Functional role of the oviduct in mammalian fertilization

(哺乳類の受精における卵管の機能解析)

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ABBREVIATION

ACN : acetonitrile
BSA : bovine serum albumin
CCD : charge-coupled device
DMEM : Dulbecco's modified Eagle's medium
dpc : day post coitus
EDTA : ethylenediaminetetraacetic acid
ET : endotheline
FBS : fetal bovine serum
hCG : human chorionic gonadotropin
HPLC : high-performance liquid chromatography
I.D. : inner diameter
KSOM : K^+ Simplex Optimization Media
NO : nitric oxide
OCC : oocyte-cumulus complex
OECs : oviductal epithelial cells
PBS : phosphate-buffered saline
PDA : photo diode array
PFA : paraformaldehyde
PG : prostaglandin
PMSG : pregnant mare's serum gonadotropin
s.c. : subcutaneous
TFA : trifluoroacetic acid
TYH : modified Krebs-Ringer bicarbonate solution [Toyoda-Yokoyama-Hoshi medium]
UTJ : uterotubal junction
ZP : zona pellucida

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GENERAL INTRODUCTION

A life begins at fertilization. Fertilization, fusion between sperm and an oocyte, is an indispensable event in reproduction. In recent decades, one in six couples of reproductive age struggle with infertility in advanced countries. Despite the lack of fundamental understanding for *in vivo* fertilization, a wide variety of assisted reproductive technologies (ART) have been rapidly put into practice for the treatment of infertility.

To accomplish mammalian fertilization, ejaculated sperm must migrate from the uterus to oviduct in the female reproductive tract, and fertilize the oocytes in the oviductal ampulla (Yanagimachi, 1994). According to current concepts, the migration of sperm from the oviductal isthmus to the ampulla is believed to be regulated by some taxes, including chemotaxis, rheotaxis, and thermotaxis (Bahat et al., 2003; Oliveira et al., 1999; Miki and Clapham, 2013; Spehr et al., 2003). However, mechanisms of the sperm migration in the oviduct have not been fully understood.

The oviduct plays a critical role for fertilization *in vivo*. The oviduct exhibits smooth muscle (myosalpinx) contractions and ciliary beating, which coordinate the regulation of oviductal fluid flows (Shi et al., 2014). Indeed, the propulsive force of fluids generated by myosalpinx contractions contributes to the transport of early embryos. Thus, myosalpinx contractions play a pivotal role in the embryo transport through the oviduct.

In my Ph.D projects, I addressed the function of myosalpinx contractions in the sperm migration and the involvement of endothelin-independent pathway in oviductal epithelial cell-contractions in mice.

CHAPTER I

Myosalpinx contractions regulate the sperm migration in the oviduct

SUMMARY

To accomplish fertilization in the oviductal ampulla, ejaculated sperm are required to migrate through the female reproductive tract. However, this fundamental process largely remains unknown. In this study, I focused on the role of oviductal smooth muscle (myosalpinx) contractions in the sperm migration. Administration of prifinium bromide, padrin, to mice effectively suppressed myosalpinx contractions, resulting in a decreased rate of fertilization in a dose-dependent manner, and an abrogation of high-speed back-and-forth/shuttling flows of oviductal fluids around the isthmus. Regardless of padrin administration, no shuttling flows were found near the ampulla. In the isthmus, sperm formed a tight assemblage that was synchronized with the shuttling flows. The sperm assemblage was gradually loosened and then completely abolished near the ampulla. No sperm assemblage was formed in the isthmus when padrin was administered. These results suggest that myosalpinx contractions contribute to the formation of sperm assemblage in the isthmus, and to the transport of assemblage to the middle region of oviduct. It is also suggested that the motility of sperm may be essential for the migration of sperm from the middle oviductal region to the ampulla.

INTRODUCTION

The oviduct, a duct connecting the periovarian space with the uterus, is indispensable for the transport of sperm and fertilized eggs/embryos to accomplish fertilization and implantation, respectively. The oviduct is comprised of four segments, the uterotubal junction (UTJ), isthmus, ampulla, and infundibulum (Coy et al., 2012; Talbot et al., 1999; Yanagimachi, 1994). The isthmus acts as a storage of ejaculated sperm ascending from the UTJ (Bedford and Breed, 1994; Suarez, 2008). The physiological changes, which render sperm competent to fertilize, are collectively called capacitation, and presumably occurs in the isthmus (DeMott and Suarez, 1992; Overstreet and Cooper, 1979; Yanagimachi, 1994). Following capacitation, sperm acquire hyperactivated motility and are released from the isthmus (Suarez and Osman, 1987; Chang and Suarez, 2012). Ovulated oocyte-cumulus complexes (OCC) are picked up by the infundibulum and stored in the ampulla, where fertilization takes place (Bedford and Breed, 1994). Fertilizing sperm enter the OCC, pass through the cumulus matrix, and penetrate the zona pellucida (ZP) to fuse with the oocyte (Chang et al., 2012). According to current concepts, the sperm migration from the isthmus to the ampulla is believed to be regulated by some taxes, including chemotaxis, rheotaxis, and thermotaxis (Bahat et al., 2003; Oliveira et al., 1999; Miki and Clapham, 2013; Spehr et al., 2003).

The action of peristaltic pumping is generated by wavelike muscular contractions, and is utilized as a fundamentally biomechanical mechanism to transport fluids and materials in the esophagus, intestine, oviduct, and ureter (Aranda et al., 2015; Teran et al., 2008). The oviduct is known to display smooth muscle (myosalpinx) contractions and ciliary

beating (Croxatto, 2002; Halbert et al., 1975). The propulsive force of mucosal fluids, which is produced by myosalpinx contractions and ciliary beating, has been suggested to participate in the transport of embryos from the ampulla to the uterus (Guidobaldi et al., 2012; Kölle et al., 2009; Talo, 1991). *Chlamydia* infection in human females results in an increased risk of ectopic pregnancy and eventually in tubal factor sterility (Dixon et al., 2009; Dixon et al., 2010). This infectious disease is attributed to the loss of the pacemaker activity of interstitial Cajal cells essential for generating spontaneously slow waves that drive myosalpinx contractions (Dixon et al., 2009; Dixon et al., 2010; Dixon et al., 2011). An excessive intake of caffeine, an agonist of ryanodine receptor and an inhibitor of cyclic nucleotide phosphodiesterase, is also associated with delayed conception, because caffeine abolishes the slow waves and concomitant myosalpinx contractions through activation of glibenclamide-sensitive K_{ATP} channels in the myosalpinx (Dixon et al., 2011). Moreover, Müllerian duct mesenchyme-derived tissues-specific conditional knockout mice lacking riboendonuclease Dicer exhibit complete female infertility due to aberrant transport of embryos, which may be caused by the defect in myosalpinx contractions (Gonzalez and Behringer, 2009). Thus, myosalpinx contractions play a vital role in the transport of embryos into the uterus. However, little is known about the mechanism of sperm migration through the oviduct; do hyperactivated sperm indeed swim in mucosal fluids of the oviduct? Are sperm as well as fertilized eggs transported by the aid of myosalpinx contractions?

In this study, I focused on the role of myosalpinx contractions in the sperm migration through the oviduct. A synthetic anticholinergic drug, prifinium bromide (padrin) (Kumada and Hitomi, 1967; Kumada et al.,

1972; Tack et al., 2006), was applied to suppress myosalpinx contractions. I found that myosalpinx contractions are involved in the formation of sperm assemblage in the isthmus, and in the transport of the assemblage to the middle region of oviduct. On the basis of the data obtained, a mechanism of sperm migration through the oviduct regulated by myosalpinx contractions is proposed.

MATERIALS AND METHODS

Animal Experiments

All animal experiments were ethically carried out according to the Guide for the Care and Use of Laboratory Animals at University of Tsukuba.

Suppression of Myosalpinx Contractions by Padrin

Padrin, 3-(diphenylmethylene)-1,1-dietyl-2-methylpyrrolidinium bromide, was purchased from Nippon Zenyaku Kogyo (Tokyo, Japan). ICR (Institute of Cancer Research) strain mice (8-wk old; Japan SLC, Shizuoka, Japan) were superovulated by intraperitoneal injection of pregnant mare's serum gonadotropin (PMSG; 5 units; ASKA Pharmaceutical, Tokyo, Japan) followed by human chorionic gonadotropin (hCG; 7.5 units; ASKA Pharmaceutical) 48 h later, as described previously (Kawano et al., 2010; Yamashita et al., 2008). The female mice were mated with vasectomized male mice (Nagy et al., 2003) 12 h after the hCG injection. The oviducts were excised 4 h after mating, placed on petri dishes containing saline (0.2 ml) at 37°C, and observed under an SZX16 stereoscopic microscope (Olympus, Tokyo, Japan) equipped with a DP70 CCD camera (Olympus). The frequencies of myosalpinx contractions per 20 sec were counted every 2 min for 16 min. To replace saline with padrin, a piece of Advantec #2 filter paper (Toyo Roshi Kaisha, Tokyo, Japan) was placed in the dish to adsorb saline. A 0.2-ml padrin solution (0.25, 1.25, 2.5, and 5.0 mM) or atropine (1 μM, 10 μM, 100 μM, 1.25 mM, 2.5 mM, and 5.0 mM) was

immediately added to the oviduct 5 min after the oviductal excision, and myosalpinx contractions were continuously counted.

Artificial Insemination

Fresh cauda epididymal sperm of ICR mice (3- to 5-mo old) were dispersed in a 50-μl drop of a modified Krebs-Ringer bicarbonate solution [Toyoda-Yokoyama-Hoshi (TYH) medium] (Toyoda et al., 1971) free of bovine serum albumin (BSA) at 37°C for 15 min. For sperm double-labeling, epididymal sperm were incubated in BSA-free TYH medium (50 μl) containing Hoechst 33342 (2.5 μg/ml; Molecular Probes, Eugene, OR) and MitoTracker Green FM (2.5 μg/ml; Molecular Probes) at 37°C for 15 min (Kimura et al., 2009). Female mice (8-wk old) were superovulated as described above, anesthetized with 0.3 ml of 2.5% tribromoethanol (Sigma-Aldrich, St. Louis, MO) in saline, and artificially inseminated with epididymal sperm 12 h after the hCG injection. An aliquot (50 μl) of epididymal sperm suspension (3×10^5 cells/μl) was directly injected into the uterus at a distance of approximately 1 cm from the UTJ using a syringe, as described previously (Kawano et al., 2010). Immediately after artificial insemination, the female mice were administered with padrin-free saline or padrin (0.5, 1.25, 2.5, or 5.0 mg/kg body weight/injection) by a total of twelve subcutaneous (s.c.) injections in 30-min intervals over a period of 5.5 h. The oocytes were recovered from the ampulla 6 h after artificial insemination, and cultured in KSOM medium (Lawitts and Biggers, 1993) for 24 h at 37°C under 5% CO_2 in air (Yamazaki et al., 2007). The numbers of two-cell embryos and unfertilized oocytes were counted and used for estimation of the success rate of

fertilization. The numbers of double-labeled sperm located in seven oviductal regions were also counted under an IX-71 fluorescence microscope (Olympus).

Observation of Oviductal Fluid Flows

Superovulated female mice (8-wk old) were mated with vasectomized mice 12 h after the hCG injection, and administered with padrin-free saline or padrin (5.0 mg/kg/injection) by a total of eight s.c. injections in 30-min intervals over a period of 3.5 h. The female mice were anesthetized with 2.5% tribromoethanol in saline, as described above. The intact oviduct connecting with the uterus, ovary, and fat pad was pulled out from the dorsal region of mice by using an artery clamp (C-17; Natsume Seisakusho, Tokyo, Japan) 4 h after mating. According to the previously published procedure (Nakagata, 1992), 1 μl of 0.2% suspension of fluorescent polystyrene microspheres, FluoSpheres (1-μm diameter; Thermo Fisher Scientific, Waltham, MA) was injected into the region near the ampulla under an SZX16 stereoscopic microscope (Olympus), using a glass microcapillary pipette (30- to 50-μm diameter). The oviduct was then excised, placed on petri dishes containing saline (0.2 ml), and immediately observed at 37°C under an IX-71 fluorescence microscope (Olympus) equipped with an ORCA-ER CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). Images were obtained at 10 frames/sec, and processed by a MetaMorph Imaging software (Molecular Devices, Sunnyvale, CA). Motions of fluorescent microspheres were analyzed by a Manual Tracking plugin of ImageJ software (http://rsbweb.nih.gov/ij/).

Sperm Migration through the Oviduct after Mating

Superovulated female mice (8-wk old) were mated with 3-mo-old transgenic male mice [*B6D2F1-Tg(CAG/su9-DsRed2, Acr3-EGFP)RBGS002Osb*] expressing green (GFP) and red fluorescent proteins (RFP) in the acrosome and mitochondria, respectively (Hasuwa et al., 2010), 12 h after the hCG injection. After mating, the female mice were administered with saline or padrin (5.0 mg/kg/injection) by a total of four s.c. injections in 30-min intervals over a period of 90 min. The females were then anesthetized with 0.3 ml of 2.5% tribromoethanol. As described above, the intact oviduct connecting with the uterus, ovary, and fat pad was pulled out from the mice 2 h after mating, placed on petri dishes containing saline (0.2 ml), and directly observed at 37°C under an SZX16 fluorescence stereoscopic microscope (Olympus) equipped with a DP80 CCD camera (Olympus). Motions of myosalpinx contractions and sperm in the living oviduct were recorded for 1 min, and the time of movies was compressed from 1 min to 15 sec by using an Image J software.

Statistical Analysis

Data are presented as the mean ± S.E.M ($n \geq 3$). The Student's *t*-test was used for statistical analysis; significance was assumed for $p < 0.05$.

