Roles of EDNs in regulating oviductal NO synthesis and smooth muscle motility in cows

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Abstract

Endothelins (EDNs) participate in various physiological events including smooth muscle contraction, nitric oxide (NO) synthesis, and embryonic development. In this study, we investigated the regional roles of EDNs produced by bovine oviductal epithelial cells in NO synthesis and smooth muscle motility. Quantification of mRNA expressions indicated that expression of EDN receptor B (*EDNRB*) in the ampullary region was higher after ovulation than before ovulation, whereas expression of *EDNRA* in the isthmus region was higher after ovulation than before ovulation. Immunohistochemistry revealed that the EDN receptors (*EDNRA* and *EDNRB*) were expressed in the epithelium, whereas smooth muscle showed positive staining only for *EDNRA*. The expressions of inducible NO synthase (iNOS) protein and its mRNA (*NOS2*) in cultured epithelial cells isolated from the ampulla were stimulated by EDN1, but not by EDN2 or EDN3, after 1 h of incubation. In isthmus epithelial cells, none of the EDNs affected the expression of *NOS2*. Isometric contraction tests indicated that spontaneous waves were strong in the isthmus region but weak in the ampullary region. EDN1 modulated smooth muscle motility in both the regions. The overall findings suggest that EDN1 plays region-specific roles in smooth muscle motility and epithelial NO synthesis, providing an optimal oviductal microenvironment for transport of gametes, fertilization, and development/transport of early embryo.

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Introduction

Mammalian oviducts play crucial roles in the first days of pregnancy (Ulbrich *et al.* 2010). Fertilized eggs are transported by two key oviductal actions: ciliary beating and smooth muscle motility (Hunter 2012). Distal parts of the oviduct including the infundibulum and ampulla have well-developed mucosal folds surrounded by abundant ciliated cells (Noreikat *et al.* 2012). Ciliary activity is more important for the transport of eggs in the distal part of the oviduct, which has a thin layer of smooth muscle. Indeed, inhibition of smooth muscle activity did not disturb the transport of eggs through the ampullary region (Halbert *et al.* 1976). By contrast, the isthmus has a few ciliated cells and thick smooth muscle layer, producing waves of contraction and relaxation that contribute to embryo transport. The amplitude, frequency, and tonus of the waves are regulated by various hormones and factors such as nitric oxide (NO) and endothelins (EDNs) (Rosselli *et al.* 1994, Priyadarsana *et al.* 2004).

Endothelins are peptide hormones composed of 21 amino acids. Endothelins have three isoforms: EDN1,

EDN2, and EDN3 (Jeoung *et al.* 2010). Endothelin-1 was first identified as a vasoactive factor in porcine aortic smooth muscle (Yanagisawa *et al.* 1988). The receptors of EDNs have two types: type A (*EDNRA*) and type B (*EDNRB*). Endothelin-1 and EDN2 bind to both *EDNRA* and *EDNRB*, whereas EDN3 only binds to *EDNRB* (Bridges *et al.* 2011). In various tissues, *EDNRA* is involved in signaling toward contraction of smooth muscle, so that only EDN1 and EDN2 promote smooth muscle motility (Rosselli *et al.* 1994, Sakamoto *et al.* 1999, Al-Alem *et al.* 2007). However, *EDNRB* signaling induces the synthesis of NO, a vasodilation factor (Hirata *et al.* 1993), and so all the EDN isoforms induce the relaxation of smooth muscle via *EDNRB*. Nitric oxide also has other functions in physiological events in the oviduct including embryonic development (Manser *et al.* 2004), epithelial secretion (Siemieniuch *et al.* 2009, Yilmaz *et al.* 2012), and ciliary beating, which are essential for pregnancy (Chiu *et al.* 2010).

