Study on the mechanism regulating bovine oviductal tonus during peri-ovulatory period

March, 2026

Sayaka KUBOTA

Graduate School of Environmental, Life,

Natural Science and Technology

(Doctor's course)

OKAYAMA UNIVERSITY

CONTENTS

PREFACE ---------- 1

ACKNOWLEDGEMENT ---------- 2

CHAPTER 1 GENERAL INTRODUCTION ---------- 3

CHAPTER 2

INTRODUCTION ---------- 6
MATERIALS AND METHODS---------- 7
RESULTS ---------- 12
DISCUSSION ---------- 28
SUMMARY ---------- 32

CHAPTER 3

INTRODUCTION ---------- 33
MATERIALS AND METHODS ---------- 34
RESULTS ---------- 37
DISCUSSION ---------- 43
SUMMARY ---------- 46

CHAPTER 4 CONCLUSIONS ---------- 47

REFERENCES ---------- 48

ABSTRACT IN JAPANESE ---------- 54

PREFACE

The experiments described in this dissertation were conducted at the Graduate School of Environmental, Life, Natural Science and Technology (Doctor's course), Okayama University, JAPAN, from April 2023 to March 2026, under the supervision of Professor Koji KIMURA.

This dissertation has not been submitted previously in whole or in part to a council, university or any other professional institution for degree, diploma or other professional qualifications.

Sayaka KUBOTA

March, 2026

ACKNOWLEDGMENTS

I wish to express my deep gratitude to Koji KIMURA, Ph.D., Professor of Graduate School of Environmental, Life, Natural Science and Technology, Okayama University, Japan, for his guidance, encouragement, constructive criticism, and excellent supervision and for providing me the opportunity to conduct this study. I also wish to thank Yuki YAMAMOTO, DVM, Ph.D., Associate Professor of Tokyo University of Agriculture and Technology, Tokyo, Japan, and Kohei KAWANO, DVM, Ph.D., Assistant Professor of Okayama University, for their support, advice, and encouragement at this study. I appreciate the Okayama Meat Center, Okayama, Japan, for providing bovine oviducts. I thank Toshimitsu HATABU, Professor of Okayama University, for supporting the isometric contraction test. It is pleasure to express my thanks to all the members of Laboratory of Animal Reproductive Physiology and my parents for helping me while carrying out this study.

CHAPTER 1
GENERAL INTRODUCTION

Oviductal physiology in reproductive events

The oviduct is essential in the reproductive process, as it facilitates fertilization, the transport of gametes and early embryos, and early embryo development [1, 2]. The timely transport of gametes and early embryos within the oviduct is crucial for the successful establishment of pregnancy [3]. This oviductal transport is regulated by the flow of oviductal fluid, which is generated by both ciliary movement and smooth muscle contractions [4]. Since the infundibulum and ampulla have well-developed mucosal folds and their luminal surfaces are covered with numerous ciliated cells, the transport of gametes and early embryos depends on ciliary movement [5]. In contrast, the transport capacity of the isthmus, characterized by fewer ciliated cells and a thicker smooth muscle layer, relies on smooth muscle contractility rather than ciliary movement [6, 7]. In the oviductal isthmus of mice and rats, the inhibition of contractility has been shown to cause failure in gamete transport, thus leading to infertility [8, 9]. Smooth muscle dysfunction in bovine oviducts is believed to contribute to lower conception rates during the summer [10]. Furthermore, the inhibition of human oviductal contractility causes ectopic pregnancies, which often cause severe complications, such as hemorrhaging [11]. Approximately 98% of ectopic pregnancies occur in the oviduct, representing 1%–2% of all pregnancies [12]. Therefore, understanding the contractile functions of the oviductal smooth muscle is essential for reducing ectopic pregnancies in humans and improving conception rates in livestock.

Oviductal smooth muscle contraction around ovulation

Oviduct contraction around ovulation facilitates the transport of gametes to the oviductal ampulla and the movement of early embryos toward the uterus. Sperm that reaches the isthmus of the oviduct by contraction of the uterus and the uterotubal junction (UTJ) bind to the oviductal epithelium, acquire

fertilizing capacity, and are subsequently transported to the oviductal ampulla where the oocyte resides. The oviductal fluid flow generated by oviductal contraction movements, directed from the uterine side toward the ovarian side, promotes sperm transport in mice [8, 13]. After fertilization, early embryos are transported toward the uterus. In mice, localized contraction directed toward the uterus is observed following fertilization. Furthermore, extended inhibition of oviductal contraction impaired oocyte transport, leading to fluid accumulation and oviductal lumen expansion that disrupted the intimate contact between the cumulus–oocyte complex and the cilia which generated ciliary movement [14]. Thus, oviductal contractions are suggested to play a role in the transport of gametes and early embryos around ovulation, however, the detailed mechanisms remain unclear.

Regulation factors of oviductal smooth muscle contraction

The oviductal contraction is regulated by some factors to ensure the efficient transport of gametes and embryos. Prostaglandin F2α ($PGF_{2\alpha}$) and endothelin (EDN) act as potent stimulators of contraction, while prostaglandin E2 (PGE_2) serve as relaxants, thereby allowing the oviduct to regulate contractility in bovine oviduct (Yamamoto, 2016). The contraction mechanisms of smooth muscle mediated by G protein-coupled receptors such as PGs and EDN receptor are regulated by the phospholipase C (PLC) β/inositol 1,4,5-trisphosphate (IP_3) and RhoA/Rho kinase (ROCK) signaling pathways in several smooth muscle tissues [15-19]. The PLCβ/IP_3 pathway induces Ca^{2+} release from the endoplasmic reticulum, leading to myosin light chain (MLC) phosphorylation and rhythmic phasic contractions that primarily influence contraction frequency and force [18]. In contrast, the RhoA/ROCK pathway not only promotes Ca^{2+} release from the endoplasmic reticulum but also activates myosin phosphatase targeting subunit 1 (MYPT1), thereby inhibiting MLC dephosphorylation [18, 19]. This process enhances and maintains MLC phosphorylation, resulting in a sustained increase in muscle tension. It is also known that the

increase in smooth muscle contractility driven by RhoA/ROCK activation is inhibited by RND proteins in other smooth muscles, such as those of the small intestine and the pregnant uterus [20-22]. Although these mechanisms are well established in other smooth muscle systems, their specific role in oviductal smooth muscle remains unclear.

Aim of the present study

This study aimed to elucidate the mechanisms of oviductal contraction in bovine oviducts around ovulation. First, we investigated the effects of estradiol-17β (E2), a steroid hormone that induces estrus, on oviductal contraction and clarified its mechanism. Second, we examined the influence of follicular fluid, which flows into the oviduct together with the oocyte at ovulation, on oviductal contraction. These findings provide novel insights into the functional roles of oviductal smooth muscle, particularly its contribution to timely fertilization and the transport of gametes and early embryos within the oviduct.

CHAPTER 2
Effect of estradiol-17β on oviductal contraction in the bovine oviduct

INTRODUCTION

Ensuring the timely transport of gametes and embryos within the oviduct is essential for the successful establishment of pregnancy [3]. This oviductal transport is regulated by the flow of oviductal fluid, which is generated by both ciliary movement and smooth muscle contractions [4]. The oviductal contractility responsible for the transport of gametes and embryos is controlled by several factors. One of these is estradiol-17β (E2), a steroid hormone secreted by the ovaries that regulates cyclical ovulation and menstruation. E2 indirectly promotes oviductal contractility by increasing the secretion of oviductal contraction factors from oviductal epithelial cells, such as prostaglandin F2α and endothelin [23]. In contrast, our previous research revealed that a Chinese medicine called Tokishakuyakusan rapidly increases oviduct tonus via G-protein-coupled estrogen receptor 1 (GPER1) [24], even though it does not contain E2. This suggests that E2, the original ligand of GPER1, directly regulates oviductal contractility.

The contraction mechanisms of smooth muscle mediated by G protein-coupled receptors such as GPER1 are regulated by the RhoA/Rho kinase (ROCK) signaling pathways in several smooth muscle tissues [15-19]. Although these mechanisms are investigated in other smooth muscle systems, their specific role in oviductal smooth muscle remains unclear. The aim of this chapter is to clarify the direct effects of E2 on oviductal contraction and to elucidate the involvement of GPER1 and the RhoA/ROCK signaling pathway.

MATERIALS AND METHODS

Collection of bovine oviducts

Bovine oviducts were transported on ice from a local slaughterhouse to our laboratory. The oviducts were distinguished into four stages—Stage I (days 1–4 after ovulation), Stage II (days 5–10 after ovulation), Stage III (days 11–17 after ovulation), and Stage IV (days 18–20 after ovulation)—based on the macroscopic observation of the ovary and the uterus [25] The oviducts were utilized on the ipsilateral side as the corpus luteum (Stages I–III) or the dominant follicle (Stage IV). The oviductal isthmus, with its well-developed and active smooth muscle layer, was selected for the subsequent experiments [26].

Isometric contraction test

The isometric contraction test of the oviductal smooth muscle was conducted following previously described methods, with a few modifications [24, 27, 28]. Briefly, bovine oviductal isthmic tissues were dissected, and three or four 5-mm longitudinal strips near the uterotubal junction (UTJ) were obtained. Each longitudinally fixed strip was equilibrated for 1 h prior to the experiment in a Magnus tube containing 10 mL of Krebs–Ringer solution (136.9 mM NaCl, 5.4 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 23.8 mM $NaHCO_3$, 5.6 mM glucose). The Krebs–Ringer solution was maintained at 38.5°C and aerated with a mixture of 95% O_2 and 5% CO_2 throughout the experiment. To confirm the functional integrity of the used smooth muscle tissues, oxytocin (4084-v, Peptide Institute, Inc., Osaka, Japan, 100 nM) and noradrenalin (143-04741, Wako, Osaka, Japan, 1 μM) were added as a contractile reagent or relaxant. The effects of E2 (1 nM or 10 nM) and the GPER1 agonist (G-1; 10008933, Cayman, Ann Arbor, USA, 1 μM or 10 μM) on oviductal contractility were investigated. We also investigated the effects of E2 (1 nM)

and pretreatment with a GPER1 antagonist (G-15; 14673, Cayman, 25 nM or 250 nM), ROCK inhibitor (Y-27632; HY-10583/CS-0878, MedChemExpress, Monmouth Junction, USA, 1 μM), and RND inhibitor (Manumycin A; 10010497, Cayman, 10 μM). The concentrations of E2, G-1, G-15, Y-27632, and Manumycin A were determined based on previous reports [29-33]. The solvent used for each reagent (0.1% DMSO or 0.1% EtOH in Krebs–Ringer solution) was added to the control group. To minimize variation among Magnus tubes, treatment groups were randomly assigned across tubes. Further, the isometric tension of each strip was measured using a force–displacement transducer (Minebea Co. Ltd., Nagano, Japan) connected to a polygraph (Yokogawa Electric Corp., Tokyo, Japan). The frequency, contraction force, and tonus were recorded every 2 min before and after the treatment, in accordance with a previous report [24].

Protein fractionation and extraction for Western blotting

The bovine oviductal isthmic smooth muscle tissues were dissected and 5-mm strips adjacent to the UTJ that were removed from the mucosal layer were obtained. The tissues were then homogenized and ultrasonicated in RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NP-40, and a protease inhibitor cocktail (1697498, Roche, Mannheim, Germany)). The supernatant was obtained by centrifugation (20,000 g × 20 minutes at 4°C). Protein concentrations were measured using the BCA method (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, Massachusetts, USA) [34].

Western blotting

Western blotting was performed in accordance with previously established protocols, but with certain modifications [24]. Briefly, the lysates from oviductal isthmic smooth muscle tissues (25 μg/lane) were prepared, separated using SDS-PAGE (7%–13%), and transferred to PVDF

membranes. After blocking, the membranes were incubated with antibodies against RhoA (sc-418, Santa Cruz Biotechnology, Santa Cruz, USA, RRID:AB_628218), ROCK I (sc-17794, Santa Cruz Biotechnology, RRID:AB_628223), ROCK II (sc-398519, Santa Cruz Biotechnology), RND1 (A06347, Boster Biological Technology, Wuhan, China), RND2 (13844-1-AP, Proteintech, Wuhan, China, RRID:AB_2181817), RND3 (66228-1-lg, Proteintech), MYPT1 (2634, Cell Signaling Technology, Massachusetts, USA, RRID:AB_915965), pMYPT1 (Thr696, 5163, Cell Signaling Technology, RRID:AB_10691830), pMYPT1 (Thr853, 4563, Cell Signaling Technology, RRID:AB_1031185), MLC (8505, Cell Signaling Technology, RRID:AB_2728760), pMLC (Thr18/Ser19, 3674, Cell Signaling Technology, RRID:AB_2147464) or GAPDH (NB300-221SS, Novus Biologicals, Briarwood Avenue, USA, RRID:AB_790028) diluted with Can Get Signal Solution 1 (NKB-201, TOYOBO) overnight at 4°C or for 2 h at room temperature. After washing with TBS-T, HRP-linked sheep anti-mouse IgG (NA931, Cytiva, RRID:AB_772210), HRP-linked donkey anti-rabbit IgG (NA934, Cytiva, RRID: AB_772206), or HRP-linked goat anti-mouse IgM (ab97230, Abcam, Cambridge, UK, RRID:AB_10688258) diluted with Can Get Signal Solution 2 (NKB-301, TOYOBO) were incubated to the membranes for 1 h at room temperature. The dilution ratio of each antibody is presented in Table 1. After washing again with TBS-T, the signals were detected using chemiluminescence. To minimize variability across replicates, the same positive control sample was included, quantified, and normalized in each run, with GAPDH used as the internal control. Band intensity was analyzed using densitometry.

ROCK activity assay

The isthmic smooth muscle tissues of bovine oviducts were dissected and 1-cm strips adjacent to the UTJ were removed to obtain the mucosal layer. These strips were equilibrated for 1 h before

sampling in a centrifuge tube filled with 10 mL of Krebs–Ringer solution. The Krebs–Ringer solution was maintained at 38.5°C and aerated with 95% O_2 and 5% CO_2. Thereafter, these strips were incubated with 1 nM E2 for 1 h and homogenized and ultrasonicated in a sampling buffer for ROCK activity assay (50 mM Tris-HCl, 150 mM NaCl, 50 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1% NP-40, phosphatase inhibitor cocktail (4906845001, Roche, Mannheim, Germany) and protease inhibitor cocktail). The solvent for E2 (0.1% EtOH) was added to the control group. The supernatant was collected by centrifugation (20,000 g × 20 min, 4°C). In addition, ROCK activity was evaluated using the ROCK activity immunoblot kit, following the manufacturer's instructions (STA-415, Cell Biolabs, San Diego, USA). The active ROCK II provided with the kit served as a positive control to verify proper assay performance. The protein expression GAPDH was used as an internal control.

Statistical analysis

All experimental data were presented as mean ± SEM. Statistical analyses were conducted using R [35]. For experiments involving the isometric contraction test, data were analyzed using a generalized linear mixed model, followed by one-way ANOVA and Tukey's multiple comparison test to compare treatment groups within the same time point. Western blot and ROCK assay data were analyzed using the Kruskal–Wallis test for comparisons between two groups. For comparisons among three or more groups, data were transformed using a generalized linear model and analyzed by one-way ANOVA with Tukey's multiple comparison test. Statistical significance was set at 5% ($P < 0.05$).