RESULTS

Padrin Suppresses Myosalpinx Contractions

A clinical anticholinergic drug, padrin (Kumada and Hitomi, 1967; Kumada et al., 1972; Tack et al., 2006), is widely used to temporarily suppress intestinal peristalsis to conduct rectal palpation and gastroduodenoscopy for diagnostic purposes, and to alleviate tonus, palsy, and hyperanacinesia of the gastrointestinal tract in clinical practices. This drug is one of the anticholinergic/antimuscarinic agents and is distinguished from the calcium channel-blocking agents such as pinaverium bromide (Tack et al., 2006). To examine whether myosalpinx contractions are suppressed by padrin, fresh oviducts were excised from female mice previously mated with vasectomized mice, put on petri dishes, and treated with padrin-free saline or various concentrations of padrin 5 min after excision of the oviduct. Although myosalpinx contractions around the isthmus persisted for 16 min, the frequency of contractions was gradually decreased in the saline-treated oviduct as time elapsed (Fig. 1, A, B, and Supplemental Movie S1). Myosalpinx contractions were inhibited by padrin in a dose-dependent manner; padrin at the concentrations of 5.0, 2.5, and 1.25 mM completely suppressed myosalpinx contractions 3, 5, and 11 min after the treatment, respectively (Fig. 1C). Thus, myosalpinx contractions are effectively suppressed by padrin at least *in vitro*. Note that atropine, (8-methyl-8-azabicyclo[3.2.1]oct-3-yl) 3-hydroxy-2-phenylpropanoate known as a typical antagonist of muscarinic acetylcholine receptors, did not affect myosalpinx contractions when the oviducts were treated with atropine at the concentrations of 1 μM to 5 mM

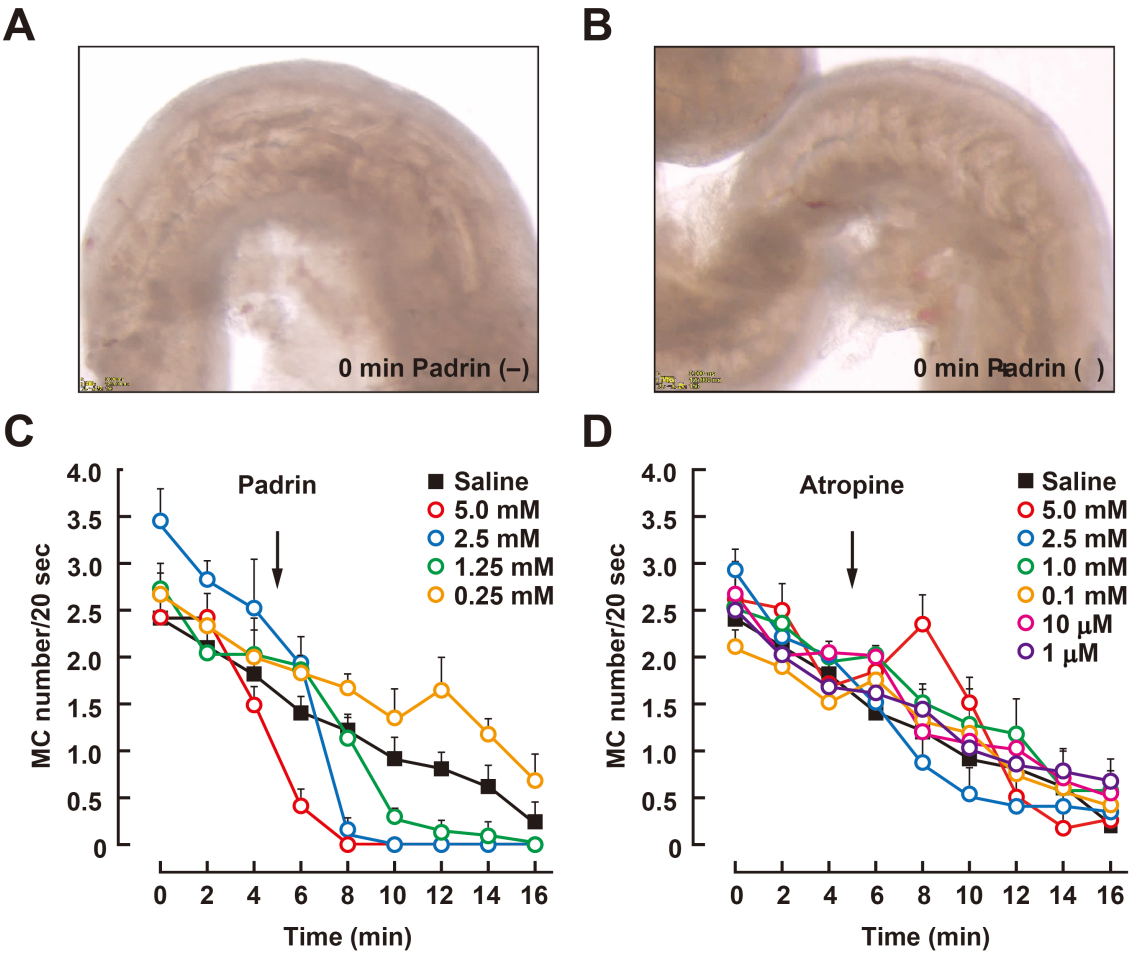

Fig. 1. Suppression of myosalpinx contractions. (A and B) Representative pictures of the oviduct treated with padrin-free saline or padrin (See Supplemental Movie S1). Following mating of female mice with vasectomized mice, the oviducts were excised and treated with padrin-free saline (−) or padrin (+). (C and D) Myosalpinx contractions in the isthmus for 20 sec were observed every 2 min for 16 min. The oviducts were treated with 0.2 ml of padrin-free saline, padrin, and atropine 5 min after the excision. (C) The concentrations of padrin at 0.25, 1.25, 2.5, and 5.0 mM were added to the oviducts. (D) The concentrations of atropine at 1 μM, 10 μM, 100 μM, 1.25 mM, 2.5 mM, and 5.0 mM were added to the oviducts. MC, myosalpinx contraction. Playback speed is 2-fold.

(Fig. 1D).

Involvement of Myosalpinx Contractions in Fertilization

I next carried out artificial insemination of superovulated mice with epididymal sperm. Different doses of padrin (0, 0.5, 1.25, 2.5, or 5.0 mg/kg/injection) were subcutaneously administered to females every 30 min for a total of 12 injections over a period of 5.5 h after insemination (Fig. 2A). The oocytes were recovered from the ampulla 6 h after insemination, and then cultured for 24 h. When 2-cell embryos were defined as fertilized oocytes, the oocytes were all fertilized without padrin administration (Fig. 2, B and C). The rate of fertilization decreased with increasing doses of padrin. Most oocytes (approximately 85%) remained unfertilized when padrin was administered at the dose of 5.0 mg/kg/injection. These results suggest that myosalpinx contractions are involved in the fertilization process.

Myosalpinx Contractions Are Involved in the Transport of Sperm from the Uterus to the Isthmus

To clarify how myosalpinx contractions contribute to the fertilization process, superovulated mice were inseminated with epididymal sperm whose nucleus and mitochondria had been prelabeled with Hoechst 33342 and MitoTracker, respectively. The inseminated females were administered with saline or padrin (5.0 mg/kg/injection) every 30 min for a total of twelve s.c. injections over a period of 5.5 h, as described in Fig. 2A. Following surgical removal of the oviducts from the mice 6 h after

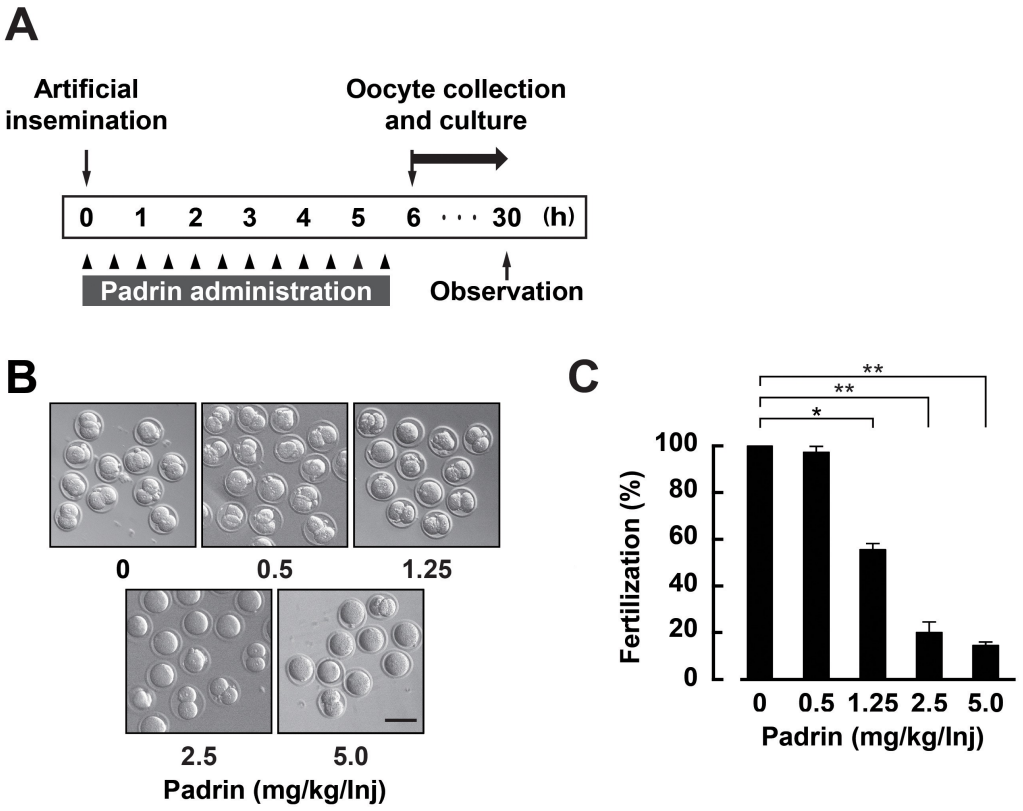

Fig. 2. Involvement of myosalpinx contractions in sperm migration. (A) Artificial insemination. Female mice were artificially inseminated with epididymal sperm, and administered with padrin-free saline or padrin (0.5, 1.25, 2.5, or 5.0 mg/kg/injection) every 30 min for a total of twelve s.c. injections over a period of 5.5 h. The oocytes were recovered from the ampulla 6 h after artificial insemination, cultured for 24 h, and observed. Prior to the 24-h culture, the fragmented oocytes recovered were excluded. (B and C) The 2-cell embryos and unfertilized oocytes were then counted. (C) The total numbers of oocytes examined were 27, 45, 39, 72, and 37 in the padrin-administered mice at the doses of 0, 0.5, 1.25, 2.5, and 5.0 mg/kg/injection, respectively. Inj, injection. Scale bar = 100 μm. *$p < 0.05$; **$p < 0.01$.

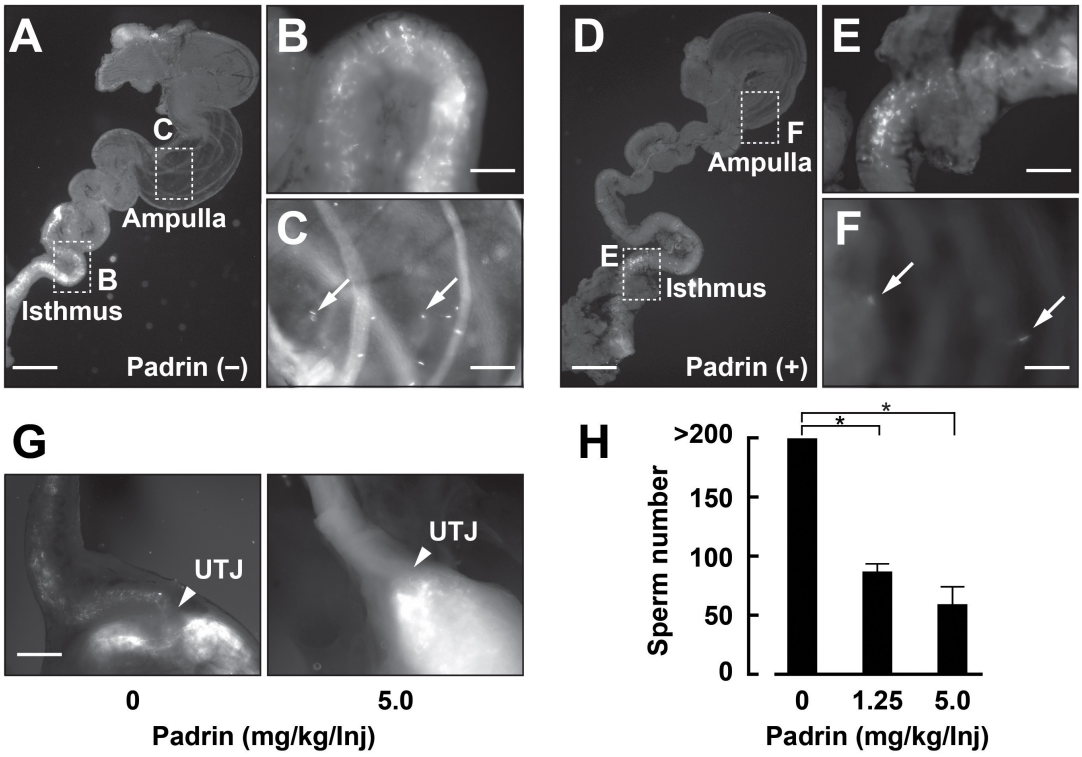

Fig. 3. Distribution of sperm in the oviduct and the UTJ. (A-F) Epididymal sperm previously double-labeled with Hoechst 33342 and MitoTracker were used for the artificial insemination. The mice were administered with padrin-free saline or padrin (5.0 mg/kg/injection). The regions surrounded by dotted lines in A and D are highlighted in B, C, E, and F. Sperm are indicated by arrows. Scale bars = 1.0 mm in A and D; 200 μm in B and E; 100 μm in C; 50 μm in F. (G and H) Abundance of sperm in the uterotubal junction (UTJ). The numbers of sperm in the UTJ (arrowheads) are counted (n = 3). Scale bars = 200 μm. *$p < 0.05$.

insemination, the localization of fluorescent dye-labeled sperm in the oviduct was observed. Without padrin administration, a large number of sperm (> 200 cells) were found in the isthmus, whereas the ampulla contained approximately 20 sperm (Fig. 3, A, B, and C). When padrin was administered, the sperm number was less than 50 in the isthmus (Fig. 3, D and E). Sperm were barely detectable (< 5 cells) in the ampulla (Fig. 3F). It is important to note that the number of sperm ascending from the uterus to the UTJ in the padrin-administered mice at the doses of 1.25 and 5.0 mg/kg/injection was much smaller than that in the control mice (Fig. 3, G and H).