Recently, we demonstrated that all the EDN isoforms are expressed in the epithelia of both the ampulla and the isthmus of the bovine oviduct (Yamamoto *et al.* 2014). Thus, EDNs may regulate oviductal smooth muscle

motility in a paracrine manner and may affect synthesis of NO in both autocrine and paracrine manners. In this study, we hypothesized that EDNs produced by epithelial cells play several roles in regulating smooth muscle motility and/or NO synthesis in the oviduct. To test this hypothesis, we investigated 1) the localizations of EDNRA and EDNRB in the ampulla and isthmus, 2) the effects of EDNs on NO synthesis in cultured oviductal epithelial cells isolated from the two regions, and 3) the effects of EDNs on smooth muscle motility in oviductal tissues.

Materials and methods

Collection of bovine oviducts

Oviducts of adult (>2 years old) non-pregnant healthy Holstein cows ($n=24$, total) were collected at a local abattoir within 10–20 min after exsanguination and the samples on ice were transported to our laboratory within 2 h. The stages of the estrous cycle were classified as Stage I (days 1–4), Stage II (days 5–10), Stage III (days 11–17), and Stage IV (days 18–20) based on a macroscopic observation of the ovary and the uterus (Ireland *et al.* 1980). The samples obtained at Stage I (after ovulation) and Stage IV (before ovulation) were utilized for the following experiments. After trimming of the oviducts being ipsilateral to the corpus luteum, the ampullary and the isthmus sections were immediately frozen and stored at -80°C until mRNA extraction. For cell culture, the oviducts ($n=9$) were submerged in ice-cold saline and transported to the laboratory.

Immunohistochemistry

Formalin–paraffin-embedded sections of the ampulla and isthmus of the oviduct obtained from three cows after ovulation were used for immunohistochemistry. Sections of $6\mu\text{m}$ were deparaffinized and rehydrated in a graded series of ethanol and washed in tap water. Antigens were retrieved by microwave in Tris–EDTA buffer (pH 9.0) for 15 min at 600W. Nonspecific binding was blocked in 2.5% horse serum (S-2012; Vector Laboratories Inc, Burlingame, CA, USA) for 20 min at room temperature. The sections were incubated with specific primary antibodies for EDNRA (1:200 dilution, NBP1-33614; Novus Biologicals, Littleton, CO, USA) or EDNRB (1:200 dilution, NBP1-31108; Novus Biologicals) overnight at 4°C , washed with PBS three times, incubated with secondary antibody for rabbit-IgG conjugated with Alexa 568 (1:500 dilution, ab175693; Abcam) for 1 h at room temperature, washed with PBS three times, covered with ProLong Gold Antifade Reagent with DAPI (36935; Life Technologies), and observed using a confocal microscope (FV1200; Olympus).

Isolation and culture of oviductal epithelial cells

Epithelial cells ($n=9$) were enzymatically isolated from the ampullary and the isthmus sections of the oviduct at the peri-ovulatory period (before and after ovulation) as described

previously (Kobayashi *et al.* 2013). The isolated cells were seeded to 24-well plates (662160; Greiner Bio-One, Frickenhausen, Germany) or 25cm^2 culture flasks (690175; Greiner Bio-One). The plates and flasks for epithelial cells were coated with collagen obtained from mouse tails before seeding. The cells were cultured at 38.5°C in a humidified atmosphere of 5% CO_2 in air. The medium was exchanged every 48 h until the cells reached confluency. When the cells reached confluency (10–11 days after the isolation of the cells), they were used for experiments.

Cell culture and treatments

Oviductal epithelial cells that had reached confluency ($n=9$) were incubated with EDN1, EDN2, or EDN3 (4198, 4209, or 4199, respectively; Peptide Institute, Osaka, Japan; 0.01, 0.1, or 1 nmol/L) in phenol red-free Dulbecco's Modified Eagle's Medium/F-12 Ham (D2906; Sigma-Aldrich) supplemented with $500\mu\text{mol/L}$ ascorbic acid (013-12061; Wako Pure Chemical Industries), $5\mu\text{g/mL}$ holo-transferrin (T4132; Sigma-Aldrich), 5ng/mL sodium selenite (S5261; Sigma-Aldrich), $2\mu\text{g/mL}$ insulin (I4011; Sigma-Aldrich), 0.1% (w/v) bovine serum albumin (A7888; Sigma-Aldrich), and 20mg/mL gentamicin (G1397; Sigma-Aldrich) for 1 and 4 h at 38.5°C . After incubations, the cells were collected to determine mRNA ($n=5$) and protein ($n=4$) expressions.

