study 2-2, and study 2-3 were compared using Steel–Dwass test.

The proportion of cows with a normal endometrial EGF profile after treatment (restoration rates) and the conception rates of each of the peptide 1, peptide 2, and peptide 3 groups in trials 1 and 2 of study 2-2 were compared to those of the negative and positive control groups using Fisher's exact

test with the Benjamini–Hochberg method. Restoration and conception rates between the negative and positive control groups in trials 1 and 2 of study 2-2 were compared using Fisher’s exact test. The restoration and conception rates between the groups in study 2-1, a total of trials 1 and 2 of study 2-2, and study 2-3 were compared using Fisher’s exact test with the Benjamini–Hochberg method.

Wilcoxon signed-rank test, Steel test, Wilcoxon rank sum test, Steel–Dwass test, and Fisher’s exact test were performed using the computer software JMP Pro 16 (SAS Institute, Tokyo, Japan). The P-values in Fisher’s exact test were adjusted for multiple comparisons using the Benjamini–Hochberg method (Benjamini and Hochberg, 1995).

Two cows infused with rOPN, two cows infused with Th-rOPN, and one cow infused with C-rOPN were culled before the pregnancy diagnosis in study 2-1. One cow infused with PBS, two cows infused with rOPN, and one cow infused with 320 nmol peptide 2 were culled before the pregnancy diagnosis in trial 1 of study 2-2. Thus, conception rates were analyzed after excluding their data. A P value < 0.05 was considered statistically significant.

Results

Verification and preparation of Th-rOPN, N-rOPN, and C-rOPN

rOPN was observed as a band via SDS-PAGE with an apparent molecular mass of 47.7×10^3 (Fig. 2-3A and 3B, lane 2). The band corresponding to rOPN disappeared, and two bands with apparent molecular masses of approximately 33.7×10^3 and 20.2×10^3 appeared as major bands via SDS-PAGE analysis in Th-rOPN (Fig. 2-3A, lane 3). The two fragments in the Th-OPN were separated by benzamidine column chromatography (Fig. 2-3B, lane 3 and 4), and their tryptic peptide masses matched with the theoretical masses calculated from the amino-acid sequences of the N- or C-terminal ends of the putative thrombin-cleavage site (Arg163–Ser164 (Sørensen *et al.*, 1995)) of rOPN (Fig. 2-3C). Furthermore, the N-terminal amino-acid sequence of the 20.2×10^3 fragment (Fig. 2-3B, lane 4) was SKKFRRS, which corresponds to the bovine OPN region of Ser164–Ser170 (Fig. 2-3C). Based on these results, we concluded that the two fragments generated by the thrombin treatment of rOPN were N-rOPN and C-rOPN, even though the C-terminal amino-acid sequence of the N-rOPN could not be determined by PMF analysis. In two batches of N-rOPN (Fig. 2-3B, lane 3) and C-rOPN preparations (Fig. 2-3B, lane 4), the bands of N-rOPN accounted for 85.6 and 93.3% in the first and second batches, respectively, while those of C-rOPN accounted for 73.3 and 79.4%, respectively, of the total protein content in the final preparation in the gel image of SDS-PAGE.

Study 2-1: Effect of Th-rOPN, N-rOPN, and C-rOPN on the restoration of the normal endometrial EGF profile

Endometrial EGF concentrations before treatment were comparable between all of the groups (Fig. 2-4). The endometrial EGF concentrations after treatment became higher than those before treatment in the rOPN, Th-rOPN, and N-rOPN groups, whereas the endometrial EGF concentrations before and after treatment were comparable in the C-rOPN group. Endometrial EGF concentrations after treatment in both Th-rOPN and N-rOPN groups were comparable, and they were at an intermediate level between the rOPN and C-rOPN groups ($P < 0.05$). Endometrial EGF concentrations after treatment in the C-rOPN group were lower than those in the rOPN group.

Restoration rates of the normal endometrial EGF profiles of the rOPN and Th-rOPN groups were comparable and higher than that of the C-rOPN group ($P < 0.05$) (Table 2-1). The restoration rate of the N-rOPN group was in a level between the rOPN and C-rOPN groups ($P < 0.05$).

Study 2-2: Effects of OPN peptides on the restoration of the normal endometrial EGF profile and fertility

In trial 1, the endometrial EGF concentrations before treatment were comparable in all of the groups (Fig. 2-5A). The endometrial EGF concentrations after treatment (Fig. 2-5A) and the restoration rates of the normal endometrial EGF profile (Table 2-2) of the 320 nmol and 1,600 nmol peptide 1 and the 1,600 nmol peptide 3 groups were higher than those of the PBS group ($P < 0.05$), and they were comparable to those of the rOPN group. The endometrial EGF concentrations after treatment and the restoration rates of the normal endometrial EGF profile of the 320 nmol and 1,600 nmol peptide 2 and the 320 nmol peptide 3 groups were lower than those of the rOPN group ($P < 0.05$), and they were similar to those of the PBS group. Endometrial EGF concentrations after treatment with 32 nmol peptide 1 were higher than those of the PBS group ($P < 0.05$), and they were comparable to those of the rOPN group. However, the restoration rate of the normal endometrial EGF profile of the 32 nmol peptide 1 group remained comparable to that of the PBS group and lower than that of the rOPN group ($P < 0.05$).

In trial 2, the endometrial EGF concentrations before treatment were comparable in all of the groups (Fig. 2-5B). The endometrial EGF concentrations after treatment in the 320 nmol and 1,600 nmol peptide 1 group were comparable to those of the rOPN group and higher than those of the PBS group ($P < 0.05$). Endometrial EGF concentrations after treatment in the 1,600 nmol peptide 3 group were similar to those of the rOPN and PBS groups. The restoration rates of the normal endometrial EGF profile and conception rates of each group in trial 2 are shown in Tables 2-2 and 2-3, respectively.

In the total of the two trials, the endometrial EGF concentrations before treatment were

comparable in all of the groups (Fig. 2-5C). The endometrial EGF concentrations after treatment (Fig. 2-5C) and the restoration rates of the normal endometrial EGF profile (Table 2-2) of the 320 nmol and 1,600 nmol peptide 1 and 1,600 nmol peptide 3 groups were comparable to the rOPN groups and higher than those of the PBS group ($P < 0.05$). In cows with a normalized endometrial EGF profile after treatment, the endometrial EGF concentrations after treatment in the 320 nmol and 1,600 nmol peptide 1 groups were comparable to those of the rOPN group and higher than those of the PBS group ($P < 0.05$). While those after treatment in the 1,600 nmol peptide 3 groups reached an intermediate level between the PBS and rOPN groups and were comparable to all other groups (Fig. 2-5C). The conception rates in cows with a normalized endometrial EGF profile were higher than those in cows whose endometrial EGF profile had not achieved normalization in each group ($P < 0.05$) (Table 2-3). Furthermore, the conception rates of the 320 nmol and 1,600 nmol peptide 1 and 1,600 nmol peptide 3 groups were comparable to that of the rOPN group and higher than that of the PBS group ($P < 0.05$).

Study 2-3: Effect of peptide 4 on the restoration of the normal endometrial EGF profile

Before treatment, the endometrial EGF concentrations of the 1,600 nmol peptide 4 group were higher than those of the PBS group ($P < 0.05$), while those of the 320 nmol peptide 1 and 320 nmol peptide 4 groups were comparable to the PBS group (Fig. 2-6). After treatment, the endometrial EGF concentrations of the 1,600 nmol peptide 4 group were comparable to those of the 320 nmol peptide 1 group and higher than those of the PBS group ($P < 0.05$). The endometrial EGF concentrations of the 320 nmol peptide 4 group were at an intermediate level between the 320 nmol peptide 1 and PBS groups. The restoration rates of the normal endometrial EGF profiles of the 320 nmol and 1,600 nmol peptide 4 groups were comparable to those of the PBS group and lower than that of the 320 nmol peptide 1 group ($P < 0.05$) (Table 2-4).

Discussion

The results of the present study demonstrated that the SVAYGLK peptide (peptide 3) is a minimal element of OPN that is required to restore the normal endometrial EGF profile in repeat breeder cows. Although the RGD is not essential for the activity of OPN to normalize the endometrial EGF profile, the addition of the RGD motif to the N-terminal end of peptide 3 (GRGDSVAYGLK, peptide 1) enhances the effect of peptide 3. In addition, the results showed that normalization of the endometrial EGF profile by peptides 1 and 3 led to improved fertility.

Interestingly, the replacement of aspartic acid (D) in RGD motif to glutamic acid (E) diminished the effect of peptide 1 to the control (PBS) levels. Similarly, it has been reported that the

replacement of the RGD motif of OPN with RGE resulted in a decrease in the effect of OPN on cell adhesion that is mediated by $\alpha 9\beta 1$ integrin, which is a target receptor of the SVVYGLR motif homologs to bovine SVAYGLK, in human melanoma cells (Smith and Giachelli, 1998).

In cattle, OPN binds to integrins via the RGD (Arg152–Asp154) and SVAYGLK (Ser155–Lys161) motifs of the N-terminal fragment that is generated by thrombin cleavage (Sørensen *et al.*, 1995; Christensen and Sørensen, 2016; Sørensen and Christensen, 2023), although the ELVTDFPTDLPAT (Glu131–Thr143) motif of human OPN, which corresponds to the EVDFPTDIPTI (Glu126–Ile136) motif of bovine OPN, has also been described as an integrin binding site (Bayless and Davis, 2001). In addition, OPN binds to CD44 via an unknown binding site on the C-terminal fragment that is generated by the thrombin cleavage (Ashkar *et al.*, 2000; Weber *et al.*, 2002) and via the Thr121–Val140 of murine OPN, which corresponds to the Val127–Ala148 of bovine OPN (Lin and Yang-Yen, 2001). The target receptors of the SVAYGLK motif are the $\alpha 4\beta 1$, $\alpha 4\beta 7$, and $\alpha 9\beta 1$ integrins (Lund *et al.*, 2009; Sørensen and Christensen, 2023) and, thus, the interaction between this motif and these integrins may be responsible for the OPN activity to normalize the endometrial EGF profile in repeat breeder cows. Therefore, information on the expression of these integrins in the vagina during the estrous cycle, especially at estrus, may provide further understanding of the receptive mechanism of OPN and the potential application of treatment using OPN or its partial peptides.

Some functions of OPN need posttranslational modifications such as glycosylation and phosphorylation for its expression (Ashkar *et al.*, 2000; Weber *et al.*, 2002; Minai-Tehrani *et al.*, 2013), whereas other functions do not require (Erikson *et al.*, 2009; Kim *et al.*, 2010; Wu *et al.*, 2022). In sheep and pigs, recombinant rat OPN without posttranslational modifications promote trophectoderm cell adhesion and migration (Erikson *et al.*, 2009; Kim *et al.*, 2010) and ion transport across the placenta (Wu *et al.*, 2022). The results of the chapter 1 and present results also suggested that OPN does not require the posttranslational modifications to exert the restoration of the normal endometrial EGF profile. Indeed, $\alpha 4$ integrin, which is a target receptor of the SVYAGLK motif, binds to OPN independently of the posttranslational modifications (Hui *et al.*, 2015). However, it is unknown whether these posttranslational modifications enhance and stabilize the function of OPN to restore the normal EGF profile in repeat breeder cows.

