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Expression of cyclooxygenase-2 and prostaglandin receptor EP4b mRNA in the ovary of the medaka fish, *Oryzias latipes*: Possible involvement in ovulation

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ABSTRACT

In vitro ovulation of mature medaka ovarian follicles was inhibited by inhibitors of cyclooxygenase (COX) or by an antagonist of the prostaglandin E₂ receptor (EP). Of the three medaka COX genes, *ptgs2* was most dominantly expressed in the fish ovary. The *ptgs2* transcript was detected in all sizes of growing follicles. In a 24-h spawning cycle, large-sized follicles contained *ptgs2* mRNA at a fairly constant level. The levels of COX enzyme activity and prostaglandin E₂ were also constant in the large-sized follicles during the spawning cycle. The expression of prostaglandin E₂ receptor EP4b (*ptger4b*) mRNA was drastically upregulated in the large-sized follicles as the ovulation time approached. The current results indicate that prostaglandin E₂, which might be produced by COX-2, is involved in the ovulation of medaka, and that EP4b is likely the receptor responsible for exerting the action of prostaglandin E₂ in the process.

1. Introduction

Prostaglandins (PGs), which are biologically important chemical substances exerting their effects by binding to specific cell surface receptors, play roles in a wide range of physiological processes (Simmons et al., 2004; Sugimoto and Narumiya, 2007). These processes include platelet aggregation, thermoregulation, wound healing, water balance, glomerular filtration, and homeostasis. PGs are also thought to be crucial factors for the normal function of the reproductive organs. For example, ovulation, the process by which a mature fertilizable ovum is liberated from the ovarian follicle (Espey and Richards, 2006), is considered analogous to proinflammatory responses (Espey, 1994; Richards et al., 2002), and thus the role of PGs in this process has been a subject of intensive investigation (Orczyk and Berhman, 1972; Armstrong, 1981; Murdoch et al., 1993; Espey and Richards, 2006). The current understanding of ovulation in mammals is that the cascade of ovarian gene expression that induces local inflammation results in follicle rupture via proteolytic disintegration of the connective tissue elements of the follicle wall and the expansion of the cumulus oocyte complex (COC) associated with production of a specialized extracellular matrix (ECM) (Espey and Richards, 2006). Each of these processes is critical for successful ovulation. Recent studies have demonstrated the role of PGE₂, the PG species that is most abundantly present in ovulating follicles of the mammalian ovary (LeMaire et al., 1973; LeMaire et al., 1975), in COC expansion (Hizaki et al., 1999; Richards et al., 2002). In contrast, the functions of PGE₂ in follicle rupture during ovulation are not fully understood.

Like that in mammalian species, ovarian PG synthesis in non-mammalian vertebrates is known to occur during spontaneous or artificially-induced ovulation. In some teleosts,

indomethacin, which is a non-selective inhibitor of the PG-producing enzyme cyclooxygenase (COX), has been reported to effectively block ovulation (Cetta and Goetz, 1982; Patino et al., 2003; Lister and Van Der Kraak, 2008). Further, other investigations have provided evidence that PGs induce *in vivo* and *in vitro* ovulation (Jalabert and Szölösi, 1975; Stacey and Pandey, 1975; Goetz and Theofan, 1979; Kagawa and Nagahama, 1981; Goetz and Nagahama, 1985; Pankhurst, 1985; Kagawa et al., 2003; Lister and Van Der Kraak, 2008). These reports indicate that PGs play an important role during ovulation in oviparous species. Possible involvement of PGs in ovulation has also been documented for amphibians (Schuetz, 1986; Chang et al., 1995; Chang et al., 1997; Ramos et al., 2008; Sena and Liu, 2008).

PGs are formed through the sequential actions of COX and specific PG synthases. It is well established that the former enzyme plays a key regulatory role in PG synthesis. Previous studies and available genomic sequence databases indicate that, like mammalian species, non-mammalian vertebrates have two paralogues of COX, a constitutive (COX-1) and an inducible enzyme (COX-2). Interestingly, teleosts possess additional copies of COX-1 and/or COX-2 that are thought to be the result of a teleost-specific genome duplication and subsequent genome loss event (Järving et al., 2004; Ishikawa and Herschman, 2007; Ishikawa et al., 2007; Havird et al., 2008). For example, the zebrafish genome contains one COX-1 gene (*ptgs1*) and two functional COX-2 genes (*ptgs2a* and *ptgs2b*), while the Japanese medaka (*Oryzias latipes*) has two COX-1 genes (*ptgs1a* and *ptgs1b*) and one COX-2 gene (*ptgs2*).

PGE₂ exerts its actions by acting on a group of G-protein-coupled receptors. In mammals, four different PGE₂ receptors, designated subtypes EP1, EP2, EP3, and EP4,

have been identified to date, and are expected to give rise to the functional specificity of PGE₂ in different tissues (Hata and Breyer, 2004; Sugimoto and Narumiya, 2007). The structures, ligand binding and signal transduction properties, tissue and cellular distribution, and physiological functions of these EP subtypes have been defined. On the other hand, only a few studies dealing with EP receptors of non-mammalian species have been published. EP4 was demonstrated to be critically involved in gastrulation during embryogenesis in zebrafish (Cha et al., 2006) and in T cell precursor development in the thymus of the fish embryo (Villablanca et al., 2007). More recently, expression of EP2 and EP4 in the tissues of chicken and their activation by PGE₂ have been reported (Kwok et al., 2008).

The medaka fish, *Oryzias latipes*, is a small egg-laying freshwater teleost that has advantages for use in various studies, including developmental, reproductive, and molecular genetic studies (Iwamatsu et al., 1988; Nagahama et al., 1994; Ozato and Wakamatsu, 1994; Ishikawa, 2000; Wittbrodt et al., 2002; Kasahara et al., 2007). This non-mammalian vertebrate species is particularly attractive to researchers conducting ovarian function studies because of the manageable cyclicity of ovarian activity in the mature medaka fish. During the reproductive season, the medaka ovary contains oocytes in all phases of oogenesis. Oocytes can be grossly divided into five phases and further into ten stages, based on major morphological characteristics of developing oocytes and follicles (Iwamatsu et al., 1988). The fish also spawns daily within 1 h of the onset of light for a number of consecutive days when kept in the laboratory under a constant long photoperiod of 14 h-light/10 h-dark at 26-28°C. This enables us to determine the timing of a sequential process of spawning, such as completion of vitellogenesis, breakdown of the germinal

vesicle and ovulation (Iwamatsu, 1978). The endocrine background for the ovarian cyclicity has been previously elucidated (Nagahama, 1994). Unlike fully-grown mammalian follicles, the follicle layer of growing and fully-grown follicles of the medaka consists of a single layer of granulosa cells surrounding an oocyte, a single layer of theca cells associated with a single layer of outermost ovary epithelial cells, and a basement membrane between the granulosa and theca cell layer (Pendergrass and Schroeder, 1976; Iwamatsu and Ohta, 1989). Only fully-grown follicles with a large oocyte (about 1 mm in diameter) ovulate. Ovulation in the medaka is also presumed to be accompanied by the destruction of ECM proteins present in the follicle layer of ovulating follicles.

We previously reported that follicle rupture during ovulation in the medaka ovary involves the cooperation of at least three matrix metalloproteinases (MMPs), together with the tissue inhibitor of metalloproteinase-2b protein (Ogiwara et al., 2005). To the best of our knowledge, the medaka is the only species in which the molecular mechanism of follicle rupture during ovulation is well documented at the present time. Taking advantage of this fact, we could effectively approach the problem of the involvement of PGs in the ovulatory process using this species. In a preliminary experiment, we found that COX inhibitors suppressed the *in vitro* ovulation of medaka ovarian follicles, indicating the relevance of the fish for solving the problem in question. However, there have been no studies addressing the PG-generating system in the medaka ovary. In the present study, we therefore examined the expression of COX and EP genes in the ovary of the mature medaka. Our data indicate that the fish ovary expresses *ptgs2* mRNA at an overwhelmingly greater level than *ptgs1a* or *ptgs1b* mRNA. In addition, we found that, among EP genes expressed in the fish ovary, the expression of *ptger4b* (the gene for the EP4b receptor) was drastically

induced in the large-sized follicles as the time of ovulation approached. These data, together with other results obtained in this study, suggest that COX-2/EP4b is involved in ovulation of the medaka.

2. Materials and methods

2.1. Animals and tissues

Individuals of the mature orange-red variant of medaka (*Oryzias latipes*) were purchased from a local dealer, and were kept in indoor tanks under artificial reproductive conditions (photoperiod, 10-h dark/14-h light; temperature, 27°C). After becoming acclimated to the conditions, mature female fish ovulated *in vivo* each day at the start of the light period. In this study, the start of the light period was designated as ovulation hour 0. Ovaries and ovarian follicles were isolated for experiments at various time points. Ovarian follicles were staged as described previously (Iwamatsu et al., 1988) into small-sized (<0.5 mm in diameter, stages I-VI), medium-sized (0.5-1.0 mm, stages VII-VIII), and large-sized (>1.0 mm, stages IX-X) follicles. Other tissues were obtained from the fish at 3 h after ovulation. The experimental procedures used in this study were approved by the Institutional Animal Use and Care Committee at Hokkaido University.