Total RNA extraction and quantitative RT-PCR

Total RNA was extracted from oviductal tissues and cells using TRIreagent according to the manufacturer's directions. Using iScript RT Supermix for RT-qPCR (170-8841; Bio-Rad Laboratories), $1\mu\text{g}$ of each total RNA was reverse transcribed. Quantifications of mRNA expressions were determined by quantitative RT-PCR using MyiQ (Bio-Rad Laboratories) and SoovAdvanced SYBR Green Supermix (1725261B10; Bio-Rad Laboratories) starting with 4 ng of reverse-transcribed total RNA as described previously (Sakumoto *et al.* 2006). All the primers were designed to amplify the specific product for *NOS2* (forward: 5'-TAC CCT CAG TTC TGC GCT TT-3'; reverse: 5'-GGG ATC TCA ATG TGG TGC TT-3'), *EDNRA* (forward: 5'-GCA TCC AGT GGA AGA ACC AT-3'; reverse: 5'-AAC CAG TCA ACC CTT CAA CG-3'), and *EDNRB* (forward: 5'-GCT CCA TCC CAC TCA GAA AA-3'; reverse: 5'-GCT CCA TCC CAC TCA GAA AA-3'). The specificity of each primer set was confirmed by running the PCR products on a 2.0% agarose gel. Protocol conditions consisted of denaturation at 95°C for 30 s, followed by 45 cycles at 95°C for 6 s, 60°C for 6 s, and 72°C for 6 s with a final dissociation (melting) curve analysis. To standardize the relative level of expression of each mRNA, three potential housekeeping genes, β -actin (*ACTB*; forward: 5'-CAG CAA GCA GGA GTA CGA TG-3'; reverse: 5'-AGC CAT GCC AAT CTC ATC TC-3'), 18S ribosomal RNA (*18S* rRNA; forward: 5'-TCG CGG AAG GAT TTA AAG TG-3'; reverse: 5'-AAA CGG CTA CCA CAT CCA AG-3'), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; forward: 5'-CAC CCT CAA GAT TGT CAG CA-3'; reverse: 5'-GGT CAT AAG TCC CTC CAC GA-3'), were initially tested. As *GAPDH* was found to be the most stable of the three genes by Normfinder software (<http://moma.dk/normfinder-software>), *GAPDH* transcripts

were selected as the internal control in our experiments. To analyze the relative level of expression of each mRNA, the $2^{-\Delta\Delta C_T}$ method was used (Livak & Schmittgen 2001).

Western blotting

Expression of iNOS protein in cultured ampullary oviductal epithelial cells ($n=4$) was detected by western blotting analysis as described previously (Schägger 2006, Nishimura *et al.* 2008) with some modifications. Briefly, the cultured epithelial cells were lysed in 200 μ L lysis buffer. The obtained protein concentrations were determined by the BCA method (Osnes *et al.* 1993). The proteins were incubated with SDS gel-loading buffer (37.5 mmol/L Tris-HCl, 3% (w/v) SDS, 7.5% glycerol, 1.5% (v/v) β -mercaptoethanol (133-14571; Wako Pure Chemical Industries), 0.0125% (w/v) Coomassie brilliant blue (B1131; Sigma-Aldrich)), pH 6.8 at 95°C for 5 min. The samples (30 μ g protein/lane) were electrophoresed on a 7.5% (v/v) SDS-PAGE gel (30 mA, 60 min). The separated proteins were electrophoretically transblotted to a 0.45- μ m PVDF membrane (RPN303F; GE Healthcare) at 250 mA for 120 min. The membranes were then incubated in PVDF Blocking Reagent for Can Get Signal (NYPBR01; Toyobo, Osaka, Japan) for 60 min at room temperature. After blocking, the membranes were incubated with specific primary antibodies to iNOS (anti-iNOS-IgG-rabbit, sc-651, 1:1000 dilution; Santa Cruz Biotechnology) or β -actin (anti- β -actin-IgG-mouse for loading control, A2228, 1:20,000 dilution; Sigma-Aldrich) in Can Get Signal Immunoreaction Enhancer Solution 1 (NKB101; Toyobo) overnight at 4°C. After incubation, the membranes were incubated with secondary antibody (anti-rabbit-IgG, horseradish peroxidase (HRP)-linked whole antibody produce in donkey, NA934, 1:10,000 dilution for iNOS; GE healthcare, or anti-mouse-IgG, HRP-linked whole antibody produced in sheep, NA931, 1:40,000 dilution for β -actin; GE healthcare) in Can Get Signal Immunoreaction Enhancer Solution 2 (NKB101; Toyobo) for 60 min at room temperature. After incubation, the membranes were incubated with Immobilon Western Chemiluminescent HRP Substrate (WBKLS0500; Merck Millipore) for 3 min, then the signals were detected using ChemiDoc XRS+ (Bio-Rad), and the intensity of the immunological reaction was estimated by measuring the optical density in the defined area by computerized densitometry using Image Lab (Bio-Rad).