Table 1. Antibody dilution ratio for western blotting

1st antibody	Dilution	2nd antibody	Dilution
Anti RhoA	1:500	HRP-linked sheep anti-Mouse IgG	1:5,000
Anti ROCK I	1:500	HRP-linked sheep anti-Mouse IgG	1:5,000
Anti ROCK II	1:1,000	HRP-linked sheep anti-Mouse IgG	1:5,000
Anti RND1	1:300	HRP-linked donkey anti-Rabbit IgG	1:5,000
Anti RND2	1:300	HRP-linked donkey anti-Rabbit IgG	1:5,000
Anti RND3	1:2,000	HRP-linked sheep anti-Mouse IgG	1:10,000
Anti MYPT1	1:2,000	HRP-linked donkey anti-Rabbit IgG	1:10,000
Anti pMYPT1 (Thr696)	1:500	HRP-linked donkey anti-Rabbit IgG	1:5,000
Anti pMYPT1 (Thr853)	1:500	HRP-linked donkey anti-Rabbit IgG	1:5,000
Anti MLC	1:2,000	HRP-linked donkey anti-Rabbit IgG	1:10,000
Anti pMLC	1:500	HRP-linked donkey anti-Rabbit IgG	1:5,000
Anti GAPDH	1:40,000	HRP-linked goat anti-mouse IgM	1:100,000

RESULTS

E2 increased tonus in the isthmus of bovine oviducts

An isometric contraction test was conducted to investigate the effect of E2 on bovine oviductal contractility. The tonus, frequency, and contraction force are presented in Figures 1, 2, and 3, respectively. E2 increased the tonus of bovine oviductal smooth muscle strips in Stage I. In stage I, a significant increase was observed in the 1 nM E2 treatment compared with that in the control and 10 nM E2 group at several time points (Figure 1; $P < 0.05$). By the end of the measurement, the tonus in the 1 nM E2 treatment group increased approximately 23-fold compared with the 1–3 min, whereas no significant change in tonus was observed in the 10 nM E2 treatment group. Moreover, there was no change in oviductal tonus in Stages II–IV in the E2 treatment group. E2 had no effect on both the frequency and contraction force at any stage (Figures 2 and 3).

E2 increased oviductal tonus via GPER1 and the RhoA/ROCK signaling pathway

Since 1 nM E2 increased oviductal tonus within 1 h in Stage I, we focused on the involvement of the transmembrane E2 receptor (GPER1) in the increase of oviductal tonus in Stage I. To determine whether the effect of the GPER1 activation on the tonus is similar to that of 1 nM E2, either G-1 or E2 was added, and tonus was measured for up to 1 h post-treatment. Figure 4A indicates the changes in tonus following G-1 or E2 treatment. In Stage I, 1 nM E2 both concentrations of G-1 group increased the tonus of the oviductal isthmus tissues compared to the control after 31 min following treatment (Figure 4A; $P < 0.05$). These data indicate that GPER1 activation produced an effect comparable to that of E2. Next, we examined the effect of the GPER1 antagonist (G-15) on oviductal contractility to verify that E2 exerts its effects through GPER1. E2 was administered 20 min after the G-15 treatment, and tonus was measured for up to

1 h following E2 administration. Both concentrations of the G-15 treatment significantly suppressed the E2-induced increase of tonus in Stage I after 41 min following E2 treatment (Figure 4B; $P < 0.05$). In addition, we investigated the effect of the ROCK inhibitor (Y-27632) on oviductal contractility to reveal the downstream mechanism of GPER1 in the E2-induced increase in oviductal tonus. E2 was administered 20 min after Y-27632 treatment, and the tonus was measured for up to 1 h following E2 administration in Stage I. Y-27632 significantly diminished the E2-induced increase in tonus after 31 min following E2 treatment (Figure 4C; $P < 0.05$). No effect of E2, G-1, G-15, or Y-27632 was observed on the frequency or contraction force (data not shown).

Expression and activity of ROCK-regulated E2 responsiveness in bovine oviductal smooth muscle

To determine the cause of the difference in responsiveness to E2 during the estrous cycle in bovine oviductal smooth muscle, RHOA, ROCK I, and ROCK II protein expression at each estrous stage in this tissue were measured using Western blotting. The protein expression of RHOA, ROCK I, and ROCK II, along with GAPDH as the internal control, was investigated in the oviductal isthmic smooth muscle in Stages I–IV. The RHOA and ROCK I protein expression levels did not change significantly throughout the estrous cycle (Figures 5A and 5B). The ROCK II protein expression was found to be significantly increased in Stage I compared to that in Stage III (Figure 5C; $P < 0.05$). Moreover, we analyzed active ROCK levels in the isthmic smooth muscle tissues of bovine oviducts at each estrous stage, sensitized by E2, to measure the amount of functional ROCK. We found that the active ROCK level in Stage I was significantly increased compared to that of the other stages after E2 treatment (Figure 5D; $P < 0.05$). Finally, we investigated whether E2 affects phosphorylation of pMYPT1 and pMLC, key downstream

components of the RhoA/ROCK signaling pathway, in Stage I oviductal smooth muscle (Figure 6A). E2 significantly increased the phosphorylation of pMYPT1 (Thr696) and pMLC compared to controls (Figures 6C–D, H–I; $P < 0.05$). In contrast, E2 did not alter total MYPT1, MLC, pMYPT1 (Thr853) expression, or pMYPT1 (Thr853) phosphorylation in Stage I oviductal smooth muscle (Figures 6B, D–G).

Expression of RND3-regulated E2 responsiveness and ROCK activity in bovine oviductal smooth muscle

To reveal the mechanism of the difference in responsiveness to E2 via the activation of ROCK during the estrous cycle in the bovine oviductal smooth muscle, the protein expression of RND1, RND2, and RND3 at each estrous stage in this tissue were measured using Western blotting. The protein expression of RND1, RND2, and RND3—with GAPDH as the internal control—was examined at all stages. The expression levels of RND1 and RND2 did not significantly change throughout the estrous cycle (Figures 7A and B). In contrast, the RND3 expression level at Stage IV significantly increased compared to that in Stage III (Figure 7C). Since these results suggested that the expression of RND3 suppressed the E2-induced increase in oviductal tonus, we investigated the effect of an RND inhibitor (Manumycin A) on the bovine oviductal tonus in Stage IV. E2 was administered 20 min after treatment with Manumycin A, and oviductal tonus was measured for up to 1 h following E2 administration. E2 and Manumycin A did not change the frequency or contraction force (data not shown). Manumycin A significantly increased tonus in Stage IV oviductal smooth muscle compared to other groups at several time points (Figure 7D; $P < 0.05$). Since Manumycin A restored E2 responsiveness in Stage IV oviducts, we further investigated the expression and activation of the RhoA/ROCK signaling pathway in isthmic smooth muscle tissues of Stage IV oviducts treated with both 1 nM E2 and 10 μM Manumycin A

(Figure 8A). Co-treatment with E2 and Manumycin A significantly increased ROCK activity and the expression and phosphorylation of pMYPT1 (Thr696) and pMLC compared to controls (Figures 8B, D–E, I–J; $P < 0.05$). In contrast, co-treatment did not alter the expression of total MYPT1, MLC, or pMYPT1 (Thr853), nor phosphorylation of pMYPT1 (Thr853) in Stage IV oviductal smooth muscle (Figures 8C, F–H).

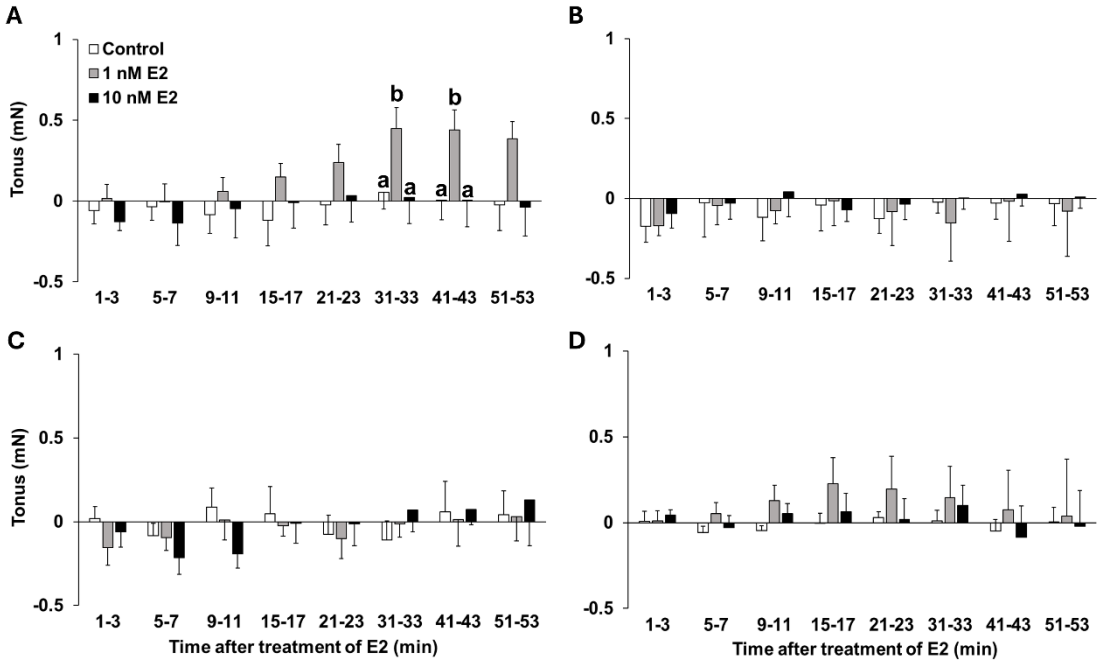

Figure 1. Effect of estradiol-17β (E2—1nM or 10 nM) on the tonus of bovine oviductal isthmic tissues in the longitudinal direction

Data were collected and averaged every 2 min post-treatment (n = 5). Each graph indicates tonus relative to pre-treatment levels in bovine oviducts at Stages I (A), II (B), III (C), and IV (D). Data are presented as mean ± SEM. Different superscripts (a, b) indicate significant differences within each treatment group at the same time point ($P < 0.05$).

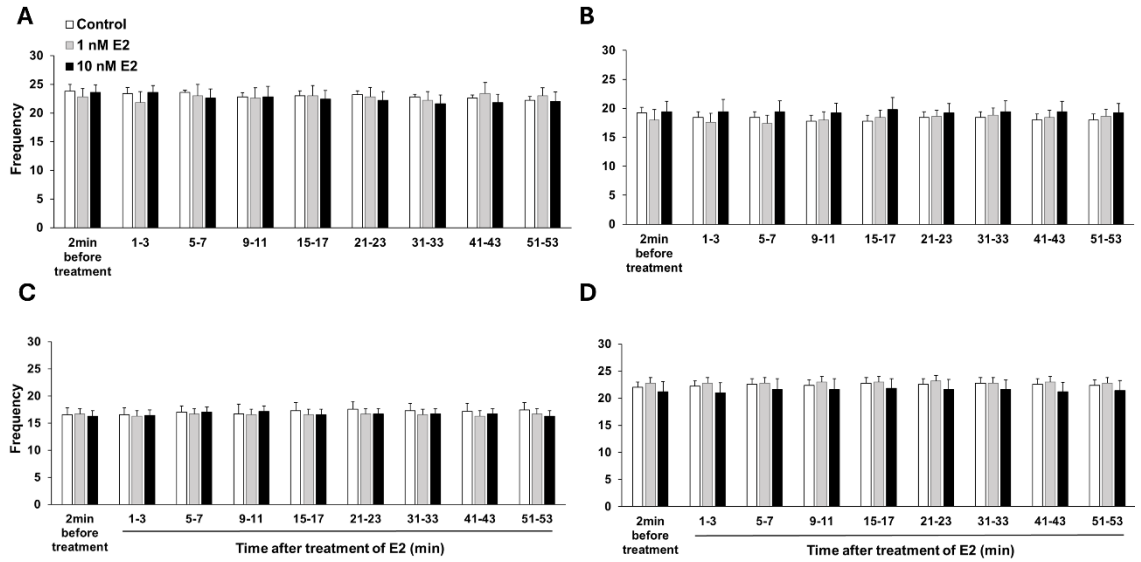

Figure 2. Effect of estradiol-17β (E2—1 nM or 10 nM) on the frequency of bovine oviductal isthmic tissues in the longitudinal direction

Data were collected every 2 min post-treatment (n = 5). Each graph indicates frequency in the oviducts of Stages I (A), II (B), III (C), and IV (D). Data are presented as mean ± SEM.

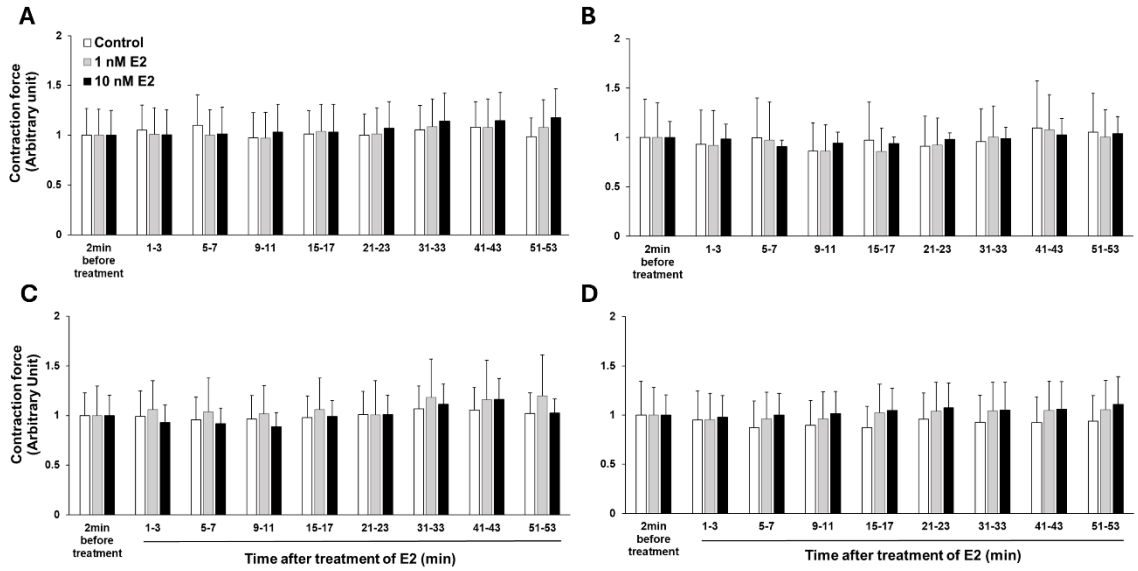

Figure 3. Effect of estradiol-17β (E2—1 nM or 10 nM) on the contraction force of bovine oviductal isthmic tissues in the longitudinal direction

Data were collected and averaged every 2 min post-treatment (n = 5). Each graph indicates contraction force relative to pre-treatment levels in bovine oviducts in Stages I (A), II (B), III (C), and IV (D). Data are presented as mean ± SEM.