The oviduct was divided into 7 regions (R1-R7), where the isthmus corresponded to R1 and R2, and the ampulla corresponded to R7 (Fig. 4). Although more than 200 sperm were found at R1 in the saline-administered mice, the number of sperm at R1 decreased with increasing doses of padrin. Approximately 20 sperm were only present at R1 in the padrin-administered mice at the dose of 5.0 mg/kg/injection. The sperm number in the region between R2 and R6 was relatively similar among the mice administered at the doses of 0, 0.5, and 1.25 mg/kg/injection. Moreover, the number of sperm at R7 decreased with increasing doses of padrin. Notably, there were less than 5 sperm at R7 at the dose of 5.0 mg/kg/injection. Thus, myosalpinx contractions may mainly function in the transport of sperm from the uterus to the isthmus.

Myosalpinx Contractions Generate Shuttling Flows of Oviductal Fluids

Because the number of sperm is decreased in the padrin-administered oviduct, myosalpinx contractions are thought to generate

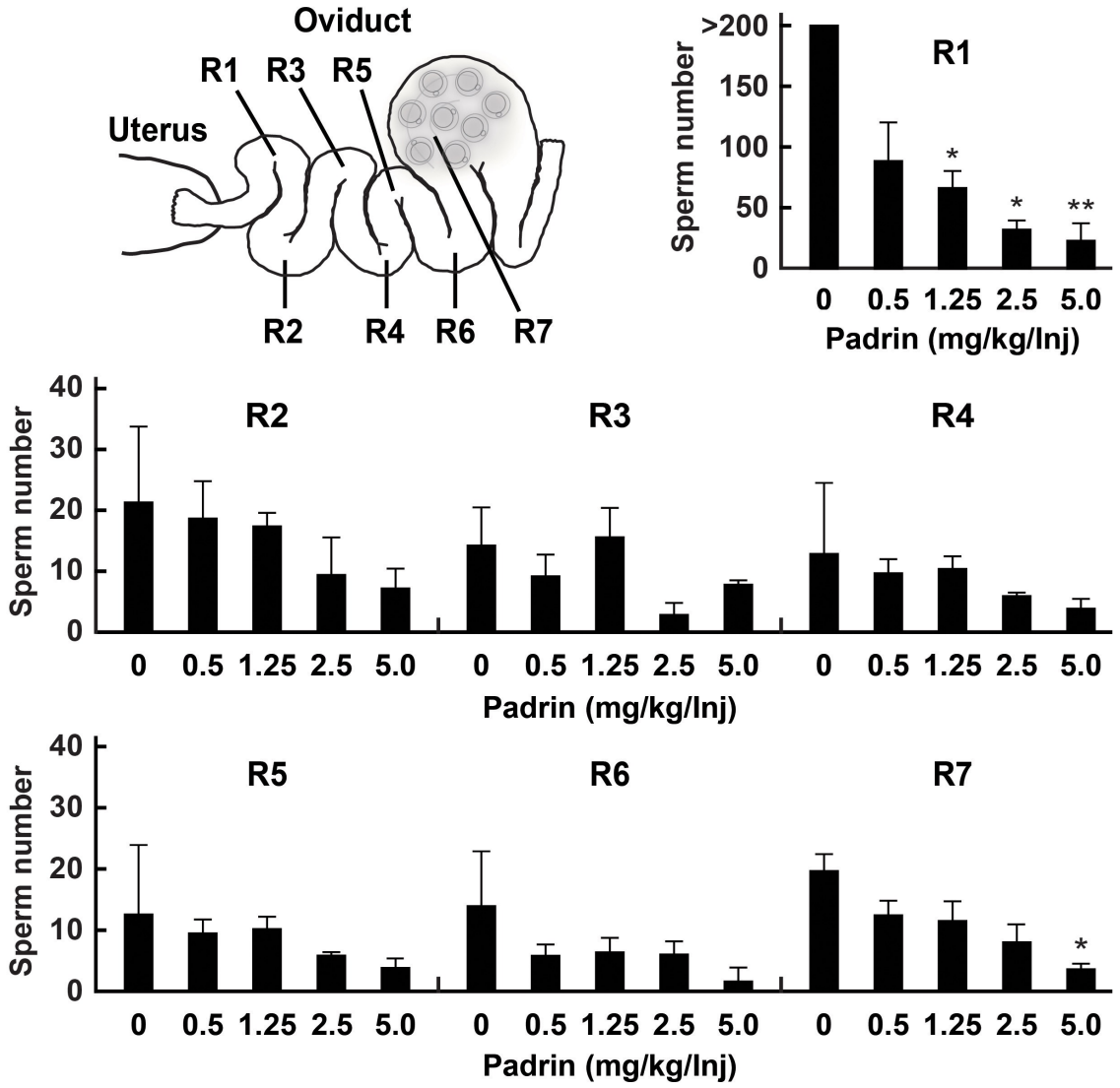

Fig. 4. Regional distribution of sperm in the oviduct. Female mice were artificially inseminated with epididymal sperm previously double-labeled with Hoechst 33342 and MitoTracker, and administered with padrin-free saline or different doses of padrin (0.5, 1.25, 2.5, or 5.0 mg/kg/injection) every 30 min for a total of twelve s.c. injections over a period of 5.5 h (see Fig. 2A). The numbers of sperm at R1-R7 were counted 6 h after artificial insemination (n = 6). Inj, injection. *$p < 0.05$; **$p < 0.01$.

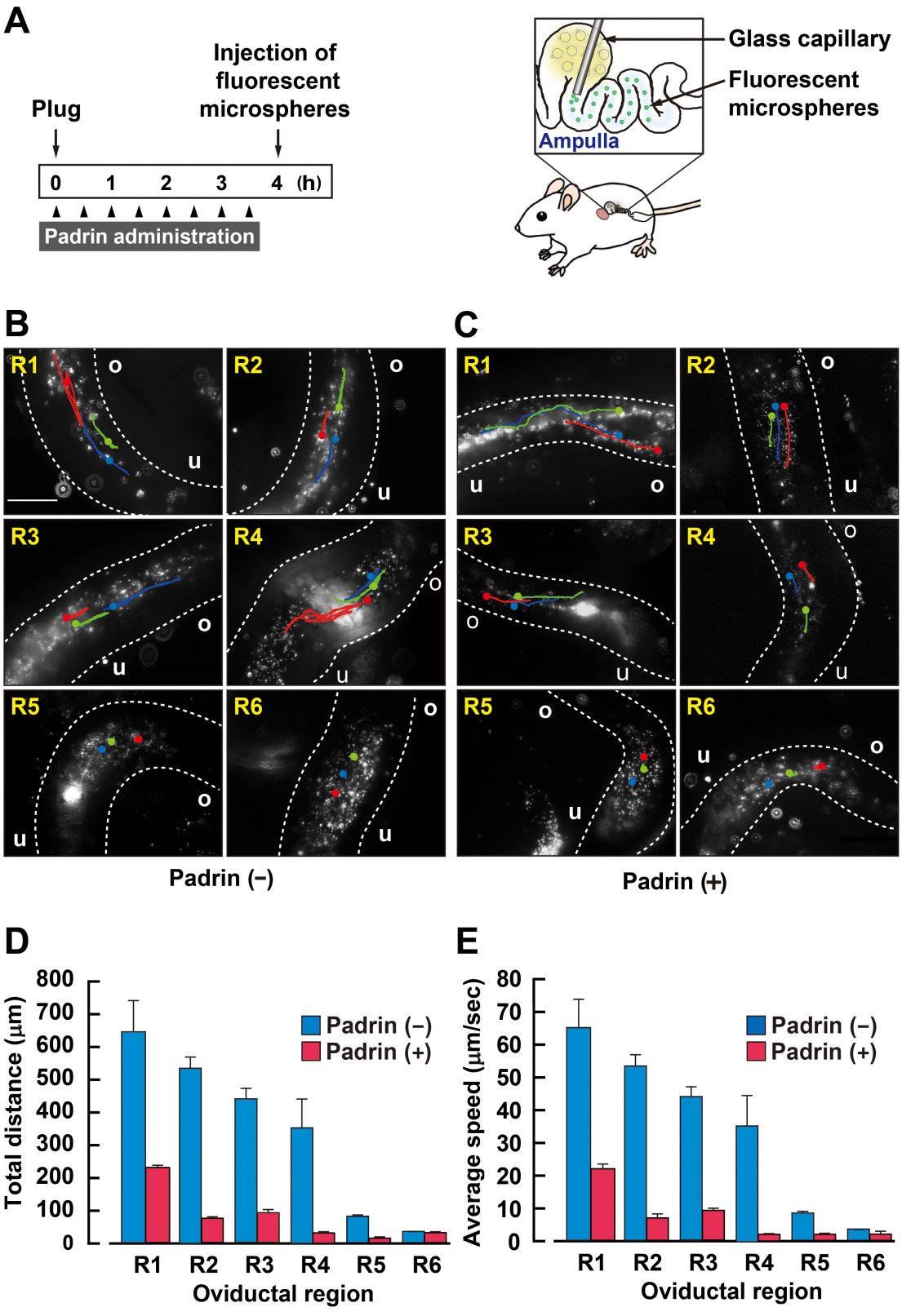

Fig. 5. Dynamic flows of oviductal fluids. (A) Experimental scheme. Female mice were mated with vasectomized mice and administered with padrin-free saline or padrin (5.0 mg/kg/injection) every 30 min for a total of eight s.c. injections over a period of 3.5 h.

The intact oviducts were pulled out 4 h after mating, and fluorescent polystyrene microspheres (1-μm diameter) were injected into the oviductal region between R6 and R7 using a glass capillary pipette. The oviducts were then excised and immediately observed. (B and C) The trajectory of each microsphere (red, blue, or green line) in the six regions (R1-R6) of saline- and padrin-administered mice was monitored (see Supplemental Movie S2). Closed circles represent a starting point for each microsphere. The sides of ovary and uterus are indicated by o and u, respectively. Scale bar = 200 μm. E and F) Repetitive distances and average speeds of microspheres. The trajectory of each microsphere in the six regions (R1-R6) was monitored for 10 sec. (D and E) Repetitive distances and average speeds of microspheres. The trajectory of each microsphere in the six regions (R1–R6) was monitored for 10 sec.

oviductal fluid flows that are implicated in the migration of sperm. To ascertain this possibility, female mice were mated with vasectomized mice and administered with saline or padrin (5.0 mg/kg/injection) every 30 min for a total of eight s.c. injections over a period of 3.5 h (Fig. 5A). Fluorescent polystyrene microspheres (1-μm diameter) were injected into the oviduct in the region between R6 and R7 4 h after mating, and then the motions of microspheres were analyzed by time-lapse imaging (Fig. 5, B, C, and Supplemental Movie S2). Most microspheres in the saline-administered mice showed repetitive motions at R1, R2, R3, and R4 in the range of 350-650 μm (Fig. 5D). The flow rates of microspheres were estimated to be approximately 65, 53, 44, and 35 μm/s at R1, R2, R3, and R4, respectively (Fig. 5E). Importantly, the repetitive motions were barely observed at R5 and R6, and the range was very narrow (less than 81 μm). In the case of mice administered with padrin, no repetitive motion of microspheres was found in all oviductal regions examined (Fig. 5C and Supplemental Movie S2). The microspheres exhibited two different motions; a unidirectional motion toward the uterus at R1, R2, R3, and R4, and a random drift motion in a very narrow range at R5 and R6 (Fig. 5, B, C, and Supplemental Movie S2). Thus, the back-and-forth/shuttling flows of oviductal fluids are generated around the isthmus by myosalpinx contractions, although the oviductal fluids remain motionless in the region near the ampulla. These data also suggest that myosalpinx contractions may not be uniform along the oviduct at least in the mouse; there are intense myosalpinx contractions only around the isthmus when ejaculated sperm migrate through the oviduct.