2.2. In vitro ovulation and effects of the COX inhibitor and PGE₂ receptor antagonist

Mature female medaka fish spawning on a 24-h cycle were used. The ovaries were removed at an intermediate time point (-12 h) of ovulation. Large-sized ovarian follicles were immediately isolated by using forceps under a dissecting microscope and were then transferred into 90% medium 199 solution (Earle's medium 199; Dainippon-Sumitomo

Seiyaku, Osaka, Japan) containing 5 µg/ml gentamycin, adjusted to pH 7.4 with NaHCO₃. About 10 follicles were cultured at 26-27°C in 1 ml of culture medium by using a 1.9 cm² tissue-culture dish. Under the *in vitro* condition, follicles started to ovulate at the expected time of ovulation (0 h) and completed ovulation in the next 6 h. Therefore, the number of oocytes that had successfully ovulated was counted 6 h after the expected ovulation time. The ovulation rate was defined as the percentage of ovulated follicles. To examine the effects of indomethacin (INDO) (known as a non-selective inhibitor for mammalian COX) or GW627368X (known as an antagonist for PGE₂ receptor EP4) on the *in vitro* ovulation, the follicles were incubated in the above medium containing various concentrations of the substances. As controls, the follicles were incubated in medium containing ethanol (for INDO) or DMSO (for GW627368X). Indomethacin was obtained from Sigma-Aldrich (St. Louis, MO). GW627368X was purchased from Cayman Chemicals (Ann Arbor, MI). In some experiments, follicles were incubated in the presence of both INDO and PGE₂. Sigma-Aldrich).

2.3. RNA isolation, reverse transcription (RT) and polymerase chain reaction (PCR)

Total RNA samples were isolated from the tissues, ovarian follicles, and cultured follicular cells using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions, and the amount and purity were determined by spectrophotometry. Reverse transcription (RT) was performed with the RNA using a SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). PCR amplification was performed in a volume of 10 µl that consisted of RT reaction, 1× PCR buffer, 0.2 mM of each dNTP, 2.5

mM MgCl₂, 0.2 µM of each primer, and 0.5U Taq polymerase. The primers used in the RT-PCR are shown in Table 1.

2.4. cDNA cloning of medaka COX-2

A gene-specific sense and antisense primer for cloning were designed using the Ensembl genome browser (http://www.ensembl.org/Oryzias_latipes/Info/Index). To obtain a 2403-bp *ptgs2* fragment, RT-PCR was conducted with KOD plus DNA polymerase (Toyobo, Osaka, Japan), using medaka ovary total RNA isolated at 3 h before ovulation. The primers used were COX2S1 and COX2AS1 (Table 1). The PCR conditions were 2 min at 94°C, followed by 30 cycles of 15 sec at 94°C, 30 sec at 60°C, and 3 min at 68°C. A fragment was cloned into pBluescript II KS(-) vector (Stratagene, La Jolla, CA) and sequenced.

The nucleotide sequences of the upstream region of the 87-bp cDNA were obtained by the 5'-RACE method (Frohman et al., 1988) using the 5'-RACE System for Rapid Amplification of cDNA Ends (Life Technologies, Rockville, MD) according to the manufacturer's protocol. The primers used were COX2AS2 (for RT), COX2AS3 (for the first PCR), and COX2AS4 (for the second PCR) (Table 1). Two rounds of PCR reactions were performed. The first PCR conditions were as follows: 94°C for 3 min, followed by 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 2 min at 72°C, and then a final extension was performed at 72°C for 7 min. The second PCR conditions were set at 94°C for 3 min, followed by 35 cycles of 30 sec at 94°C, 30 sec at 57°C, and 2 min at 72°C, and then a final extension at 72°C for 7 min. The amplification products (171-bp *ptsg2* fragment) were cloned into pBluescript II KS(-) vector (Stratagene) and sequenced.

A 3' portion of medaka *ptgs2* was obtained by the 3'-RACE method (Frohman et al., 1988) using the 3'-full RACE Core Set (Takara, Tokyo, Japan) according to the manufacturer's protocol. The sense primers used were COX2S2, COX2S3 and COX2S4 (Table 1). Two rounds of PCR reactions were performed. The first PCR conditions were set at 94°C for 3 min, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, and then a final extension at 72°C for 7 min. The second PCR conditions were set at 94°C for 3 min, followed by 35 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C, and then a final extension at 72°C for 7 min. The final PCR products (700-bp *ptgs2* fragment) were subcloned into pBluescript II plasmid for sequencing. The sequencing reaction was performed with the BigDye Terminator Cycle Sequencing Kit v3.1 and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). As a result, a 2,584-bp complete medaka *ptgs2* cDNA clone (DDBJ/EMBL/GenBank accession no. **AB516993**) was obtained.

2.5. Northern blotting

Northern blot hybridization was performed as described in our previous study (Ogiwara et al., 2004). Briefly, total RNA (60 µg for *ptgs1a* and *ptgs1b*; 30 µg for *ptgs2*) from various tissues were electrophoretically fractionated on a 1.2% denaturing agarose gel that contained 2.2 M formaldehyde, transferred to a Nytran-plus membrane (Schleicher and Schuell, Dassel, Germany), and UV cross-linked. cDNA probes for Northern blotting were prepared by PCR using the two specific primers for *ptgs1a*, *ptgs1b*, and *ptgs2* (Table 1) for the respective cDNA templates. The membranes were separately hybridized with the ³²P-labeled probes in a buffer containing 50% formaldehyde, 5 × 0.15 M NaCl/8.65 mM

NaH₂PO₄/1.25 mM EDTA (SSPE), 1% SDS and 5 × Denhardt's solution. The membranes were washed twice in 2 × saline-sodium citrate (SSC) buffer/0.05% SDS and then in 0.1 × SSC/0.1% SDS at 50°C. As a control, medaka cytoplasmic actin (*bactin*) mRNA was detected with a ³²P-labeled 312-bp *bactin* cDNA fragment (Kusakabe et al., 1999). Washed membranes were then subjected to autoradiography.

2.6. Real-time RT-PCR of medaka ovary COX-1a, COX-1b, and COX-2 and PGE₂ receptors

Total RNAs (2 µg) from each sample was reverse-transcribed in a volume of 20 µl, and the reaction was diluted to 80 µl with MilliQ water. The PCR reaction mixture (10 µl) contained 1 × SYBR Green PCR Master Mix (Applied Biosystems), 0.1 µM of each primer, 0.5 µl of sample cDNA or standard cDNA and the PCR was performed using an ABI 7300 Sequence Detector (Applied Biosystems). Reactions were carried out at 50°C for 2 min, followed by 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and then a final extension for 1 min at 60°C. In real-time PCR, the threshold cycle (C_t) is the cycle at which a significant increase in fluorescence takes place. The sequences of the real-time PCR primers for the medaka genes examined are shown in Table 1. Standard cDNAs for quantification were synthesized *in vitro* using pBluescript II KS(-) vector (Stratagene) constructs containing the respective cDNAs for COX-1a (*ptgs1a*), COX-1b (*ptgs1b*), COX-2 (*ptgs2*), and six PGE₂ receptors (EP1a (*ptger1a*), EP1b (*ptger1b*), EP2 (*ptger2*), EP3 (*ptger3*), EP4a (*ptger4a*), and EP4b (*ptger4b*)), cytoplasmic actin (*bactin*), and 18S rRNA (*18S rRNA*). The standards were processed in parallel with the cDNA samples and

were used to generate a standard curve. Dissociation curves were examined after each PCR run to ensure that a single PCR product had been amplified. PCR reactions were performed in triplicate on three separate RNA preparations from independent samples. The sizes of cDNA inserts in the vector were 140 bp (364 to 503, Ensembl gene ID: **ENSORLG00000005095** for *ptgs1a*), 93 bp (1278 to 1370, Ensembl gene ID: **ENSORLG00000000509** for *ptgs1b*), 122 bp (1471 to 1592, DDBJ/EMBL/GenBank accession no. **AB516993** for *ptgs2*), 179 bp (669 to 847, Ensembl gene ID: **ENSORLG00000016366** for *ptger1a*), 105 bp (799 to 903, Ensembl gene ID: **ENSORLG00000014518** for *ptger1b*), 132 bp (797 to 928, Ensembl gene ID: **ENSORLG00000003901** for *ptger2*), 127 bp (797 to 923, Ensembl gene ID: **ENSORLG00000007950** for *ptger3*), 103 bp (1047 to 1149, Ensembl gene ID: **ENSORLG00000003780** for *ptger4a*), 137 bp (957-1093, DDBJ/EMBL/GenBank accession no. **AB563504** for *ptger4b*), 64 bp (654 to 717, DDBJ/EMBL/GenBank accession no. **D89627** for *bactin*), and 56 bp (1415 to 1470, DDBJ/EMBL/GenBank accession no. **AB105163** for *18S rRNA*). The mRNA levels of target genes were normalized to *bactin* or *18S rRNA* mRNA levels.

2.7. In situ hybridization

A fragment of *ptgs2* cDNA (604 bp), which was the same nucleotide as employed for the Northern blotting, and a fragment of *ptger4b* cDNA (202 bp), which corresponded to the nucleotide sequence 627-828 (**AB563504**), were used to generate both antisense and sense digoxigenin (DIG)-labeled riboprobes with a DIG RNA Labeling Mix (Roche Diagnostics, Mannheim, Germany). The ovaries were isolated from mature medaka at 6 h

after ovulation (for *ptgs2*) or at 3 h before ovulation (for *ptger4b*), frozen, and sectioned at 14- μ m thickness. The sections were acetylated and postfixed. Prehybridization, hybridization, and washing of the sections were carried out according to the previously reported protocol (Ogiwara and Takahashi, 2007). The hybridized probes were detected with anti-DIG-alkaline phosphatase (AP) conjugate in a buffer containing nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in darkness.