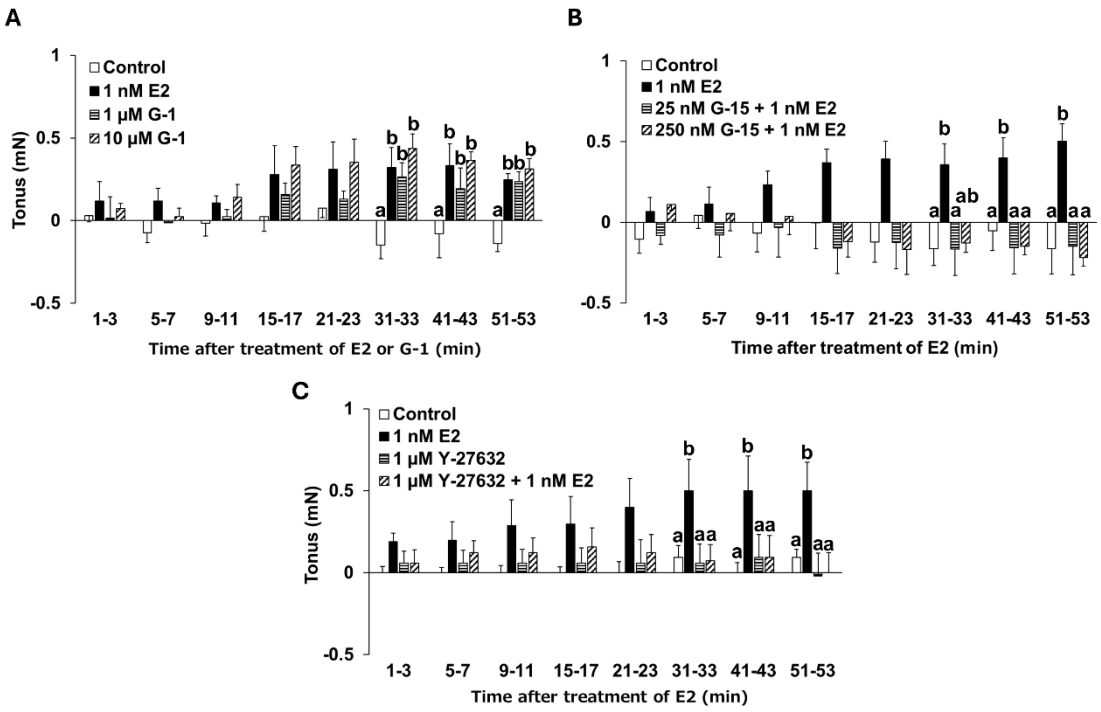

Figure 4. Effect of estradiol-17β (E2), G protein-coupled estrogen receptor 1 (GPER1) agonist (G-1), GPER1 antagonist (G-15), and Rho kinase inhibitor (Y-27632) on the tonus of bovine oviductal isthmic tissues in a longitudinal direction

Data were collected and averaged every 2 min post-treatment (n = 5). Each graph indicates tonus compared to before E2 or G-1 treatment (A), G-15 pretreatment and E2 treatment (B), and Y-27632 pretreatment and E2 treatment (C) in the oviducts in Stage I (n = 6). Data are presented as mean ± SEM. Different superscripts (a, b) indicate significant differences within each treatment group at the same time point ($P < 0.05$).

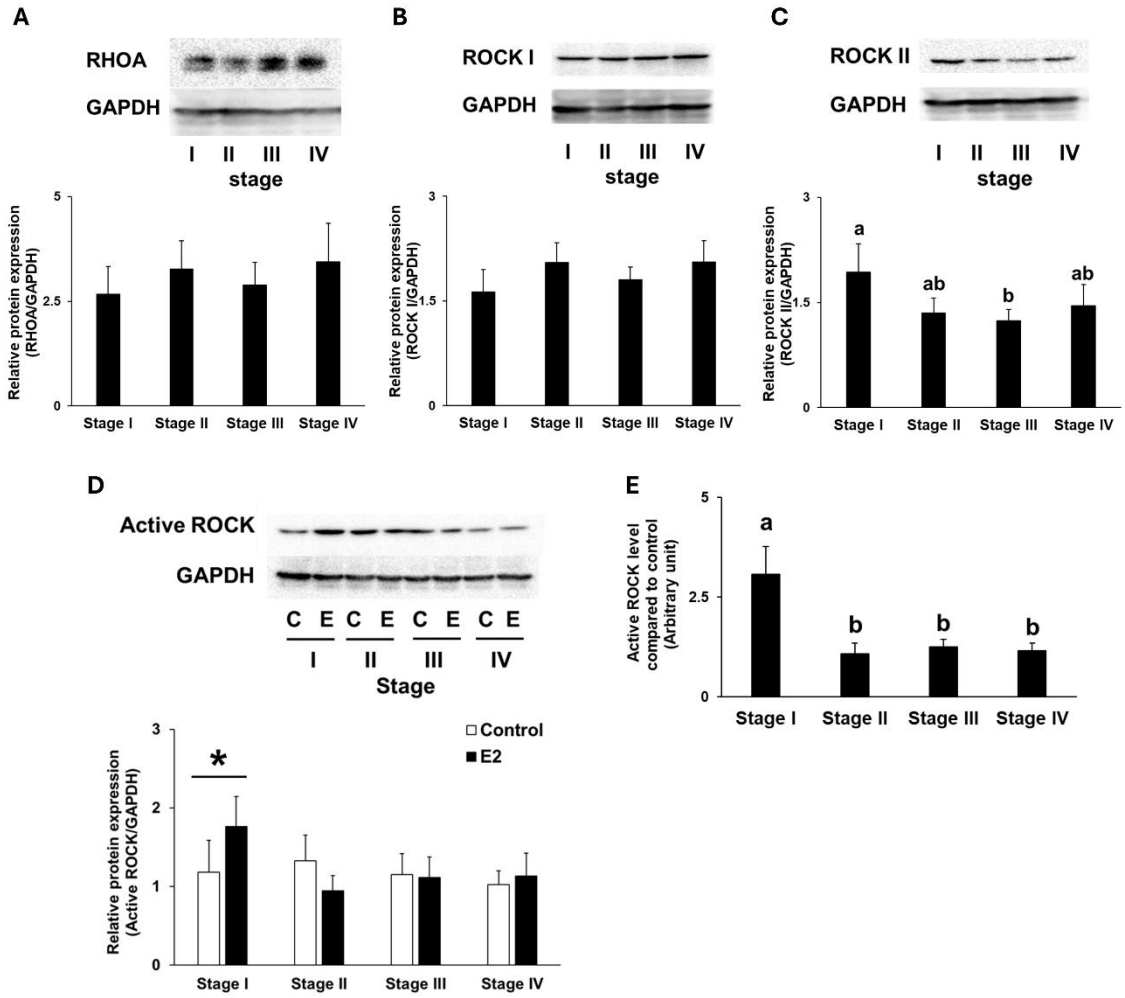

Figure 5. Protein expression of RHOA, Rho kinase (ROCK) I, ROCK II, active ROCK in bovine oviductal isthmic smooth muscle tissues

The protein expression levels of RHOA (A), ROCK I (B), and ROCK II (C) in bovine oviductal isthmic smooth muscle tissues throughout the estrous cycle are indicated (n = 18). The figure presents the active ROCK (D) protein expression levels in bovine oviductal isthmic smooth muscle tissues during the estrous cycle sensitized by 1 nM estradiol-17β (E2) (n = 16; C, without E2 treatment; E, with 1 nM E2 treatment). These protein expression levels were normalized by the GAPDH protein expression level. Data are presented as mean ± SEM. Different superscripts (a, b) indicate significant differences in each estrous stage ($P < 0.05$). Asterisk indicates significant differences between control group and E2 treatment group at the same estrous stage ($P < 0.05$).

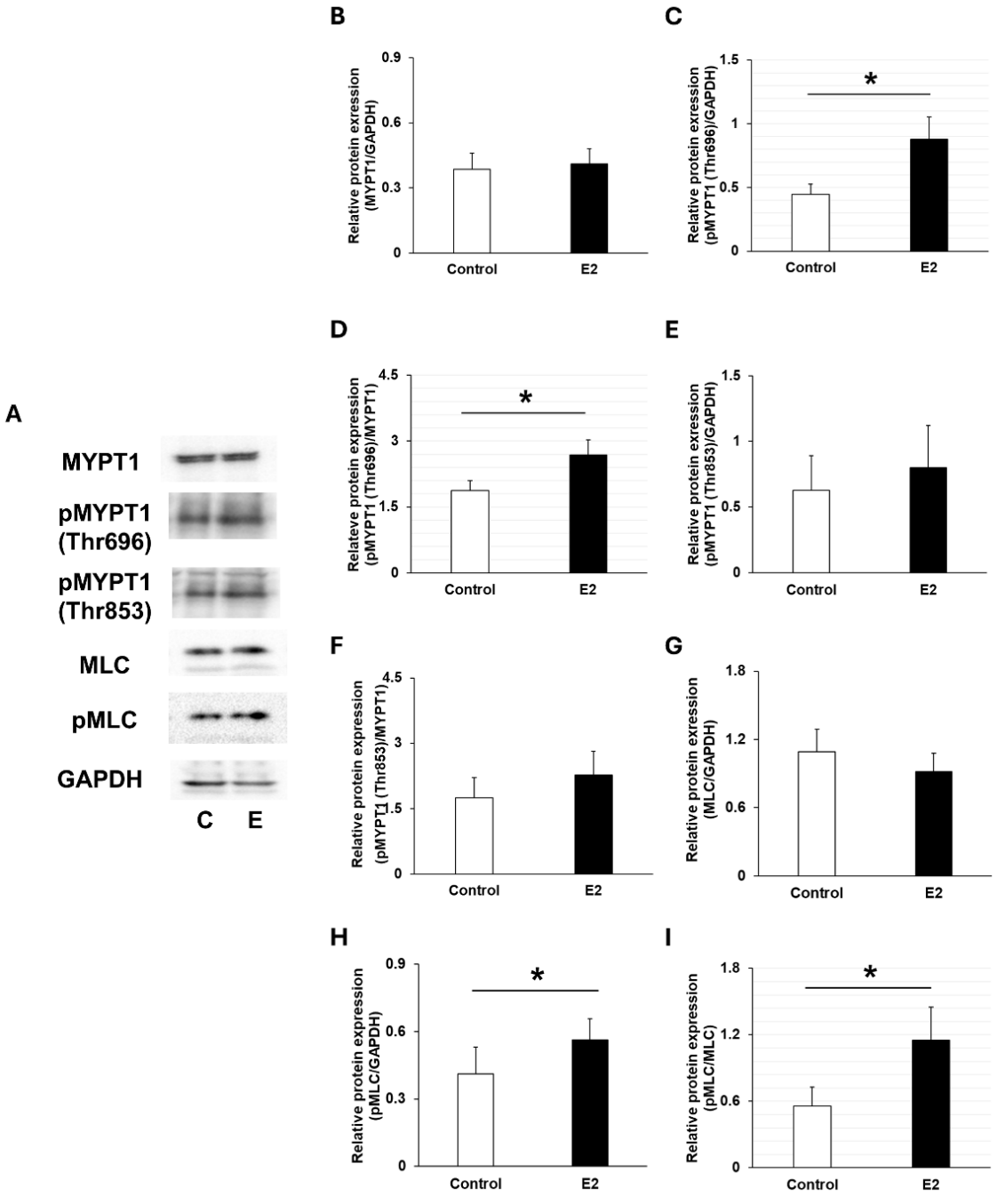
A
MYPT1
pMYPT1 (Thr696)
pMYPT1 (Thr853)
MLC
pMLC
GAPDH
C
E
B
Relative protein exression (MYPT1/GAPDH)
0.9
0.6
0.3
0
Control
E2
C
Relative protein exression (pMYPT1 (Thr696)/GAPDH)
1.5
1
0.5
0
*
Control
E2
D
Relateve protein expression (pMYPT1 (Thr696)/MYPT1)
4.5
3
1.5
0
*
Control
E2
E
Relative protein exression (pMYPT1 (Thr853)/GAPDH)
1.5
1
0.5
0
Control
E2
F
Relative protein expression (pMYPT1 (Thr853)/MYPT1)
4.5
3
1.5
0
Control
E2
G
Relative protein exression (MLC/GAPDH)
1.8
1.2
0.6
0
Control
E2
H
Relative protein expression (pMLC/GAPDH)
0.9
0.6
0.3
0
*
Control
E2
I
Relative protein expression (pMLC/MLC)
1.8
1.2
0.6
0
*
Control
E2

Figure 6. Protein expression of the downstream factor of RHOA/Rho kinase (ROCK) signaling pathway in bovine oviductal isthmic smooth muscle tissues in Stage I sensitized by 1 nM E2

(A) Representative Western blot images of downstream factors. Protein expression of MYPT1 (B), pMYPT1 (Thr696) (C), pMYPT1 (Thr853) (E), MLC (G), and pMLC (H) in Stage I tissues (n = 20; C, without E2; E, with 1 nM E2). Phosphorylation levels of pMYPT1 (Thr696) (D), pMYPT1 (Thr853) (F), and pMLC (I) are shown. Protein expression was normalized to GAPDH. Data are presented as mean ± SEM. Asterisks indicate significant differences between control and E2-treated groups ($P < 0.05$).