Although the mechanisms by which OPN normalized the endometrial EGF profile are unknown, the site of action of OPN for the restoration of the normal endometrial EGF profile in repeat breeder cows has been thought to be the vagina (Badrakh *et al.*, 2020a; Kyaw *et al.*, 2022), as discussed in chapter 1. OPN activates immune cells, including macrophages, dendritic cells, and lymphocytes, and induces a pro-inflammatory response (Lund *et al.*, 2009). Endometrial functions are regulated by pro-

inflammatory cytokines, such as IL-6 (Campanile *et al.*, 2021) and TNF α (Gori *et al.*, 2011). Therefore, it can be hypothesized that OPN activates immune cells in the vagina, and these cells enter the adjacent lymph node via lymph, thus causing further immune cell activation in the lymph nodes (Kyaw *et al.*, 2022). The activated immune cells in the vagina and lymph nodes then infiltrate into the uterus via lymph and blood circulation. This may, in turn, normalize the endometrial EGF profile in repeat breeder cows. In support of this hypothesis, the SLAYGLR motif, the mouse homolog of the SVAYGLK motif, mediates pro-inflammatory responses by OPN in mice (Yamamoto *et al.*, 2003; Kourepini *et al.*, 2014). OPN induces a pro-inflammatory response via the SLAYGLR motif in rheumatoid arthritis (Yamamoto *et al.*, 2003) and inflammatory bowel disease (Kourepini *et al.*, 2014) murine models. The receptive mechanism of the SVAYGLK motif in the vagina and immune cells needs to be investigated in cows to confirm the hypothesis.

The interaction between OPN and integrin expressed in the conceptus and endometrium have known to have important roles in mammalian pregnancy (Johnson *et al.*, 2023). At the phase of the conceptus attachment, OPN directly promote adhesion and migration of trophoblast cells to uterine luminal epithelial cells via integrins expressed in these cells (Johnson *et al.*, 2023). In addition, it has been assumed that integrins may utilize other signaling cascades within the integrin adhesome to influence the uterine-placental environment (Wing *et al.*, 2020; Wu *et al.*, 2022; Johnson *et al.*, 2023). OPN affect angiogenesis (Wing *et al.*, 2020) and ion transport (Wu *et al.*, 2022) in porcine uterine and placental tissue via integrins. However, it seems unlikely that OPN infused into the vagina directly affect the uterus in cows, as discussed in chapter 1, and we hypothesized that OPN may act as cytokine which utilizes integrin signal cascade as discussed above.

The present study demonstrated that the infusion of N-rOPN alone had a lower effect on the normalization of the endometrial EGF profile than that of rOPN and Th-rOPN, which contain both the N-rOPN and C-rOPN fragments, in repeat breeder cows. In addition, peptide 1 required larger doses than rOPN to induce a similar effect. The following hypotheses may explain these results: (1) The amino-acid sequences other than the RGD and SVAYGLK motifs might have a supportive role in the normalization of the endometrial EGF profile. (2) The number of amino acids of peptide 1 (Gly151–Lys161, eleven amino-acid residues) is far less than that of rOPN (Leu17–Asn278, 262 amino-acid residues) and, thus, the peptide may be susceptible to denaturation or loss of activity by peptidases present in the vagina (Hussain and Ahsan, 2005). Therefore, we chose higher dose of peptides than that of rOPN. (3) The reason N-rOPN was less effective than rOPN and Th-rOPN may be because the C-terminal end corresponding to peptide 1 of a part of N-rOPN was excessively cleaved by thrombin treatment. In fact, LC-MS/MS analysis indicated that the Th-rOPN solution contained N-rOPN, which

lacked a part of the C-terminal amino acids (data not shown).

In conclusion, the present study demonstrated that the SVAYGLK motif is the structural element of OPN required to restore the normal endometrial EGF profile and fertility in repeat breeder cows. The present results also suggest that the RGD motif may play a role in enhancing the effect of the SVAYGLK motif. This knowledge provides a foundation for the elucidation of the mechanism by which OPN regulates endometrial function and for the development of a treatment for improved fertility in repeat breeder cows.

Tables and figures

Table 2-1. Effect of Th-rOPN, N-rOPN, and C-rOPN on the restoration of the normal endometrial epidermal growth factor (EGF) profile in repeat breeder cows.

Samples	(n)	No. (%) of cows with the normal endometrial EGF profile after treatment	No. of cows subjected to pregnancy diagnosis on days 30-35	No. (%) of cows conceived
rOPN	(18)	10 (55.6) ^A	16	8 (50.0)
Th-rOPN	(14)	9 (64.3) ^A	12	5 (41.7)
N-rOPN	(20)	5 (25.0) ^{AB}	20	6 (30.0)
C-rOPN	(13)	1 (7.7) ^B	12	4 (33.3)

rOPN: Recombinant osteopontin, Th-rOPN: the mixture of fragments generated by thrombin cleavage of rOPN, N-rOPN: N-terminal fragment generated by thrombin cleavage of rOPN, C-rOPN: C-terminal fragment generated by thrombin cleavage of rOPN. Doses of rOPN, N-rOPN and C-rOPN were 16 nmol. The dose of Th-rOPN was fragments generated by 16 nmol of rOPN. ^{A, B} Proportions in a same column differ significantly ($P < 0.05$).

Table 2-2. Effect of osteopontin (OPN) peptides on the restoration of the normal endometrial epidermal growth factor (EGF) profile in repeat breeder cows.

Samples	Dose	Trail 1		Trial 2		Total	
		No. of cows treated	No. (%) of cows with the normal endometrial EGF profile after treatment	No. of cows treated	No. (%) of cows with the normal endometrial EGF profile after treatment	No. of cows treated	No. (%) of cows with the normal endometrial EGF profile after treatment
PBS	—	45	7 (15.6) ^a	30	9 (30.0)	75	16 (21.3) ^A
rOPN	9.5 nmol	83	53 (63.9) ^b	28	15 (53.6)	111	68 (61.3) ^B
Peptide 1	32 nmol	25	8 (32.0) [†]	—	—	—	—
	320 nmol	25	13 (52.0) [*]	25	15 (60.0)	50	28 (56.0) ^B
	1600 nmol	25	11 (44.0) [*]	25	15 (60.0)	50	26 (52.0) ^B
Peptide 2	320 nmol	20	4 (20.0) [†]	—	—	—	—
	1600 nmol	20	5 (25.0) [†]	—	—	—	—
Peptide 3	320 nmol	25	9 (36.0) [†]	—	—	—	—
	1600 nmol	23	13 (56.3) [*]	32	15 (46.9)	55	28 (50.9) ^B

rOPN: Recombinant OPN, Peptide 1: the GRGDSVAYGLK peptide, Peptide 2: the GRGDS peptide, Peptide 3: the SVAYGLK peptide. ^{*}, [†] Proportions of cows with the normal endometrial EGF profile after treatment of peptide 1, peptide 2, and peptide 3 groups were compared to those of PBS (negative control) and rOPN (positive control) groups in trial 1 and 2. Asterisks (^{*}) and daggers ([†]) indicate the values differ from the PBS and rOPN groups, respectively ($P < 0.05$). ^{a, b} Proportions differ between PBS and rOPN groups in a same column ($P < 0.05$). ^{A, B} Proportion of cows with the normal endometrial EGF profile after treatment of PBS, rOPN, peptide 1 (320 and 1,600 nmol) and peptide 3 (1,600 nmol) groups were calculated and compared between groups in total of trials. The proportions with different uppercase letters differ significantly in a same column ($P < 0.05$).

Table 2-3. Effect of osteopontin (OPN) peptides on the restoration of fertility in repeat breeder cows.

Samples	Dose	Endometrial EGF profile after treatment	Trial 1		Trial 2		Total of trials	
			No. of cows subjected to pregnancy diagnosis on days 30-35	No. (%) of cows conceived	No. of cows subjected to pregnancy diagnosis on days 30-35	No. (%) of cows conceived	No. of cows subjected to pregnancy diagnosis on days 30-35	No. (%) of cows conceived
PBS	—	Normalized	7	4 (57.1) ^C	9	5 (55.6)	16	9 (56.3) ^C
		Unnormalized	37	3 (8.1) ^D	21	4 (19.0)	58	7 (12.1) ^D
		Total	44	7 (15.9) ^a	30	9 (30.0)	74	16 (21.6) ^A
rOPN	9.5 nmol	Normalized	52	33 (63.5) ^C	15	10 (66.7) ^C	67	43 (64.2) ^C
		Unnormalized	29	6 (20.7) ^D	13	3 (23.1) ^D	42	9 (21.4) ^D
		Total	81	39 (48.1) ^b	28	13 (46.4)	109	52 (47.7) ^B
Peptide 1	32 nmol	Normalized	8	3 (37.5)	—	—	—	—
		Unnormalized	17	2 (11.8)	—	—	—	—
		Total	25	5 (20.0)	—	—	—	—
	320 nmol	Normalized	13	9 (69.2) ^C	15	9 (60.0)	28	18 (64.3) ^C
		Unnormalized	12	2 (16.7) ^D	10	2 (20.0)	22	4 (18.2) ^D
		Total	25	11 (44.0) [*]	25	11 (44.0)	50	22 (44.0) ^B
	1600 nmol	Normalized	11	6 (54.5)	15	11 (73.3) ^C	26	17 (65.4) ^C
		Unnormalized	14	6 (42.9)	10	2 (20.0) ^D	24	8 (33.3) ^D
		Total	25	12 (48.0) [*]	25	13 (52.0)	50	25 (50.0) ^B
Peptide 2	320 nmol	Normalized	4	3 (75.0) ^C	—	—	—	—
		Unnormalized	15	1 (6.7) ^D	—	—	—	—
		Total	19	4 (21.1)	—	—	—	—
	1600 nmol	Normalized	5	3 (60.0)	—	—	—	—
		Unnormalized	15	3 (20.0)	—	—	—	—
		Total	20	6 (30.0)	—	—	—	—
Peptide 3	320 nmol	Normalized	9	7 (77.8) ^C	—	—	—	—
		Unnormalized	16	3 (18.8) ^D	—	—	—	—
		Total	25	10 (40.0)	—	—	—	—
	1600 nmol	Normalized	13	8 (61.5)	15	10 (66.7) ^C	28	18 (64.3) ^C
		Unnormalized	10	2 (20.0)	17	3 (17.6) ^D	27	5 (18.5) ^D
		Total	23	10 (43.5) [*]	32	13 (40.6)	55	23 (41.8) ^B

rOPN: Recombinant OPN, Peptide 1: the GRGDSVAYGLK peptide, Peptide 2: the GRGDS peptide, Peptide 3: the SVAYGLK peptide. EGF: epidermal growth factor. * Conception rates of peptide 1, peptide 2, and peptide 3 groups were compared to those of PBS (negative control) and rOPN (positive control) groups in trial 1 and 2. Asterisks (*) indicate the values differ from the PBS group, respectively ($P < 0.05$). ^{a, b} Conception rates differ significantly between PBS and rOPN groups in a same column ($P < 0.05$). ^{A, B} Overall conception rates of cows of PBS, rOPN, peptide 1 (320 and 1,600 nmol), and peptide 3 (1,600 nmol) groups were calculated and compared between groups in total of trials. The conception rates with different uppercase letters differ significantly in a same column ($P < 0.05$). ^{C, D} Proportions differ significantly between the different endometrial EGF profile within a same group ($P < 0.05$).