Isometric contraction test

Isometric contraction test of oviductal smooth muscle was performed as described previously (Sogawa *et al.* 2010, Ning *et al.* 2014) with some modifications. Briefly, the ampullary ($n=5$) and isthmus ($n=6$) tissues of bovine oviducts obtained from cows after ovulation were cut open and 3-mm-length strips were prepared. Each strip was incubated in a Magnus tube filled with 10 mL 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 , and 5.6 mM glucose). The Krebs-Ringer solution was kept at 38.5°C and aerated with 95% O_2 and 5% CO_2 during the experiment. The strips were equilibrated for 1 h before the experiment. The tension of each strip was measured under a resting tension of 1 g. Oxytocin (0.1 μ M) and noradrenalin (1 μ M)

were utilized as a constrictor and a relaxant respectively. Then, the strips responded to the constrictor and relaxant utilized for incubation with EDN1, EDN2, or EDN3 (0.1 nM, 1 nM, or 10 nM). The isometric tension of each strip was recorded using a force-displacement transducer (Minebea Co. Ltd., Nagano, Japan) connected to a polygraph (Yokogawa Electric Corp., Tokyo, Japan) with the chart running at 1000 mm/h.

Statistical analysis

All experimental data are shown as the mean $\pm$ S.E.M. The statistical significance of differences was assessed by analysis of variance (ANOVA) followed by Mann-Whitney U test for the dataset shown in Fig. 1A, Tukey-Kramer test for the dataset shown in Fig. 2B, or Dunn's multiple comparison test for the dataset shown in Figs 2A and 3, and Tables 1 and 2 using GraphPad Prism (GraphPad Software). P values <0.05 were considered to be statistically significant.

Figure 1 (A) Changes of endothelin receptor A (EDNRA) and EDNRB mRNA expressions in tissues collected from the ampulla (black bar) and the isthmus (white bar) of the oviduct around ovulation (mean $\pm$ S.E.M., $n=6$, before ovulation; $n=12$, after ovulation). Significant differences were indicated by $*P<0.05$ and $**P<0.01$. (B) Distributions of EDNRA and EDNRB proteins in ampullary and isthmus tissues obtained from cows after ovulation (L: lumen, EP: epithelium, SM: smooth muscle). Red (Alexa 568) indicates each target protein (EDNRA or EDNRB), and blue (DAPI) indicates nuclei of the cells. All the scale bars indicate 50 μ m.

Results

Expressions of EDN receptors

In the ampulla, *EDNRB* mRNA expressions were higher after ovulation than before ovulation ($P < 0.05$, Fig. 1A), whereas *EDNRA* mRNA expressions did not significantly change around ovulation ($P > 0.05$, Fig. 1A). In the isthmus, mRNA expressions of *EDNRA* were higher after ovulation than before ovulation ($P < 0.05$), whereas mRNA expressions of *EDNRB* did not change around ovulation ($P > 0.05$, Fig. 1A). *EDNRA* protein was distributed in most of the epithelial cells and in some of the smooth muscle cells, whereas *EDNRB* protein was localized only in the epithelial cells in both the ampulla and isthmus (Fig. 1B).