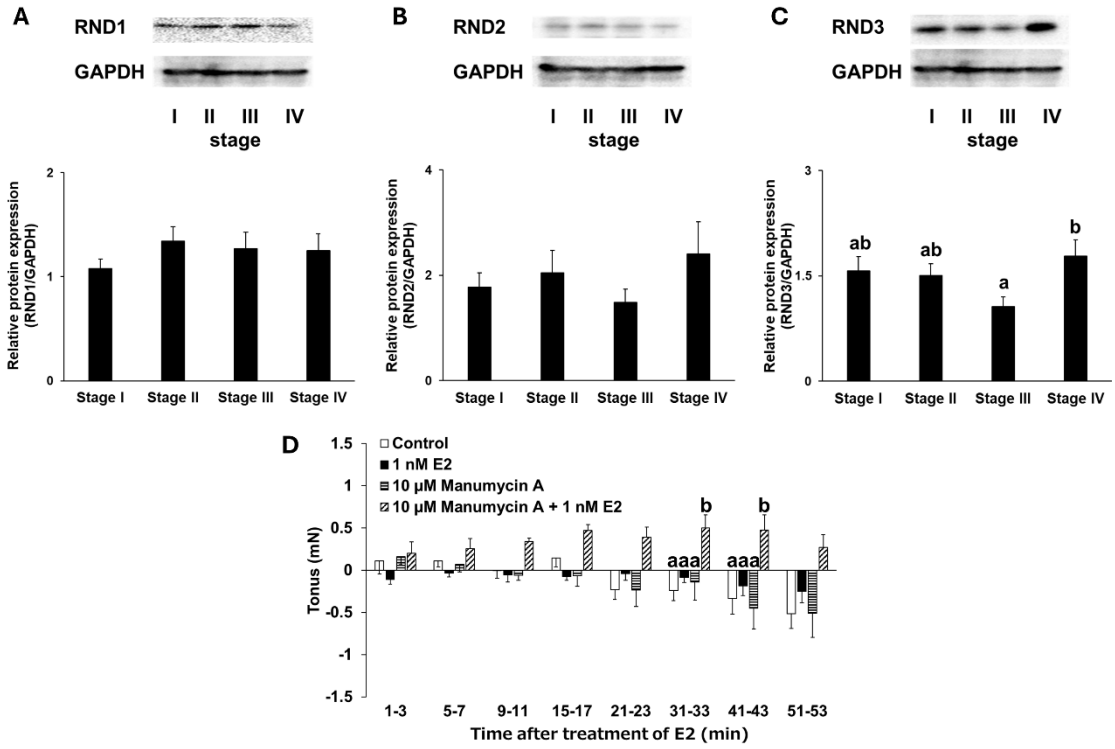

Figure 7. Protein expression of RNDs and the effect of the RNDs inhibitor in bovine oviductal isthmic smooth muscle tissues

The protein expression levels of RND1 (A), RND2 (B), and RND3 (C) in bovine oviductal isthmic smooth muscle tissues throughout the estrous cycle are indicated (n = 18). These protein expression levels were normalized by the GAPDH protein expression level. Data are presented as mean ± SEM. Different superscripts (a, b) indicate significant differences within each estrous stage ($P < 0.05$). The effects of estradiol-17β (E2) and RNDs inhibitor (Manumycin A) on the tonus of bovine oviductal isthmic tissues in the longitudinal direction in Stage IV are indicated (D). Data were collected and averaged every 2 min after treatment. This graph depicts the tonus compared to before Manumycin A pretreatment and E2 treatment in the oviducts in Stage I (n = 6). Data are presented as mean ± SEM. Different superscripts (a, b) indicate significant differences in each treatment group at the same time point ($P < 0.05$).

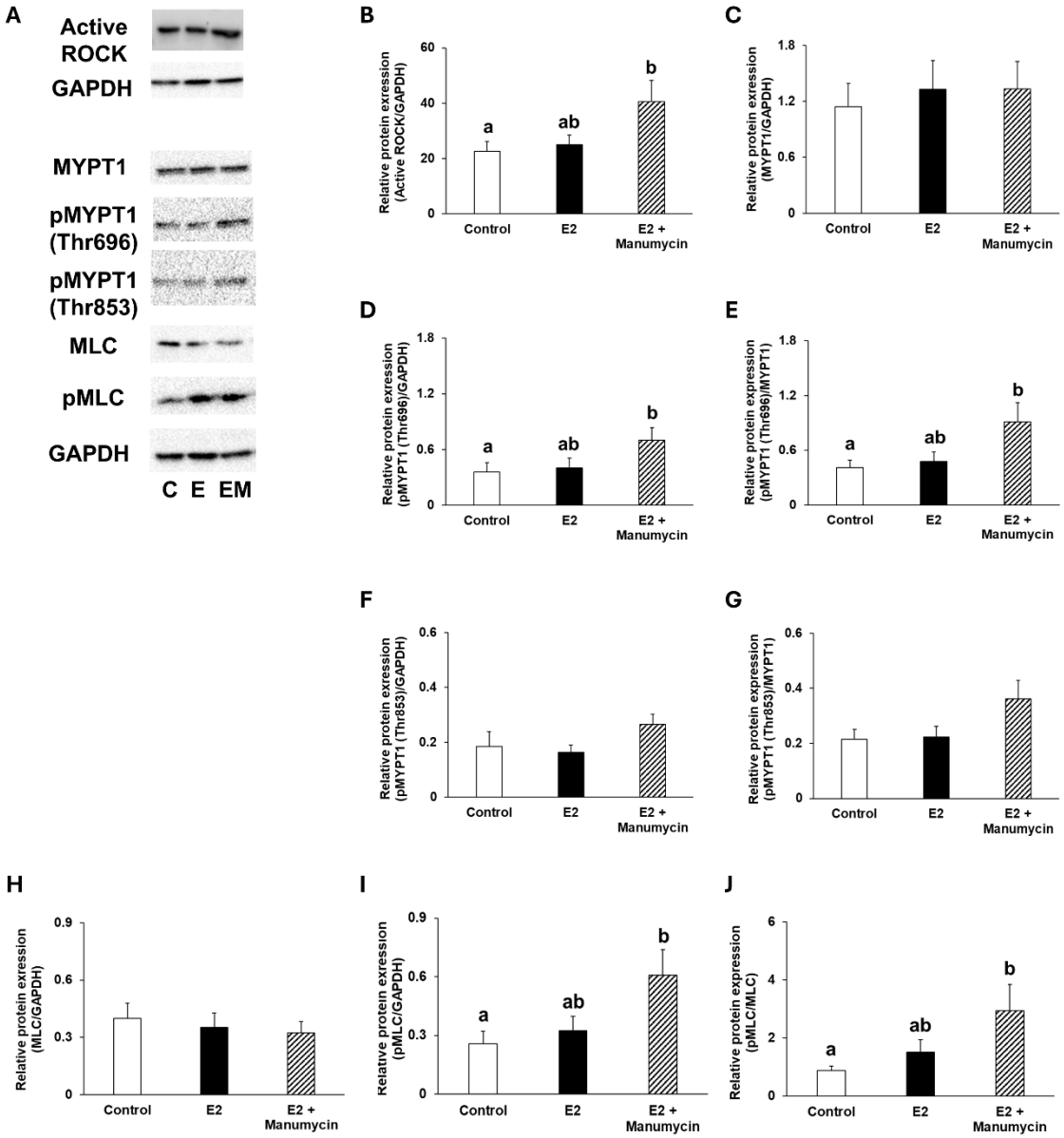
A
Active ROCK
GAPDH
MYPT1
pMYPT1 (Thr696)
pMYPT1 (Thr853)
MLC
pMLC
GAPDH
C E EM
B
Relative protein exression (Active ROCK/GAPDH)
C
Relative protein expression (MYPT1/GAPDH)
D
Relative protein exression (pMYPT1 (Thr696)/GAPDH)
E
Relative protein expression (pMYPT1 (Thr696)/MYPT1)
F
Relative protein exression (pMYPT1 (Thr853)/GAPDH)
G
Relative protein expression (pMYPT1 (Thr853)/MYPT1)
H
Relative protein exression (MLC/GAPDH)
I
Relative protein exression (pMLC/GAPDH)
J
Relative protein expression (pMLC/MLC)
Control
E2
E2 + Manumycin
a
ab
b

Figure 8. Protein expression of the downstream factor of RHOA/Rho kinase (ROCK) signaling pathway in bovine oviductal isthmic smooth muscle tissues in Stage IV tissue sensitized by 1 nM estradiol-17β (E2) and RNDs inhibitor (Manumycin A).

(A) Representative Western blot images. Protein expression of MYPT1 (C), pMYPT1 (Thr696) (D), pMYPT1 (Thr853) (F), MLC (H), and pMLC (I) in Stage IV tissues (n = 14; C, without E2; E, with 1 nM E2; EM, with 1 nM E2 + 10 μM Manumycin A). Activation/phosphorylation levels of ROCK (B), pMYPT1 (Thr696) (E), pMYPT1 (Thr853) (G), and pMLC (J) are shown. Protein expression was normalized to GAPDH. Data are presented as mean ± SEM. Different superscripts (a, b) indicate significant differences between treatment groups ($P < 0.05$).

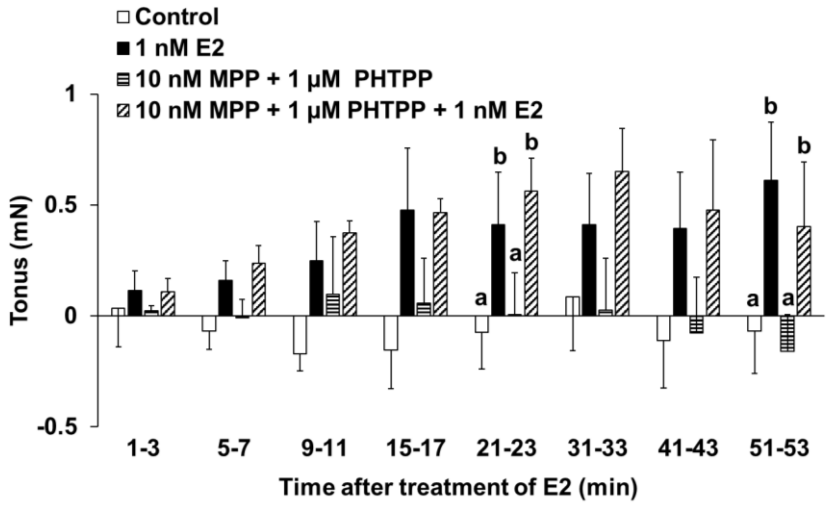

Figure 9. Effects of estradiol-17β (E2, 1 nM), Estrogen receptor (ER) α antagonist (MPP, 10 nM) and ERβ antagonist (PHTPP, 1 μM) on the tonus of bovine oviductal isthmic tissues in a longitudinal direction

Data were collected and averaged every 2 min post-treatment (n=5). This graph indicates tonus relative to pre-treatment levels in bovine oviducts at Stage I. Data are presented as mean ± SEM. Different superscripts (a, b) indicate significant differences in each treatment group at the same time point ($P < 0.05$).

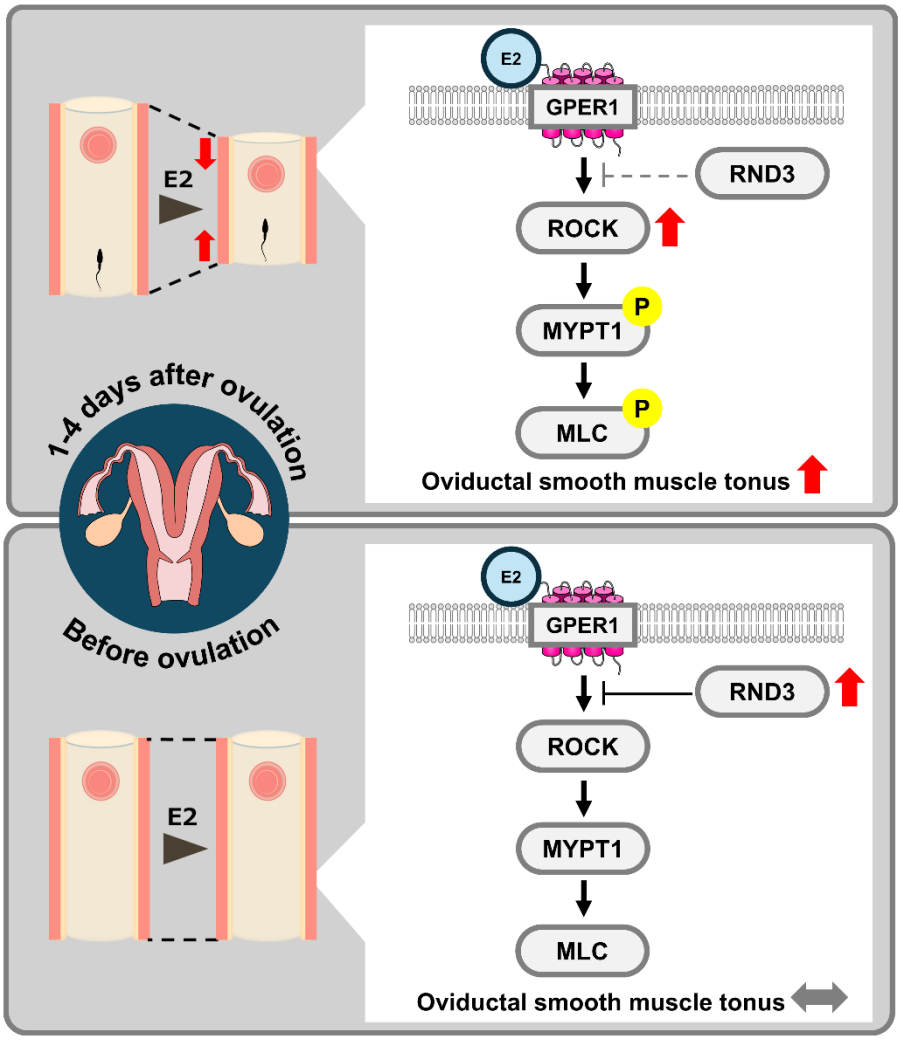

Figure 10. Summary of estradiol-17β (E2) regulation in oviductal contractility.

E2 directly increases oviductal tonus by activating the RhoA/Rho kinase (ROCK)/Myosin phosphatase targeting subunit 1(MYPT1)/Myosin light chain (MLC) signaling pathway via G protein coupled receptor 1 (GPER1) at 1–4 days after ovulation. In contrast, a high expression of RND3 inhibits E2-induced activation of the RhoA/ROCK/MYPT1/MLC signaling pathway in the preovulatory oviduct.

DISCUSSION

This is the first study to investigate the direct effect of E2 on bovine oviductal contractility and its mechanism. Our study clarified that 1 nM E2 increased bovine oviductal tonus at 1–4 days after ovulation. Steroid hormones such as E2 exert their effects through nuclear receptor-mediated genomic pathways and transmembrane receptor-mediated nongenomic pathways, with the latter typically inducing a faster response [36]. GPER1, a transmembrane E2 receptor, has been identified in the bovine oviductal smooth muscle [29]. In our previous research, Tokishakuyakusan—a traditional Chinese medicine—was increased oviduct tonus via GPER1 [24]. Taken together, we hypothesized that E2 directly increased oviductal tonus via GPER1, but not nuclear estrogen receptors. The addition of the GPER1 agonist as well as E2 induced an increase in oviductal tonus, and the GPER1 antagonist significantly suppressed the effect of E2. On the other hand, nuclear estrogen receptor antagonists did not suppress the effect of E2 on bovine oviductal tonus in Stage I (Figure 9). Therefore, E2 increased oviductal tonus via GPER1 and shortened the oviduct in the longitudinal direction. Next, since E2 affected only oviductal tonus via GPER1, we focused on RhoA/ROCK signaling pathway, which regulated smooth muscle tone. The ROCK inhibitor significantly suppressed the increase in oviductal tonus caused by E2. ROCK II protein expression and ROCK activity levels after exposure to E2 were significantly higher in the bovine oviduct at 1–4 days after ovulation compared to that in other stages. These results suggest that E2 increases bovine oviductal tonus by activating ROCK. This study did not clarify the regulation of ROCK expression and activity, however, E2 influences both the expression of ROCK in the uterus [37], and ROCK gene expression tends to increase in bovine oviductal epithelium before ovulation [38]. The RhoA/ROCK signaling pathway, mediated by a G-protein-coupled receptor (GPCR)—such as GPER1—is also involved in the regulation of

contractility in smooth muscles of blood vessels, the internal anal sphincter, and the uterus [17-19]. In these smooth muscles, RhoA/ROCK induces the activation of MYPT1 and inhibition of MLC dephosphorylation [18, 19]. This mechanism promotes and maintains the phosphorylation of MLC, thereby causing a sustained increase in muscle tension. In the present study, E2 increased ROCK activity and phosphorylation of pMYPT1 (Thr696) and pMLC in Stage I bovine oviductal smooth muscle. Notably, Thr696 phosphorylation of MYPT1 is thought to be more effective than Thr853 in maintaining smooth muscle tone due to its stronger inhibition of myosin light chain phosphatase [39]. Taken together, these findings indicate that E2 increases bovine oviductal tonus through the GPER1-mediated RhoA/ROCK/MYPT1/MLC signaling pathway.