Table 2-4. Effect of the peptide 4 on the restoration of the normal endometrial epidermal growth factor (EGF) profile in repeat breeder cows.

Samples	Dose	(n)	No. (%) of cows with the normal endometrial EGF profile after treatment	No. of cows	
				subjected to pregnancy diagnosis on days 30-35	No. (%) of cows conceived
PBS	—	(24)	4 (16.7) ^A	24	5 (20.8)
Peptide 1	320 nmol	(78)	35 (44.9) ^B	78	31 (39.7)
Peptide 4	320 nmol	(50)	8 (16.0) ^A	50	10 (20.0)
	1600 nmol	(26)	5 (19.2) ^A	26	8 (30.8)

Peptide 1: the GRGDSVAYGLK peptide. Peptide 4: the GRGESVAYGLK peptide. ^{A, B} Proportions differ significantly in a same column ($P < 0.05$).

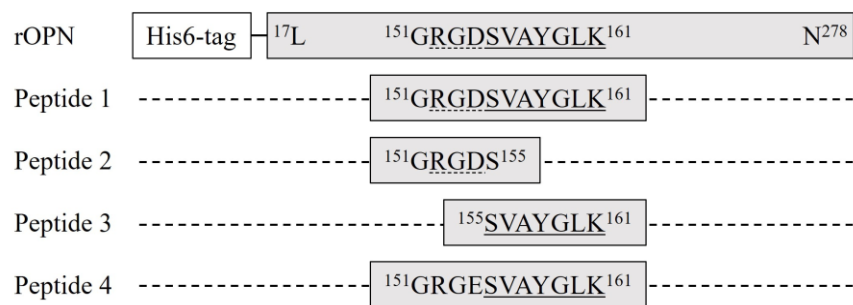


Fig. 2-1. A schematic structure of the recombinant osteopontin (OPN) and OPN peptides.

Recombinant OPN (rOPN) was expressed in *Escherichia. coli* as an N-terminally His6-tagged recombinant protein. The amino-acid sequences of peptide 1, peptide 2, and peptide 3 were designed to correspond to Gly151–Lys161, Gly151–Ser155, and Ser155–Lys161 of bovine OPN, respectively. Dashed and solid underlined sections indicate the RGD (Arg152–Asp154) and SVAYGLK (Ser155–Lys161) motifs, respectively. Peptide 4 was a peptide 1 with the aspartic acid (D) replaced with glutamic acid (E).

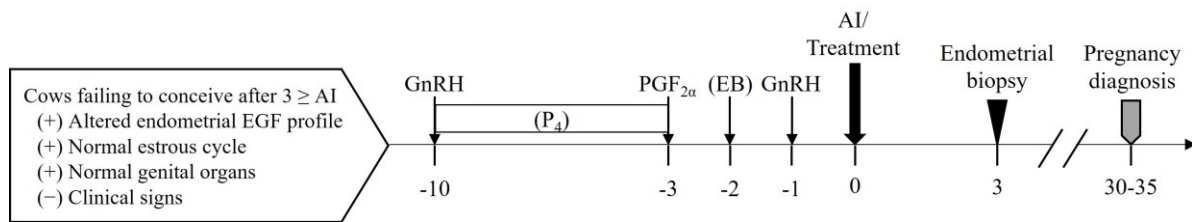


Fig. 2-2. The outline of activities.

Before enrollment in the study, repeat breeder cows were recruited by the criteria described in the figure. They were examined for the epidermal growth factor (EGF) profile by a single examination of the endometrial EGF concentration on day 3 of the estrous cycle. Those showing an altered endometrial EGF profile were used for further study. They were synchronized for ovulation and subjected to AI 16–20 h after the second gonadotropin-releasing hormone (GnRH) treatment of the ovulatory synchronization protocol (day 0). Immediately after AI, the cows were infused with treatment samples into the vagina. The endometrial EGF concentration was determined for a second time on day 3, and pregnancy was diagnosed 30–35 days after AI. P₄: progesterone; PGF_{2α}: prostaglandin F_{2α}; EB: estradiol benzoate.

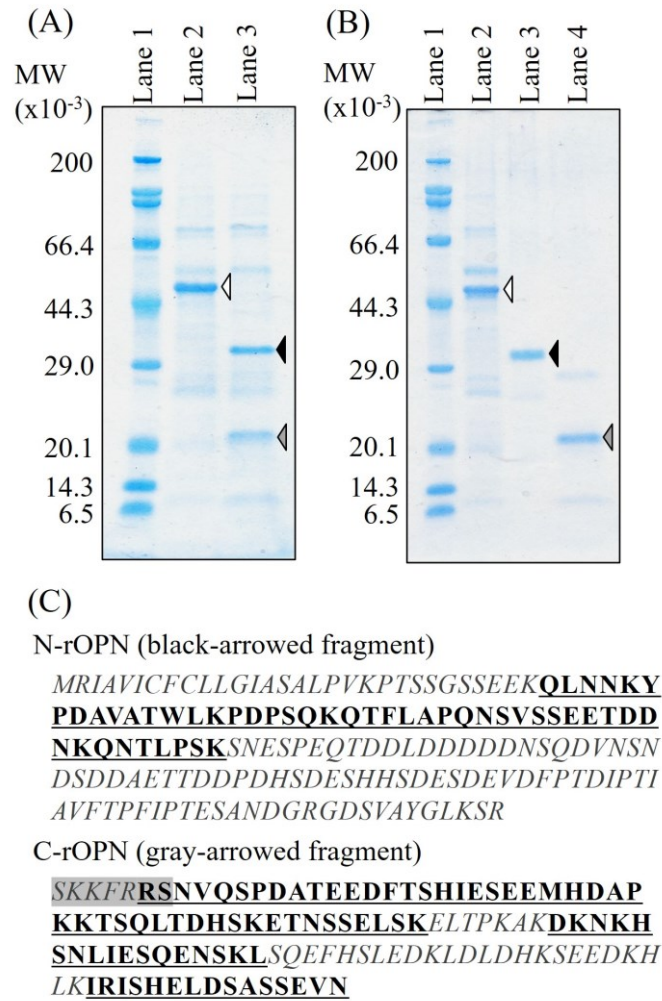


Fig. 2-3. Recombinant proteins for the infusion study.

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to the recombinant protein preparations. White arrowhead: the recombinant osteopontin (rOPN) identified by western blotting in chapter 1. Black and gray arrowheads: N-terminal (N-rOPN) and C-terminal (C-rOPN) fragments of rOPN cleaved by thrombin determined by peptide-mass fingerprinting (PMF). (A) Lane 1, molecular mass marker. Lane 2, rOPN preparation. Lane 3, thrombin-cleaved rOPN (Th-rOPN) preparation. (B) Lane 1, molecular mass marker. Lane 2, rOPN preparation. Lane 3 and 4, Th-rOPN fragments separated on a benzamidine column: the bound and unbound fraction, respectively. (C) Protein sequence coverage of N-rOPN and C-rOPN determined by PMF. The amino acids covered by the analysis are indicated with bold characters and a solid underline. Seven amino-acid residues in C-rOPN fragment shown in light shading were determined by N-terminal amino-acid sequence analysis of the fragment indicated with a gray arrowhead.

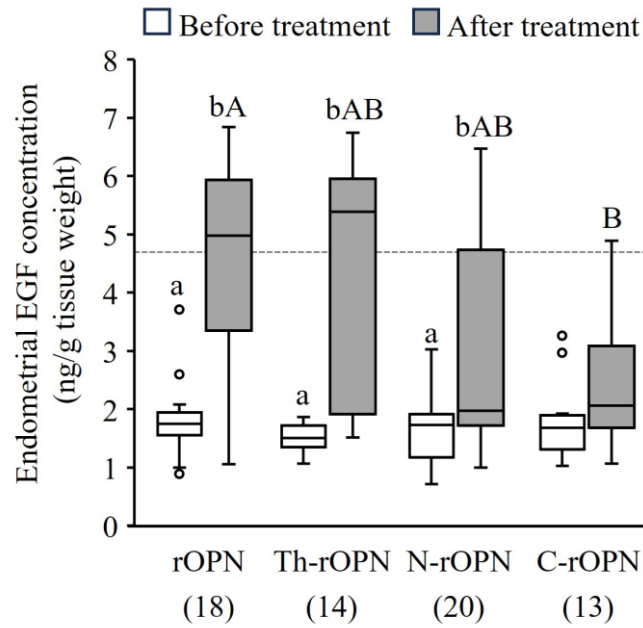


Fig. 2-4. The effect of Th-rOPN, N-rOPN, and C-rOPN on the endometrial epidermal growth factor (EGF) concentrations in repeat breeder cows.

rOPN: Recombinant osteopontin, Th-rOPN: the mixture of fragments generated by thrombin cleavage of rOPN, N-rOPN: N-terminal fragment generated by thrombin cleavage of rOPN, C-rOPN: C-terminal fragment generated by thrombin cleavage of rOPN. Doses of rOPN, N-rOPN and C-rOPN were 16 nmol. The dose of Th-rOPN was fragments generated by 16 nmol of rOPN. The gray dot line indicates the threshold concentration of the normal endometrial EGF profile (4.7 ng/g tissue weight). Numbers in parenthesis below each samples indicate the number of cows for each group. a, b Values differ before and after treatment within the same group ($P < 0.05$). A, B Values between groups differ after treatment ($P < 0.05$).