2.8. COX activity assay

Tissues were isolated from mature fish at 3 h before ovulation. Each determination was made using only a single ovary, but using the combined livers or intestines obtained from two fish. After rinsing with phosphate buffered saline (PBS), the tissues were homogenized with 500 μ l of 100 mM Tris-HCl (pH 7.5) and centrifuged at 4°C with $10000 \times g$ for 15 min. The supernatants were used for the enzyme activity assay. For the activity assay of large-sized follicles, the follicles were isolated from mature fish ovaries every 4 h. From a single ovary, 15-20 follicles were obtained at the indicated time point, pooled, homogenized, and centrifuged as described above. The total COX activities of the samples were measured using a COX Activity Assay Kit (Cayman Chemicals) according to the manufacturer's instructions. One unit (U) of the activity was defined as the amount of the enzyme sample that produces 1 nmol of PGH_2 per min at 22°C. The protein concentrations of the samples were determined using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Three determinations were conducted.

2.9. *PGE₂ determination*

The tissue levels of PGE₂ were determined using a PGE₂ EIA Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's instructions. Each determination was made using a single ovary, but the combined livers or intestines from three fish. For determination of the ovarian follicle PGE₂ level, follicles (6-10 in number) were collected from the ovary of a single fish at each time point, combined, and used. Tissues were homogenized with 500 μ l of assay buffer containing 1 mM EDTA and 10 μ M indomethacin and centrifuged at 4°C with 10000 $\times$ g for 20 min. The supernatant was measured for the PGE₂ level. The protein concentrations of the samples were determined using a BCA Protein Assay Reagent Kit (Pierce). Three determinations (for the ovary, liver and intestine) or five determinations (for the ovarian follicle) were conducted.

2.10. *Statistical analysis*

All the experiments were repeated at least three times, except for the Northern blot analysis, in which two independent experiments were performed. Data are presented as the mean values $\pm$ S.E.M. Differences between groups were evaluated by Student's *t*-test, and the level of statistical significance was set at $P < 0.05$ or $P < 0.01$.

3. Results

3.1. *Effects of COX inhibitor on the in vitro ovulation of medaka ovarian follicles*

Large-sized follicles, which were to ovulate in 12 h, were collected from the ovaries and incubated in the absence or presence of inhibitors. At a concentration of 10 μ M INDO

(non-selective COX inhibitor), ovulation was strongly inhibited (Fig. 1). Addition of the inhibitor to the medium at 1 μ M inhibited ovulation by 80%. The concentration of the inhibitor required to inhibit the rate of ovulation by 50% was calculated to be about 450 nM. When dissected follicles were incubated in the presence of both INDO (10 μ M) and PGE₂ (10 μ M), the inhibitory effect of INDO was completely nullified. These results indicate that COX enzyme(s) and its product PGE₂ are involved in the ovulation of the medaka, and that the fish could be a suitable animal model for elucidating the role of PGs in ovulation.

3.2. *cDNA cloning and structure of the medaka ptgs2*

The full-length medaka *ptgs2* cDNA was cloned (DDBJ/EMBL/GenBank accession no. **AB516993**). The cDNA consisted of an 87 bp 5'-untranslated region, an open reading frame of 1824 bp, and a 673 bp 3'-untranslated region. This clone encoded a protein with a predicted length of 607 amino acids (Fig. 2A). A unique domain structure for COX-2 (Simmons et al., 2004) was found in the sequence: a signal peptide (21 residues), epidermal growth factor-like domain/dimerization domain (36 residues), membrane-binding domain (50 residues), catalytic domain (492 residues), and ER retention signal (4 residues) (Fig. 2B).

The catalytic domain of medaka COX-2 contains active site residues (Tyr-374 and Val-512), arachidonic acid binding residues (Arg-109 and Tyr-337), and residues crucial for peroxidase activity (His-377). The protein has four potential N-glycosylation sites (Asn-56, Asn-133, Asn-399, and Asn-584).

We compared our sequence (DDBJ/EMBL/GenBank accession no. **AB516993**) with

that of medaka COX-2 available from the draft medaka genome sequence database (http://www.ensembl.org/Oryzias_latipes/Info/Index). Amino acid residue substitutions were found at three positions: Ala-167 (our sequence) for Thr (genome database sequence), Thr-401 (our sequence) for Ser (genome database sequence), and Ala-478 (our sequence) for Val (genome database sequence). In addition, our COX-2 protein sequence was two residues shorter than the sequence deduced from the database. An insertion of dipeptide (Gly-Leu), which is missing in our sequence, was present between Gly-20 and Gly-21 in the database sequence of the protein. The current sequence was highly homologous to those of lower vertebrate COX-2 proteins; medaka COX-2 shared 84.6% identity with killifish COX-2 (**AAS21313**), 70.2-79.2% identity with zebrafish COX-2a (**NM153657**) and COX-2b (**NM001025504**), and 72.8% identity with rainbow trout COX-2 (**NP001117820**). As shown in Fig. 2A, the deduced amino acid sequence of medaka COX-2 is homologous to those of medaka COX-1a (59.5% identity) or COX-1b (59.4% identity).

3.3. Ovarian expression of medaka *ptgs2* mRNA

Northern blot analysis was conducted for *ptgs1a*, *ptgs1b*, and *ptgs2* using a blot of total RNA isolated from various tissues (Fig. 3A). The tissue expression patterns of the COX genes were different from each other. They were all expressed in the ovary, although the signals were not very strong. A single band of 3.5 kb was detected for *ptgs2*, while two transcript forms were seen for *ptgs1a* (1.7 and 2.4 kb) and *ptgs1b* (1.9 and 2.7 kb). The signal intensities of the *ptgs* genes relative to the control cytoplasmic actin (*bactin*) were in the following decreasing order: *ptgs2* > *ptgs1b* > *ptgs1a* (data not shown). We next

determined the ovarian expression levels of the *ptgs1a*, *ptgs1b*, and *ptgs2* genes by real-time PCR analysis. Among the three genes, *ptgs2* was found to be expressed most abundantly in the fish ovary (Fig. 3B). The *ptgs2* mRNA level was about 20 and 109 times greater than those of *ptgs1a* and *ptgs1b*, respectively. Changes in the *ptgs2* expression levels were examined by real-time PCR analysis using the ovaries of mature medaka fish with an established 24 h-spawning cycle. The transcript of *ptgs2* was detected throughout the period, and no significant changes in the *ptgs2* expression level were seen (Fig. 3C).

3.4. *In situ* detection of *ptgs2* mRNA in the ovary of the medaka

An *in situ* hybridization analysis of *ptgs2* mRNA in the medaka ovary sections was carried out. In ovaries isolated at 6 h after ovulation, strong signals associated with the oocyte cytoplasm of small-sized growing follicles were detected with the *ptgs2* antisense probe (Fig. 4A and B). The sizes of *ptgs2* mRNA-positive follicles were less than 100 μm in diameter. Medium- and large-sized follicles did not show a clear signal. In addition, no clear mRNA expression was seen in the follicular tissue that had ovulated (data not shown). The same morphological result was obtained when ovary sections were prepared at 3 h before ovulation (data not shown). The results indicate that the oocytes of very small-sized growing follicles contain *ptgs2* mRNA in the cytoplasm at a highly concentrated level.

3.5. Demonstration of *ptgs2* mRNA expression in the follicle cells

Having observed that only small-sized follicles exhibited clear positive signals for *ptgs2* mRNA in the ovary sections, as described above, we next examined whether or not medium- and large-sized follicles expressed *ptgs2* mRNA by real-time PCR. Small-sized

follicles appeared to contain *ptgs2* mRNA at the highest level, and the transcript level was lowered as the follicles grew (Fig. 5A). We further examined the expression of *ptgs2* mRNA using large follicles. The follicle layers and oocytes were isolated from follicles at -2 h of ovulation, and their total RNAs were prepared for real-time RT-PCR analysis. As shown in Fig. 5B, the level of *ptgs2* mRNA expression was about 3-fold higher in the oocytes than in the layers. Large-sized follicles, which were to ovulate at the next coming ovulation cycle, were analyzed for *ptgs2* mRNA expression every 4 h. The *ptgs2* transcript appeared to be most abundantly present in the follicle isolated at -7 h of ovulation, although the difference was not statistically significant. Both the *ptgs1a* and *ptgs1b* expressions in the follicle were very low and could be detected only when RT-PCR was conducted with 35 cycles of amplification (data not shown). These results indicate that the large-sized, preovulatory follicles of the medaka ovary expressed *ptgs2* mRNA.

3.6. COX activity in the medaka ovary

COX activity was measured using the extracts of the mature fish ovary obtained at 3 h before ovulation, and the results are shown in Fig. 6, where the activities of the liver and intestinal extracts are included for comparison. Ovarian COX activity was greater than COX activity in the liver, but lower than COX activity in the intestine. This order of enzyme activity levels among the three organs appeared to correspond to that of the *ptgs2* mRNA expression levels as examined by Northern blot analysis (Fig. 3A). The enzyme activity was also determined with large-sized ovarian follicles isolated from mature fish every 4 h (Fig. 6B). COX activity was the highest at -19 h and lowest at -7 h of ovulation, although such activity changes in the 24-h spawning cycle were not statistically significant.