A Combination of Oviductal Shuttling Flows and Cell Motility Is Involved

in the Migration of Sperm from the Isthmus to the Ampulla

To examine the migration of ejaculated sperm through the oviduct further, female mice were mated with transgenic mice expressing GFP and RFP in the acrosome and mitochondria, respectively (Hasuwa et al., 2010). The female mice were then administered with saline or padrin (5.0 mg/kg/injection) every 30 min for a total of four s.c. injections over a period of 90 min. When the living oviducts were pulled out from the saline-administered mice 2 h after mating and directly observed without surgical excision (Fig. 6), tightly packed and loosely packed assemblages of sperm were found at R1 and R2 and at R3 and R4, respectively (Fig. 6A and Supplemental Movie S3). Importantly, the sperm assemblage was synchronized with the shuttling flows of oviductal fluids in the region between R1 and R4. I also observed occasionally that a small portion of the sperm assemblage is incorporated into another assemblage between R1 and R2. At R3 and R4, some sperm were released from the sperm assemblage, and swam with their own motility. Sperm also swam at R5 and R6 where no assemblage was found. In the case of padrin-administered mice, both myosalpinx contractions and sperm assemblage were not observed at R1 (Fig. 6B and Supplemental Movie S3). Indeed, sperm randomly swam in this region. Moreover, consistent with the observation shown in Figs. 1 and 2, a small number of sperm were found in the region between R2 and R6. Most sperm bound on the surface of epithelial cells and exhibited flagellar motility at R2, R3, and R4 (Supplemental Movie S3). These results suggest that the shuttling flows of oviductal fluids produced by myosalpinx contractions may play an important role in the transport of sperm as an assemblage from the isthmus to the middle region of oviduct (Fig. 7). It

also suggests that the sperm motility may be required for the migration of sperm to the ampulla.

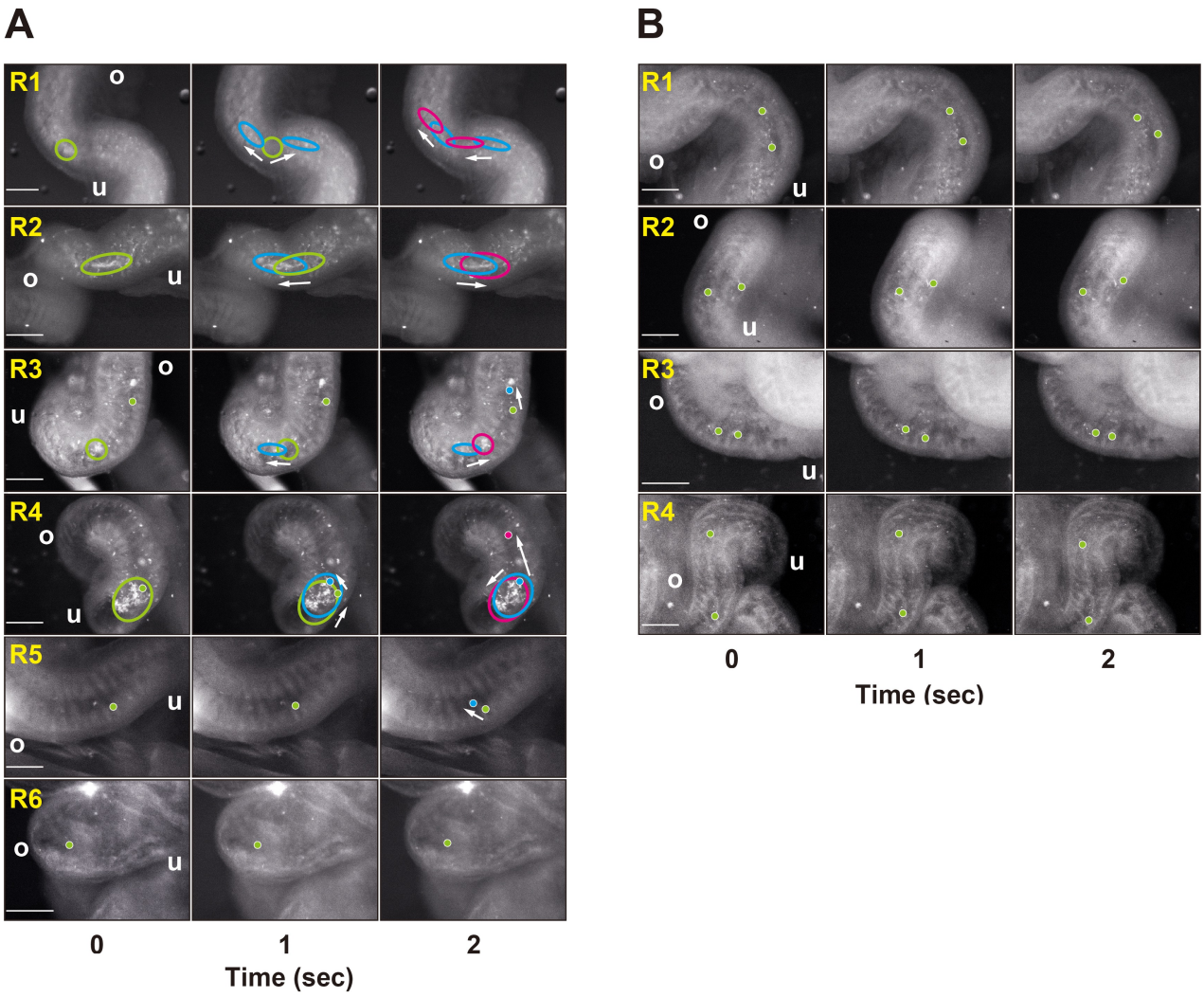

Fig. 6. Live-imaging of sperm motions in the oviduct. (A and B) Female mice were mated with transgenic mice expressing GFP and RFP in the acrosome and mitochondria, respectively, and administered with padrin-free saline (A) or padrin (B; 5.0 mg/kg/injection) every 30 min for a total of four s.c. injections over a period of 90 min. The oviducts were pulled out from the saline- or padrin-administered mice 2 h after mating, and immediately observed without surgical excision. The motion of sperm assemblage (green open circles) is successively shown by blue and red open circles after 1 and 2 sec, respectively. Tightly packed and loosely packed sperm assemblages were found in R1-R2 and R3-R4 (R1-R4 in A, see Supplemental Movie S3), respectively. The sperm motions are also indicated by green, blue, and red dots. Arrows indicate the positional changes of sperm and sperm assemblage. The sides of ovary and uterus are indicated by o and u, respectively. Scale bars = 200 μm.

DISCUSSION

This study describes the role of myosalpinx contractions in the migration of sperm through the oviduct: the formation of sperm assemblage in the isthmus and the transport of the assemblage from the isthmus to the middle region of oviduct. Ejaculated sperm have been believed to be capacitated on the surface of epithelial cells in the isthmus, detach from the cells, and swim to reach the oocytes in the ampulla (DeMott and Suarez, 1992; Yanagimachi, 1994). My data show that suppression of myosalpinx contractions by padrin administration results in a remarkably decreased number of sperm ascending from the uterus to the isthmus via the UTJ. Myosalpinx contractions produce unique shuttling flows of oviductal fluids between the isthmus and the middle region of oviduct. Moreover, regardless of padrin administration, the region near the ampulla displays little or no shuttling flows. Thus, my data suggest that the transport of sperm from the isthmus to the upper oviductal region is mainly dependent on oviductal fluid flows generated by myosalpinx contractions, and the sperm migration toward the ampulla is switched by the sperm motility in the middle region of oviduct.

As ejaculated sperm approach the ampulla, the number of sperm has been reported to dramatically decrease; the sperm numbers in the isthmus and ampulla are found to be >100 and 10-20, respectively, although sperm are abundantly present in the UTJ after mating (Fujihara et al., 2013; Hasuwa et al., 2010; Kawano et al., 2014; Muro et al., 2016; Spina et al., 2016). Our present data obtained by artificial insemination appear consistent with the above findings. Despite the abundance of sperm in the UTJ 6 h after mating, more than 200 and approximately 20 sperm are

found in the isthmus (R1 and R2) and ampulla (R7), respectively. Importantly, the number of sperm transported from the uterus to R1 via the UTJ was decreased by padrin administration in a dose-dependent manner. Thus, myosalpinx contractions contribute to the transport of sperm from the uterus to the UTJ and subsequently to the isthmus.

Chemotaxis, thermotaxis, and rheotaxis have been suggested to guide sperm to the oocytes in the ampulla (Bahat et al., 2003; Miki and Clapham, 2013; Spehr et al., 2003). Because high-speed shuttling flows of oviductal fluids are produced by myosalpinx contractions around the isthmus, these three taxes appear to be unlikely involved in the migration of sperm at least in the isthmus region. The mode of sperm transport, which is assisted by the shuttling flows around the isthmus, may be explained as follows. Sperm ascending from the uterus form an assemblage in the isthmus, as described above. The high-speed shuttling flows carry the sperm assemblage back and forth around the isthmus. The sperm assemblage is then transported gradually to the upper region of oviduct under the back-and-forth motions of oviductal flows. I emphasize that the gradual movement of sperm assemblage is the predominant mechanism for the transport of sperm around the isthmus. However, it is still unclear at present whether the sperm assemblage is already formed in the uterus and UTJ, although myosalpinx contractions participate in the sperm transport from the uterus to the UTJ. I also note that sperm of deer mice (genus *Peromyscus*) form motile aggregations *in vitro* (Fisher and Hoekstra, 2010; Fisher et al., 2014). The sperm aggregates are generated by unrelated conspecific sperm in a monogamous strain, whereas sperm of a highly promiscuous strain show the tendency to aggregate with the same male sperm. If the aggregations are implicated in the formation of sperm

assemblage in the isthmus, comparative studies between the monogamous and promiscuous strains may provide an intriguing hint for the migration of sperm in the female reproductive tract.

The assemblage of sperm is loosened at R3 and R4, and consequently some sperm are released from the assemblage. Since reduced shuttling flows of oviductal fluids are still produced in these regions, the released sperm are required to swim upstream against the current. Rather, sperm may resist the back-flow of oviductal fluids to avoid the return to the original location. If so, both motility and rheotaxis of sperm may play a crucial role in the resistance to the back-flow. On the other hand, the status of oviductal flows in the region near the ampulla is totally different from that in the isthmus region. Noteworthy is that oviductal fluids very gently flow through R5 and R6, and hence the sperm motility is essential for the migration from these regions to the ampulla. I speculate that the chemotaxis, possibly in addition to rheotaxis, may start near R5 and R6, which is necessary for the sperm access to the oocytes. Taken together, my data suggest that the migration of sperm is mediated by myosalpinx contractions around the isthmus, and then by sperm motility near the ampulla.

As described above, peristaltic pumping of myosalpinx contractions has been demonstrated to function in the transport of fertilized eggs/embryos through the oviduct (Croxatto, 2002; Halbert et al., 1975; Talo 1991). In the mouse, the embryos need 3.5 days to be transported from the ampulla to the uterus (Finn and Martin, 1974). Our preliminary data indicate that approximately 20 and 100% of mouse oocytes are fertilized 2 and 6 h after natural mating, respectively (Usui, master's thesis). Despite the same duct, the time of sperm transit differs from that of embryo

transport. This may be explained by the differences in the cell size and motility between sperm and embryos. Indeed, the sizes of embryo and sperm head are 80 and 7 μm in the mouse, respectively (Yanagimachi, 1994). Since the embryo size is much bigger than the sperm head size, the transport of embryos through the oviduct may require a stronger peristaltic action and/or a faster flow of oviductal fluids. Another possibility is that myosalpinx contractions, which are produced during the sperm and embryo transports, are totally different from each other. Enhanced myosalpinx contractions may be generated around the isthmus when sperm migrate through the oviduct. In contrast, the embryos may be transported from the ampulla to the uterus by relatively mild myosalpinx contractions that occur uniformly throughout the oviduct. The mechanism underlying the embryo transport in the oviduct still remains to be fully elucidated.

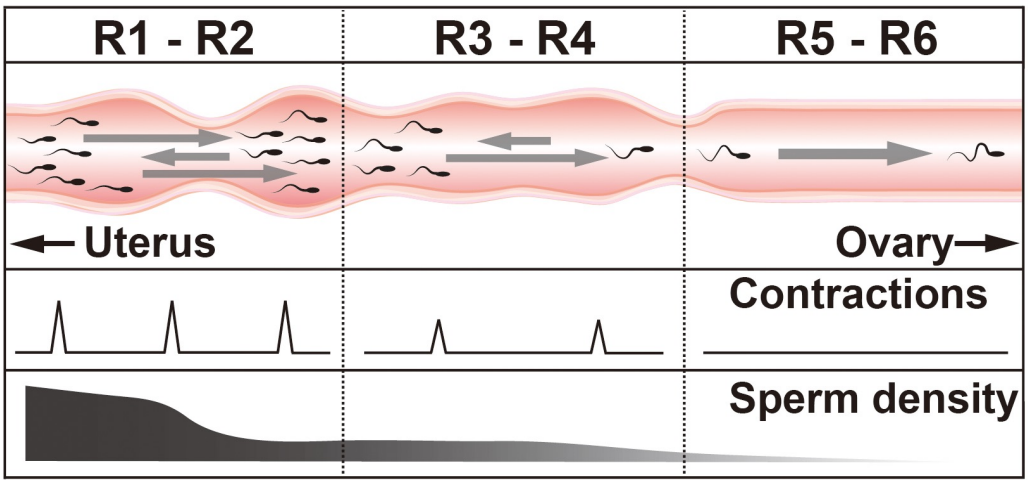

Fig. 7. A schematic model for sperm migration through the oviduct. The assemblage of sperm is transported by shuttling flows of oviductal fluids around the isthmus (R1 and R2). Some sperm are released from the assemblage at R3 and R4. Then, at R5 and R6, sperm are required to swim toward the ampulla because no shuttling flows are generated in these regions.