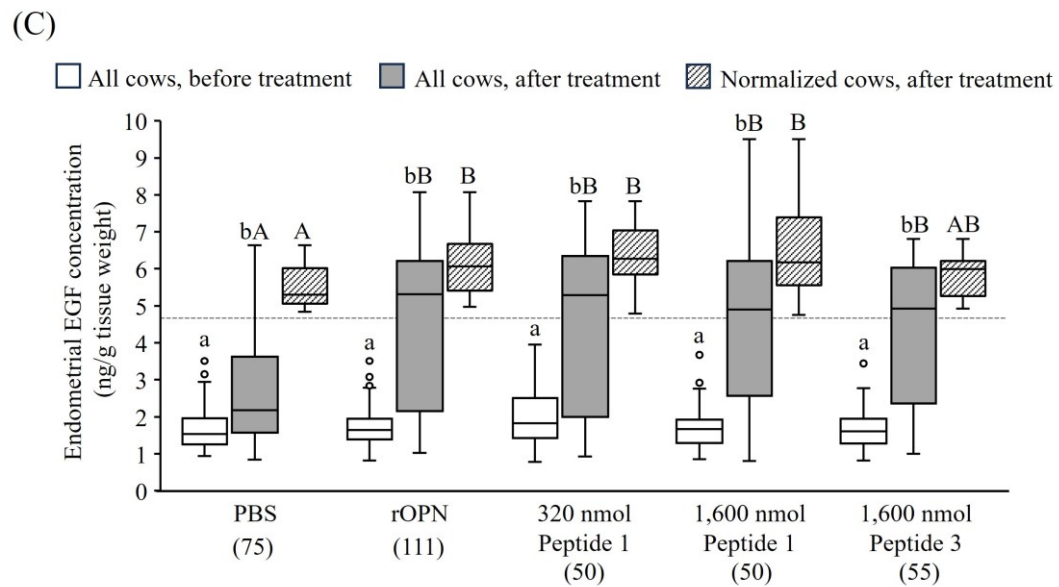
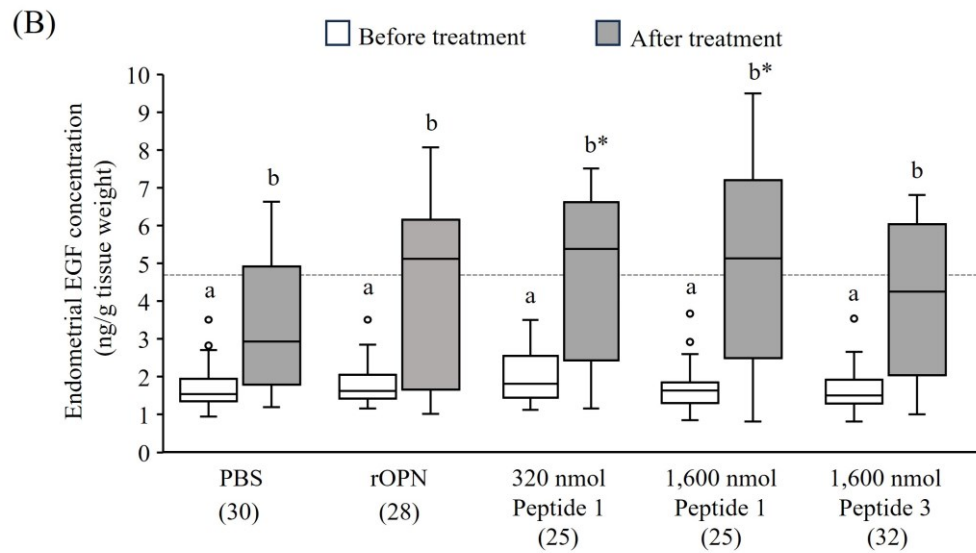
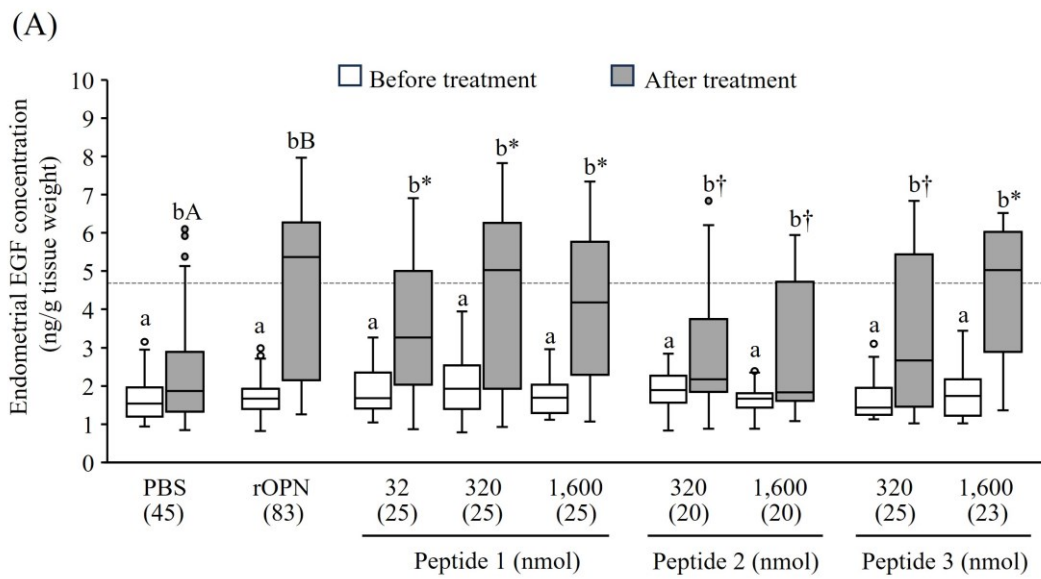


Fig. 2-5. The effect of osteopontin (OPN) peptides on the endometrial epidermal growth factor (EGF) concentrations in repeat breeder cows in trial 1 (A), trial 2 (B), and combined data from the two trials (C) for study 2-2.

rOPN: recombinant OPN. Peptide 1: the GRGDSVAYGLK peptide. Peptide 2: the GRGDS peptide. Peptide 3: the SVAYGLK peptide. The gray dot line indicates the threshold concentration of the normal endometrial EGF profile (4.7 ng/g tissue weight). Numbers in parenthesis below each samples indicate the number of cows for each group. (A) Asterisks (*) and daggers (†) indicate the values differ from the PBS and rOPN groups, respectively ($P < 0.05$). a, b Values differ before and after treatment within the same group ($P < 0.05$). A, B Values in the PBS and rOPN groups differ after treatment ($P < 0.05$). (B) Asterisks (*) indicate the values differ from the PBS group ($P < 0.05$). a, b Values differ before and after treatment within the same group ($P < 0.05$). (C) a, b Values differ before and after treatment within all cows of the same group ($P < 0.05$). A, B Values between groups differ within all cows or cows with a normalized EGF profile (normalized cows) after treatment ($P < 0.05$).

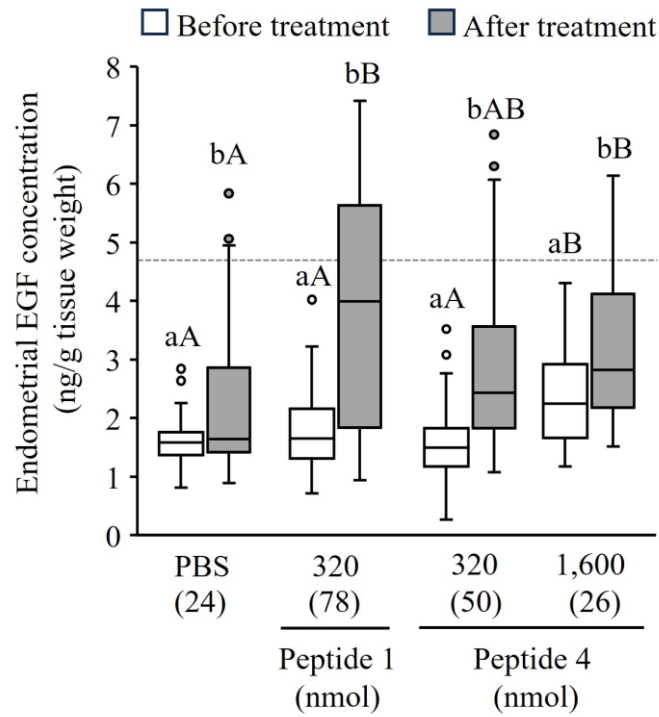


Fig. 2-6. The effect of peptide 4 on the endometrial epidermal growth factor (EGF) concentrations in repeat breeder cows.

Peptide 1: the GRGDSVAYGLK peptide. Peptide 4: the GRGESVAYGLK peptide. The gray dot line indicates the threshold concentration of the normal endometrial EGF profile (4.7 ng/g tissue weight). Numbers in parenthesis below each samples indicate the number of cows for each group. a, b Values differ between before and after treatment within the same group ($P < 0.05$). A, B Values differ between groups before and after treatment ($P < 0.05$).

Summary

The loss of a cyclic change with two peaks of increased endometrial EGF concentration on days 2–4 and 13–14 during the estrous cycle has been linked to low fertility in repeat breeder cows. An intravaginal infusion of osteopontin (OPN) restored the normal endometrial EGF profile in repeat breeder cows. The present study aimed to determine a structural element of OPN to restore the normal EGF profile and fertility. Holstein repeat breeder cows were diagnosed the EGF profile by a single examination of the endometrial EGF concentration on day 3 of the estrous cycle. Those with an altered EGF profile were intravaginally infused with OPN and its fragments on the day of insemination (day 0); the concentration of endometrial EGF was measured on day 3, and pregnancy was diagnosed on days 30–35. In study 2-1, recombinant OPN (rOPN) (16 nmol), thrombin-cleaved N- and C-terminal fragments of rOPN (N-rOPN and C-rOPN, respectively), and a combination of these fragments (Th-rOPN) were infused (n = 13–20). The restoration rate of the normal EGF profile of the N-rOPN group (25.0%) was a level in between the C-rOPN group (7.7%) and both the rOPN (55.6%) and Th-rOPN (64.3%) groups. In study 2-2, PBS (n = 47), rOPN (9.5 nmol, n = 83), and peptides of integrin binding motifs, GRGDSVAYGLK (peptide 1; 32, 320, and 1,600 nmol), GRGDS (peptide 2; 320 and 1,600 nmol), and SVAYGLK (peptide 3; 320 and 1,600 nmol), were infused (n = 20–25). Restoration rates of the normal EGF profile of peptide 1 (320 and 1,600 nmol) and peptide 3 (1,600 nmol) groups (44.0–56.3%) were comparable with those of the rOPN group (63.9%) and higher than those of the PBS group (15.6%). Restoration rates of the other groups were similar to those of the PBS group. Additional cows received infusions to determine the effect on fertility. Conception rates of the peptide 1 (320 and 1,600 nmol; n = 50 each), peptide 3 (1,600 nmol; n = 55), and rOPN (n = 111) groups (41.8–50.0%) were comparable and higher than that of the PBS group (21.6%, n = 75). In study 2-3, PBS (n = 24), peptide 1 (320 nmol; n = 78), and GRGESVAYGLK peptide (peptide 4; 320 and 1,600 nmol; n = 50 and 26, respectively) were infused. Restoration rates of the normal EGF profile of peptide 4 and PBS groups (16.0–19.2%) were comparable and lower than that of the peptide 1 group (44.9%). Thus, the SVAYGLK motif may be an OPN structural element to restore the normal EGF profile and fertility in repeat breeder cows, and the RGD motif may enhance its effect.

Summary and conclusions

The presence of repeat breeder cows is a hurdle to achieve the sustainable dairy industry. The loss of the peaks of endometrial EGF concentrations on days 2-4 and days 13-14 during the estrous cycle has been linked to low fertility in repeat breeder cows. Infusion of OPN in SP and milk into the vagina restores the normal endometrial EGF profile and fertility in repeat breeder cows. However, SP and milk may not be suitable as raw materials to prepare OPN at production sites from the perspective of the availability in quantity and the transmission of the infectious diseases. Thus, alternative sources of this protein without these disadvantages are needed. Furthermore, the mechanism by which OPN restores the normal EGF profile and fertility remains unknown. Understanding this mechanism is important for the improvement of treatment to repeat breeder cows.

OPN is a protein having multiple functions in the body, and this protein switches its function by the presence of posttranslational modifications and the binding to various receptors via multiple binding sites on this protein. However, the significance of these modifications for the expression of the OPN function to restore the normal endometrial EGF profile and the primary structure which is responsible for its action are unknown. The clarifying these points would contribute to understand the mechanism of OPN action in normalizing the EGF profile and improving the treatment with OPN. Thus, in the present study, I aimed to identify the structural element of OPN to restore the normal endometrial EGF profile and fertility in repeat breeder dairy cows. For this purpose, I investigated the significance of post-translational modifications of OPN (chapter 1) and determined the element of the primary structure of this protein (chapter 2) for these actions.