In contrast, E2 did not affect oviductal contractility in the preovulatory bovine oviduct, despite the E2 addition of concentrations found in the preovulatory oviductal tissue and fluid. The protein expression of GPER1 did not change during the estrous cycle in our previous study [24], and the activation of the RhoA/ROCK signal pathway was not observed in the preovulatory oviduct in the present study. However, in the preovulatory bovine oviduct, the E2 concentration of oviductal plasma, tissues, and fluid is higher and there is increased sensitivity of E2 compared to that in the other stages [40-42]. To clarify this contradiction, we focused on the expression of RNDs, which have three isoforms: RND1, RND2, and RND3 [43]. RNDs inhibit the RhoA/ROCK signaling pathway [43]. In the present study, E2 increased oviductal tonus in Stage I but not in Stage IV. Protein expression of RND1 and RND2 did not differ between Stage I and Stage IV, suggesting that these isoforms are minimally involved in the E2-induced increase in oviductal tonus. Consequently, inhibition of RND1 and RND2 by Manumycin A does not contribute to restoring E2 responsiveness. In contrast, RND3 was highly expressed specifically at Stage IV, which can explain the absence of E2-induced tonus increases at this stage. Indeed, treatment with Manumycin A in Stage IV oviducts restored the E2-induced increase in oviductal tonus, ROCK

activity, and phosphorylation of pMYPT1 (Thr696) and pMLC, indicating effective inhibition of highly expressed RND3. These results suggest that a high expression of RND3 in the oviduct before ovulation suppresses the E2-induced increase in oviductal tonus, although this study does not directly investigate the effect of the overexpression or a loss of expression of RND3 on the E2-induced increase in oviductal tonus. It is known that the increase in smooth muscle contractility due to RhoA/ROCK signal activation is also inhibited by RNDs in other smooth muscles, such as the small intestine and pregnant uterus [20-22]. The expression of RNDs varies depending on species and tissues. In the small intestine of rats, RND2/3 has an inhibitory effect on E2-induced contractility, whereas in the human uterus, RND1/2/3 has an inhibitory effect [20-22]. Our study revealed that RND3 plays an important role in the mechanism of the regulation of bovine oviductal tonus by E2. RND3 expression in the uterine smooth muscle of pregnant rabbits is significantly increased compared to that in nonpregnant rabbits [33]. Moreover, ileal smooth muscle tissue isolated from ovariectomized rats treated with E2 also increases RND3 protein expression [20]. Although the regulation of RND3 expression in the bovine oviduct has not been manifested in this study, it is possible that steroid hormones are involved in the expression.

This study revealed that the effect of E2 on oviductal contractility differed before and after ovulation. What is the physiological significance of this phenomenon in the bovine oviduct? It is known that the oviduct plays a role in transporting gametes and early embryos at the appropriate time for it to be considered an established pregnancy [3]. The ejaculated sperm is incapacitated and normally deposited in the vagina or uterus. These sperm attach to, and detach from, the epithelium of the oviduct isthmus, thereby suggesting that spermatozoa bind and unbind several times as they migrate through the oviduct [44, 45]. The detachment of sperm is caused by hyperactivated motility [46]. Thereafter, they are distributed toward the oviductal ampulla, where fertilization occurs. Our study suggests that the high expression of RND3 in the oviduct

suppresses the excessive increase in oviductal tonus caused by E2, thus resulting in the retention of sperm in the isthmus of the oviduct before ovulation. This prevention of excessive sperm transport before ovulation supports fertilization at the appropriate time.

However, after ovulation, excess E2 prevents the establishment of pregnancy. In normal cows, the E2 concentration in oviductal tissue and fluid at 1–4 days after ovulation is approximately 300 pM [41, 42]; thus, an addition of 1 nM E2 is an abnormally high concentration for the oviduct at 1–4 days after ovulation. Nevertheless, in the present study, 1 nM E2 increased oviductal tonus in the bovine oviduct at 1–4 days after ovulation. One of the events that causes abnormally high concentrations of E2 after ovulation is superovulation treatment. In various animals, superovulation increases E2 concentrations after ovulation and reduces pregnancy rates [47, 48]. It has also been reported that superovulation treatment causes abnormal acceleration of oocyte transport in rats, thus resulting in infertility [47]. Based on these findings, it is possible that the increase in E2 concentration due to abnormal hormone secretion causes the abnormal acceleration of gametes and early embryo transport, thus resulting in infertility in humans and livestock.

E2 directly increases oviductal tonus by activating the RhoA/ROCK/MYPT1/MLC signaling pathway via GPER1 at 1–4 days after ovulation. It is possible that this phenomenon promotes excessive gametes and early embryo transport in the oviduct lead to infertility (Figure 10). In the preovulatory oviduct, a high expression of RND3 inhibits E2-induced activation of the RhoA/ROCK/MYPT1/MLC signaling pathway, thereby retaining sperm transport in the isthmus of the oviduct before ovulation (Figure 10). These mechanisms contribute to supporting fertilization at the appropriate time and establishing pregnancy in human and livestock species.

SUMMARY

Ensuring the timely transport of gametes and embryos within the oviduct is essential for the successful establishment of pregnancy. This study investigated the direct effect of estradiol-17β (E2) on bovine oviductal contractility and the differences in responsiveness to E2 during the estrous cycle. Bovine isthmic tissues from four estrous stages were analyzed using the Magnus method to assess contractile responses to E2 and related reagents. Protein expression of G-protein-coupled estrogen receptor 1 (GPER1) and components of the RhoA/Rho kinase (ROCK) signaling pathway were also evaluated. E2 and a GPER1 agonist significantly increased oviductal tonus at 1–4 days after ovulation. This effect was significantly suppressed by treatment with a GPER1 antagonist and a ROCK inhibitor. At 1–4 days after ovulation, both ROCK II expression and ROCK activity were elevated. E2 also enhanced phosphorylation of myosin phosphatase targeting subunit 1 (MYPT1) and myosin light chain (MLC), key downstream targets of ROCK. Before ovulation, when endogenous E2 levels peak, the expression of RND3—a ROCK inhibitor—was upregulated. The application of an RNDs inhibitor restored E2 responsiveness in oviductal tonus, ROCK activity, and the phosphorylation of MYPT1 and MLC in oviductal tissues before ovulation. These findings suggest that E2 directly increases oviductal tonus via GPER1 and ROCK/MYPT1/MLC activation at 1–4 days after ovulation. Differences in oviductal responsiveness to E2 during the estrous cycle appear to be mediated by the expression of ROCK and RND3.

CHAPTER 3
Influence of follicular fluid on bovine oviductal contractility

INTRODUCTION

The oviductal fluid flow generated by oviductal contraction movements promotes sperm transport in mice [8, 13]. Moreover, extended inhibition of oviductal contraction impaired oocyte transport, leading to fluid accumulation and oviductal lumen expansion that disrupted the intimate contact between the cumulus–oocyte complex and the cilia which generated ciliary movement [14]. These findings suggested that mechanisms exist to promote sperm transport in coordination with ovulation, although the detailed processes remain unclear.

Follicular fluid is the liquid found inside ovarian follicles that surrounds and nourishes the oocyte. It contains hormones, growth factors, nutrients, and antioxidants that regulate oocyte maturation and competency. The composition of follicular fluid strongly influences fertilization potential of oocytes and early embryo development. In some mammalian species, it has been observed that follicular fluid flows into the oviduct together with the oocyte at the time of ovulation [49-51]. Previous studies have suggested that ovulatory products are involved in the transport of sperm to the ampulla by oviductal contraction [8, 13, 52]. The combined action of ovulatory products and oviductal contraction movements may facilitate sperm transport to the ampulla [52-54]. Furthermore, activation of oviductal contraction by steroid hormones contained in follicular fluid promotes sperm transport to the ampulla [52, 54, 55]. Taken together, it is suggested that follicular fluid and oviductal contraction movements are involved in sperm transport within the oviduct immediately prior to fertilization. The aim of this chapter is to elucidate the effect of follicular fluid on oviductal contraction and its mechanism in the bovine.

MATERIALS AND METHODS

Collection of bovine oviducts and follicular fluid

The oviducts of bovine were transported on ice from a local slaughterhouse to our laboratory. The oviducts were distinguished into four stages—Stage I (days 1–4 after ovulation), Stage II (days 5–10 after ovulation), Stage III (days 11–17 after ovulation), and Stage IV (days 18–20 after ovulation)—based on the macroscopic observation of the ovary and the uterus [25]. This study utilized Stage IV oviducts to exclude the influence of follicular fluid under *in vivo* conditions. The oviducts ipsilateral to the dominant follicle in Stage IV were used in the present study. The oviductal isthmus, with well-developed and active smooth muscle layer, was selected for the subsequent experiments [26]. Follicular fluid was aspirated from dominant follicles (diameter 10–15 mm) at Stage IV and centrifuged (4 °C, 15,000 rpm, 20 min). The supernatant was stored at –80 °C until use in experiments. In each experiment, follicular fluid was diluted in Krebs–Ringer solution so that the final concentration reached 10%.

Enzyme immunoassay (EIA)

To confirm whether the collected follicular fluid was pre- or post- LH surge, the concentrations of PGE_2 in the follicular fluid were determined via EIA, as described previously [56, 57]. The PGE_2 standards ranged from 0.039 to 10 ng/ml, and the median effective dose (ED50) of the assay was 0.625 ng/ml. The intra- and inter-assay coefficients of variation, on average, were 7.83% and 13.02%, respectively. Based on the results of the EIA, the follicular fluid was classified as pre- or post-LH surge using 2 ng/ml as the criterion [58]. The concentrations of platelet activating factor (PAF) in follicular fluid were measured by EIA using a Bovine PAF ELISA Reagent Kit (MBS740718; MyBioSource, San Diego, USA). The intra-assay coefficients of variation was

5.25%.

Isometric contraction test

The isometric contraction test of the oviductal smooth muscle was conducted following the methods previously described, with a few modifications [24, 27, 28]. Stage IV bovine oviductal isthmic tissues near the UTJ were dissected into 5-mm strips and equilibrated in Krebs–Ringer solution (38.5 °C, 95% O_2/5% CO_2). To confirm the functional integrity of the smooth muscle tissues used, oxytocin (100 nM) and noradrenaline (1 μM) were added as a contractile reagent or relaxant before each experiment. The effects of pre- or post- LH surge follicular fluid (pre-LH FF or post-LH FF) on oviductal contractility were then examined. We also investigated the effects of post-LH FF and pretreatment with PAF receptor antagonist (WEB 2086; 2339, TOCRIS Bioscience, Bristol, UK, 10 μM), ROCK inhibitor (Y-27632; HY-10583/CS-0878, MedChemExpress, Monmouth Junction, USA, 1 μM), and protein kinase C α (PKCα) inhibitor (Go6976; S7119, Selleckchem, Houston, USA, 1 μM). The concentrations of WEB2086, Y-27632, and Go6976 were determined based on previous reports [29-33, 59]. For the control, bovine serum albumin (BSA; 12657, Millipore, Billerica, MA, USA, 8.271 mg/ml) was supplemented at a concentration equivalent to the protein content of the follicular fluid. The solvent used for each inhibitor (DMSO) was added to the control group at 0.1%. To minimize variation among Magnus tubes, treatment groups were randomly assigned across tubes. Further, measurement of the isometric tension in each strip was used a force–displacement transducer (Minebea Co. Ltd., Nagano, Japan) connected to a polygraph (Yokogawa Electric Corp., Tokyo, Japan). Frequency, force of contraction, and tonus were monitored at 2-min intervals before and after treatment, according to previous studies [24].

Contraction assay using a tubular oviductal tissue segment

Bovine oviductal isthmic tissues in Stage IV were dissected, and 8 cm longitudinal tubular tissue segment near the UTJ were obtained. The excised oviduct was connected to a tube, and Krebs–Ringer solution was perfused for 1 h at a speed of 0.2 mm/sec using a perfusion apparatus. The Krebs–Ringer solution was maintained at 38.5°C and aerated with a mixture of 95% O_2 and 5% CO_2 throughout the experiment. From the ampullary side of the oviduct, post-LH FF (1 ml) stained using dyed purple reagent (B7024A, New England Biolabs, Ipswich, MA, USA) was injected. And then, the oviductal length after these had passed through the oviductal tissue was measured. BSA (8.271 mg/ml) was added to the control group.

Statistical analysis

All experimental data were presented as mean ± SEM. Statistical analyses were conducted using R [35]. Isometric contraction tests were evaluated with a generalized linear mixed model, followed by one-way ANOVA and Tukey's post hoc test for group comparisons at each time point. Contraction assays with tubular oviductal segments were analyzed using the Kruskal–Wallis test. PAF EIA data was transformed using a generalized linear model and analyzed by one-way ANOVA with Tukey's multiple comparison test. Statistical significance was defined as $P < 0.05$.

RESULTS

Post- luteinizing hormone surge follicular fluid (LH FF) increased tonus in the bovine oviductal isthmic tissues

An isometric contraction assay was conducted to evaluate how pre- or post- LH FF influences bovine oviductal contractility. Tonus, contraction frequency, and force are shown in Figure 11. Post-LH FF significantly increased the tonus of oviductal smooth muscle strips before ovulation after 1 min following treatment compared with the other treatment groups (Figure 11; $P < 0.05$). In contrast, pre-LH FF did not show any effects on tonus. Neither contraction frequency nor force was affected by follicular fluid treatment (Figure 11). Next, to examine whether follicular fluid exerts its effect from inside the oviduct, post-LH FF was injected into the oviduct lumen, and contraction assays were performed. Luminal administration of post-LH FF significantly reduced oviduct length relative to the pre-treatment levels compared to control group (Figure 12; $P < 0.05$). These results indicate that post-LH FF enhances oviductal tonus from the luminal side.

Platelet-activating factor (PAF) contained in post-LH FF increased oviductal tonus

Post-LH FF increased oviductal tonus within 5 min, but not pre-LH FF. These observations suggest that factors that rise in follicular fluid after the LH surge regulate oviductal smooth muscle tonus. Platelet-activating factor (PAF) is known to increase in ovine follicular fluid following the LH surge and to enhance smooth muscle tonus in other smooth muscle tissues [60-62]. However, since it remains unclear whether the same phenomenon occurs in bovine, we measured PAF concentrations in bovine follicular fluid. Consistent with other species, PAF concentrations in bovine follicular fluid were significantly increased after the LH surge compared with before the surge (Figure 13A; $P < 0.05$). Based on this result, we hypothesized that PAF present in follicular

fluid contributes to the increase in oviductal tonus. We examined the effect of the PAF receptor antagonist (WEB 2086) on oviductal contractility to verify that post-LH FF shows its effects through PAF receptor. WEB 2086 treatment significantly suppressed the post-LH FF-induced increase of tonus in oviductal isthmus at Stage IV after 1 min following post-LH FF treatment (Figure 13B; $P < 0.05$). Post-LH FF did not change contraction the frequency or contraction force (data not shown).