3.7. Ovarian level of PGE₂

PGE₂ levels were determined for the ovary, liver, and intestine of mature medaka. In the ovary isolated from the fish at 3 h before ovulation, the PG was reproducibly detected. The amounts of PGE₂ were 2920, 218, and 22.0 pg/mg protein for the intestine, ovary, and liver, respectively (Fig. 7A). Next, the PGE₂ levels of large-sized follicles that were destined to ovulate in 24 h were determined every 4 h. The follicular level of PGE₂ was fairly constant throughout this measurement period (Fig. 7B). Follicles isolated at -15 h of ovulation seemed to contain the substance at a higher level, but this increase was not statistically significant.

3.8. Expression of PGE₂ receptor mRNA in the medaka ovary

A search of PGE₂ receptor genes using the Ensembl website for the medaka genome (http://www.ensembl.org/Oryzias_latipes/Info/Index) revealed that the fish contains six different genes coding for the receptor: *ptger1a* (for EP1a), *ptger1b* (for EP1b), *ptger2* (for EP2), *ptger3* (for EP3), *ptger4a* (for EP4a), and *ptger4b* (for EP4b). Using a set of primers synthesized based on the putative transcript sequences for the respective receptors, RT-PCR amplifications were performed. With a fixed 25-cycle reaction using total RNA isolated from the ovary at 3 h before ovulation, only a PCR product corresponding to *ptger4b* was detected (data not shown). Real-time PCR using the same cDNA was conducted to compare the expression levels of EP genes in the ovary. Fixing the *ptger1b* expression level to 1, those of *ptger1a*, *ptger2*, *ptger3*, *ptger4a*, and *ptger4b* were 1.3, 2.5, 10.4, 9.8, and 141, respectively (Fig. 8A). Next, the expression of *ptger4b* was examined by real-time RT-PCR

for large-sized preovulatory follicles of mature medaka ovaries (Fig. 8B). The transcript of *ptger4b* was present in the follicle at a very low level during the first half of the 24-h spawning cycle, but its levels were remarkably elevated thereafter, and reached a maximum at 3 h before ovulation. The relative expression levels of *ptger4b* in the follicles were 1 (-23 h), 2.2 (-19 h), 1.8 (-15 h), 47.5 (-11 h), 315 (-7 h), and 431 (-3 h) when the level at -23 h was set to 1. These results indicate that that expression of *ptger4b* in the large-sized follicles is increasingly upregulated as the time of ovulation approaches.

3.9. *In situ* detection of *ptger4b* mRNA in the ovary of the medaka

In situ detection of *ptger4b* mRNA was performed using sections of the fish ovaries isolated 3 h before ovulation. Clear signals were detected with the antisense probe in the oocyte cytoplasm of small-sized follicles having a diameter greater than 250 μm (Fig. 9A and 9B, asterisks). Specific staining was also observed in the follicle layer of the large-sized follicles (Fig. 9B, large arrows). Strong signals associated with small-sized follicles of a diameter less than 80 μm were detected (Fig. 9A and 9C, small arrows). However, a comparison with sections treated with the *ptger4b* sense probe indicated that these signals are nonspecific.

3.10. Inhibition of *in vitro* ovulation by an EP4 antagonist

Finally, we tested whether the EP4 antagonist GW627368X has an inhibitory effect on the *in vitro* ovulation of medaka ovarian follicles. A progressive decrease of the rate of ovulation was observed with increasing concentrations of the antagonist (Fig. 10). Under the conditions employed, the IC_{50} value was calculated to be about 150 nM. These results

indicate that the EP4 receptor may be responsible for exerting the action of PGE₂ in the ovulation of the medaka.

4. Discussion

A database search indicates that medaka contains two COX-1 genes (*ptgs1a* and *ptgs1b*) and one COX-2 gene (*ptgs2*) (Ishikawa et al., 2007; Havird et al., 2008). However, there has been no scientific report dealing with the cloning and/or expression of any of these medaka COX genes. In the present study, cDNA cloning of medaka *ptgs2* was conducted because *ptgs2* was demonstrated to be a dominant COX gene expressed in the mature fish ovary. The putative medaka COX-2 contains a unique set of domain structures found in the mammalian counterparts and possesses conserved amino acid residues essential for its catalytic activity (Simmons et al., 2004). Therefore, medaka COX-2 should serve as a functional enzyme producing PGH₂, from which various bioactive PG isomers, such as PGE₂ and PGF_{2α} are subsequently generated by the actions of isomerases and oxidoreductases (Simmons et al., 2004).

We observed that, compared with the *ptgs1a* and *ptgs1b* mRNA levels, *ptgs2* mRNA was always expressed at a dominant level throughout the whole ovary: the *ptgs2* mRNA level was approximately 20-fold higher than the *ptgs1a* mRNA level and 109-fold higher than the *ptgs1b* mRNA level. The same was true for the large-sized preovulatory follicles. This dominant expression of *ptgs2* over *ptgs1a* or *ptgs1b* in the medaka ovary is rather distinct from the findings in other teleosts; adult female zebrafish (Grosser et al., 2002; Ishikawa et al., 2007; Lister and Van Der Kraak, 2008) and brook trout (Roberts et al.,

2000) rarely have detectable levels of *ptgs2* in ovarian samples, while *cox1* is constitutively expressed. Assuming that active COX proteins are generated in proportion to the amounts of the respective *ptgs* transcripts, COX-2 could be virtually the only enzyme responsible for PG production in the ovaries of the mature medaka.

In the *in situ* hybridization analysis of mature medaka ovary sections, clear signals for *ptgs2* mRNA expression were detected in the oocytes of small-sized follicles having a diameter of less than 100 μm . These *ptgs2*-expressing follicles were at the primary growth/early-previtellogenic stage (stages I and II) of medaka folliculogenesis (Iwamatsu et al., 1988). Localization of *ptgs2* transcript in the follicle at this stage suggests that medaka *ptgs2* might be involved in follicle development, particularly at the early stages. Such a spatiotemporal expression pattern of medaka ovary *ptgs2* resembles that of *growth differentiation factor 9 (gdf9)* recently reported for zebrafish (Liu and Ge, 2007). In that study, *in situ* detection of the zebrafish *gdf9* transcript showed that the oocytes at the primary growth stage exhibited the strongest staining, and the signals declined progressively with the growth of the follicles.

Because we detected *ptgs2* mRNA signals only for small-sized follicles by the morphological analysis of ovary sections, we initially suspected that *ptgs2* would not be expressed in the fully-grown follicles of spawning fish. However, real-time RT-PCR analysis revealed that *ptgs2* mRNA was expressed in the large follicles. Further analyses revealed that not only oocytes but also follicle cells synthesized *ptgs2* mRNA in the large-sized follicles. This finding strongly suggests the involvement of COX-2 in oocyte maturation and/or ovulation of the medaka. This, in turn, raises an intriguing question as to how and to what extent germ cell-expressed COX-2, somatic cell-expressed COX-2, or

both contribute to these biological processes of the fish. To answer this question, further studies, including the measurement of COX-2 at the protein level and the estimation of catalytic activity for the enzyme localized in the two different cell types, are necessary. At any rate, a plausible explanation for our failure to demonstrate *ptgs2* mRNA expression in the medium- and large-sized follicles by *in situ* hybridization experiments is that only small-sized follicles express the *ptgs2* transcript in quantities sufficient to produce clear signals.

Previous studies using mammalian species have established that *ptgs2* is inducible (Espey and Richards, 2006). Evidence for the inducible nature of *ptgs2* has also been reported for zebrafish (Grosser et al., 2002; Ishikawa et al., 2007; Lister and Van Der Kraak, 2009), rainbow trout (Zou et al., 1999; Ishikawa and Herschman, 2007), and brook trout (Roberts et al., 2000). However, in the present study the *ptgs2* transcript levels were fairly constant in the ovary as well as in the large-sized follicles of mature medaka during the 24-h spawning cycle. These observations would seem to argue against the notion that medaka *ptgs2* is an inducible gene. We should note that many immediate-early genes often contain the AUUUA Shaw-Kamen motif (Shaw and Kamen, 1985), a pentameric sequence, in the AU-rich elements (AREs) of the 3'-untranslated region (UTR) of the mRNA (Dean et al., 2004; Espel, 2005). Indeed, multiple AREs motifs are present in common in the extended 3' UTR of zebrafish, rainbow trout, and brook trout *ptgs2* mRNA (Roberts et al., 2000). Interestingly, seven AUUUA Shaw-Kamen motifs were also found in the 3' UTR of medaka *ptgs2* mRNA. Obviously, medaka ovary *ptgs2* expression is presumed to be regulated in a manner distinct from the ovary *ptgs2* expression of the other three teleosts.

We detected COX activity in the extract of the fish ovary. In our *in vitro* ovulation

experiments using large-sized ovarian follicles, we found that ovulation was effectively suppressed by the COX inhibitor indomethacin, and that this suppression was prevented by concomitant addition of PGE₂. These findings strongly suggest that intra-follicular generation of PGE₂ is essential for successful ovulation of the fish. As discussed above, we conclude that COX-2 may be responsible for the production of PGE₂ in the large-sized follicles.