Figure 2 Effects of endothelin-1 on *NOS2* mRNA (A, $n = 5$ oviducts) and iNOS protein (B, $n = 4$ oviducts) expressions in cultured oviductal epithelial cells isolated from the ampulla and isthmus of the oviduct (mean $\pm$ S.E.M.). Asterisks indicate significant differences ($P < 0.05$, compared with the groups without EDN1).

Effects of EDNs on iNOS expressions

Incubating ampullary epithelial cells with EDN1 increased *NOS2* mRNA expression after 1 h ($P < 0.05$, Fig. 2A), but not after 4 h ($P > 0.05$, Fig. 2A), and increased iNOS protein expression after 1 h ($P < 0.05$, Fig. 2B). Neither EDN2 nor EDN3 affected *NOS2* expression in the ampullary cells ($P > 0.05$, Table 1). *NOS2* expression in isthmic epithelial cells was not affected by EDN1, EDN2, or EDN3 ($P > 0.05$, Fig. 2 and Table 1).

Effects of EDNs on oviductal smooth muscle motility

Weak spontaneous waves of contraction and relaxation were observed in the ampullary region (Fig. 3A). In that region, EDN1 increased tonus (average values of top and bottom of the waves, $P < 0.05$), amplitude ($P < 0.05$), and frequency ($P < 0.01$) of smooth muscle motility (Fig. 3C, D, and E). By contrast, the isthmus region showed strong spontaneous waves (Fig. 3B). Endothelin-1 increased the tonus of spontaneous waves in the isthmus ($P < 0.01$, Fig. 3B and C). Neither ampullary nor isthmic smooth muscle motility was significantly affected by EDN2 and EDN3 (Fig. 3A, B, and Table 2, $P > 0.05$).

Discussion

The preceding results show that EDN1 affect at least two oviductal functions: NO synthesis (Fig. 2) and spontaneous waves of contraction and relaxation of oviductal smooth muscle (Fig. 3). These effects are schematically shown in Fig. 4. Spontaneous waves of smooth muscle are responsible for the transport of embryo through the isthmus (Hunter 2012). Nitric oxide promotes ciliary beating (Chiu *et al.* 2010) and embryo development (Manser *et al.* 2004). The structures of the oviductal regions are dramatically different. Numerous motile cilia surround the luminal epithelium in the distal parts of the oviduct, including the infundibulum and ampulla. Beating of these cilia produces a stream of oviductal fluid, transporting the ovulated oocyte and the embryo toward the site of fertilization and uterus respectively (Kölle *et al.* 2009). By contrast, in the isthmus, a few mucosal folds are surrounded by thick smooth muscle layer that produces waves of contraction and relaxation. These spontaneous waves of smooth muscle allow the early embryo to pass through the narrow isthmic cavity. The present results suggest that the specific functions of the ampulla and isthmus are regulated by EDNs.

Two types of receptors for EDNs showed different distribution in this study, i.e. smooth muscle cells expressed EDNRA but not EDNRB (Fig. 1). Binding of EDNs to EDNRA has been demonstrated to immediately activate phospholipase C, generate inositol triphosphate, mobilize extracellular Ca^{2+} , and induce contraction in vascular smooth muscle cells

Figure 3 Effects of endothelins (EDNs) on spontaneous waves of contraction and relaxation in the ampulla (A) and isthmus (B) obtained from cows after ovulation. Horizontal long lines indicate tonus of the waves at the start of the test. Horizontal and vertical scale bars indicate 2 min and 10 mN respectively. Effects of EDN1 on tonus (C), amplitude (D), and frequency (E) of contractile motility in the ampulla (solid lines, $n=5$) and isthmus (dashed lines, $n=6$). Significant differences were indicated by * $P<0.05$ and ** $P<0.01$ compared with the groups without EDN1.