Post-LH FF regulated oviductal tonus via Rho kinase (ROCK) and protein kinase C α (PKCα) activation

PAF receptor is a member of G protein–coupled receptors (GPCRs), and the RhoA/ROCK signaling pathway is one of the mechanisms by which GPCR regulate smooth muscle tonus [18, 19, 63]. Therefore, we examined whether PAF present in follicular fluid activates the RhoA/ROCK signaling pathway via the PAF receptor and consequently increases oviductal tonus. Y-27632 significantly suppressed the post-LH FF-induced increase in tonus after 3 min following treatment (Figure 14A; $P < 0.05$). In addition, GPCRs such as PAF receptor activates the protein kinase (PKC), and PKCα participates in the RhoA/ROCK signaling pathway [59, 64-66]. We investigated the effect of PKCα inhibitor (Go6976) on oviductal contractility to determine whether PKCα contributes to post-LH FF-induced increase in oviductal tonus. Go6976 significantly reduced the post-LH FF-induced increase in oviductal tonus after 2 min following treatment (Figure 14B; $P < 0.05$). No effects of post-LH FF, Y-27632, and Go6976 were observed on the frequency or contraction force (data not shown).

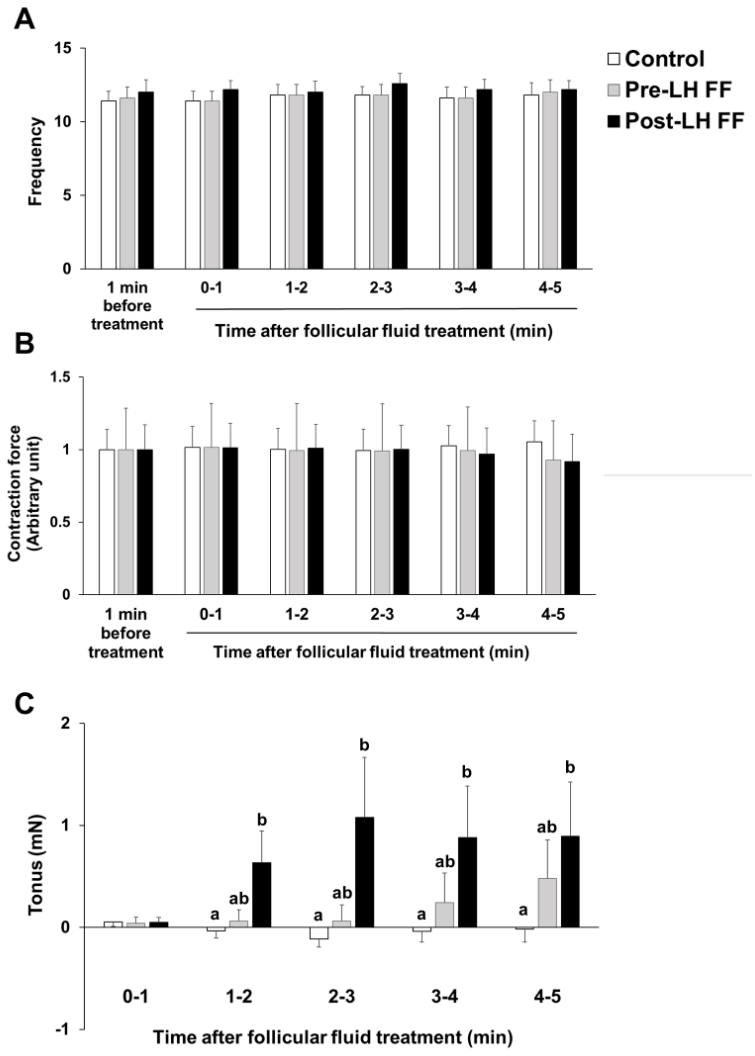

Figure 11. Effect of pre- or post- luteinizing hormone surge follicular fluid (pre-LH FF or post-LH FF) on the frequency, contraction force and tonus of bovine oviductal isthmic tissues in the longitudinal direction

Data were collected and averaged every 1 min post-treatment (n = 5). Each graph indicates frequency (A), contraction force (B) and tonus relative to pre-treatment levels (C) in bovine oviductal isthmic tissues at Stages IV, respectively. Data are presented as mean ± SEM. Different superscripts (a, b) indicate significant differences within each treatment group at the same time point ($P < 0.05$).

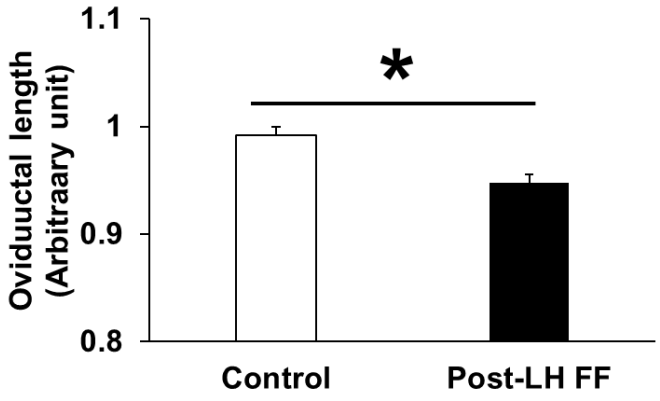

Figure 12. Effect of post- luteinizing hormone surge follicular fluid (post-LH FF) on the length of a tubular bovine oviductal isthmic tissue segment

The graph indicates the length of bovine oviductal isthmic tissues relative to pre-treatment levels at Stages IV (n=5). Data are presented as mean ± SEM. Asterisks indicate significant differences between control and E2-treated groups ($P < 0.05$).

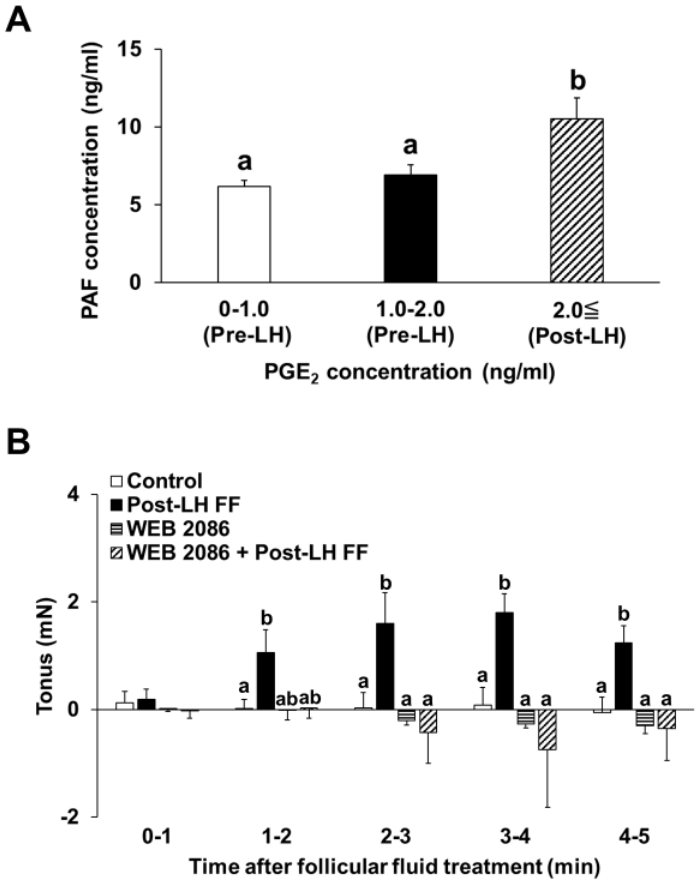

Figure 13. Platelet activating factor (PAF) concentration in follicular fluid and Effect of post- luteinizing hormone surge follicular fluid (post-LH FF) and PAF receptor antagonist (WEB 2086) on the tonus of bovine oviductal isthmic tissues in a longitudinal direction

The PAF concentration in bovine follicular fluid before and after LH surge are measured (A, n = 12). Different superscripts (a, b) indicate significant differences within each group ($P < 0.05$). The effects of post-LH FF and WEB 2086 on the tonus of bovine oviductal isthmic tissues at Stage IV in the longitudinal direction are shown in (B). Data were collected and averaged every 1 min post-treatment (n=5). Data are presented as mean ± SEM. Different superscripts (a, b) indicate significant differences in each treatment group at the same time point ($P < 0.05$).

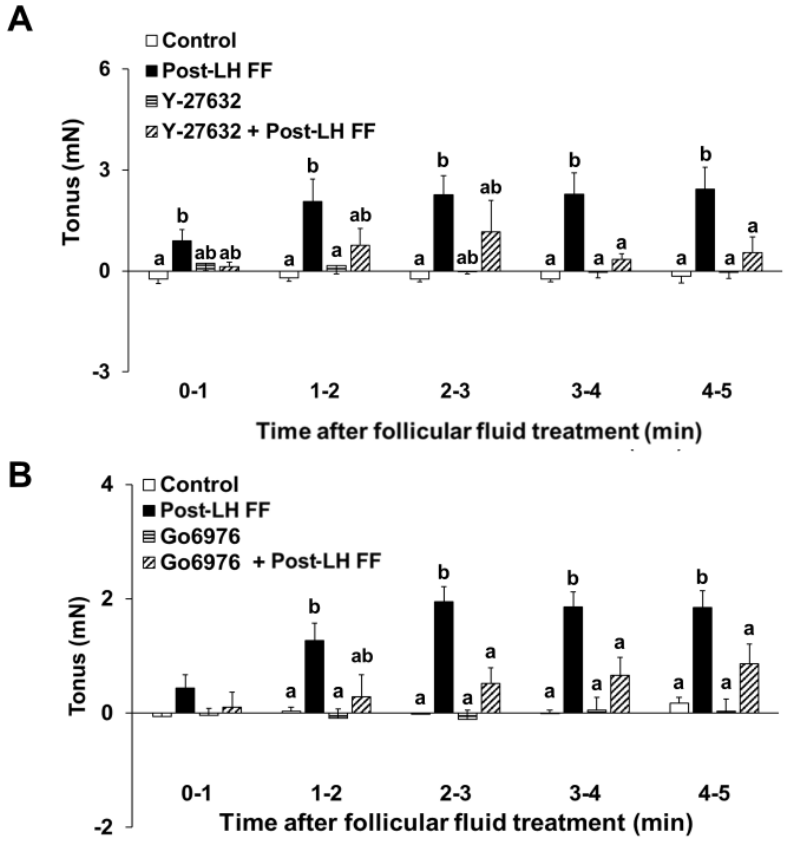

Figure 14. Effect of post- luteinizing hormone surge follicular fluid (post-LH FF), Rho kinase (ROCK) inhibitor (Y-27632), and protein kinase C α (PKCα) inhibitor (Go6976) on the tonus of bovine oviductal isthmic tissues in a longitudinal direction

Data were collected and averaged every 1 min post-treatment. Each graph indicates tonus compared to before Y-27632 pretreatment and post-LH FF treatment (A), and Go6976 pretreatment and post-LH FF treatment (B) in bovine oviductal isthmic tissues at Stage IV (n = 4). Data are presented as mean ± SEM. Different superscripts (a, b) indicate significant differences in each treatment group at the same time point ($P < 0.05$).

DISCUSSION

This study is the first report to show that follicular fluid regulates oviductal tonus in cattle. In bovine oviducts at Stage IV, pre-luteinizing hormone surge follicular fluid (LH FF) did not affect oviductal contraction, whereas post-LH FF rapidly increased oviductal tonus and shortened oviductal length. Follicular fluid contains hormones, enzymes, bioactive lipids, antioxidants, and other factors essential for oocyte development, and its composition is known to differ before and after the LH surge [67-69]. Since only post-LH FF enhanced oviductal tonus in this study, we hypothesized that factors in follicular fluid whose production increases after the LH surge contribute to smooth muscle contraction. One of such factors that increase in follicular fluid after the LH surge is platelet activating factor (PAF) in some species [60, 62]. Consistent with other species, PAF concentrations in bovine follicular fluid also were significantly increased after the LH surge compared with before the surge. PAF is a bioactive lipid that induces platelet aggregation and is known to enhance contractile activity in other smooth muscles [61]. In the present study, inhibition of PAF significantly suppressed the increase in oviductal tonus induced by post-LH FF. Furthermore, a ROCK inhibitor also significantly suppressed the oviductal tonus enhancing effect of post-LH FF. The RhoA/ROCK signaling cascade, activated through GPCRs such as PAF receptor, also contributes to the control of smooth muscle contractility in tissues including blood vessels, the internal anal sphincter, and the uterus [17-19]. In these muscles, RhoA/ROCK facilitates the activation of myosin phosphatase targeting subunit 1 (MYPT1) and suppresses the dephosphorylation of myosin light chain (MLC) [18, 19]. This process enhances and sustains MLC phosphorylation, resulting in prolonged elevation of muscle tone. In the previous chapter, we demonstrated that activation of ROCK increases the phosphorylation levels of MYPT1 and MLC in the oviductal isthmic smooth muscle. Therefore, follicular fluid may

likewise regulate oviductal tonus through the ROCK/MYPT1/MLC pathway.

However, we showed that the oviducts at Stage IV did not exhibit an E2-induced increase in tonus via GPER1, a member of the G protein coupled receptors (GPCRs) family, due to the high expression of RND3. Therefore, we considered that post-LH FF contains factors that suppress RND3 activity and activate the RhoA/ROCK signaling pathway. One of such candidates is protein kinase C (PKC), which is known to be activated by increases in diacylglycerol (DAG) and calcium generated downstream of GPCRs such as the PAF receptor [59, 64-66]. In mice, inhibition of PKCα changes the intracellular localization of RND3 and consequently activates the RhoA/ROCK pathway [59, 64-66]. In the present study, pretreatment with a PKCα inhibitor significantly reduced the increase of oviductal tonus by post-LH FF, suggesting that PKCα-mediated suppression of RND3 also occurs in bovine oviductal smooth muscle. To further clarify the relationship between PKCα, RND3, and RhoA/ROCK signaling pathway, it is necessary to examine RND3 protein expression and ROCK activity in bovine oviductal smooth muscle tissue following PKCα inhibition.

In this study, post-LH FF increased oviductal tonus and induced shortening of oviductal length when applied from the luminal side of the oviduct. These rapid responses suggested that factors of post-LH FF act directly on oviductal smooth muscle. Although this could not be examined in the present study, PAF receptors have been identified in smooth muscle cells in several other species [70-72]. PAF is a phospholipid with a short acyl chain, high lipid solubility, and relatively high membrane permeability [73, 74]. In bladder smooth muscle, PAF-induced increase of contractility is observed even after removal of the epithelial layer [61]. Estradiol-17β (E2) levels are high in the Stage IV oviducts used in this study, and E2 has been reported to loosen tight junctions in intestinal and uterine epithelia [75-77]. Therefore, E2 may modulate the epithelial barrier of the oviduct, thereby allowing PAF in oviductal lumen to pass

through the epithelial layer and reach the smooth muscle layer. Then, PAF may directly bind to PAF receptors on smooth muscle cells and increase oviductal tonus.