CHAPTER II

Endothelin-independent pathway induces contractions of mouse oviductal epithelial cells

SUMMARY

The female reproductive tract is indispensable for the transport of ejaculated sperm and early embryos to accomplish fertilization and implantation, respectively. Radially symmetrical contractions and relaxations of smooth muscles (myosalpinx) generate oviductal peristalsis and function in the embryo transport from the oviductal ampulla to the uterus. Shuttling flows of oviductal fluids regulated by myosalpinx contractions contribute to the transport of sperm from the isthmus to the middle region of oviduct. Although a well-known vasoactive peptide, endothelin, has been reported to induce oviductal peristalsis during the embryo transport, the key molecules involved in the oviductal contractions during the sperm migration are still unknown. This study aims to identify oviductal contraction-inducing factors by a collagen gel contraction assay system using oviductal epithelial cells (OEC). As compared with endothelin-3 isoform, oviductal extracts greatly induced contractions of OEC. The OEC contractions were not inhibited by endothelin receptor antagonist tezosentan. When oviductal extracts were separated by reverse-phase high-performance liquid chromatography (HPLC), several peaks exhibiting the contraction activity were found. Moreover, microarray of the oviducts indicated that genes involved in muscular contractions were upregulated in the period of sperm migration. These results suggest that oviductal extracts may contain OEC contraction-inducing factors that act independently of the endothelin pathway in sperm migration.

INTRODUCTION

The female reproductive tract, including the vagina, cervix, uterus, and oviduct, plays essential roles in the reproductive process. The oviduct, a narrow tube connecting the periovarian space with the uterus, renders a specific microenvironment to accomplish fertilization (Yanagimachi, 1994). Rhythmic symmetrical contraction/relaxation of the myosalpinx and beating of epithelial cilia generate oviductal fluid flows, which assist the transport of gametes and embryo through the oviduct (Fig. 8A; Talo, 1991).

Oviductal contractions are regulated by several factors, including endothelin (ET), prostaglandin (PG), nitric oxide (NO), and thromboxane by autocrine/paracrine system during the embryo transport, (Bridges et al., 2011; Martinez et al., 1998; Nabekura et al., 1994). Endothelin was identified as a potent vasoactive peptide regulating vascular tone and blood pressure (Yanagisawa et al., 1988). There are three isoforms of endothelins (ET1, ET2, and ET3) encoded by three different genes (Bridges et al., 2011). Recent studies have demonstrated that ETs stimulate release of PGs (PGE_2 and $PGF_{2\alpha}$) that induce muscular contractions of the oviduct in the human, bovine, and rat (Al-Alem et al., 2007; Wanggren et al., 2008; Wijayagunawardane et al., 2001) (Fig. 8B). These findings suggest that ETs have an important role in the regulation of oviductal contractions.

Sperm motility is crucial for migration through the female reproductive tract to reach the oocytes in the oviductal ampulla for fertilization (Yanagimachi, 1994). My data in CHAPTER I demonstrated that shuttling flows of oviductal fluids regulated by myosalpinx contractions also contribute to the transport of sperm from the isthmus to the middle region of oviduct (Fig. 8A). Endothelins and endothelin

receptors are expressed in oviductal epithelial cells in mice at the ovulation period, suggesting that requirement of endothelin-regulated function in the epithelial cells (Jeoung et al., 2010). Although ETs induce oviductal contractions in the embryo transport, it is still unclear whether these molecules are involved in contractions in sperm migration (Fig. 8B).

In this study, I attempted to identify oviductal contraction-inducing factors during sperm migration in the mouse oviduct. To evaluate contraction-inducing activity, a collagen gel contraction assay using primary mouse oviductal epithelial cells (OEC) has been established (Bell et al., 1979). Purified oviductal extracts by reverse-phase high-performance liquid chromatography (HPLC) induced decreased area of OEC collagen gels even in the presence of vasoactive peptide inhibitor, tezosentan. These results suggest that OEC contraction-inducing factors other than ETs may be involved in this process. A possible mechanism of oviductal contractions during sperm migration through the oviduct is discussed.

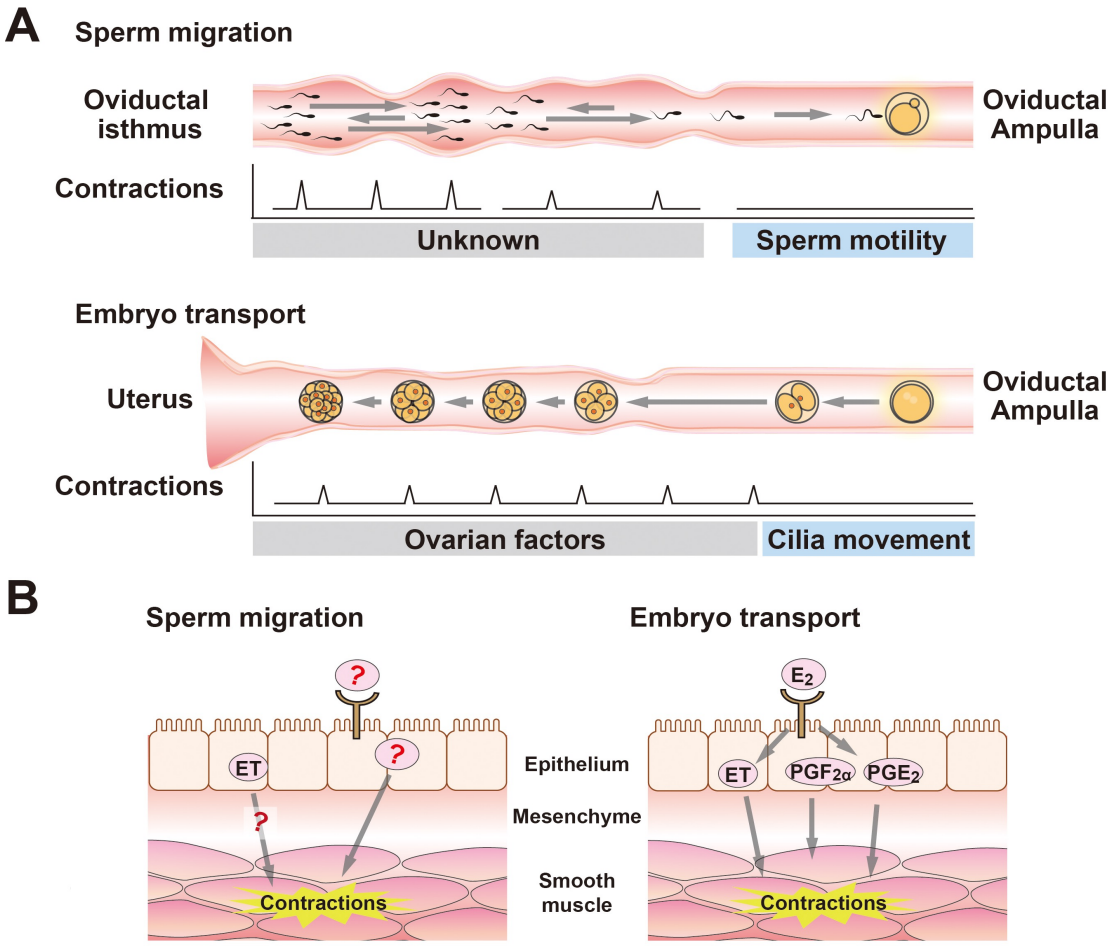

Fig. 8. Schematic diagram for the transport of sperm and embryos in the oviduct in mice. (A) Ejaculated sperm ascend from the uterus to the ampulla to fertilize the oocytes (Upper panel). Shuttling flows of oviductal fluids are generated by myosalpinx contractions and function in the transport of sperm. The sperm motility may be required for the migration of sperm with the approach to the ampulla. Oviductal peristalsis and ciliary motility play an important role in the embryo transport (Lower panel). (B) A well-known vasoactive peptide, endothelin (ET), and prostaglandin (PGE_2 and $PGF_{2\alpha}$) under the control of estrogen (E_2) have been reported to induce oviductal peristalsis during the embryo transport (Right panel). However, it is still unknown whether ET induces oviductal contractions not only in embryo transport but also in sperm migration (Left panel).

MATERIALS AND METHODS

Animal Experiments

All animal experiments were ethically carried out according to the Guide for the Care and Use of Laboratory Animals at University of Tsukuba and NIH Animal Care and Use Committee guidelines.

Preparation of the Oviductal Extracts

Six ICR strain mice (8-wk old; Japan SLC, Shizuoka, Japan) were superovulated by intraperitoneal injection of pregnant mare's serum gonadotropin (PMSG; 5 units; ASKA Pharmaceutical, Tokyo, Japan) followed by human chorionic gonadotropin (hCG; 7.5 units; ASKA Pharmaceutical) 48 h later, as described previously (Ishikawa et al., 2016). The oviducts were excised 14 h after injection of hCG corresponded to 0.1 dpc (dpc, day post coitus), perfused to remove oviductal fluids, boiled for 10 min in 200 µl of MilliQ water (Merck, Burlington, MA). Boiled oviducts (approximately 0.2 g) were homogenized with 10 times extraction buffer (0.1 M acetic acid, 20 mM HCl) at 4°C by using Dounce homogenizer. The oviductal extracts were obtained by sequential centrifugation at 3,300 × *g* and 13,000 × *g* for 30 min at 4°C, and filtered by a Millix PETF membrane (Merck) to remove cellular debris, insoluble proteins, and lipid of the oviducts. The extracts were precipitated with acetone at -80°C. The supernatant fluids including peptides were prepared at 4°C by centrifugation at 16,000 × *g* for 60 min and dissolved in 20 µl of MilliQ.

Separation of the Oviductal Extracts by Reverse-phase liquid chromatography

Extracts of 24 oviducts from mice (8-wk old) were dissolved in 150 μl of 0.05% trifluoroacetic acid (TFA)/H_2O (Wako, Tokyo, Japan), filtered by 0.45 μm of a syringe filter (Milliex®-LH, Merck), and then the extracts (100 μl) were separated on reverse-phase liquid chromatography by a Waters Empower2 software (Waters Corporation, Milford, MA) equipped with binary solvent manager, sampler manager, column compartment, photo diode array (PDA) detector. A COSMOSIL® Protein-R (4.6 mm I.D. × 250 mm; nakarai tesque, Kyoto, Japan) with a COSMOSIL Protein-R Guard Column (4.6 mm I.D. × 10 mm ; nakarai tesque) were used for separation. The column temperature was maintained at 30°C. The retention was carried out at 1.0 ml/min with 0.05% TFA/H_2O for 10 min. Elution was achieved with a gradient of 0-60% 0.05% TFA/Acetonitrile (ACN; Wako) in 30 min (1 ml/min) and 60-100% for 10 min (1 ml/min) by using a Waters 2695 Separation Module. Eluates were monitored by UV absorbance at 210 nm and collected for 40 min. By using FDU-830 (Tokyo Rikakikai Co, Ltd, Tokyo, Japan), fractions were then lyophilized and stored at −80°C until use.

Collection and Culture of the Oviductal Epithelial Cells

Five ICR strain mice (8-wk old) were sacrificed 48 h after the injection of PMSG (Umezu et al., 2003). The oviducts were excised and placed on petri dishes containing cold PBS (2 ml) to remove blood, soaked with 0.25% trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA) by

injection using a 30 G needle inserted from the infundibulum and soaked for 30 min on ice followed by incubation for 30 min at 37°C. Oviductal epithelial cells (OEC) were then prepared from the oviduct by using a 30 G needle, collected by centrifugation at 180 × *g* for 5 min, and cultured in Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM/F12) (Wako) containing 10% fetal bovine serum (10% FBS; Gibco, Thermo Fisher Scientific, Waltham, MA), 100 U/ml of penicillin streptomycin (Gibco), and 0.1 mg/ml of L-glutamine (Gibco) at 37°C under 5% CO_2 in air.

Immunostaining of OEC

Monolayers of OEC were grown on cover slips for 48 h. The OEC were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS, pH 7.4, at 4°C for 60 min, washed with cold PBS, and treated with 0.1% Triton X-100 in PBS at room temperature for 30 min. Permeabilized cells were blocked with 3% (v/v) normal goat serum in PBS containing 0.05% Tween-20 on ice for 60 min, washed with cold PBS, incubated with anti-E-cadherin (Takara Bio Inc., Shiga, Japan), anti-β-catenin (Sigma-Aldrich, St. Louis, MO), anti-vimentin (Sigma-Aldrich), and anti-estrogen receptor α (Sigma-Aldrich) antibodies for 60 min, and reacted with Alexa Fluor 488 or Alexa Fluor 568-conjugated anti-mouse, or anti-rabbit IgG antibodies for 60 min. After washing with PBS, the nuclei were stained with Hoechst 33342 (2.5 μg/ml) for 5 min, washed with PBS, mounted, and observed under an IX-71 fluorescence microscope (Olympus, Tokyo, Japan). The images of OEC were obtained by a MetaMorph Imaging software (Molecular Devices, Sunnyvale, CA).

OEC Contraction Assay

The OEC were recovered from a culture dish using 0.05% trypsin/EDTA, and washed with and suspended in DMEM/F12 (Wako). According to the manufacture's instruction, Type I collagen, DMEM (5 times concentrated), and reconstituting buffer (Nitta Gelatin, Osaka, Japan) were mixed carefully on ice at a ratio of 8:1:1. The mixture was placed in 24-well plates and allowed to gel for 20 min at 37°C under 5% CO_2 in air (Jia et al., 2016; Sakota et al., 2014). The OEC (5.5×10^4 cell/well) was then added onto the gel in each well. The gel was floated from a wall by using a sterile spatula 2 h after incubation. For collagen contraction assay, 5 µl oviductal extracts dissolved in 20 µl of MilliQ, 100 nM of endothelin-3 (ET3, human) peptides (Peptide Institute Inc., Osaka, Japan), and 200 nM tezosentan (Actelion) were added to each well. A collagen gel size was observed every 12 h for 36 h, captured from the upper of plate, and analyzed by using an ImageJ software (http://rsbweb.nih.gov/ij/).