In chapter 1, the effects of the recombinant OPN expressed in *E. coli* (rOPN) that had no posttranslational modifications on the restoration of the normal endometrial EGF profile and fertility was investigated in repeat breeder dairy cows. In study 1-1, PBS, SP (0.5 ml), and rOPN (0.3 mg) were infused into the vagina at estrus (day 0) in 74, 37, and 105 repeat breeder Holstein cows with an altered EGF profile, respectively. The endometrial EGF concentrations were measured on day 3. Some cows (n = 58, 20, and 83, respectively) were inseminated immediately before the infusion and then diagnosed for pregnancy on days 30–35. The restoration rate of the normal endometrial EGF profile and conception rate in the rOPN group (58.1% and 47.0%, respectively) were not significantly different from those in the SP group (62.2% and 45.0%, respectively) but higher than those in PBS group (29.7% and 28.1%, respectively) ($P < 0.05$). In study 1-2, repeat breeder cows with an altered EGF profile were infused with PBS (n = 18) and rOPN (n = 17), while fertile controls with a normal EGF profile (n = 18) were infused with PBS on day 0. Then, the endometrial EGF concentrations were measured on day 3. Two or three embryos were transferred into cows on day 7 and then recovered on day 14. Embryo recovery rates of

the rOPN and fertile groups were comparable (58.7% vs. 58.3%) but higher than that of the PBS group (32.0%) ($P < 0.05$). The embryo recovery rate of cows with a normal EGF profile after treatment was higher than that of cows with an altered EGF profile after treatment (64.4% vs. 16.7%) ($P < 0.05$). The embryo sizes of cows in the rOPN and fertile groups were comparable but larger than those in the PBS group ($P < 0.05$). However, the embryo size was not correlated to the corresponding endometrial EGF concentrations. These results indicate that posttranslational modifications of OPN were not essential for the restoration of the normal endometrial EGF profile and fertility in repeat breeder cows and the primary structure of OPN may play an important role in the expression of these OPN actions. Improved fertility by restoration of the normal EGF profile could be attributed partly to the increased embryo survival to day 14. Meanwhile, the present results also indicated that there may be an unknown mechanism by which OPN infused into the vagina enhances the embryo elongation in the uterus, independent of the restoration of the normal EGF profile.

In chapter 2, the element of primary structure which is responsible for OPN action to restore the normal endometrial EGF profile and fertility was identified. Firstly, the effects of the fragments of rOPN cleaved by thrombin on the restoration of the normal endometrial EGF profile were examined in repeat breeder dairy cows (study 2-1). Holstein repeat breeder cows with an altered EGF profile were intravaginally infused with rOPN (16 nmol), thrombin-cleaved N- and C-terminal fragments of rOPN (N-rOPN and C-rOPN, respectively), and a combination of these fragments (Th-rOPN) ($n = 13$ – 20) on the day of insemination (day 0); the concentration of endometrial EGF was measured on day 3, and pregnancy was diagnosed on days 30–35. The restoration rate of the normal endometrial EGF profile of the N-rOPN group (25.0%) was in a level between the C-rOPN group (7.7%) and both the rOPN (55.6%) and Th-rOPN (64.3%) groups. Then, the effects of the chemically synthesized peptides of integrin binding motifs of OPN on the restoration of the normal endometrial EGF profile and fertility were examined. Holstein repeat breeder cows with an altered EGF profile were intravaginally infused with the peptides. The concentration of endometrial EGF and pregnancy was measured and diagnosed, respectively, same as schedule of study 2-1. In study 2-2, PBS ($n = 47$), rOPN (9.5 nmol, $n = 83$), and peptides of integrin binding motifs, GRGDSVAYGLK (peptide 1; 32, 320, and 1,600 nmol), GRGDS (peptide 2; 320 and 1,600 nmol), and SVAYGLK (peptide 3; 320 and 1,600 nmol), were infused ($n = 20$ – 25). Restoration rates of the normal EGF profile of peptide 1 (320 and 1,600 nmol) and peptide 3 (1,600 nmol) groups (44.0–56.3%) were comparable with that of the rOPN group (63.9%) and higher than that of the PBS group (15.6%). Restoration rates of the other groups were similar to that of the PBS group. Additional cows received infusions to determine the effect on fertility. Conception rates of the peptide 1 (320 and 1,600 nmol; $n = 50$ each), peptide 3 (1,600 nmol; $n = 55$), and rOPN ($n = 111$) groups (41.8

–50.0%) were comparable and higher than that of the PBS group (21.6%, n = 75). In study 2-3, PBS (n = 24), peptide 1 (320 nmol; n = 78), and GRGESVAYGLK peptide (peptide 4; 320 and 1,600 nmol; n = 50 and 26, respectively) were infused. Restoration rates of the normal EGF profile of peptide 4 and PBS groups (16.0–19.2%) were comparable and lower than that of the peptide 1 group (44.9%). These results suggest that the SVAYGLK motif may be an OPN structural element to restore the normal EGF profile and fertility in repeat breeder cows, and the RGD motif may enhance its effect.

In conclusion, the present study revealed that the post-translational modifications were not essential in the OPN action to restore the normal endometrial EGF profile and fertility in repeat breeder cows and an integrin binding motif is the structural element for the OPN action. These results suggest that infusion of the peptide or protein moiety containing the integrin binding motifs of OPN could become a treatment option to improve fertility in repeat breeder dairy cows. Further, the mechanism by which intravaginal infusion of OPN normalizes the EGF profile in the uterus via the interaction with integrins remains to be elucidated for the improvement of treatment to repeat breeder cows.

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisor, Professor Seiji KATAGIRI of Laboratory of Theriogenology, Department of Clinical Sciences, Faculty of Veterinary Medicine, Hokkaido University, for supporting my research and studying throughout my doctoral program, teaching me the valuable knowledge and skills of research and education.

I would also like to express my sincere gratitude to Professor Toshio TSUBOTA of Laboratory of Wildlife Biology and Medicine, Department of Environmental Veterinary Sciences, Faculty of Veterinary Medicine, Hokkaido University and Professor Yuko OKAMATSU-OGURA of Laboratory of Biochemistry, Department of Basic Veterinary Sciences, Faculty of Veterinary Medicine, Hokkaido University for reviewing my thesis and making insightful comments and opinions.

I am with my sincere gratefulness for Associate Professor Yojiro YANAGAWA of Laboratory of Theriogenology, Department of Clinical Sciences, Faculty of Veterinary Medicine, Hokkaido University, for teaching me important laboratory skills and knowledge, reviewing my manuscript, and giving insightful comments and opinions.

My special thankfulness goes to Associate Professor Takayoshi TAGAMI of Laboratory of Molecular Enzymology, Research Faculty of Agriculture, Hokkaido University for supporting my research, for teaching me important laboratory skills and knowledge and reviewing my manuscript and helpful discussion throughout of my study. In addition, I would like to thank sincerely to Professor emeritus Kazuhiro KIMURA for making insightful comments and opinions for my study.

I would like to express my sincere gratefulness to the farmers, practitioners and veterinarians who have enabled the present study at commercial dairy farms. I also deeply grateful to the Instrumental Analysis Division, Global Facility Center, Creative Research Institution, and Hokkaido University for providing insight, expertise and technology that greatly assisted the research. My special thanks also go to Frontier Institute for their technical assistance.

This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 19H00964, 22KJ0112 and 23H00353), Grants-in-Aid for the Promotion of Livestock Industry from the Japan Racing Association, Grants-in-Aid from the Japan Science and Technology Agency [Support for Pioneering Research Initiated by the Next Generation (SPRING) program (No. JPMJSP2119)], and Grant-in-Aid for Graduate Students from the Ministry of Education, Culture, Sports, Science and Technology [the World-leading Innovative and Smart Education (WISE) Program (No. 1801)]. I am also sincerely grateful for the financial supports provided by JSPS Research Fellowships for Young Scientists, the WISE Program and SPRING program.

Finally, I wish to express my greatest thanks to my family for their encouragement.

References

- Alrabiah NA, Evans ACO, Fahey AG, Cantwell N, Lonergan P, McCormack J, Browne JA and Fair T** (2021) Immunological aspects of ovarian follicle ovulation and corpus luteum formation in cattle. *Reproduction* **162** 209–225.
- Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, Rittling SR, Denhardt DT, Glimcher MJ and Cantor H** (2000) Eta-1 (osteopontin): An early component of type-1 (cell-mediated) immunity. *Science* **287** 860–864.
- Badrakh D, Yanagawa Y, Nagano M and Katagiri S** (2020a) Effect of seminal plasma infusion into the vagina on the normalization of endometrial epidermal growth factor concentrations and fertility in repeat breeder dairy cows. *Journal of Reproduction and Development* **66** 149–154.
- Badrakh D, Shirasawa A, Yanagawa Y, Nagano M and Katagiri S** (2020b) Identification of bovine seminal plasma proteins with an activity to normalize endometrial epidermal growth factor concentrations in repeat breeder cows. *Japanese Journal of Veterinary Research* **68** 91–103.
- Båge R, Gustafsson H, Larsson B, Forsberg M and Rodríguez-Martínez H** (2002) Repeat breeding in dairy heifers: Follicular dynamics and estrous cycle characteristics in relation to sexual hormone patterns. *Theriogenology* **57** 2257–2269.
- Barry ST, Ludbrook SB, Murrison E and Horgan CMT** (2000) Analysis of the $\alpha 4\beta 1$ integrin-osteopontin interaction. *Experimental Cell Research* **258** 342–351.
- Bartlett P., Kirk J. and Mather EC** (1986) Repeated insemination in Michigan Holstein-Friesian cattle: Incidence, descriptive epidemiology and estimated economic impact. *Theriogenology* **26** 309–322.
- Bayless KJ and Davis GE** (2001) Identification of dual $\alpha 4\beta 1$ integrin binding sites within a 38 amino acid domain in the N-terminal thrombin fragment of human osteopontin. *Journal of Biological Chemistry* **276** 13483–13489.
- Benjamini Y and Hochberg Y** (1995) Controlling the false discovery rate : A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)* **57** 289–300.
- Betteridge KJ, Eaglesome MD, Randall GCB and Mitchell D** (1980) Collection, description and transfer of embryos from cattle 10-16days after oestrus. *Journal of Reproduction and Fertility* **59** 205–216.
- Boccia L, Di Francesco S, Neglia G, De Blasi M, Longobardi V, Campanile G and Gasparrini B** (2013) Osteopontin improves sperm capacitation and invitro fertilization efficiency in buffalo

- (*Bubalus bubalis*). *Theriogenology* **80** 212–217.
- Brigstock DR** (1991) Growth factors in the uterus: Steroidal regulation and biological actions. *Bailliere's Clinical Endocrinology and Metabolism* **5** 791–808.
- Brito LF, Bedere N, Douhard F, Oliveira HR, Arnal M, Peñagaricano F, Schinckel AP, Baes CF and Miglior F** (2021) Review: Genetic selection of high-yielding dairy cattle toward sustainable farming systems in a rapidly changing world. *Animal* **15** 100292.
- Bromfield JJ, Schjenken JE, Chin PY, Care AS, Jasper MJ and Robertson SA** (2014) Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proceedings of the National Academy of Sciences of the United States of America* **111** 2200–2205.
- Brunswig-Spickenheier B and Mukhopadhyay AK** (2003) Expression of osteopontin (OPN) mRNA in bovine ovarian follicles and corpora lutea. *Reproduction in Domestic Animals* **38** 175–181.
- Campanile G, Baruselli PS, Limone A and D'Occhio MJ** (2021) Local action of cytokines and immune cells in communication between the conceptus and uterus during the critical period of early embryo development, attachment and implantation – Implications for embryo survival in cattle: A review. *Theriogenology* **167** 1–12.
- Casida LE** (1961) Present status of the repeat-breeder cow problem. *Journal of Dairy Science* **44** 2323–2329.
- Castello LM, Raineri D, Salmi L, Clemente N, Vaschetto R, Quaglia M, Garzaro M, Gentili S, Navalesi P, Cantaluppi V et al.** (2017) Osteopontin at the crossroads of inflammation and tumor progression. *Mediators of Inflammation* **2017** 4049098.
- Chelenga M, Sakaguchi K, Abdel-Ghani MA, Yanagawa Y, Katagiri S and Nagano M** (2020) Effect of increased oxygen availability and astaxanthin supplementation on the growth, maturation and developmental competence of bovine oocytes derived from early antral follicles. *Theriogenology* **157** 341–349.
- Christensen B and Sørensen ES** (2016) Structure, function and nutritional potential of milk osteopontin. *International Dairy Journal* **57** 1–6.
- Chu TM, Nocera M, Flanders KC and Kawinski E** (1996) Localization of seminal plasma transforming growth factor- β 1 on human spermatozoa: An immunocytochemical study. *Fertility and Sterility* **66** 327–330.
- Clemente M, De La Fuente J, Fair T, Al Naib A, Gutierrez-Adan A, Roche JF, Rizos D and Lonergan P** (2009) Progesterone and conceptus elongation in cattle: A direct effect on the embryo or an indirect effect via the endometrium? *Reproduction* **138** 507–517.