In contrast to the previous observation that PGs were produced at the time of ovulation in the brook trout ovary (Goetz and Cetta, 1983; Goetz and Garczynski, 1997), in the present study PGE₂ was always detectable in the large-sized follicles of the medaka and its levels did not change significantly during the 24-h spawning cycle. Since PGE₂ is synthesized by a series of enzymatic reactions involving phospholipase A₂, COX, and PGE synthase, the quantity of the metabolite is undoubtedly the result of the concerted actions of these enzymes. Insights into the mechanisms by which the PGE₂ levels in the large-sized follicles are determined await further investigations. In the present work, inclusion of indomethacin in the culture medium of the *in vitro* follicle ovulation experiments during the last 12 h prior to ovulation effectively suppressed the release of oocytes. This observation strongly suggests that exposure to PGE₂ at this time point is critical for the fully grown follicles to ovulate.

In order for PGE₂ to exert such an effect, the target cells must have specific receptors (EP) on the cell surface (Sugimoto and Narumiya, 2007). In this study, we demonstrated the dominant expression of the *ptger4b* gene in the fish ovary. In addition, the expression of *ptger4b* was dramatically increased in the large-sized follicles, which are destined to ovulate as the time of ovulation approaches. More importantly, the EP4 antagonist

(GW627368X) drastically reduced the rate of *in vitro* ovulation. These facts tempt us to speculate that EP4b may be the receptor responsible for mediating the actions of PGE₂ and that its expression on the surface of target cells may be critical for exerting an ovulation-inducing effect of the prostanoid in the medaka ovarian follicles. As clearly shown by our *in situ* hybridization analysis, *ptger4b* mRNA was localized in the follicle layer of the ovulating large-sized follicles. Transcripts of *ptgs2* were also demonstrated to be present in the same layer of the large-sized follicles. Therefore, the translated products of both COX-2 and EP4b should be associated with the follicle layer cells (granulosa cells or theca cells) of ovulating follicles. Identification of cells expressing COX-2 and/or EP4b in the follicle will be important for understanding how PGE₂ is involved in the fish ovulation.

In summary, the present study reports for the first time the expression of PGE₂-producing enzyme COX-2 (*ptgs2*) and PGE₂ receptor EP4b (*ptger4b*) mRNA in the medaka ovary. The current results strongly suggest the involvement of COX-2/EP4b in the ovulatory process of the fish. This study has established the basis for further investigations to define the mechanism of the role of PGE₂ in the ovulation of the medaka.

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FIGURE LEGENDS

Fig. 1. Effect of COX inhibitor on *in vitro* ovulation in the medaka ovarian follicles.

Large-sized follicles (at least 10 follicles/experiment) isolated from mature fish ovaries were incubated with a non-selective COX inhibitor indomethacin (INDO) at concentrations ranging from 0.01 to 10 μ M. In some experiments, the follicles were incubated in the medium containing both INDO (10 μ M) and PGE₂ (10 μ M). In parallel experiments, follicles were incubated in medium containing ethanol alone as a control. The ovulation rates were determined at 6 h after ovulation. The results are shown as the mean $\pm$ S.E.M. (n=3). Asterisks indicate significant differences at $P < 0.01$ (*) as compared to the control.

Fig. 2. Deduced amino acid sequence of medaka COX-2 and its domain structure.

(A) The primary sequence of medaka COX-2 (the present study) is compared with those of medaka COX-1a (Ensembl gene ID: ENSORLG00000005095) and COX-1b (Ensembl gene ID: ENSORLG0000000509). The putative signal peptide cleavage site is indicated by an arrowhead. Identical residues in the three COX proteins are boxed and shaded in gray. The residues (Tyr-374 and Val-512) of the Cox active site are indicated by asterisks. The residues essential for peroxidase activity (His-377) and for binding arachidonic acid (Arg-109 and Tyr-337) are indicated by filled circles and open circles, respectively. The asparagine residues (Asn-56, Asn-133, Asn-399, and Asn-584) for putative *N*-glycosylation are indicated by triangles. (B) The domain structure of medaka COX-2 is shown. SP, signal peptide; EGF, epidermal growth factor-like domain/dimerization domain; MBD, membrane-binding domain; and ER, ER retention signal. The numbers of amino acid residues in each domain are indicated at the bottom of

the figure.

Fig. 3. Expression of *ptgs2* mRNA in the medaka ovary.

(A) Northern blot analyses were conducted using a membrane loaded with 60 μ g (for *ptgs1a* and *ptgs1b*) or 30 μ g (for *ptgs2*) of total RNA isolated from various tissues of the mature medaka fish. The membranes were separately hybridized with 32 P-labeled cDNA fragments of *ptgs* genes. The lower panel of each set shows the detection of medaka cytoplasmic actin (*bactin*) mRNA with the 32 P-labeled *bactin* cDNA fragment. The results of one representative experiment out of two independent experiments with similar findings are presented. (B) Real-time RT-PCR analysis was conducted for *ptgs1a*, *ptgs1b*, and *ptgs2* using total RNA isolated from the mature medaka ovary. Data are presented as the mean $\pm$ S.E.M. (n=3). (C) Real-time RT-PCR analysis was performed for *ptgs2* using total RNA isolated from the ovaries of mature medaka with a 24 h-cycle of spawning. The expression levels were normalized to that of *bactin* and expressed as the fold change of the levels of the -23 h ovary. Data are presented as the mean $\pm$ S.E.M. (n=3).

Fig. 4. *In situ* detection of *ptgs2* mRNA in the medaka ovary.

Medaka ovaries isolated at 6 h after ovulation were used. Neighboring sections of the ovaries were hybridized with DIG-labeled *ptgs2* antisense (A and B) or sense (C and D) RNA probes. The boxed areas in (A) and (C) are shown at higher magnification in (B) and (D), respectively. Positively stained oocytes of small-sized growing follicles are indicated by an arrow. #, medium-sized follicles; +, large-sized follicles that are destined to ovulate at

the next cycle of ovulation. The scale bars represent 500 μm (A and C) and 200 μm (B and D). The reproducibility of the findings was confirmed by conducting three independent experiments, and the results of a representative experiment are presented.

Fig. 5. Expression of *ptgs2* mRNA in the ovarian follicles.

(A) Real-time RT-PCR analysis of *ptgs2* mRNA using different sizes of ovarian follicles. Total RNA was isolated from small-, medium- and large-sized follicles of the fish ovaries at 3 h before ovulation. The expression levels of the *ptgs2* mRNA were normalized with those of the medaka cytoplasmic actin (*bactin*) transcript that was measured on the same cDNAs. The results are presented as the mean $\pm$ S.E.M. (n=3), and the mRNA levels are shown relative to that of large-sized follicles. (B) Real-time RT-PCR analysis of *ptgs2* mRNA using oocytes and follicle layers of ovarian follicles. Oocytes and follicle layers were prepared from large-sized ovarian follicles isolated from mature medaka at 2 h before ovulation, and the total RNAs were separately prepared. The expression levels of the *ptgs2* mRNA were normalized with medaka *bactin*, and expressed as the fold change of the levels of the follicle layer. The results are presented as the mean $\pm$ S.E.M. (n=3). (C) Real-time RT-PCR analysis of *ptgs2* mRNA using large-sized follicles. The large-sized follicles, which were destined to ovulate in 24 h, were isolated from the fish ovaries at the indicated time points. The expression levels were normalized to that of *bactin* and expressed as the fold change of the levels of the -23 h follicle. Data are presented as the mean values $\pm$ S.E.M. (n=3).

Fig. 6. COX activity in the mature medaka ovary.

(A) Ovaries, livers and intestines were obtained from the mature female medaka at 3 h before ovulation. (B) Large-sized follicles were isolated from mature fish ovaries at the indicated time points. Enzyme samples were prepared as described in the **Materials and methods**. COX activity assays were conducted according to the manufacturer's protocol using tissue and follicle extracts containing approximately 30 µg protein/reaction. The results are shown as the mean ± S.E.M. (n=3).

Fig. 7. PGE₂ levels in the medaka ovary.

(A) Ovaries, livers and intestines were obtained from mature female medaka at 3 h before ovulation, and used for PGE₂ determination. PGE₂ were determined according to the manufacturer's protocol using the extracts. The results are shown as the mean ± S.E.M. (n=3). (B) Large-sized follicles were isolated from mature female medaka ovaries every 4 h, and were used for PGE₂ determination. The results are shown as the mean ± S.E.M. (n=5).

Fig. 8. Expression of PGE₂ receptor mRNA in the medaka ovary.

(A) Real-time RT-PCR analysis of *ptger* mRNA using total RNA isolated from the ovaries of mature fish at 3 h before ovulation. The expression levels of the *ptger* mRNA were normalized with medaka *bactin* and expressed as the fold change of the levels of the *ptger1a*. The results are shown as the mean ± S.E.M. (n=3). (B) Real-time RT-PCR analysis of *ptger4b* mRNA using large-sized follicles. The large-sized follicles, which were destined to ovulate in 24 h, were isolated from the fish ovaries at the indicated time points. The expression levels of *ptger4b* mRNA were normalized to that of *bactin* and expressed as the

fold change of the levels of the -23 h follicle. Data are presented as the mean values $\pm$ S.E.M. (n=3).

Fig. 9. *In situ* detection of *ptger4b* mRNA in the medaka ovary.