Microarray of the Oviducts

CF-1 female mice (8-wk old; Harlan Laboratories) were determined as the proestrus and estrus using vaginal smear cytology. Naturally cycling female mice were mated with B6D2F1/J males (8- to 12-wk old; Jackson Laboratory) at 6:30-8:30 AM, and plugs were checked at 8:30 AM. The oviducts were collected at the proestrus, 0.1 dpc, 0.5 dpc, and 1.5 dpc and total RNAs were extracted as previously described (Hewitt and Korach, 2011). Gene expression analysis was conducted using Agilent

Whole Mouse Genome 4 × 44 multiplex format oligo arrays (Agilent Technologies, Santa Clara, CA). ANOVA was used to detect differentially expressed genes between groups and fold changes based on error-weighted ratios built in OmicSoft Array Studio (QIAGEN®, Cary, NC). Gene lists were generated using a false discovery rate < 0.05 and absolute value fold ≥ 2.0. After normalized data were transformed to log(2), hierarchical clustering was done using Morpheus clustering method (https://software.broadinstitute.org/morpheus/). Gene Ontology Consortium (http://geneontology.org/) and Reactome (https://reactome.org/) were used for static analyses.

Measurement of Myosalpinx Contraction

The oviducts at the proestrus and 0.1, 0.5, and 1.5 dpc were excised, placed on petri dishes containing 5 ml of DMEM/F-12 (Thermo Fisher Scientific) supplemented with 10% FBS, 100 U/ml penicillin streptomycin, and 0.1 mg/ml L-glutamine at 37°C under 5% CO_2, and observed under an EVOS® FL Auto Cell Imaging System (Thermo Fisher Scientific). The frequencies of myosalpinx contractions were counted for 60 min.

Statistical Analysis

Data are presented as the mean ± S.E.M ($n \geq 3$). The Student's *t*-test was used for statistical analysis; significance was assumed for $p < 0.05$.

RESULTS

Frequency of Oviductal Contractions in 0.1 dpc and 1.5 dpc

Oviductal contraction, generated by radially symmetrical contraction and relaxation of smooth muscles, has been reported to function in embryo transport and sperm migration (Talo, 1991; Ishikawa et al., 2016). To examine frequencies of contractions in sperm migration and the embryo transport, fresh oviducts were excised from mice at the proestrus, and 0.1, 0.5, and 1.5 dpc (Fig. 9, A-D). The number of oviductal contractions in 0.1 dpc (sperm migration) and 1.5 dpc (embryo transport) was larger than the proestrus and 0.5 dpc (Fig. 9E and Supplemental Movie 4). No significant difference of oviductal contraction in number was found between the 0.1 and 1.5 dpc oviducts. It is noted that contractions number at the proestrus (prior to the ovulation) were limited compared to other samples. The oviducts exhibited two patterns of myosalpinx contractions at 0.1 and 1.5 dpc: peristaltic movement and non-propulsive peristalsis. Peristaltic movements were wave-like muscular contractions along the oviduct. Non-propulsive peristalsis simultaneously occurred at several positions without propagation (Supplemental Movie 4). These results imply that the contraction frequencies are increased during the sperm and embryo transport in the oviduct.

Collagen Gel Contraction Assays Using Oviductal Epithelial Cells

As described above, the layers of epithelial cells contract in coordination with smooth muscle contractions. Since oviductal epithelial

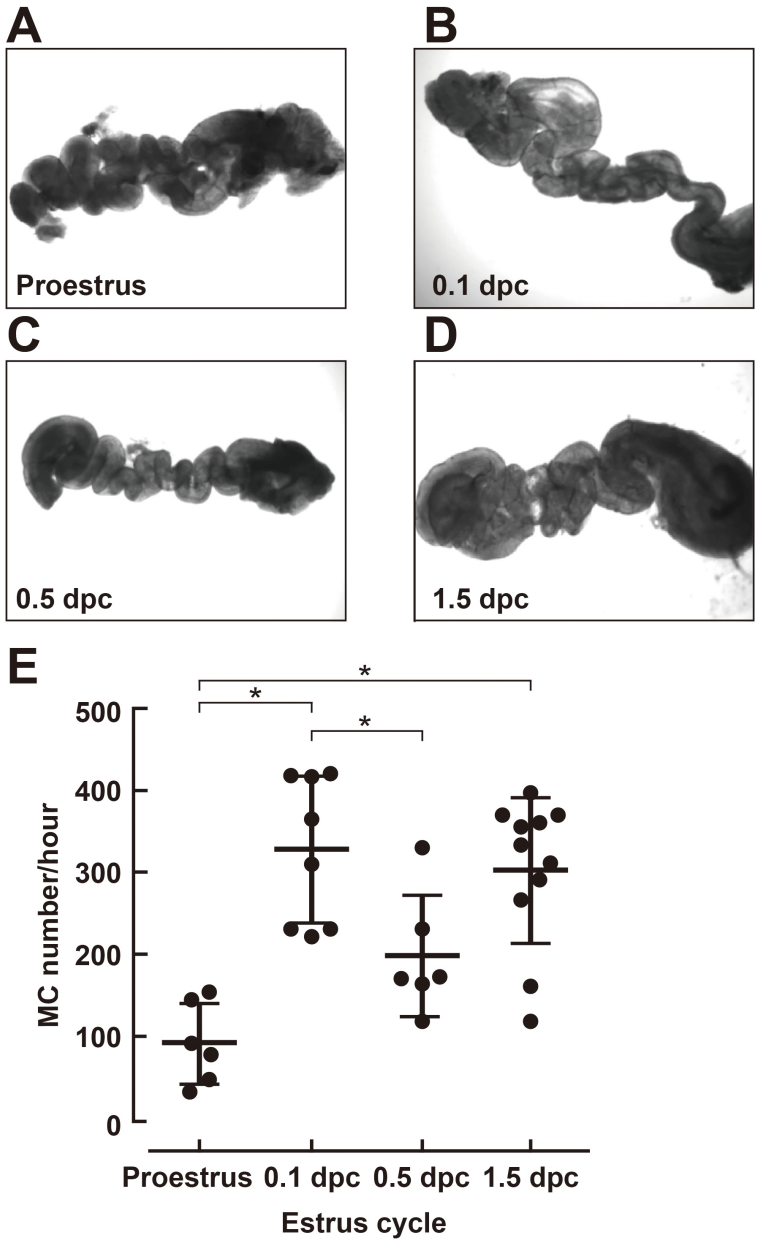

Fig. 9. Frequencies of myosalpinx contractions. (A-D) The oviducts were excised at the proestrus (A), 0.1 dpc (B), 0.5 dpc (C), and 1.5 dpc (D), and incubated in conditioned medium. (E) The frequencies of myosalpinx contractions (MC) in the whole oviduct were counted for 60 min. Each dot represents an oviduct. *, $p < 0.05$.

cells interact with gamete and embryos and maintain microenvironment in the oviduct (Coy et al., 2012), epithelial cells may also participate in oviductal contractions.

To examine whether the oviduct possesses endogenous factors that induce oviductal contractions via the epithelial cells during the sperm migration, I attempted to culture OEC *in vitro* (Fig. 10A). Primary cells prepared from the oviducts were typical epithelial cell morphology with rounded shape (Fig. 10B). These cells initially consisted of both ciliated and nonciliated secretory cells (Abe and Hoshi, 1997). Although ciliated cells were recognized by an active movement in the first day of culture, these cells were not observed in the second day. Cultured OEC were characterized by a female reproductive cell marker, estrogen receptor α, and epithelial cell markers, E-cadherin and ß-catenin, and intermediate filament protein, vimentin. Cultured cells strongly cross-reacted with anti-estrogen receptor α, anti-E-cadherin, and anti-β-catenin antibodies, but not anti-vimentin antibody (Fig. 10C).

Induction of OEC Contractions by Oviductal Extracts

To verify whether the oviduct possesses contraction factors, an OEC contraction assay system was established. A collagen gel contraction assay system has been developed to assess contractions of cells embedded in collagen gel matrices by measuring the gel size (Sakota et al., 2014). It is widely used for fibroblast, epithelium, myocyte, and cardiac cells to evaluate activity of contractions (Bell et al., 1979; Lazar-Karsten et al., 2016; Martin, 1997; Mochitate et al., 1991; Stupak and Harris, 1982; Tian et al., 2002). Contraction assays were carried out using OEC embedded

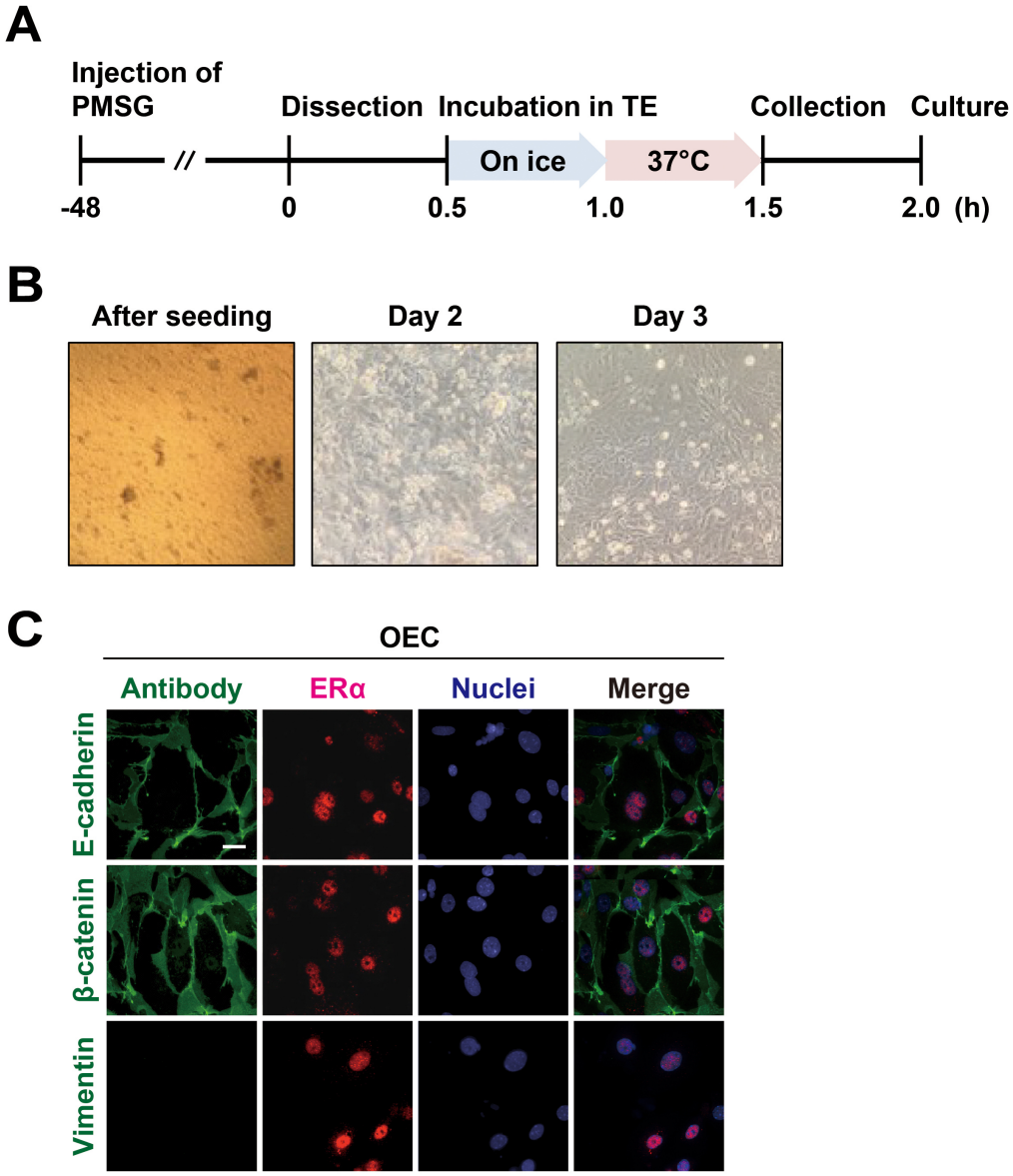

Fig. 10. Establishment of collagen contraction assay of oviductal epithelial cells. (A) Experimental scheme. The oviducts were dissected from female ICR mice 48 h after injection of PMSG and soaked in 0.25% trypsin-EDTA (TE) for 30 min on ice, and incubated for 30 min at 37°C. Oviductal epithelial cells (OEC) were then collected and cultured in DMEM/F12 containing 10% FBS at 37°C under 5% CO_2 in air. (B) Morphology of OEC after seeding and at 2nd and 3rd day of incubation. (C) Immunostaining of OEC. The OEC were stained with antibodies against E-cadherin, β-catenin, vimentin (green), and estrogen receptor α (red). The nucleus was stained with Hoechst 33342 (blue). Scale bar = 30 μm.

in collagen gel in DMEM/F12 medium without 10% FBS after addition of contractile factors (Fig. 11A). The OEC suspension (5.5×10^4 cells) was placed on the collagen gel in 24-well plates, and the area of collagen was measured every 12 h for 36 h (Fig. 11B). In the case of endothelin-3 (ET3), the OEC contractions were observed. When extracts from the oviduct prepared 14 h after hCG injection, which corresponded to 0.1 dpc, were added to the OEC collagen, the size of collagen was decreased compared in the absence of extracts (Fig. 11, C and D). ET3-induced contractions were inhibited by tezosentan, a dual endothelin receptor antagonist. However, tezosentan failed to suppress extracts-induced collagen gel contraction. These results suggest that the oviductal extracts may include OEC contraction-inducing factors.