- Denhardt DT and Guo X** (1993) Osteopontin: a protein with diverse functions. *The FASEB Journal* **7** 1475–1482.
- Dochi O, Takahashi K, Hirai T and Hayakawa H** (2008) The use of embryo transfer to produce pregnancies in repeat-breeding dairy cattle. **69** 124–128.
- Dubuc J, Duffield TF, Leslie KE, Walton JS and LeBlanc SJ** (2010) Definitions and diagnosis of postpartum endometritis in dairy cows. *Journal of Dairy Science* **93** 5225–5233.
- Erb RE, Garverick HA, Randel RD, Brown BL and Callahan CJ** (1976) Profiles of reproductive hormones associated with fertile and nonfertile inseminations of dairy cows. *Theriogenology* **5** 227–242.
- Erikson DW, Way AL, Chapman DA and Killian GJ** (2007) Detection of osteopontin on Holstein bull spermatozoa, in cauda epididymal fluid and testis homogenates, and its potential role in bovine fertilization. *Reproduction* **133** 909–917.
- Erikson DW, Burghardt RC, Bayless KJ and Johnson GA** (2009) Secreted phosphoprotein 1 (SPP1, osteopontin) binds to integrin α v β 6 on porcine trophectoderm cells and Integrin α v β 3 on uterine luminal epithelial cells, and promotes trophectoderm cell adhesion and migration. *Biology of Reproduction* **81** 814–825.
- Fair T** (2015) The contribution of the maternal immune system to the establishment of pregnancy in cattle. *Frontiers in Immunology* **6** 1–8.
- Fernandez-Fuertes B** (2023) Review: The role of male reproductive tract secretions in ruminant fertility. *Animal* **17** 100773.
- Forde N, Beltman ME, Duffy GB, Duffy P, Mehta JP, O’Gaora P, Roche JF, Lonergan P and Crowe MA** (2011) Changes in the endometrial transcriptome during the bovine estrous cycle: Effect of low circulating progesterone and consequences for conceptus elongation. *Biology of Reproduction* **84** 266–278.
- Forde N, Mehta JP, Minten M, Crowe MA, Roche JF, Spencer TE and Lonergan P** (2012) Effects of low progesterone on the endometrial transcriptome in cattle. *Biology of Reproduction* **87** 1–11.
- Gabler C, Chapman DA and Killian GJ** (2003) Expression and presence of osteopontin and integrins in the bovine oviduct during the oestrous cycle. *Reproduction* **126** 721–729.
- Garcia-Ispuerto I and López-Gatius F** (2017) Progesterone supplementation in the early luteal phase after artificial insemination improves conception rates in high-producing dairy cows. *Theriogenology* **90** 20–24.
- Garlow JE, Ka H, Johnson GA, Burghardt RC, Jaeger LA and Bazer FW** (2002) Analysis of

- osteopontin at the maternal-placental interface in pigs. *Biology of Reproduction* **66** 718–725.
- Garvey M** (2020) Mycobacterium avium paratuberculosis: A disease burden on the dairy industry. *Animals* **10** 1–11.
- Givens MD** (2018) Review: Risks of disease transmission through semen in cattle. *Animal* **12**:S1 s165–s171.
- Gori I, Pellegrini C, Staedler D, Russell R, Jan C and Canny GO** (2011) Tumor necrosis factor- α activates estrogen signaling pathways in endometrial epithelial cells via estrogen receptor α . *Molecular and Cellular Endocrinology* **345** 27–37.
- Haile-Mariam M and Goddard ME** (2008) Genetic and phenotypic parameters of lactations longer than 305 days (extended lactations). *Animal* **2** 325–335.
- Harada M, Miyano T, Matsumura K, Osaki S, Miyake M and Kato S** (1997) Bovine oocytes from early antral follicles grow to meiotic competence in vitro: Effect of FSH and hypoxanthine. *Theriogenology* **48** 743–755.
- Herlekar DA, Shashikant CS, Gurjar AA and Jayarao BM** (2013) Presence of viral and bacterial organisms in milk and their association with somatic cell counts. *Journal of Dairy Science* **96** 6336–6346.
- Huet-Hudson YM, Chakraborty C, De SK, Suzuki Y, Andrews GK and Dey SK** (1990) Estrogen regulates the synthesis of epidermal growth factor in mouse uterine epithelial cells. *Molecular Endocrinology* **4** 510–523.
- Hui T, Sørensen ES and Rittling SR** (2015) Osteopontin binding to the $\alpha 4$ integrin requires highest affinity integrin conformation, but is independent of post-translational modifications of osteopontin. *Matrix Biology* **41** 19–25.
- Hussain A and Ahsan F** (2005) The vagina as a route for systemic drug delivery. *Journal of Controlled Release* **103** 301–313.
- Icer MA and Gezmen-Karadag M** (2018) The multiple functions and mechanisms of osteopontin. *Clinical Biochemistry* **59** 17–24.
- Ikeuchi M, Takio K and Inoue Y** (1989) N-terminal sequencing of photosystem II low-molecular-mass proteins 5 and 4.1 kDa components of the O₂-evolving core complex from higher plants. *FEBS Letters* **242** 263–269.
- Johnson DC and Chatterjee S** (1993a) Embryo implantation in the rat uterus induced by epidermal growth factor. *Journal of Reproduction and Fertility* **99** 557–559.
- Johnson DC and Chatterjee S** (1993b) Epidermal growth factor (EGF) replaces estradiol for the initiation of embryo implantation in the hypophysectomized rat. *Placenta* **14** 429–438.

- Johnson GA, Burghardt RC, Spencer TE, Newton GR, Ott TL and Bazer FW** (1999) Ovine osteopontin: II. osteopontin and $\alpha\text{v}\beta 3$ integrin expression in the uterus and conceptus during the periimplantation period. *Biology of Reproduction* **61** 892–899.
- Johnson GA, Burghardt RC, Bazer FW and Spencer TE** (2003) Osteopontin: Roles in implantation and placentation. *Biology of Reproduction* **69** 1458–1471.
- Johnson GA, Burghardt RC, Bazer FW, Seo H and Cain JW** (2023) Integrins and their potential roles in mammalian pregnancy. *Journal of Animal Science and Biotechnology* **14** 1–19.
- Joyce MM, González JF, Lewis S, Woldesenbet S, Burghardt RC, Newton GR and Johnson GA** (2005) Caprine uterine and placental osteopontin expression is distinct among epitheliochorial implanting species. *Placenta* **26** 160–170.
- Katagiri S** (2006) Relationship between endometrial epidermal growth factor and fertility after embryo transfer. *Journal of Reproduction and Development* **52(suppl)** 133–137.
- Katagiri S and Moriyoshi M** (2013) Alteration of the endometrial EGF profile as a potential mechanism connecting the alterations in the ovarian steroid hormone profile to embryonic loss in repeat breeders and high-producing cows. *Journal of Reproduction and Development* **59** 415–420.
- Katagiri S and Takahashi Y** (2004a) Changes in EGF concentrations during estrous cycle in bovine endometrium and their alterations in repeat breeder cows. *Theriogenology* **62** 103–112.
- Katagiri S and Takahashi Y** (2004b) Relationship between endometrial concentrations of epidermal growth factor (EGF) and preimplantation embryo development in dairy cattle. *Biology of Reproduction* **SI** 211.
- Katagiri S and Takahashi Y** (2006) Potential relationship between normalization of endometrial epidermal growth factor profile and restoration of fertility in repeat breeder cows. *Animal Reproduction Science* **95** 54–66.
- Katagiri S and Takahashi Y** (2008) A progestin-based treatment with a high dose of estradiol benzoate normalizes cyclic changes in endometrial EGF concentrations and restores fertility in repeat breeder cows. *The Journal of Reproduction and Development* **54** 473–479.
- Katagiri S, Moriyoshi M and Takahashi Y** (2013) Low incidence of an altered endometrial epidermal growth factor (EGF) profile in repeat breeder Holstein heifers and differential effect of parity on the EGF profile between fertile Holstein (dairy) and Japanese Black (beef) cattle. *Journal of Reproduction and Development* **59** 575–579.
- Katagiri S, Moriyoshi M and Yanagawa Y** (2016) Endometrial epidermal growth factor profile and its abnormalities in dairy cows. *Journal of Reproduction and Development* **62** 465–470.