(Pollock *et al.* 1995, Vignon-Zellweger *et al.* 2012). As EDNRA has a low affinity for EDN3 (Yanagisawa 1994), only EDN1 and EDN2 seems to bind to EDNRA on smooth muscle cells of the oviduct. This is supported by the finding that EDN1, but not EDN3, stimulated the activity of smooth muscle (Fig. 3 and Table 2). As EDN1 is synthesized in epithelial cells of the oviduct (Yamamoto *et al.* 2014), these EDNs are suggested to be released from epithelial cells, to bind EDNRA expressed on smooth muscle cells, to activate motility of smooth muscle, and to contribute to the transport of embryo toward the uterus.

As the smooth muscle layer of the ampullary region is not well developed, ciliary activity in the ampullary epithelium is required for the transport of oocyte/embryo. In fact, inhibition of ciliary beating suppresses egg transport (Halbert *et al.* 1976). The ampullary lumen is surrounded by numerous cilia whose motility produces the stream toward the uterus (Kölle *et al.* 2009). Ciliary beating is regulated by several factors including NO (Chiu *et al.* 2010). As EDN1 stimulated *NOS2* mRNA and *iNOS* protein expressions in ampullary epithelial cells in this study (Fig. 2), EDN1 may participate in the regulation of ciliary activity. Immediately after ovulation, the ovulated

oocyte with follicular fluid containing EDN1 is allowed to enter the oviduct and to be transported to the site of fertilization within a few hours (Acosta *et al.* 1998). EDN1 derived from not only oviductal epithelium but also ruptured follicle seems to facilitate ciliary motility for oocyte transport by stimulating NO synthesis.

The binding of EDN1 to EDNRB increases endothelial NOS (eNOS)-derived NO in vascular endothelial cells (Hirata *et al.* 1993, Tsukahara *et al.* 1994). This eNOS derived NO relax vascular smooth muscle. However, a specific antagonist of EDNRB (BQ-788) suppresses *iNOS* expression/NO production, which is stimulated by EDN1 via activation of nuclear factor κ B (NF- κ B) in rat brain astrocytes (Wang *et al.* 2011). Activation of NF- κ B signaling induces *iNOS* expressions in some cell types in the brain (Harris *et al.* 2009, Pérez-Rodríguez *et al.* 2009). In the oviduct, several factors are known to activate NF- κ B signaling (Gabler *et al.* 2008, Shaw *et al.* 2011). Our previous findings that *iNOS* mRNA expression is highest on the day of ovulation in the ampullary region during the estrous cycle (Kobayashi *et al.* 2016) could be an evidence that EDN1 mediates *iNOS* expression/NO production via NF- κ B in the oviduct. Other isoforms of NOS, eNOS

Table 1 Effects of endothelin (EDN) 2 and EDN3 on NOS2 mRNA expression in cultured epithelial cells obtained from the ampulla and isthmus of bovine oviducts (n=5, mean±s.e.m.). There were no significant differences (P>0.05).

Region/treatment (nmol/L)	Incubation period (fold changes)	
	1 h	4 h
Ampulla		
EDN2		
0	1.00±0.42	1.00±0.39
0.01	1.18±0.56	0.82±0.14
0.1	1.43±0.67	0.56±0.18
1	0.83±0.22	0.73±0.29
EDN3		
0	1.00±0.68	1.00±0.31
0.01	0.84±0.61	0.67±0.21
0.1	0.79±0.47	0.76±0.25
1	1.33±0.88	0.74±0.31
Isthmus		
EDN2		
0	1.00±0.47	1.00±0.20
0.01	0.86±0.48	1.18±0.32
0.1	0.75±0.45	0.92±0.33
1	1.17±0.83	0.97±0.24
EDN3		
0	1.00±0.15	1.00±0.22
0.01	1.22±0.19	0.99±0.16
0.1	0.96±0.06	0.95±0.30
1	1.02±0.48	1.03±0.21

and neuronal NOS (nNOS), are also expressed in the bovine oviduct (Lapointe *et al.* 2006, Yilmaz *et al.* 2012). Yilmaz *et al.* describes that eNOS and nNOS are possible to participate in secretions from epithelial cells because eNOS and nNOS are also distributed in the epithelial layer of the oviduct. Therefore, these isoforms regulated by EDNs might affect oviductal functions. Further studies are required to determine the regulatory mechanism of NO synthesis by EDN system in the oviduct.