Reverse-phase liquid chromatography of oviductal extracts

To purify and identify contraction factors, oviductal extracts were separated on a reverse-phase liquid chromatography of COSMOSIL® Protein-R with a linear gradient of 0-60% ACN. Several peaks monitored by UV absorbance at 210 nm were detected in fraction numbers 24-26 and 28-34 (Fig. 12A). When the collagen gel contractions were observed for 48 h, the fraction numbers 24, 25, and 26 exhibited higher activities of contraction than any other fraction examined (Fig. 12B). While the OEC contractions in fraction 24 were inhibited by 200 nM tezosentan, fractions 25 and 26 induced OEC collagen contractions even in the presence of tezosentan, (Fig. 13). These results suggest that fraction 24 contains ETs. It is also suggested that fractions 25 and 26 may contain oviductal epithelial cell contraction-inducing factors that act independently of the ET-mediated

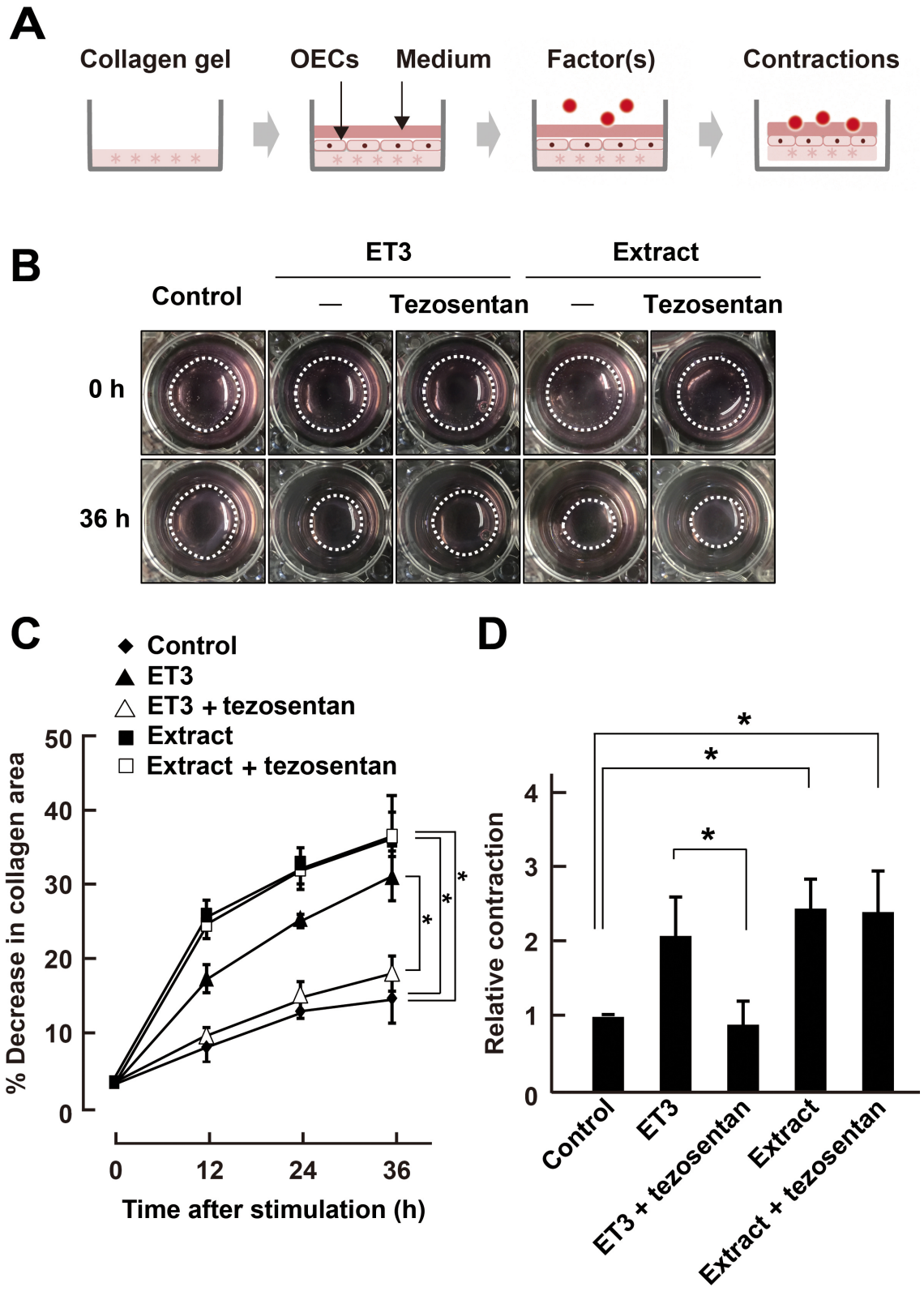

Fig. 11. The collagen contraction assay of oviductal extracts. (A) Experimental scheme of collagen contraction assay. The area of collagen after addition of contractile factors was measured to evaluate the contraction activity as time elapsed. (B) Representative pictures of collagen assays at 0 and 36 h. The OCEs were treated with 100 nM of ET3 peptide and oviductal extracts in the absence (−) and presence of 200 nM of endothelin receptor antagonist tezosentan. MilliQ was used as a control. Dotted circles indicate the gel area. (C) Decreased area of collagen area (n = 3). (D) Ratio of relative contraction at 36 h after stimulation. *, $p < 0.05$.

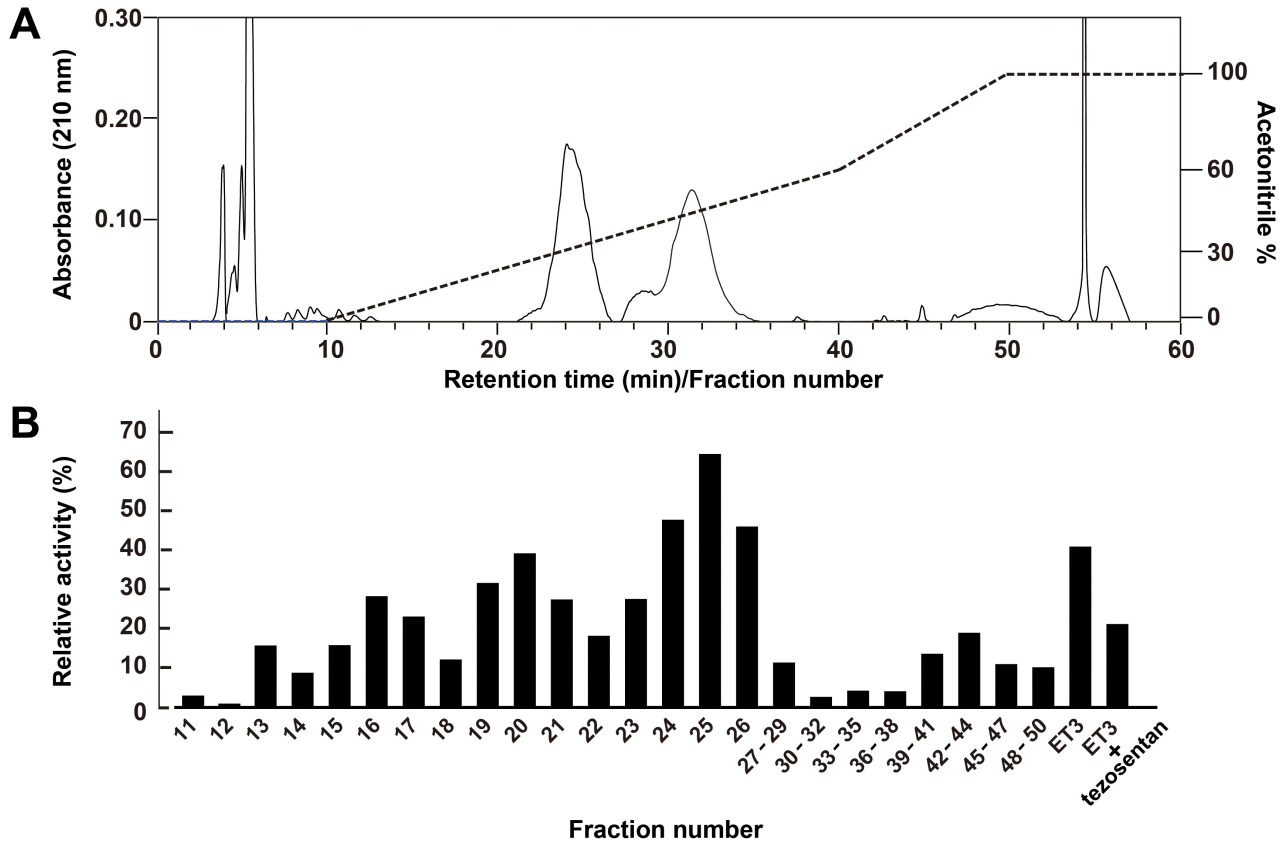

Fig. 12. Reverse-phase liquid chromatography of oviductal extracts on (A) Oviductal extracts were separated on COSMOSIL® Protein-R with a linear gradient of 0-60% acetonitrile (broken line). Eluates were monitored by UV absorbance at 210 nm (gray line). (B) Collagen contractions. The fractions were subjected to collagen contraction assays. ET3 in the absence and presence of 200 nM tezosentan are used as positive and negative controls for contractions, respectively.

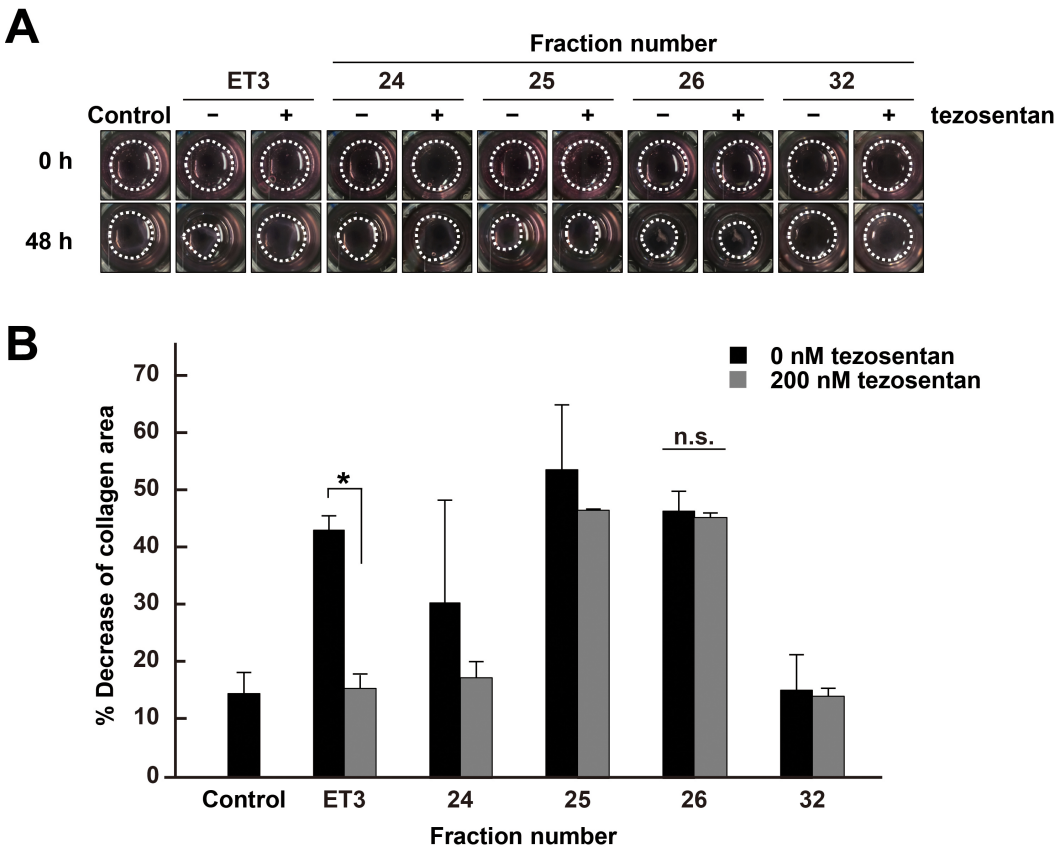

Fig. 13. The collagen contraction assays. (A) Representative pictures of collagen assays at 0 and 48 h after stimulation. Fractions in the presence 0 (−) or 200 nM (+) tezosentan were subjected to collagen contraction assays. MilliQ was used as a control. Dotted circles indicate gel areas. (B) Rate of decrease in collagen area. Black and gray bars indicate in the presence of tezosentan 0 and 200 nM, respectively. n.s., not significant. *$p < 0.05$.

pathway.

Regulation of Smooth Muscle Contraction-Inducing Factors in the Oviduct

Microarray analysis is widely used to determine global gene expression profiling of tissues for certain functions. To identify oviductal contraction-inducing factor(s), microarray analysis of the oviducts was carried out. As described in Fig. 9, the frequencies of myosalpinx contractions were altered at the proestrus, 0.1, 0.5, and 1.5 dpc. Hierarchical clustering indicated the signal intensities from microarray of 4 groups, the proestrus, and 0.1, 0.5, and 1.5 dpc of the oviduct (Fig. 14). The heat map of oviductal transcriptome indicated that biological functions responsible for inflammatory response, cell communication, signal transduction, metabolism, cell cycle, and muscle contraction were upregulated in the 0.1-dpc oviductal samples (Fig. 15, A-C). The genes related to cell contractions at 0.1 dpc were listed in Tables 1. The level of gene expression related to muscular contractions excluding *Rgs2* at 0.1 dpc was not upregulated in the 1.5-dpc oviducts (Fig. 15C). These results suggest that candidate gene(s) may induce oviductal contractions during the sperm migration.