Medaka ovaries isolated at 3 h before ovulation were used. Neighboring sections of the ovaries were hybridized with DIG-labeled *ptger4b* antisense (A and B) or sense (C and D) RNA probes. The boxed areas in (A) and (C) are shown at higher magnification in (B) and (D), respectively. Positively stained oocytes of small-sized growing follicles are indicated by an asterisk. Positive staining associated with the follicle layers of large-sized follicles are indicated by a large arrow. Small arrows indicate non-specific staining associated with small-sized follicles with a diameter less than 80 μ m. +, large-sized follicles that are destined to ovulate at the next cycle of ovulation. The scale bars represent 500 μ m (A and C) and 200 μ m (B and D). The reproducibility of the findings was confirmed by conducting three independent experiments, and the results of a representative experiment are presented.

Fig. 10. Effect of EP4 antagonist on *in vitro* ovulation in the medaka ovarian follicles.

Large-sized follicles (at least 10 follicles/experiment) isolated from mature fish ovaries were incubated with GW627368X (EP4 antagonist) at concentrations ranging from 0.01 to 10 μ M. In parallel experiments, follicles were incubated in medium containing DMSO alone as a control. The ovulation rates were determined at 6 h after ovulation. The results are shown as the mean $\pm$ S.E.M. (n=3). Asterisks indicate significant differences at $P < 0.01$ (*) as compared to the control.

Table 1. Primers used for cloning, Northern blotting, in situ hybridization, and real-time PCR.

Primer name	Gene	Primer sequence	Accession No.
<u>Cloning / 3'-RACE / 5'-RACE</u>			
COX2S1	<i>ptgs2</i>	5'-ATGAACTCACTTAGAGCTGTG -3'	AB516993
COX2S2	<i>ptgs2</i>	5'-GCTCGTTTGTGACAATGTCAG-3'	
COX2S3	<i>ptgs2</i>	5'-CTCTCGCCTAGCAGATGTCAA-3'	
COX2S4	<i>ptgs2</i>	5'-CTGAAGGAAAGGACTACTGAA-3'	
COX2AS1	<i>ptgs2</i>	5'-TCAGGTTTTACCTCTCCCA -3'	
COX2AS2	<i>ptgs2</i>	5'-CCCAAACCTTGTGCACAC-3'	
COX2AS3	<i>ptgs2</i>	5'-GCACACACCTCTGTTCTGGCAAGG-3'	
COX2AS4	<i>ptgs2</i>	5'-AGGCCATGAGCAGCACGGATTAC-3'	
<u>Northern blotting / <i>in situ</i> hybridization</u>			
COX2S4	<i>ptgs2</i>	5'-CTGAAGGAAAGGACTACTGAA-3'	AB516993
COX2AS1	<i>ptgs2</i>	5'-TCAGGTTTTACCTCTCCCA-3'	
COX1aS1	<i>ptgs1a</i>	5'-GGCTACCTCAACTGGGAATC-3'	ENSORLG00000005095
COX1aAS1	<i>ptgs1a</i>	5'-CAGACCCAAACCTGGTAGGAG-3'	
COX1bS1	<i>ptgs1b</i>	5'-GGAGAAACTGGTGGATGCTT-3'	ENSORLG00000000509
COX1bAS1	<i>ptgs1b</i>	5'-TCCTGCCTCTTCCTGGTTTGG-3'	
EP4bSS1	<i>ptger4b</i>	5'-GCAGCGCTCTCTCTTCTTGG-3'	AB563504
EP4bAS1	<i>ptger4b</i>	5'-TGCAGATGACCG-3'	
<u>RT-PCR</u>			
EP1aSS1	<i>ptger1a</i>	5'-TTCTGCTTCCAGGGGGACCCA-3'	ENSORLG00000016366
EP1aAS1	<i>ptger1a</i>	5'-AGATCCGTGGCTTTGGTGCCA-3'	
EP1bSS1	<i>ptger1b</i>	5'-TTACCAAACCGCTGCCACATA-3'	ENSORLG00000014518

Table 1. (continued)

Primer name	Gene	Primer sequence	Accession No.
EP1bAS1	<i>ptger1b</i>	5'-CAGCTTCAGTACGCTGACTGT-3'	
EP2SS1	<i>ptger2</i>	5'-GCTGCAGGCATCTTCGGCAAC-3'	ENSORLG00000003901
EP2AS1	<i>ptger2</i>	5'-CGATCGGTTTGCTCACAGTGA-3'	
EP3SS1	<i>ptger3</i>	5'-GACAGCAAAGTGCCAGGAAAC-3'	ENSORLG00000007950
EP3AS1	<i>ptger3</i>	5'-CAGAGTCCTGCTGTTATTAGC-3'	
EP4aSS1	<i>ptger4a</i>	5'-AGTTGGCCGGATTCACTCTG-3'	ENSORLG00000003780
EP4aAS1	<i>ptger4a</i>	5'-GACATCCTGCGCACAAAGCG-3'	
EP4bSS1	<i>ptger4b</i>	5'-GCAGCGCTCTCTCTTCTTGG-3'	AB563504
EP4bAS2	<i>ptger4b</i>	5'-GGTCTTCATTGATCAGTGAGG-3'	
OLCA1SS	<i>bactin</i>	5'-CAGACACGTATTTGCCTCTG-3'	D89627
OLCA1AS	<i>bactin</i>	5'-CAAGTCGGAACACATGTGCA-3'	
<u>Real-time RT-PCR</u>			
COX2S5	<i>ptgs2</i>	5'-AAGCCCTATGCGTCTTTTGA-3'	AB516993
COX2AS5	<i>ptgs2</i>	5'-CGGGGTTTCTCAACCAGTAG-3'	
COX1aS1	<i>ptgs1a</i>	5'-GGCTACCTCAACTGGGAATC-3'	ENSORLG00000005095
COX1aAS2	<i>ptgs1a</i>	5'-AGCCTCTCCACCACAACCT-3'	
COX1bS1	<i>ptgs1b</i>	5'-GGAGAAACTGGTGGATGCTT-3'	ENSORLG00000000509
COX1bAS2	<i>ptgs1b</i>	5'-ATCTGGGGCCACCTTAAGAAC-3'	
EP1aSS2	<i>ptger1a</i>	5'-CGTAAGGTCCCACGATACAG-3'	ENSORLG00000016366
EP1aAS2	<i>ptger1a</i>	5'-TTCTGACGCCGGTCACCATC-3'	
EP1bSS2	<i>ptger1b</i>	5'-ACGGTCACCTCCTGCATC-3'	ENSORLG00000014518
EP1bAS2	<i>ptger1b</i>	5'-TTTGTAGCTGCACAGCTTGTC-3'	

Table 1. (continued)

Primer name	Gene	Primer sequence	Accession No.
EP2SS2	<i>ptger2</i>	5'-AGGAGGTGGAGCATCTCATC-3'	ENSORLG00000003901
EP2AS2	<i>ptger2</i>	5'-CCGTCAGATCCAAAATGTGA-3'	
EP3SS2	<i>ptger3</i>	5'-CTCCTCTGCTGGTACTGATG-3'	ENSORLG00000007950
EP3AS2	<i>ptger3</i>	5'-AGACGAATCGCTGTCAGAAA-3'	
EP4aSS2	<i>ptger4a</i>	5'-GCAAGATGCCACAAACACTT-3'	ENSORLG00000003780
EP4aAS2	<i>ptger4a</i>	5'-GAGTGCGGGATCTTTTGAAT-3'	
EP4bSS2	<i>ptger4b</i>	5'-CAGATGGTGATCCTGCTCAT-3'	AB563504
EP4bAS3	<i>ptger4b</i>	5'-GCCAGGAGGTCTTCATTGAT-3'	
OLCA1S2	<i>bactin</i>	5'-TGACGGAGCGTGGCTACTC-3'	D89627
OLCA1AS2	<i>bactin</i>	5'-TCCTTGATGTCACGGACAATTT-3'	
18SS1	<i>18S rRNA</i>	5'- CGTTCAGCCACACGAGATTG-3'	AB105163
18SAS1	<i>18S rRNA</i>	5'-CCGGACATCTAAGGGCATCA -3'	