This study revealed that the effect of post-LH FF on oviductal contractility. What is the physiological significance of this phenomenon in the bovine oviduct? The oviduct is recognized as a key organ that transports gametes and early embryos at the appropriate timing required for successful establishment of pregnancy [3]. After ejaculation, sperm are initially incapacitated and typically deposited in the vagina or uterus. The sperm repeatedly attach to and detach from the oviductal isthmus epithelium, undergoing multiple binding–release cycles during their passage [44, 45]. Their detachment is triggered by the initiation of hyperactivated motility [46]. Following this process, sperm moves toward the oviductal ampulla, where fertilization takes place. Follicular fluid enters the oviduct together with the cumulus oocyte complex (COCs) at ovulation in several mammalian species [49-51]. In addition, the combined influence of ovulatory components and oviductal motility may therefore enhance sperm progression toward the ampulla [52-54]. Our findings indicate that follicular fluid–mediated regulation of oviductal tonus may promote passive sperm movement within the oviduct. In mice, stimulation of oviductal contractions generates a luminal fluid flow from the isthmus toward the ampulla [13]. Taken together, the entry of follicular fluid might increase oviductal tonus and drive capacitated sperm toward the ampulla at ovulation, potentially ensuring that sperm reaches the fertilization site at the optimal time.

Post-LH FF increases PAF concentrations, and oviductal tonus by activating ROCK signaling pathway via PAF receptor. Although RND3, which inhibits RhoA/ROCK signaling, was highly expressed in the bovine oviducts at Stage IV, our data indicates that PKCα activation induced by post-LH FF may reduce this inhibition. It is possible that this phenomenon promotes sperm transport during ovulation and contributes to supporting fertilization at the appropriate time and establishing pregnancy in human and livestock species.

SUMMARY

It is suggested that follicular fluid and oviductal contraction movements are involved in sperm transport within the oviduct immediately prior to fertilization. The aim of this study is to elucidate the effect of follicular fluid on oviductal contraction and its mechanism in the bovine. The collected follicular fluid was classified into pre-LH FF and post-LH FF based on its PGE_2 concentration. Bovine isthmic tissues before ovulation were analyzed using contraction tests to assess contractile responses to pre- or post- luteinizing hormone surge follicular fluid (LH FF) and related reagents. The PAF concentration in the follicular fluid was measured by enzyme immunoassay (EIA). Post-LH FF significantly increased the tonus of oviductal smooth muscle strips before ovulation after 1 min following treatment compared with the other treatment groups. In contrast, pre-LH FF did not show any effects on tonus. Luminal administration of post-LH FF significantly reduced oviduct length relative to the pre-treatment levels compared to control group. The PAF concentrations in bovine follicular fluid were significantly increased after the LH surge compared with before the surge. Pre-treatment of platelet activating factor (PAF) receptor antagonist, Rho kinase (ROCK) inhibitor, and protein kinase C α (PKCα) inhibitor significantly suppressed the post-LH FF-induced increase of tonus in oviductal isthmus before ovulation. Based on these findings, post-LH FF increased PAF concentrations, and oviductal tonus through PKCα and ROCK activation via PAF receptor. This suggests that follicular fluid entering the oviduct at ovulation may rapidly contract the oviductal smooth muscle and thereby facilitate sperm transport.

CHAPTER 4
CONCLUSIONS

This study aimed to elucidate the mechanisms of oviductal contraction by estradiol-17β (E2) and follicular fluid bovine oviducts around ovulation. E2 directly increases oviductal tonus by activating Rho kinase (ROCK), myosin phosphatase targeting subunit 1 (MYPT1), and myosin light chain (MLC) via G protein coupled estrogen receptor 1 (GPER1) at 1–4 days after ovulation. It is possible that this phenomenon promotes excessive gametes and early embryo transport in the oviduct leads to infertility. In the preovulatory oviduct, a high expression of RND3 inhibits E2-induced activation of the RhoA/ROCK/MYPT1/MLC signaling pathway, thereby retaining sperm transport in the isthmus of the oviduct before ovulation. On the other hand, post- luteinizing hormone surge follicular fluid (post-LH FF) increased platelet activating factor (PAF) concentrations, and oviductal tonus ROCK activation via PAF receptor before ovulation. Although RND3, which inhibits RhoA/ROCK signaling, was highly expressed in the bovine oviducts before ovulation, our data indicates that post-LH FF induced protein kinase C α (PKCα) activation may reduce this inhibition. This suggests that follicular fluid entering the oviduct at ovulation may rapidly contract the oviductal smooth muscle and thereby facilitate sperm transport. These mechanisms contribute to supporting fertilization at the appropriate time and establishing pregnancy in human and livestock species.

REFERENCES

1. Greve T, Callesen H: **Rendez-vous in the oviduct: implications for superovulation and embryo transfer.** *Reprod Nutr Dev* 2001, **41:**451-459.
2. Menezo Y, Guerin P: **The mammalian oviduct: biochemistry and physiology.** *Eur J Obstet Gynecol Reprod Biol* 1997, **73:**99-104.
3. Talbot P, Shur BD, Myles DG: **Cell adhesion and fertilization: steps in oocyte transport, sperm-zona pellucida interactions, and sperm-egg fusion.** *Biol Reprod* 2003, **68:**1-9.
4. Hunter RH: **Components of oviduct physiology in eutherian mammals.** *Biol Rev Camb Philos Soc* 2012, **87:**244-255.
5. Noreikat K, Wolff M, Kummer W, Kölle S: **Ciliary activity in the oviduct of cycling, pregnant, and muscarinic receptor knockout mice.** *Biol Reprod* 2012, **86:**120.
6. Croxatto HB: **Physiology of gamete and embryo transport through the fallopian tube.** *Reprod Biomed Online* 2002, **4:**160-169.
7. Yuan S, Wang Z, Peng H, Ward SM, Hennig GW, Zheng H, Yan W: **Oviductal motile cilia are essential for oocyte pickup but dispensable for sperm and embryo transport.** *Proc Natl Acad Sci U S A* 2021, **118**.
8. Ishikawa Y, Usui T, Yamashita M, Kanemori Y, Baba T: **Surfing and Swimming of Ejaculated Sperm in the Mouse Oviduct.** *Biol Reprod* 2016, **94:**89.
9. Dixon RE, Hwang SJ, Hennig GW, Ramsey KH, Schripsema JH, Sanders KM, Ward SM: **Chlamydia infection causes loss of pacemaker cells and inhibits oocyte transport in the mouse oviduct.** *Biol Reprod* 2009, **80:**665-673.
10. Kobayashi Y, Wakamiya K, Kohka M, Yamamoto Y, Okuda K: **Summer heat stress affects prostaglandin synthesis in the bovine oviduct.** *Reproduction* 2013, **146:**103-110.
11. Zhang D, Zhu Q, Xia W, Zhu C, Zhao X, Zhang Y, He C, Ji S, Li X, Zhang J: **The role of SK3 in progesterone-induced inhibition of human fallopian tubal contraction.** *Reprod Biol Endocrinol* 2022, **20:**73.
12. Madhra M, Otify M, Horne AW: **Ectopic pregnancy.** *Obstet Gynaecol Reprod Med* 2017, **27**:245-250.
13. Hino T, Yanagimachi R: **Active peristaltic movements and fluid production of the mouse oviduct: their roles in fluid and sperm transport and fertilization†.** *Biol Reprod* 2019, **101:**40-49.
14. Hino T: **Oocyte transport against fluid flow to the fertilization site in mice: contributions of cilia beating and peristalsis†.** *Biol Reprod* 2025, **113:**557-567.
15. Mita M, Walsh MP: **alpha1-Adrenoceptor-mediated phosphorylation of myosin in rat-tail**

arterial smooth muscle. *Biochem J* 1997, **327 (Pt 3):**669-674.

16. Feng J, Ito M, Ichikawa K, Isaka N, Nishikawa M, Hartshorne DJ, Nakano T: **Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase.** *J Biol Chem* 1999, **274:**37385-37390.
17. Tahara M, Morishige K, Sawada K, Ikebuchi Y, Kawagishi R, Tasaka K, Murata Y: **RhoA/Rho-kinase cascade is involved in oxytocin-induced rat uterine contraction.** *Endocrinology* 2002, **143:**920-929.
18. Swärd K, Mita M, Wilson DP, Deng JT, Susnjar M, Walsh MP: **The role of RhoA and Rho-associated kinase in vascular smooth muscle contraction.** *Curr Hypertens Rep* 2003, **5:**66-72.
19. Rattan S: **Ca2+/calmodulin/MLCK pathway initiates, and RhoA/ROCK maintains, the internal anal sphincter smooth muscle tone.** *Am J Physiol Gastrointest Liver Physiol* 2017, **312:**G63-g66.
20. Shimomura A, Ohama T, Hori M, Ozaki H: **17Beta-estradiol induces gastrointestinal motility disorder by decreasing CPI-17 phosphorylation via changes in rho-family G-protein Rnd expression in small intestine.** *J Vet Med Sci* 2009, **71:**1591-1597.
21. Kim YS, Hori M, Yasuda K, Ozaki H: **Differences in the gestational pattern of mRNA expression of the Rnd family in rat and human myometria.** *Comp Biochem Physiol A Mol Integr Physiol* 2005, **142:**410-415.
22. Lartey J, Gampel A, Pawade J, Mellor H, Bernal AL: **Expression of RND proteins in human myometrium.** *Biol Reprod* 2006, **75:**452-461.
23. Wijayagunawardane MP, Miyamoto A, Taquahashi Y, Gabler C, Acosta TJ, Nishimura M, Killian G, Sato K: **In vitro regulation of local secretion and contraction of the bovine oviduct: stimulation by luteinizing hormone, endothelin-1 and prostaglandins, and inhibition by oxytocin.** *J Endocrinol* 2001, **168:**117-130.
24. Kubota S, Yamamoto Y, Kimura K: **A Chinese Medicine, Tokishakuyakusan, Increases Bovine Oviductal Tonus via G Protein-Coupled Estrogen Receptor 1.** *Biol Pharm Bull* 2022, **45:**1133-1141.
25. Ireland JJ, Murphee RL, Coulson PB: **Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum.** *J Dairy Sci* 1980, **63:**155-160.
26. Barton BE, Herrera GG, Anamthathmakula P, Rock JK, Willie A, Harris EA, Takemaru KI, Winuthayanon W: **Roles of steroid hormones in oviductal function.** *Reproduction* 2020, **159:**R125-r137.
27. Sogawa C, Abe A, Tsuji T, Koizumi M, Saga T, Kunieda T: **Gastrointestinal tract disorder in natriuretic peptide receptor B gene mutant mice.** *Am J Pathol* 2010, **177:**822-828.
28. Ning N, Zhu J, Du Y, Gao X, Liu C, Li J: **Dysregulation of hydrogen sulphide metabolism**

impairs oviductal transport of embryos. *Nat Commun* 2014, **5:**4107.

29. Nishie T, Kobayashi Y, Kimura K, Okuda K: **Acute stimulation of a smooth muscle constrictor by oestradiol-17 β via GPER1 in bovine oviducts.** *Reprod Domest Anim* 2018, **53:**326-332.
30. Bologa CG, Revankar CM, Young SM, Edwards BS, Arterburn JB, Kiselyov AS, Parker MA, Tkachenko SE, Savchuck NP, Sklar LA, et al: **Virtual and biomolecular screening converge on a selective agonist for GPR30.** *Nat Chem Biol* 2006, **2:**207-212.
31. Dennis MK, Burai R, Ramesh C, Petrie WK, Alcon SN, Nayak TK, Bologa CG, Leitao A, Brailoiu E, Deliu E, et al: **In vivo effects of a GPR30 antagonist.** *Nat Chem Biol* 2009, **5:**421-427.
32. Nakahara T, Moriuchi H, Yunoki M, Sakamato K, Ishii K: **Y-27632 potentiates relaxant effects of beta 2-adrenoceptor agonists in bovine tracheal smooth muscle.** *Eur J Pharmacol* 2000, **389:**103-106.
33. Cario-Toumaniantz C, Reillaudoux G, Sauzeau V, Heutte F, Vaillant N, Finet M, Chardin P, Loirand G, Pacaud P: **Modulation of RhoA-Rho kinase-mediated Ca2+ sensitization of rabbit myometrium during pregnancy - role of Rnd3.** *J Physiol* 2003, **552:**403-413.
34. Osnes T, Sandstad O, Skar V, Osnes M, Kierulf P: **Total protein in common duct bile measured by acetonitrile precipitation and a micro bicinchoninic acid (BCA) method.** *Scand J Clin Lab Invest* 1993, **53:**757-763.
35. Ihaka R, Gentleman R: **R: A Language for Data Analysis and Graphics.** *J Comput Graph Stat* 1996, **5**.
36. Migliaccio A, Rotondi A, Auricchio F: **Calmodulin-stimulated phosphorylation of 17 beta-estradiol receptor on tyrosine.** *Proc Natl Acad Sci U S A* 1984, **81:**5921-5925.
37. Aktas S, Un I, Omer Barlas I, Ozturk AB, Ilkay Karagul M: **Evaluation of the Rho A/Rho-kinase pathway in the uterus of the rat model of polycystic ovary syndrome.** *Reprod Biol* 2019, **19:**45-54.
38. Lopera-Vásquez R, Uribe-García F, Rondón-Barragán I: **Effect of estrous cycle phases on gene expression in bovine oviduct epithelial cells.** *Vet World* 2022, **15:**1665-1675.
39. Khasnis M, Nakatomi A, Gumpper K, Eto M: **Reconstituted human myosin light chain phosphatase reveals distinct roles of two inhibitory phosphorylation sites of the regulatory subunit, MYPT1.** *Biochemistry* 2014, **53:**2701-2709.
40. Glencross RG, Munro IB, Senior BE, Pope GS: **Concentrations of oestradiol-17 beta, oestrone and progesterone in jugular venous plasma of cows during the oestrous cycle and in early pregnancy.** *Acta Endocrinol (Copenh)* 1973, **73:**374-384.
41. Wijayagunawardane MP, Miyamoto A, Cerbito WA, Acosta TJ, Takagi M, Sato K: **Local distributions of oviductal estradiol, progesterone, prostaglandins, oxytocin and**

endothelin-1 in the cyclic cow. *Theriogenology* 1998, **49:**607-618.