- Katagiri S, Yanagawa Y and Nagano M** (2019) Repeat breeding: The cause and the possible treatment to restore fertility. In *Proceedings of the 17th International Conference on Production Diseases in Farm Animals*, p 173. Eds RM Bruckmaier and JJ Gross. Bern: Bern Open Publishing.
- Kim J, Erikson DW, Burghardt RC, Spencer TE, Wu G, Bayless KJ, Johnson GA and Bazer FW** (2010) Secreted phosphoprotein 1 binds integrins to initiate multiple cell signaling pathways, including FRAP1/mTOR, to support attachment and force-generated migration of trophectoderm cells. *Matrix Biology* **29** 369–382.
- Kimura M, Nakao T, Moriyoshi M and Kawata K** (1987) Luteal phase deficiency as a possible cause of repeat breeding in dairy cows. *Brit. Vet. J.* **143** 560–566.
- Kliem A, Tetens F, Klonisch T, Grealy M and Fischer B** (1998) Epidermal growth factor receptor and ligands in elongating bovine blastocysts. *Molecular Reproduction and Development* **51** 402–412.
- Kourepini E, Aggelakopoulou M, Alissafi T, Paschalidis N, Simoes DCM and Panoutsakopoulou V** (2014) Osteopontin expression by CD103⁺ dendritic cells drives intestinal inflammation. *Proceedings of the National Academy of Sciences of the United States of America* **111** E856–E865.
- Kuczewski A, Orsel K, Barkema HW, Mason S, Erskine R and van der Meer F** (2021) Invited review: Bovine leukemia virus—Transmission, control, and eradication. *Journal of Dairy Science* **104** 6358–6375.
- Kuwabara Y, Katayama A, Tomiyama R, Piao H, Kurihara S, Ono S, Mine K, Akira S, Orimo H and Takeshita T** (2015) Gonadotropin regulation and role of ovarian osteopontin in the periovulatory period. *Journal of Endocrinology* **224** 49–59.
- Kyaw HM, Sato H, Tagami T, Yanagawa Y, Nagano M and Katagiri S** (2022) Effects of milk osteopontin on the endometrial epidermal growth factor profile and restoration of fertility in repeat breeder dairy cows. *Theriogenology* **184** 26–33.
- Lafi SQ, Kaneene JB, Black JR and Lloyd JW** (1992) Epidemiological and economic study of the repeat breeder syndrome in Michigan dairy cattle. II. Economic modeling. *Preventive Veterinary Medicine* **14** 99–114.
- Larson JE, Lamb GC, Stevenson JS, Johnson SK, Day ML, Geary TW, Kesler DJ, DeJarnette JM, Schrick FN, DiCostanzo A *et al.*** (2006) Synchronization of estrus in suckled beef cows for detected estrus and artificial insemination and timed artificial insemination using gonadotropin-releasing hormone, prostaglandin F2 α , and progesterone. *Journal of Animal Science* **84** 332–342.

- Levine HD** (1999) The repeat breeder cow. *The Bovine Practitioner* **33** 97–105.
- Lin YH and Yang-Yen HF** (2001) The osteopontin-CD44 survival signal involves activation of the phosphatidylinositol 3-kinase/akt signaling pathway. *Journal of Biological Chemistry* **276** 46024–46030.
- Liu Q, Xie QZ, Zhou Y and Yang J** (2014) Osteopontin is expressed in the oviduct and promotes fertilization and preimplantation embryo development of mouse. *Zygote* **760** 622–630.
- Lonergan P and Forde N** (2014) Maternal-embryo interaction leading up to the initiation of implantation of pregnancy in cattle. *Animal* **8** 64–69.
- Lonergan P, Woods A, Fair T, Carter F, Rizos D, Ward F, Quinn K and Evans A** (2007) Effect of embryo source and recipient progesterone environment on embryo development in cattle. *Reproduction, Fertility and Development* **19** 861–868.
- Lund SA, Giachelli CM and Scatena M** (2009) The role of osteopontin in inflammatory processes. *Journal of Cell Communication and Signaling* **3** 311–322.
- Luo Y, Matejic T, Ng CK, Nunnally B, Porter T, Raso S, Rouse J, Shang T and Steckert J** (2011) Characterization and analysis of biopharmaceutical proteins. *Separation Science and Technology* **10** 283–359.
- Mateo-Otero Y, Sánchez JM, Recuero S, Bagés-Arnal S, McDonald M, Kenny DA, Yeste M, Lonergan P and Fernandez-Fuertes B** (2020) Effect of exposure to seminal plasma through natural mating in cattle on conceptus length and gene expression. *Frontiers in Cell and Developmental Biology* **8** 341.
- Mee JF, Buckley F, Ryan D and Dillon P** (2009) Pre-breeding ovario-uterine ultrasonography and its relationship with first service pregnancy rate in seasonal-calving dairy herds. *Reproduction in Domestic Animals* **44** 331–337.
- Mezu-Ndubuisi OJ and Maheshwari A** (2021) The role of integrins in inflammation and angiogenesis. *Pediatric Research* **89** 1619–1626.
- Minai-Tehrani A, Chang SH, Park SB and Cho MH** (2013) The O-glycosylation mutant osteopontin alters lung cancer cell growth and migration in vitro and in vivo. *International Journal of Molecular Medicine* **32** 1137–1149.
- Mizuno T, Mori H, Ito H, Matsui H, Kimura A and Chiba S** (1999) Molecular cloning of isomaltotriose-dextranase gene from *Brevibacterium fuscum* var. *dextranolyticum* strain 0407 and its expression in *Escherichia coli*. *Bioscience, Biotechnology, and Biochemistry* **63** 1582–1588.
- Monaco E, Gasparrini B, Boccia L, De Rosa A, Attanasio L, Zicarelli L and Killian G** (2009) Effect of osteopontin (OPN) on in vitro embryo development in cattle. *Theriogenology* **71** 450–

- Montes F, Meinen R, Dell C, Rotz A, Hristov AN, Oh J, Waghorn G, Gerber PJ, Henderson B, Makkar HPS *et al.*** (2013) SPECIAL TOPICS-Mitigation of methane and nitrous oxide emissions from animal operations: II. A review of manure management mitigation options. *Journal of Animal Science* **91** 5070–5094.
- Murphy EM, Kelly AK, O'Meara C, Eivers B, Lonergan P and Fair S** (2018) Influence of bull age, ejaculate number, and season of collection on semen production and sperm motility parameters in holstein friesian bulls in a commercial artificial insemination centre. *Journal of Animal Science* **96** 2408–2418.
- Nelson KG, Takahashi T, Bossert NL, Walmer DK and McLachlan J a** (1991) Epidermal growth factor replaces estrogen in the stimulation of female genital-tract growth and differentiation. *Proceedings of the National Academy of Sciences of the United States of America* **88** 21–25.
- Ninpetch N** (2022) Role of metabolic and endocrine factors in an alteration of the endometrial epidermal growth factor concentration in repeat breeder dairy cows. Hokkaido university.
- Noble JE and Bailey MJA** (2009) Quantitation of protein. *Methods in Enzymology* **463** 73–95.
- Norby B, Bartlett PC, Byrem TM and Erskine RJ** (2016) Effect of infection with bovine leukemia virus on milk production in Michigan dairy cows. *Journal of Dairy Science* **99** 2043–2052.
- O'Callaghan E, Sánchez JM, McDonald M, Kelly AK, Hamdi M, Maicas C, Fair S, Kenny DA and Lonergan P** (2021) Sire contribution to fertilization failure and early embryo survival in cattle. *Journal of Dairy Science* **104** 7262–7271.
- O'leary S, Jasper MJ, Warnes GM, Armstrong DT and Robertson SA** (2004) Seminal plasma regulates endometrial cytokine expression, leukocyte recruitment and embryo development in the pig. *Reproduction* **128** 237–247.
- Odhiambo JF, Poole DH, Hughes L, DeJarnette JM, Inskeep EK and Dailey RA** (2009) Pregnancy outcome in dairy and beef cattle after artificial insemination and treatment with seminal plasma or transforming growth factor beta-1. *Theriogenology* **72** 566–571.
- OECD/FAO** (2024) Dairy and dairy products. In *OECD-FAO Agricultural Outlook 2024-2033*, pp 178–189. Ed OECD/FAO. Paris/Rome: OECD Publishing/FAO.
- Okegawa Y and Motohashi K** (2015) Evaluation of seamless ligation cloning extract preparation methods from an Escherichia coli laboratory strain. *Analytical Biochemistry* **486** 51–53.
- Ortiz WG, Rizo JA, Carvalheira LR, Ahmed BMS, Estrada-Cortes E, Harstine BR, Bromfield JJ and Hansen PJ** (2019) Effects of intrauterine infusion of seminal plasma at artificial insemination on fertility of lactating Holstein cows. *Journal of Dairy Science* **102** 6587–6594.

- Overton TW** (2014) Recombinant protein production in bacterial hosts. *Drug Discovery Today* **19** 590–601.
- Paria BC and Dey SK** (1990) Preimplantation embryo development in vitro: cooperative interactions among embryos and role of growth factors. *Proceedings of the National Academy of Sciences of the United States of America* **87** 4756–4760.
- Paria BC, Song H and Dey SK** (2001) Implantation: Molecular basis of embryo-uterine dialogue. *International Journal of Developmental Biology* **45** 597–605.
- Parkinson TJ** (2019) Infertility in the Cow Due to Functional and Management Deficiencies. In *Veterinary Reproduction & Obstetrics*, 10th ed, pp 361–407. Eds DE Noakes, TJ Parkinson and GCW England. Amsterdam: Elsevier Ltd.
- Pérez-Marín CC and Quintela LA** (2023) Current insights in the repeat breeder cow syndrome. *Animals* **13** 2187.
- Perkins JR, Olds D and Seath DM** (1954) A Study of 1,000 Bovine Genitalia. *Journal of Dairy Science* **37** 1158–1163.
- Perkins DN, Pappin DJC, Creasy DM and Cottrell JS** (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20** 3551–3567.
- Poole DH, Ndiaye K and Pate JL** (2013) Expression and regulation of secreted phosphoprotein 1 in the bovine corpus luteum and effects on T lymphocyte chemotaxis. *Reproduction* **146** 527–537.
- Ray DE, Halbach TJ and Armstrong D V.** (1992) Season and Lactation Number Effects on Milk Production and Reproduction of Dairy Cattle in Arizona. *Journal of Dairy Science* **75** 2976–2983.
- Robertson SA** (2005) Seminal plasma and male factor signalling in the female reproductive tract. *Cell and Tissue Research* **322** 43–52.
- Robertson I and Nelson RE** (2010) Certification and identification of embryos. In *Manual of the International Embryo Transfer Society, 4th Ed*, pp 86–105. Eds DA Stringfellow and MD Givens. Champaign: International Embryo Transfer Society.
- Sahdev S, Khattar SK and Saini KS** (2008) Production of active eukaryotic proteins through bacterial expression systems: A review of the existing biotechnology strategies. *Molecular and Cellular Biochemistry* **307** 249–264.
- Sánchez JM, Passaro C, Forde N, Browne JA, Behura SK, Fernández-Fuertes B, Mathew DJ, Kelly AK, Butler ST, Spencer TE et al.** (2019) Do differences in the endometrial transcriptome between uterine horns ipsilateral and contralateral to the corpus luteum influence conceptus

- growth to day 14 in cattle? *Biology of Reproduction* **100** 86–100.
- Senger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiou A, Perruzzi CA and Detmar M** (1996) Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the $\alpha(v)\beta 3$ integrin, osteopontin, and thrombin. *American Journal of Pathology* **149** 293–305.
- Sguizzato ALL, da Silva TE, Chagas JCC, Argüelo AM, Gonçalves NM and Marcondes MI** (2024) Understanding the dynamics of mastitis in milk yield: decoding onset and recovery patterns in response to mastitis occurrence. *JDS Communications* **5** 669–673.
- Shelton K, Gayerie De Abreu MF, Hunter MG, Parkinson TJ and Lamming GE** (1990) Luteal inadequacy during the early luteal phase of subfertile cows. *Journal of Reproduction and Fertility* **90** 1–10.
- Smith LL and Giachelli CM** (1998) Structural requirements for $\alpha 9\beta 1$ -mediated adhesion and migration to thrombin-cleaved osteopontin. *Experimental Cell Research* **242** 351–360.
- Sodek J, Ganss B and Mckeel D** (2000) Osteopontin. *Critical Reviews in Oral Biology & Medicine* **11** 279–303.
- Sørensen ES and Christensen B** (2023) Milk osteopontin and human health. *Nutrients* **15**.
- Sørensen ES, Petersen TE and Højrup P** (1995) Posttranslational modifications of bovine osteopontin: Identification of twenty-eight phosphorylation and three O-glycosylation sites. *Protein Science* **4** 2040–2049.
- Spencer TE, Forde N and Lonergan P** (2016) The role of progesterone and conceptus-derived factors in uterine biology during early pregnancy in ruminants. *Journal of Dairy Science* **99** 5941–5950.
- Suarez SS and Pacey AA** (2006) Sperm transport in the female reproductive tract. *Human Reproduction Update* **12** 23–37.
- Tagami T, Miyano E, Sadahiro J, Okuyama M, Iwasaki T and Kimura A** (2016) Two novel glycoside hydrolases responsible for the catabolism of cyclobis-(1→6)- α -nigerosyl. *Journal of Biological Chemistry* **291** 16438–16447.
- Vasavada PC** (1988) Pathogenic Bacteria in Milk — A Review. *Journal of Dairy Science* **71** 2809–2816.
- Weber GF, Zawaideh S, Hikita S, Kumar VA, Cantor H and Ashkar S** (2002) Phosphorylation-dependent interaction of osteopontin with its receptors regulates macrophage migration and activation. *Journal of Leukocyte Biology* **72** 752–761.
- Wiltbank MC and Pursley JR** (2014) The cow as an induced ovulator : Timed AI after

synchronization of ovulation. *Theriogenology* **81** 170–185.