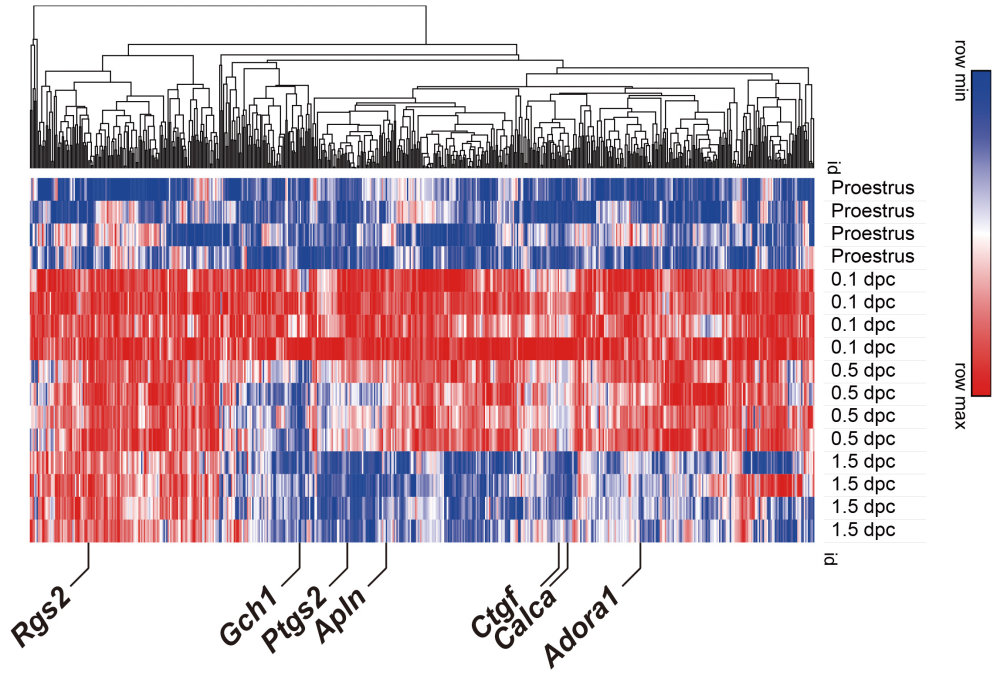

Fig. 14. Expressions of the oviductal genes. Hierarchical clustering of microarray data from the proestrus, and 0.1, 0.5, and 1.5 dpc. Using a 2.0-fold cutoff, 593 probes in the 0.1 dpc were significantly different from other oviducts. The heat map shows log2 in Morphalus. Blue and red color represents probes with intensity less than mean and more than mean, respectively. Represents data from a single animal; n = 4 mice/group.

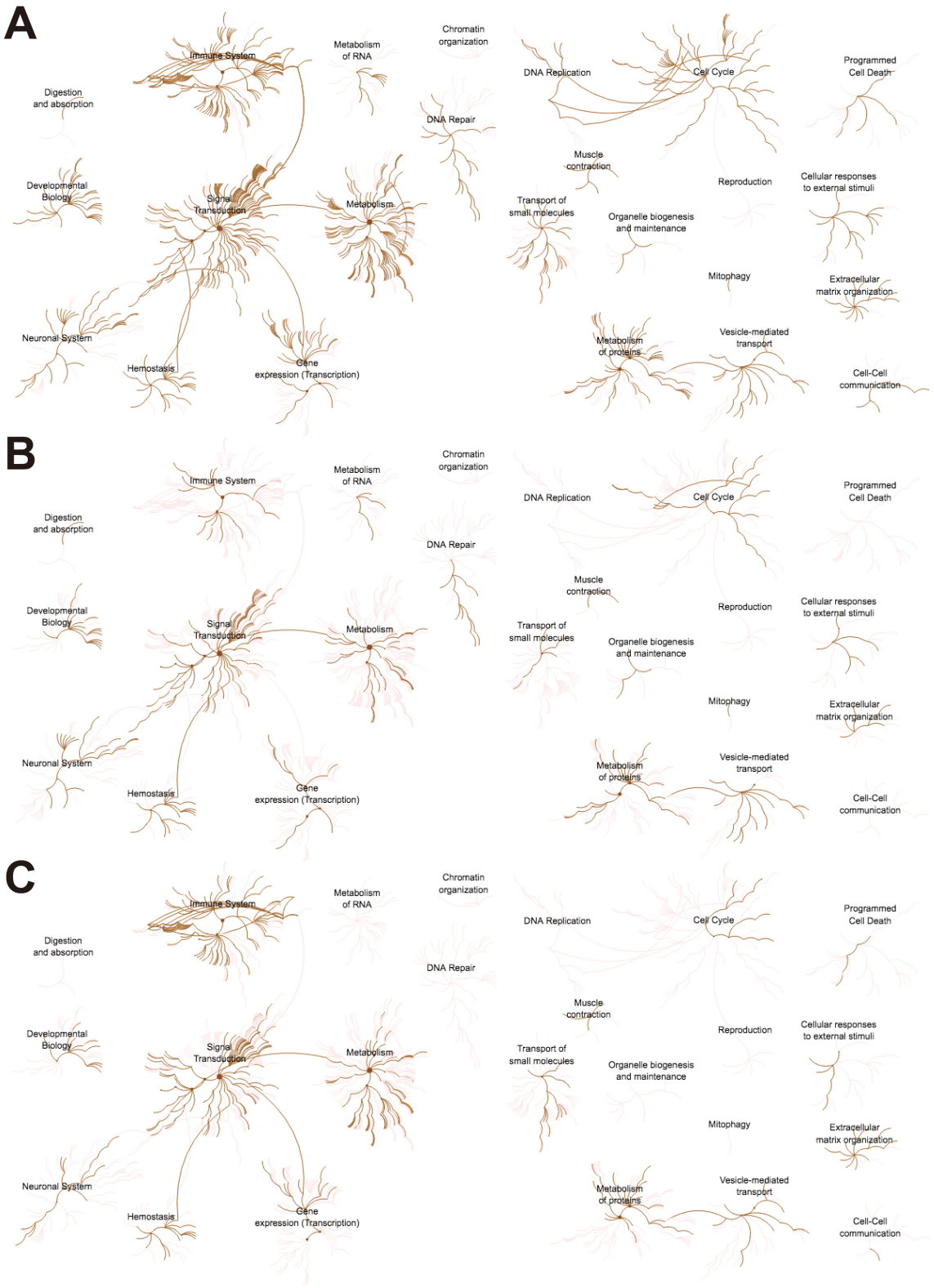

Fig. 15. Pathway analysis of upregulated gene in the 0.1 dpc oviducts. Each cluster represented biological functions from the reactome functional interaction network data. (A) Upregulated gene in the 0.1 dpc oviducts. (B) Upregulated genes of oviducts from 0.1 dpc to 1.5 dpc compared to the proestrus. (C) Upregulated genes of the 0.1 dpc oviducts compared to the 1.5 dpc oviducts. A single line of orange color indicates a gene.

Table 1. List of candidate factors for muscular contraction upregulated in the 0.1 dpc oviducts

Symbol	Name	Protein class
Rgs2	**Regulator of G-protein signaling 2**	**G-protein modulator**
Ctgf	**Connective tissue growth factor**	**Growth factor**
Apln	**Apelin**	**Peptide hormone**
Gch1	**GTP cyclohydrolase 1**	**Hydrolase**
Adora1	**Adenosine receptor A1**	**G-protein coupled receptor**
Ptgs2	**Prostaglandin G/H synthase 2**	**Oxygenase**
Calca	**Calcitonin**	**Peptide hormone**

DISCUSSION

This study was performed to identify oviductal contraction-inducing factors by using a collagen contraction assay of oviductal epithelial cells (OEC) in mice. Myosalpinx contractions were observed in the excised oviducts at 0.1 dpc (sperm migration) and 1.5 dpc (embryo transport) and were barely found in the proestrus. The frequencies of myosalpinx contractions were not significantly different between 0.1 dpc and 1.5 dpc. When extracts from the oviducts 14 h after hCG injection corresponded to 0.1 dpc were analyzed by HPLC, several peaks exhibiting the contraction activity were found. Oviductal extracts induced high contractile activities of OEC than ET3 did. The endothelin receptor antagonist, tezosentan, failed to inhibit contractions of OEC. Moreover, candidate factors for contractions were indicated by microarray data. These data suggest that oviductal epithelial cell contraction-inducing factors may act independently of the endothelin pathway during sperm migration.

It has been suggested that epithelium, the cell layer lining the luminal surface in organs, plays an important role in regulating smooth muscle contractions (Ruan et al., 2008; Ruan et al., 2010). Indeed, separation of the epithelium from the smooth muscle cells in the uterus resulted in decreased contractile responses of myometrium (Whalley, 1978). However, the mechanism underlying the regulation of smooth muscle contractions by the epithelial cells remains largely unknown (Ruan et al., 2010). This study indicates that factors including oviductal extracts induce epithelial contractions and the established bioassay is a valuable method to find candidate factors that induce contractions via epithelial cells in the oviduct (Fig. 11D and 13B). Further improvement of this bioassay system

is required to examine contractions in cultured epithelial cells with smooth muscle cells.

Smooth muscles play central roles in rhythmic contraction and relaxation (smooth muscle contractions) in several organs including the blood vessel, gastrointestinal tracts, and female reproductive tracts. Vascular endothelium and gastrointestinal epithelium produce hormonal peptides, endothelin, motilin, and ghrelin (Brown et al., 1979; Kojima et al., 1999; Yanagisawa et al., 1988). Secreted peptides bind receptors expressed on the surface of smooth muscle cells for activation of contractions via autocrine and paracrine systems. Oviductal contraction-inducing factors may be identical to well-known peptides and receptors in other tissues. Indeed, ETs have been reported to express in epithelial cells in the oviduct and to function in oviductal contractions during the embryo transport (Al-Alem et al., 2007; Jeoung et al., 2010). In contrast, the OEC collagen contraction assay indicated that the oviduct contains contraction-inducing factor besides ET3. The present study may raise the possibility that oviductal contractions utilize unidentified factors for regulating in sperm migration.

Despite the similarity in contraction frequencies in the oviduct at 0.1 dpc and 1.5 dpc (Fig. 9), expression of muscular contraction-related genes, including *Ctgf*, *Apln*, *Gch1*, *Adora1*, *Ptgs2*, and *Calca*, were only upregulated at 0.1 dpc and decreased at 1.5 dpc (Fig. 14). This result may be attributed to the difference between *in vivo* and *in vitro* states of contractions. In mice, ejaculated sperm migrate from the isthmus to the middle region of the oviduct by the aid of myosalpinx contractions. Sperm swim toward the ampulla by their motility at the minimum 2 h (Ishikawa et al., 2016). Embryos are transported from the ampulla to the isthmus, and it

takes 3.5 days to enter the uterus after fertilization (Humphrey, 1968), suggesting that contraction frequencies in the 1.5 dpc oviduct are different from 0.1 dpc. In this study, the oviducts were excised from mice and were incubated in cultured medium to count myosalpinx contractions *in vitro*. Excised oviducts at 1.5 dpc did not necessarily reflect physiological state of myosalpinx contractions *in vivo*.

Oviductal contractions play an important role in the sperm migration and embryo transport in the oviduct (Finn and Martin, 1974; Ishikawa et al., 2016). The oviduct plays an important role in the transport of sperm and embryos in opposite directions (Parker, 1931). These transport processes of are dissimilar from the swallowing of food by the gastrointestinal tracts. For instance, circulation system in our body maintains a one-way flow to prevent the increase of gastric acid by eating in the gastrointestinal tract. In *Drosophila melanogaster*, the oocytes are produced in the ovaries and move through the oviduct to the uterus where they are fertilized with sperm (Heifetz et al., 2005). Thus, the mammalian oviduct takes advantage of distinct features from other organs and species. However, questions remain; why does the oviduct have an inward-outward strategy for reproduction? Do oviductal contractions regulate flow directions to accomplish the appropriate transport of sperm and embryos? The mechanism underlying myosalpinx contractions between sperm migration and embryo transport need to be explored further.

GENERAL CONCLUSION AND PROSPECTIVE

This study demonstrates the importance of myosalpinx contractions contributing to regulation in the transport of sperm for fertilization in the mouse oviduct. Chapter I describes the involvement of myosalpinx contractions in the sperm migration. Administration of prifinium bromide, padrin, to mice suppressed myosalpinx contractions, resulting in a decreased rate of fertilization in a dose-dependent manner, and in an abrogation of high-speed back-and-forth/shuttling flows of oviductal fluids around the isthmus (Fig. 1-3). Interestingly, a tight assemblage of sperm was transported by the aid of shuttling flows generated by myosalpinx contractions in the isthmus (Fig. 5). Sperm motility was required to approach to the oocyte in the ampulla. In the chapter II, I examined whether the oviduct possesses contraction factors of oviductal epithelial cells during the sperm migration. Although ET3 induced the OEC collagen contractions, my results suggest that the oviductal extracts possibility include oviductal epithelial cell contraction-inducing factor(s) beside ET3 during sperm migration.

In my Ph.D projects, I elucidated functions of the oviduct in the sperm migration. The results may contribute to understanding of the fertilization process *in vivo*. By taking assisted reproductive technologies, it has been believed that the oviductal function is perhaps unnecessary for reproduction. However, basic/fundamental knowledge of the oviduct is provided from actual phenomena of myosalpinx contractions regulating the sperm migration. A clue of optimal environment for *in vitro* fertilization possibly accrues by analysis of the oviductal epithelial cells. I emphasize that our research stands for not “how to make a life” but “Why a life

begins ”. I hope that this research contributes to making a link between *in vitro* and *in vivo* fertilization in mammals.

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