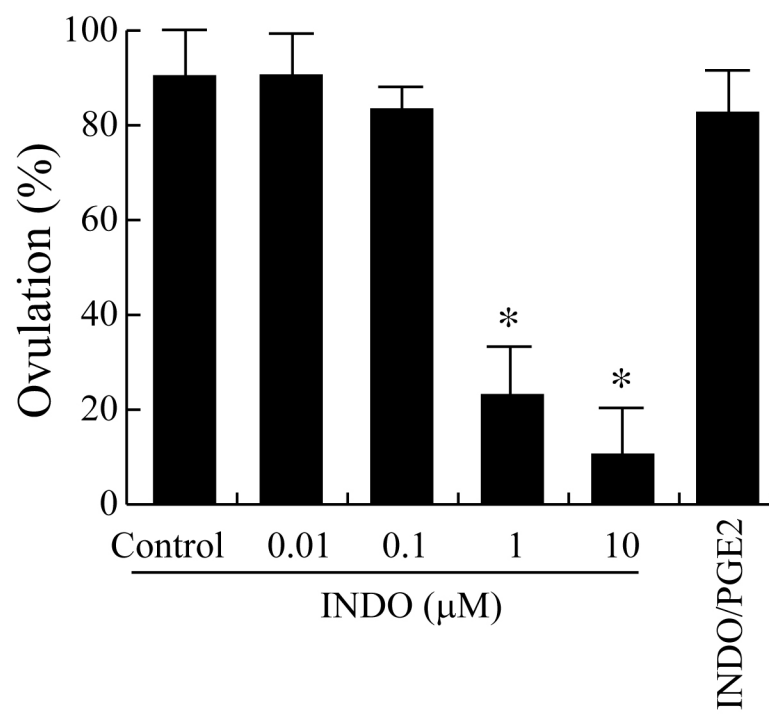
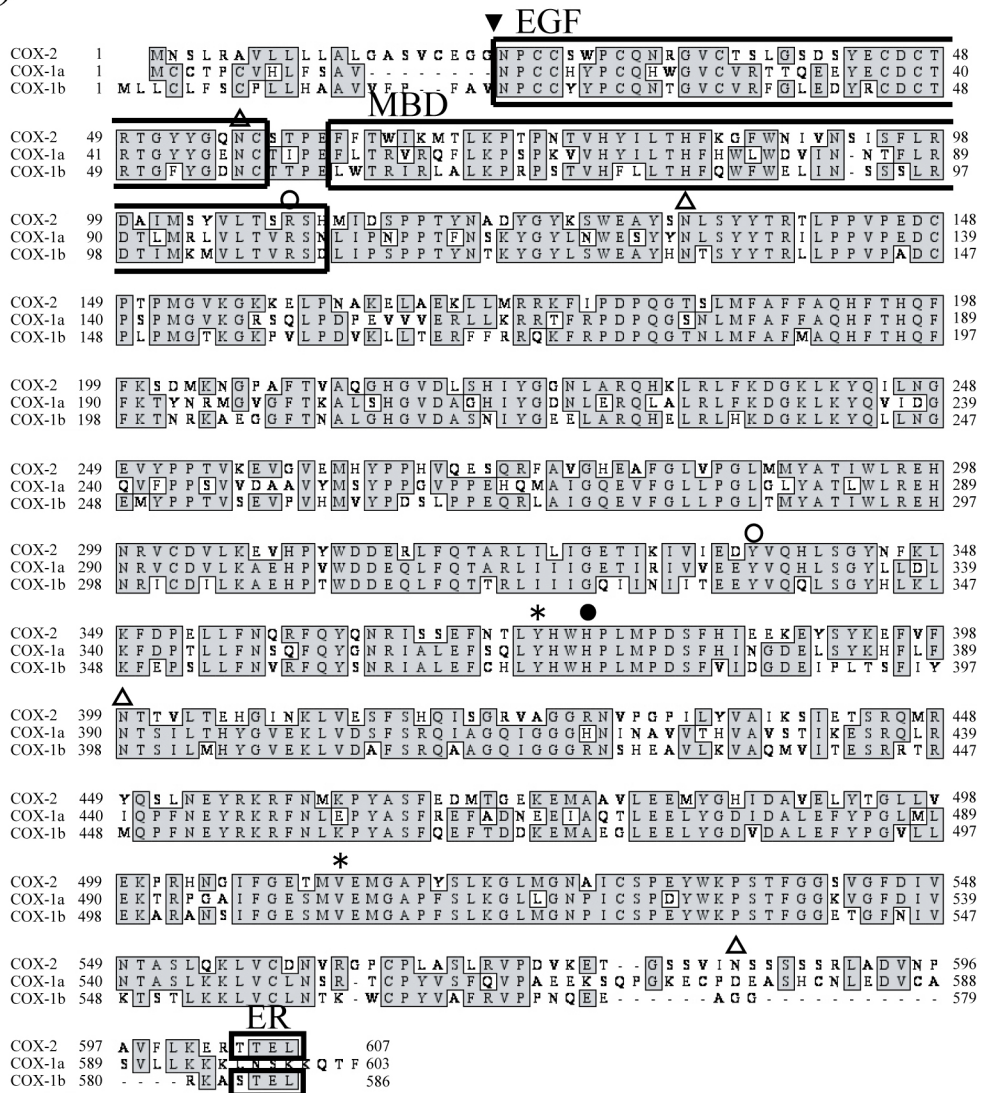


Figure 1 Fujimori et al.

(A)



(B)

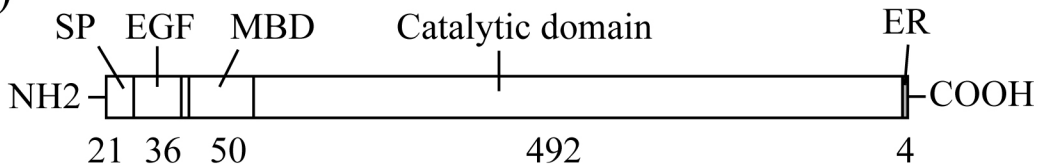
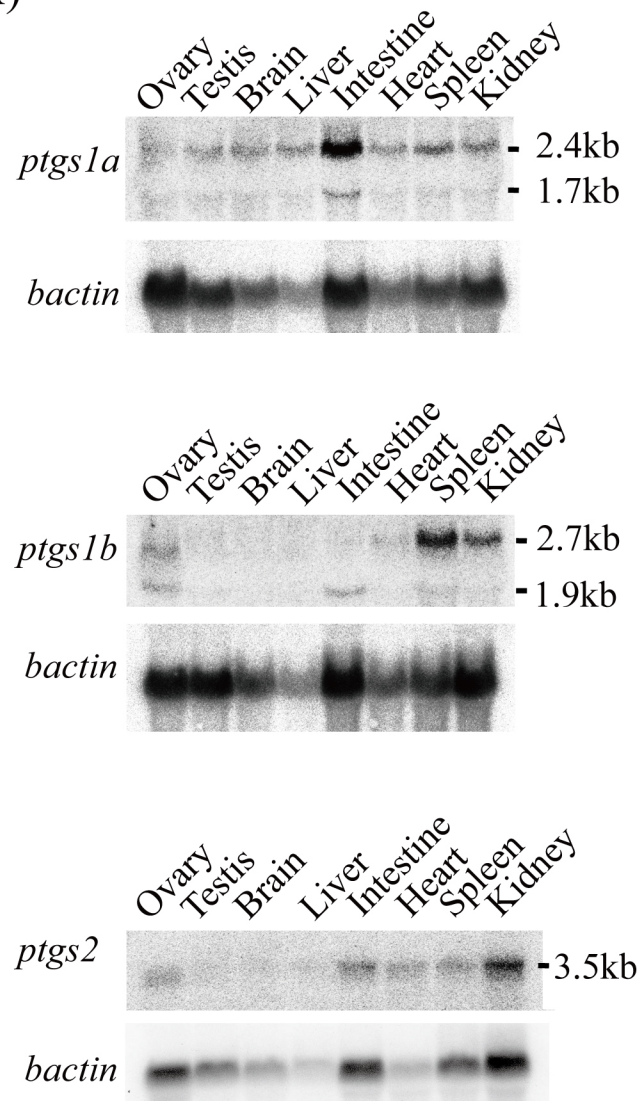
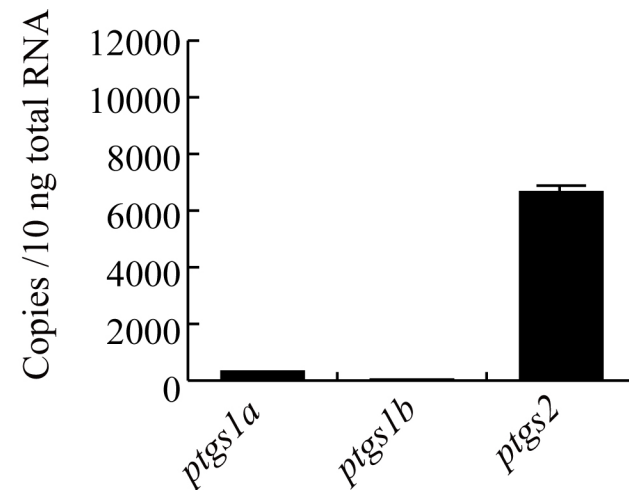


Figure 2 Fujimori et al.

(A)



(B)



(C)

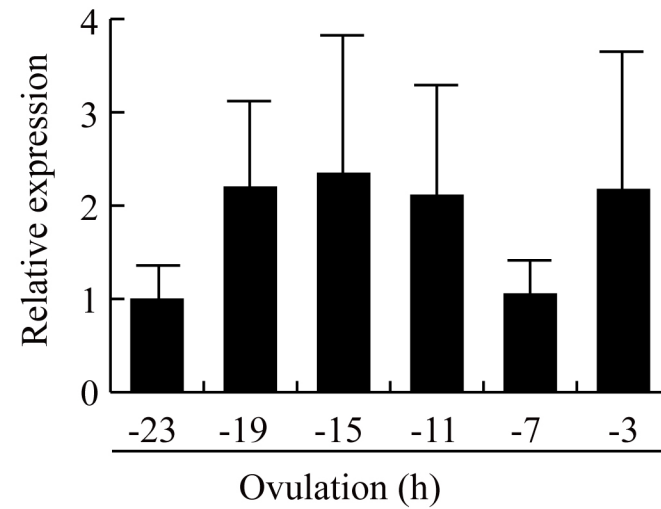


Figure 3 Fujimori et al.