Wing TT, Erikson DW, Burghardt RC, Bazer FW, Bayless KJ and Johnson GA (2020) OPN binds alpha V integrin to promote endothelial progenitor cell incorporation into vasculature. *Reproduction* **159** 465–478.

Wu G, Li X, Seo H, McLendon BA, Kramer AC, Bazer FW and Johnson GA (2022) Osteopontin (OPN)/secreted phosphoprotein 1 (SPP1) binds integrins to activate transport of ions across the porcine placenta. *Frontiers in Bioscience - Landmark* **27** 117.

Yamamoto N, Sakai F, Kon S, Morimoto J, Kimura C, Yamazaki H, Okazaki I, Seki N, Fujii T and Uede T (2003) Essential role of the cryptic epitope SLAYGLR within osteopontin in a murine model of rheumatoid arthritis. *Journal of Clinical Investigation* **112** 181–188.

Yokosaki Y, Matsuura N, Sasaki T, Murakami I, Schneider H, Higashiyama S, Saitoh Y, Yamakido M, Taooka Y and Sheppard D (1999) The integrin $\alpha 9\beta 1$ binds to a novel recognition sequence (SVVYGLR) in the thrombin-cleaved amino-terminal fragment of osteopontin. *Journal of Biological Chemistry* **274** 36328–36334.

Summary in Japanese

臨床的な異常が認められないにもかかわらず、授精を繰り返しても受胎しないリピートブリーダー牛の存在は持続的な酪農経営を達成するにあたって障壁となる。正常な受胎性を示す牛では、発情後 2~4 日目および 13~14 日目にそれぞれ子宮内膜上皮成長因子（EGF）濃度の上昇がみられるが、多くの乳用リピートブリーダー経産牛ではこれらの濃度上昇は消失もしくは低下しており、この異常と低受胎との関連が示されている。精漿および生乳中に含まれるオステオポンチン（OPN）を腔内に投与すると、リピートブリーダー牛における子宮内膜 EGF 発現が正常化し、受胎性も回復することから、OPN の作用を活用した本処置の実用化が望まれている。しかし、供給量および感染症伝播リスクの観点から、精漿と生乳は治療用製剤開発における OPN の供給源としてふさわしくなく、他の供給源が望まれる。また、OPN が子宮内膜における EGF 発現異常を正常化する機序は明らかになっておらず、リピートブリーダー牛に対する治療法を改良していくためにも本機序の解明が求められている。

OPN は多様な機能を有するタンパク質であり、翻訳後修飾の有無や、一次構造に含まれる複数の結合部位を介して多様な受容体（インテグリンおよび CD44）に結合することでその機能を変化させる。しかし、子宮内膜 EGF 発現を正常化させる機能においては、翻訳後修飾の必要性や本機能の発現に関与する一次構造の部位（最小単位）は明らかになっていない。これらの不明点を解明することで、OPN が子宮内膜の EGF 発現を正常化させる機序の解明や、本タンパク質を用いた治療法の改良につながる可能性がある。そこで本研究では、乳用リピートブリーダー牛の子宮内膜 EGF 発現と受胎性を回復させる OPN の構造的な最小単位を同定することを目的とした。本目的を達成するため、第 1 章では、本機能における翻訳後修飾の必要性を調べ、第 2 章では一次構造の最小単位を決定した。

第 1 章では、翻訳後修飾をもたない大腸菌由来組換えオステオポンチン（rOPN）が、リピートブリーダー牛の子宮内膜 EGF 発現と受胎性の回復に及ぼす効果を調べることで、本機能の発現において OPN が翻訳後修飾を必要とするかを調べた。まず、子宮内膜 EGF 発現異常を示すホルスタイン種リピートブリーダー牛の腔内に、PBS（n = 74）、SP（0.5 ml、n = 37）または rOPN（0.3 mg、n = 105）を発情時に投与した（実験 1-1）。そして、発情後 3 日目に子宮内膜 EGF 濃度を測定した。また、PBS 群の 58 頭、SP 群の 20 頭および rOPN 群の 83 頭については、試料投与直前に人工授精を行い、授精後 30~35 日目に妊娠鑑定を行った。その結果、rOPN 群の EGF 発現正常化率および受胎率（58.1%および 47.0%）は、SP 群（62.2%および 45.0%）と同程度であり、PBS 群と比べて高かった（29.7%および 28.1%）（ $P < 0.05$ ）。次いで、子宮内膜 EGF 発現異常を示すホルスタイン種リピートブリーダー牛に対し、PBS（PBS 群、n = 18）または rOPN（rOPN 群、n = 17）を発情時の腔内に投与した（実験 1-2）。また、正常な子宮内膜 EGF 発現および受胎性を示す牛（正常群、n = 18）に対し、PBS を同様に投与した。子宮内膜 EGF 濃度を発

情後 3 日目に測定後、1 頭あたり 2 個または 3 個の胚を発情後 7 日目に移植し、発情後 14 日目に回収した。その結果、rOPN 群の胚回収率 (58.7%) は、正常群と同程度 (58.3%) であったが、PBS 群 (32.0%) と比べて高値を示した ($P < 0.05$)。また、試料投与後に正常な子宮内膜 EGF 発現を示した牛の胚回収率 (64.4%) は、異常な EGF 発現を示した牛 (16.7%) と比べて高値を示した ($P < 0.05$)。一方、rOPN 群と正常群から回収された胚の大きさは同程度であったが、PBS 群と比べて大きかった ($P < 0.05$)。しかし、胚の大きさと子宮内膜 EGF 濃度との間に相関は認められなかった。以上の結果から、子宮内膜 EGF 発現と受胎性を回復させる機能において、OPN は翻訳後修飾を必要とせず、一次構造がその機能の発現に重要な役割を果たすことが示唆された。また、子宮内膜 EGF 発現の回復が受胎性の向上につながる理由として、妊娠 14 日目における胚の生存性が向上することが考えられた。一方、腔内に投与された OPN は、子宮内膜 EGF 発現の正常化とは関係しない未知の機序を介して子宮内における胚の伸長を促進することが示唆された。

第 2 章では、子宮内膜 EGF 発現正常化および受胎性回復機能の発現における、OPN の一次構造の最小単位を決定することを目的とした。まず、トロンビンで切断された rOPN の断片が、リピートブリーダー牛の子宮内膜 EGF 発現の正常化に及ぼす効果を調べた (実験 2-1)。子宮内膜 EGF 発現異常を示すホルスタイン種リピートブリーダー牛に対し、16 nmol の rOPN、トロンビンで切断された rOPN の N 末端および C 末端断片 (それぞれ N-rOPN および C-rOPN)、またはこれら切断断片の混合物 (Th-rOPN) を、授精時の腔内に投与した ($n = 13 \sim 20$)。そして、授精後 3 日目に子宮内膜 EGF 濃度を測定し、授精後 30~35 日目に妊娠鑑定を実施した。その結果、N-rOPN 群の EGF 発現正常化率 (25.0%) は、C-rOPN 群 (7.7%) と rOPN 群 (55.6%) または Th-rOPN 群 (64.3%) の中間の値を示した。次いで、OPN のインテグリン結合モチーフを含む化学合成部分ペプチドが、子宮内膜 EGF 発現と受胎性の回復に及ぼす効果を調べた。子宮内膜 EGF 発現異常を示すホルスタイン種リピートブリーダー牛に対し、OPN 部分ペプチドを授精時の腔内に投与し、実験 2-1 と同様、子宮内膜 EGF 濃度測定および妊娠鑑定を実施した。実験 2-2 では、PBS ($n = 47$)、rOPN (9.5 nmol, $n = 83$) またはインテグリン結合モチーフを含むペプチド [GRGDSVAYGLK (ペプチド 1; 32, 320 および 1600 nmol)、GRGDS (ペプチド 2; 320 および 1600 nmol) および SVAYGLK (ペプチド 3; 320 および 1600 nmol)] を投与した ($n = 20 \sim 25$)。その結果、ペプチド 1 群 (320 および 1600 nmol) およびペプチド 3 群 (1600 nmol) の EGF 発現正常化率 (44.0~56.3%) は rOPN 群 (63.9%) と同程度であり、PBS 群 (15.6%) と比べて高かった。その他の群の EGF 発現正常化率は PBS 群と同程度であった。受胎性への影響を調べるため、EGF 発現正常化効果が認められたペプチドについて、投与試験を追加実施したところ、ペプチド 1 群 (320 および 1600 nmol、それぞれ $n = 50$)、ペプチド 3 群 (1600 nmol、 n

= 55) および rOPN 群 (n = 111) の受胎率 (41.8~50.0%) は同程度であり、PBS 群 (21.6%, n = 75) と比べて高かった。実験 2-3 では、PBS (n = 24)、ペプチド 1 (320 nmol, n = 78) または GRGESVAYGLK ペプチド (ペプチド 4 ; 320 および 1600 nmol、それぞれ n = 50 および n = 26) を投与した。その結果、ペプチド 4 群および PBS 群の EGF 発現正常化率 (16.0~19.2%) は同程度であり、ペプチド 1 群 (44.9%) と比べて低かった。これらの結果から、SVAYGLK モチーフが乳用リピーター牛の子宮内膜 EGF 発現と受胎性を回復させる OPN の構造的最小単位であり、RGD モチーフが本効果の活性増強に参与する可能性が示唆された。

以上より、乳用リピーター牛の子宮内膜 EGF 発現と受胎性を回復させる機能において、OPN はその機能の発現に翻訳後修飾を必要としないことが明らかとなった。また、インテグリン結合モチーフが本機能における OPN の構造的最小単位であることが示された。これらの結果から、OPN のインテグリン結合モチーフを含む部分タンパク質やペプチドを投与することが、リピーター牛の受胎性を向上させる治療法になりうることが示唆された。今後の方向性として、リピーター牛に対する治療法を改良していくために、OPN がインテグリンとの相互作用を介して子宮内膜 EGF 発現を正常化させる機序を引き続き解明していくことが求められる。