An antagonist of both types of EDNs (tezosentan) decreases early embryonic development in mice (Jeoung *et al.* 2010). Transforming growth

Table 2 Effects of endothelin (EDN) 2 and EDN3 on tonus, amplitude, and frequency of contractile motility (mean±s.e.m.) in the ampulla (n=5) and isthmus (n=6). There were no significant differences (P>0.05).

Region/treatment (nmol/L)	ΔTonus (μN)	Amplitude (%)	Frequency (mHz)
Ampulla			
EDN2			
0	0.00±0.00	100.0±0.00	66.8±6.25
10 ⁻¹⁰	84.1±149.08	95.6±6.65	64.1±5.03
10 ⁻⁹	74.7±130.05	116.3±13.57	66.4±7.19
10 ⁻⁸	126.7±162.90	131.6±11.72	71.3±7.05
EDN3			
0	0.00±0.00	100.0±0.00	55.3±3.26
10 ⁻¹⁰	-151.0±66.16	104.2±7.17	62.6±2.33
10 ⁻⁹	-57.6±89.24	118.9±7.43	60.9±4.32
10 ⁻⁸	-143.7±108.18	117.9±11.05	59.8±5.00
Isthmus			
EDN2			
0	0.00±0.00	100.0±0.00	91.0±6.10
10 ⁻¹⁰	-20.8±63.42	98.2±2.43	94.4±5.54
10 ⁻⁹	35.0±79.81	102.7±2.26	93.7±5.88
10 ⁻⁸	248.22±150.22	103.6±3.42	94.4±6.60
EDN3			
0	0.00±0.00	100.0±0.00	95.2±7.60
10 ⁻¹⁰	-28.0±87.50	100.4±1.67	94.0±7.66
10 ⁻⁹	171.97±88.52	100.0±1.61	93.3±5.27
10 ⁻⁸	140.47±77.54	102.7±1.52	93.7±5.14

factorβ(TGFβ) family regulated by EDNs may be involved in embryonic development according to a microarray analysis of tezosentan-treated mice (Jeoung *et al.* 2010). In fact, TGFβ-SMAD signaling is required for early embryonic development in cattle (Zhang *et al.* 2015), and TGFβ type I, II, and III are expressed in human oviductal epithelium (Zhao *et al.* 1994). We also propose that ET-1-EDNRB signaling via iNOS-derived NO affects embryonic development because NO promotes early embryonic development (Manser *et al.* 2004). In the oviductal cavity, various factors derived from oviductal cells, follicular fluid, and the circulation participate in fertilization and embryonic development. Mechanisms of formation of oviductal milieu to make

Figure 4 Region-specific roles of endothelins (EDNs) in the ampulla and isthmus of the oviduct. In the ampulla, EDN1 stimulates iNOS expression and NO synthesis via EDNRB, which is highly expressed in the epithelial cells at the day of ovulation. As NO contributes to survival of oocyte and embryos (Manser *et al.* 2004) and ciliary beating (Chiu *et al.* 2010), EDN1 may promote early embryonic development and ciliary beating in the ampullary region. By contrast, EDN1 promote the contractility of isthmus smooth muscle via EDNRA expressed on smooth muscle cell surface, participating in the complicated regulatory mechanism of smooth muscle motility regulated by various factors and contributing to successful embryo transport toward the uterus.

sure successful pregnancy are expected to be clarified by further studies.

In conclusion, the present results indicate that EDN1 has specific effects on 1) NO synthesis in the ampulla and 2) smooth muscle motility in the ampulla and isthmus. These roles of EDN1 could be involved in region-specific physiological events, ciliary beating for oocyte transport, and fertilization in the ampulla, and waves of contraction and relaxation of oviductal smooth muscle that are needed to transport the early embryo to the uterus.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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