42. Ray JA, Kushnir MM, Rockwood AL, Meikle AW: **Direct Measurement of Free Estradiol in Human Serum and Plasma by Equilibrium Dialysis-Liquid Chromatography-Tandem Mass Spectrometry.** *Methods Mol Biol* 2016, **1378:**99-108.
43. Basbous S, Azzarelli R, Pacary E, Moreau V: **Pathophysiological functions of Rnd proteins.** *Small GTPases* 2021, **12:**336-357.
44. La Spina FA, Puga Molina LC, Romarowski A, Vitale AM, Falzone TL, Krapf D, Hirohashi N, Buffone MG: **Mouse sperm begin to undergo acrosomal exocytosis in the upper isthmus of the oviduct.** *Dev Biol* 2016, **411:**172-182.
45. Muro Y, Hasuwa H, Isotani A, Miyata H, Yamagata K, Ikawa M, Yanagimachi R, Okabe M: **Behavior of Mouse Spermatozoa in the Female Reproductive Tract from Soon after Mating to the Beginning of Fertilization.** *Biol Reprod* 2016, **94:**80.
46. Suarez SS: **Mammalian sperm interactions with the female reproductive tract.** *Cell Tissue Res* 2016, **363:**185-194.
47. Akira S, Sanbuissho A, Lin YC, Araki T: **Acceleration of embryo transport in superovulated adult rats.** *Life Sci* 1993, **53:**1243-1251.
48. Valle GR, Cassali GD, Nogueira JC, Castro AC, Reis AM, Cardoso FM, Figueiredo CB, Nascimento EF: **Nuclear estrogen and progesterone receptors in the oviduct of heifers under natural and superovulated estrous cycles.** *Anim Reprod Sci* 2007, **101:**28-37.
49. Hansen C, Srikandakumar A, Downey BR: **Presence of follicular fluid in the porcine oviduct and its contribution to the acrosome reaction.** *Mol Reprod Dev* 1991, **30:**148-153.
50. Ezzati M, Djahanbakhch O, Arian S, Carr BR: **Tubal transport of gametes and embryos: a review of physiology and pathophysiology.** *J Assist Reprod Genet* 2014, **31:**1337-1347.
51. Wang S, Larina IV: **In vivo dynamic 3D imaging of oocytes and embryos in the mouse oviduct.** *Cell Rep* 2021, **36:**109382.
52. Ito M, Smith TT, Yanagimachi R: **Effect of ovulation on sperm transport in the hamster oviduct.** *J Reprod Fertil* 1991, **93:**157-163.
53. Boling JL, Blandau RJ: **Egg transport through the ampullae of the oviducts of rabbits under various experimental conditions.** *Biol Reprod* 1971, **4:**174-184.
54. Guidobaldi HA, Teves ME, Uñates DR, Giojalas LC: **Sperm transport and retention at the fertilization site is orchestrated by a chemical guidance and oviduct movement.** *Reproduction* 2012, **143:**587-596.
55. Battalia DE, Yanagimachi R: **The change in oestrogen and progesterone levels triggers adovarian propulsive movement of the hamster oviduct.** *J Reprod Fertil* 1980, **59:**243-247.
56. Uenoyama Y, Hattori S, Miyake M, Okuda K: **Up-regulation of oxytocin receptors in porcine endometrium by adenosine 3',5'-monophosphate.** *Biol Reprod* 1997, **57:**723-728.

57. Tanikawa M, Acosta TJ, Fukui T, Murakami S, Korzekwa A, Skarzynski DJ, Piotrowska KK, Park CK, Okuda K: **Regulation of prostaglandin synthesis by interleukin-1alpha in bovine endometrium during the estrous cycle.** *Prostaglandins Other Lipid Mediat* 2005, **78:**279-290.

58. Acosta TJ, Miyamoto A, Ozawa T, Wijayagunawardane MP, Sato K: **Local release of steroid hormones, prostaglandin E2, and endothelin-1 from bovine mature follicles In vitro: effects of luteinizing hormone, endothelin-1, and cytokines.** *Biol Reprod* 1998, **59:**437-443.

59. Madigan JP, Bodemann BO, Brady DC, Dewar BJ, Keller PJ, Leitges M, Philips MR, Ridley AJ, Der CJ, Cox AD: **Regulation of Rnd3 localization and function by protein kinase C alpha-mediated phosphorylation.** *Biochem J* 2009, **424:**153-161.

60. Alexander BM, Van Kirk EA, Murdoch WJ: **Secretion of platelet-activating factor by periovulatory ovine follicles.** *Life Sci* 1990, **47:**865-868.

61. Obara K, Yoshioka K, Tanaka Y: **Effects of Platelet-Activating Factor (PAF) on the Mechanical Activities of Lower Urinary Tract and Genital Smooth Muscles.** *Biol Pharm Bull* 2024, **47:**1467-1476.

62. Kawano Y, Harada E, Yamashita Y, Itonaga Y, Inoue N, Narahara H: **Inflammatory Mediators in Ovarian Follicles: The Possible Role of Platelet-Activating Factor and its Metabolic Enzyme.** *Journal of Mammalian Ova Research* 2017, **34:**47-55.

63. Fan W, Xu Y, He X, Luo P, Zhu J, Li J, Wang R, Yuan Q, Wu K, Hu W, et al: **Molecular basis for the activation of PAF receptor by PAF.** *Cell Rep* 2024, **43:**114422.

64. Guasch RM, Blanco AM, Pérez-Aragó A, Miñambres R, Talens-Visconti R, Peris B, Guerri C: **RhoE participates in the stimulation of the inflammatory response induced by ethanol in astrocytes.** *Exp Cell Res* 2007, **313:**3779-3788.

65. Woodsome TP, Eto M, Everett A, Brautigan DL, Kitazawa T: **Expression of CPI-17 and myosin phosphatase correlates with Ca(2+) sensitivity of protein kinase C-induced contraction in rabbit smooth muscle.** *J Physiol* 2001, **535:**553-564.

66. Riou P, Kjær S, Garg R, Purkiss A, George R, Cain RJ, Bineva G, Reymond N, McColl B, Thompson AJ, et al: **14-3-3 proteins interact with a hybrid prenyl-phosphorylation motif to inhibit G proteins.** *Cell* 2013, **153:**640-653.

67. Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M, Rinaudo P: **Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics.** *Reprod Biol Endocrinol* 2009, **7:**40.

68. Ambekar AS, Nirujogi RS, Srikanth SM, Chavan S, Kelkar DS, Hinduja I, Zaveri K, Prasad TS, Harsha HC, Pandey A, Mukherjee S: **Proteomic analysis of human follicular fluid: a new perspective towards understanding folliculogenesis.** *J Proteomics* 2013, **87:**68-77.

69. Hessock EA, Edwards JL, Schrick FN, Payton RR, Campagna SR, Pollock AB, Clark HM, Stokes AE, Klabnik JL, Hill KS, et al: **Metabolite abundance in bovine preovulatory follicular fluid is influenced by follicle developmental progression post estrous onset in cattle.** *Front Cell Dev Biol* 2023, **11:**1156060.

70. Velasquez LA, Maisey K, Fernandez R, Valdes D, Cardenas H, Imarai M, Delgado J, Aguilera J, Croxatto HB: **PAF receptor and PAF acetylhydrolase expression in the endosalpinx of the human Fallopian tube: possible role of embryo-derived PAF in the control of embryo transport to the uterus.** *Hum Reprod* 2001, **16:**1583-1587.

71. Lash GE, Legge M: **Localization and distribution of platelet activating factor receptors in the mouse ovary and oviduct during the estrous cycle and early pregnancy.** *Am J Reprod Immunol* 2001, **45:**123-127.

72. Lawson EF, Ghosh A, Grupen C, Netherton J, Aitken RJ, Smith ND, Lim R, Drury HR, Pickford R, Gibb Z, et al: **Investigations into the role of platelet-activating factor in the peri-conception period of the mare.** *Reproduction* 2024, **168**.

73. Prescott SM, Zimmerman GA, Stafforini DM, McIntyre TM: **Platelet-activating factor and related lipid mediators.** *Annu Rev Biochem* 2000, **69:**419-445.

74. Demopoulos CA, Pinckard RN, Hanahan DJ: **Platelet-activating factor. Evidence for 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine as the active component (a new class of lipid chemical mediators).** *J Biol Chem* 1979, **254:**9355-9358.

75. Braniste V, Jouault A, Gaultier E, Polizzi A, Buisson-Brenac C, Leveque M, Martin PG, Theodorou V, Fioramonti J, Houdeau E: **Impact of oral bisphenol A at reference doses on intestinal barrier function and sex differences after perinatal exposure in rats.** *Proc Natl Acad Sci U S A* 2010, **107:**448-453.

76. Murphy CR: **Uterine receptivity and the plasma membrane transformation.** *Cell Res* 2004, **14:**259-267.

77. Zhang S, Kong S, Lu J, Wang Q, Chen Y, Wang W, Wang B, Wang H: **Deciphering the molecular basis of uterine receptivity.** *Mol Reprod Dev* 2013, **80:**8-21.

ABSTRACT IN JAPANESE

排卵前後におけるウシ卵管緊張度制御メカニズムに関する研究

窪田早耶香

卵管は卵巣と子宮の間に位置する細管で、精子の受精能獲得および初期胚発生を促進する環境を提供するとともに、配偶子および初期胚の輸送経路である。この卵管の輸送機能には、卵管平滑筋の収縮弛緩運動が大きく関与しており、特に卵管峡部においては平滑筋収縮弛緩運動が胚や精子の輸送に影響する。卵管平滑筋の輸送機能が低下すると、正常な受精が行われないだけでなく、初期胚発生障害を引き起こす。卵管平滑筋の機能障害がウシにおいては夏季の受胎率低下、ヒトにおいては異所性妊娠の原因となることが示唆されており、卵管平滑筋における収縮弛緩機能の解明はウシの受胎率向上やヒトの不妊症の改善に不可欠である。卵管収縮弛緩運動は、リズミカルな収縮弛緩を生み出す収縮の頻度、大きさ (振幅) に加え、筋に備わっている張力を示す緊張度により構成される。その中で、緊張度の制御は、正常な妊娠をもたらす適切なタイミングでの卵管輸送に寄与しているにもかかわらず、収縮頻度や振幅と比較して緊張度に関する研究は軽視されており、その報告は非常に少ない。本研究では、配偶子や初期胚の卵管内輸送が生じる排卵前後のウシ卵管における緊張度制御メカニズムを解明することを目的とし、排卵前に分泌が増加するエストラジオール-17β (E2)、および排卵時に卵管内に流入する卵胞液が卵管緊張度に与える影響を検討した。

１．E2 による卵管緊張度増加制御メカニズムの解明

ウシ卵管峡部をステージ I (排卵後 1-4 日)、ステージ II (排卵後 5-10 日)、ステージ III (排卵後 11-17 日)、ステージ IV (排卵後 18-20 日) の 4 つのステージに分け、1 nM および 10 nM E2 添加後 1 時間までの各ステージの収縮能 (収縮頻度、収縮弛緩力、緊張度) をマグヌス法により測定した。E2 は、全ステージの卵管の収縮頻度および収縮弛緩力に有意な変化を及ぼさなかった一方、排卵直後であるステージ I の卵管では、1nM E2 添加区でのみコントロールと比較し有意な緊張度の増加が観察された。また、ステージ I の卵管におけるGタンパク質共役型 E2 受容体 (GPER1) アゴニストの添加は 1 nM E2 の添加同様に卵管緊張度の増加を引き起こし、GPER1 アンタゴニストおよび Rho キナーゼ (ROCK) 阻害剤の添加により、この E2 誘導性の卵管緊張度増加は有意に抑制された。排卵直後であるステージ I の卵管平滑筋組織における ROCK II タンパク質発現量は、ステージ III の卵管と比較して有意に高かったことに加えて、1 nM E2 に感作させたステージ I の卵管平滑筋組織の ROCK 活性は、他のステージの

卵管よりも有意に高いレベルを示した。また、ステージ I の卵管は、1 nM E2 に感作することでコントロールと比較して RhoA/ROCK シグナル経路の下流因子であるミオシンホスファターゼ標的サブユニット 1 (MYPT1) およびミオシン軽鎖 (MLC) のリン酸化レベルを有意に増加させた。さらに、ROCK の阻害効果をもつRho 関連 GTP 結合タンパク質 (RND) 3 は、ステージ III と比較して排卵前であるステージ IV の卵管平滑筋組織で有意に高く発現していた。RND 阻害剤は、ステージ IV の卵管における RhoA/ROCK シグナル経路の活性化を促進し、1 nM E2 誘導性の卵管緊張度増加効果を回復させた。以上のことから、排卵直後の卵管においてのみ E2 の卵管緊張度増加効果が見られるのは、ROCK のタンパク質発現量および活性レベルに依存する可能性が示された。一方、実際の生体内で E2 に感作される排卵前の卵管において E2 の卵管緊張度増加効果が見られないのは、RND3 が高発現することで E2 誘導性の ROCK 活性レベル増加を阻害しているためであると考えられた。

２．卵胞液による卵管緊張度増加制御メカニズムの解明

生体内において卵胞液に感作されていないステージ IV のウシ卵管峡部組織を用いて、卵胞液添加後 5 分間の収縮能をマグヌス法により測定した。黄体形成ホルモン (LH) サージ前の卵胞液は収縮能に有意な変化をもたらさなかったが、LH サージ後の卵胞液はコントロールと比較して添加後急速に卵管緊張度を増加させた。また、卵管内に、LH サージ後の卵胞液を注入すると、コントロールと比較して有意に卵管長が短縮した。LH サージ後の卵胞液のみが卵管緊張度を増加させたため、LH サージ後に卵胞内で産生量が増加することが報告されている血小板活性化因子 (PAF) に着目した。LH サージ後のウシ卵胞液中の PAF 濃度は、LH サージ前と比較し有意に高い値を示した。PAF 受容体アンタゴニストでステージ の卵管を前処理したところ、LH サージ後の卵胞液による卵管緊張度の増加を有意に抑制した。同様に、ROCK およびタンパク質キナーゼC α (PKCα) 阻害剤による前処理も、LH サージ後の卵胞液による卵管緊張度増加効果を阻害した。以上の結果から、LH サージ後の卵胞液は、PAF 濃度を増加させるとともに、PAF 受容体を介した ROCK および PKCα の活性化によりウシ卵管緊張度を増加させる可能性が示された。

本研究より、E2 は排卵直後のウシ卵管において GPER1 を介した ROCK/MYPT1/MLC の活性化により緊張度を増加させた一方、卵胞液は PAF 受容体を介した ROCK の活性化により排卵前の卵管緊張度を増加させた。生体内の排卵直後の卵管における E2 濃度は 300 pM 程度であるにもかかわらず、本研究では 1 nM E2 が排卵直後の卵管において緊張度を増加させた。ウシでは過排卵処理により排卵直後に

おける E2 濃度が異常に高くなることや、マウスでは排卵直後に E2 濃度が増加すると妊娠率の低下を招くことが報告されている。そのため、異常なホルモン環境による E2 濃度の増加が、過剰な卵管緊張度の増加を引き起こし、適切なタイミングでの配偶子や初期胚の卵管内輸送を妨げることで不妊を生じる可能性がある。一方で、LH サージ後の卵胞液は E2 とは異なり、排卵前の卵管緊張度を増加させた。これまでの研究において、卵管峡部の急速な平滑筋収縮によるポンピングが、卵管内の精子輸送に関与することが報告されている。本研究から、卵胞液は排卵時に卵母細胞とともに卵管内に流れ込み、卵管峡部における緊張度を増加させて精子の卵管内輸送を促進することが示唆される。これらは、家畜動物やヒトにおける卵管輸送機能の理解および繁殖効率向上に重要な知見を示す。