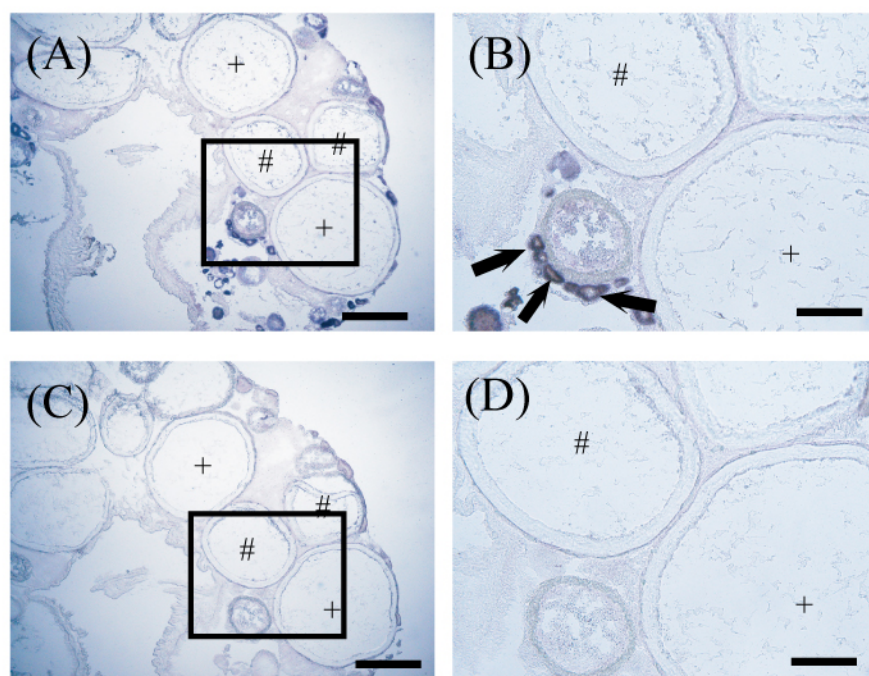
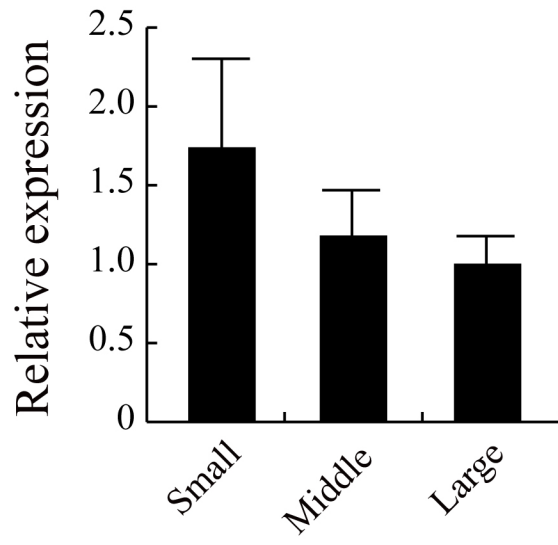
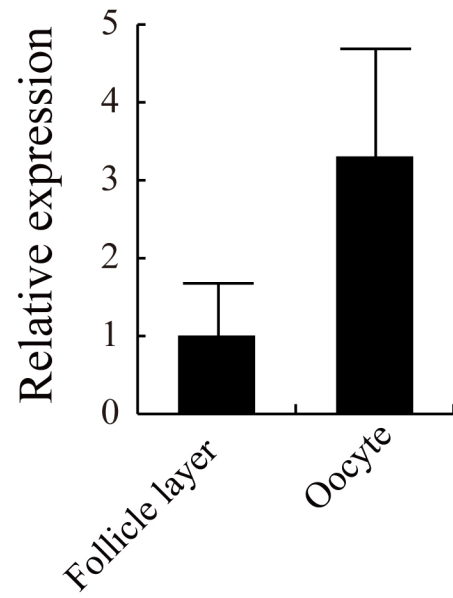


Figure 4 Fujimori et al.

(A)



(B)



(C)

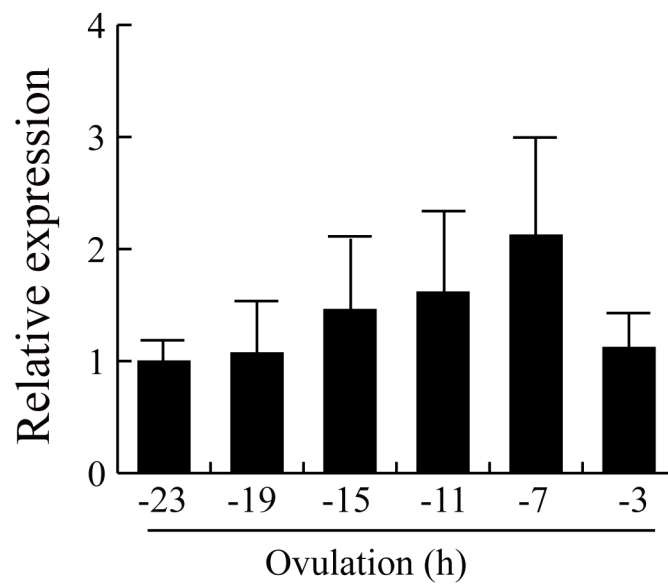
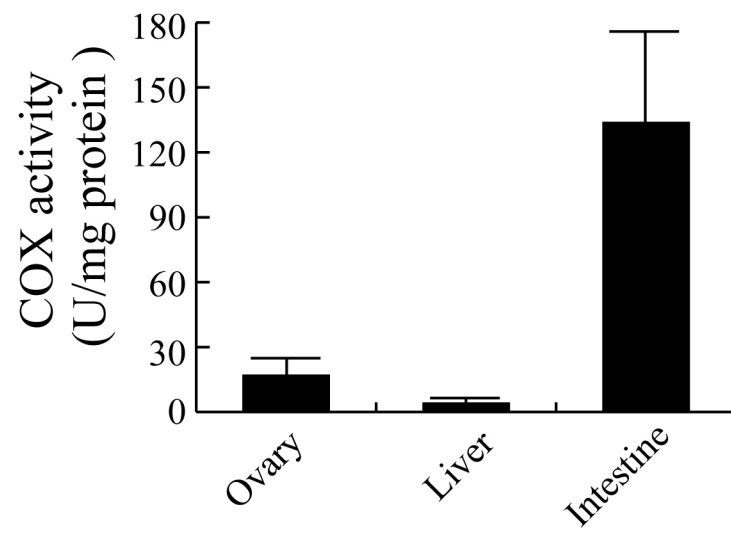


Figure 5 Fujimori et al.

(A)



(B)

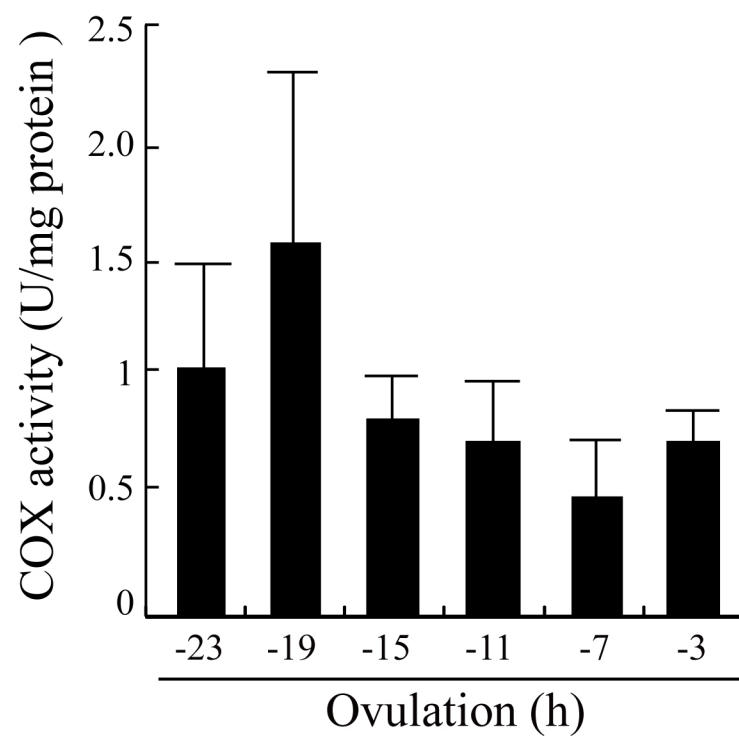
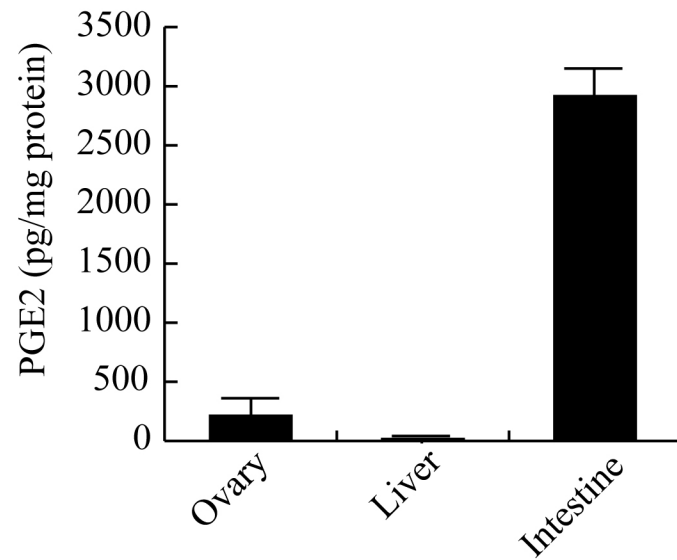


Figure 6 Fujimori et al.

(A)



(B)

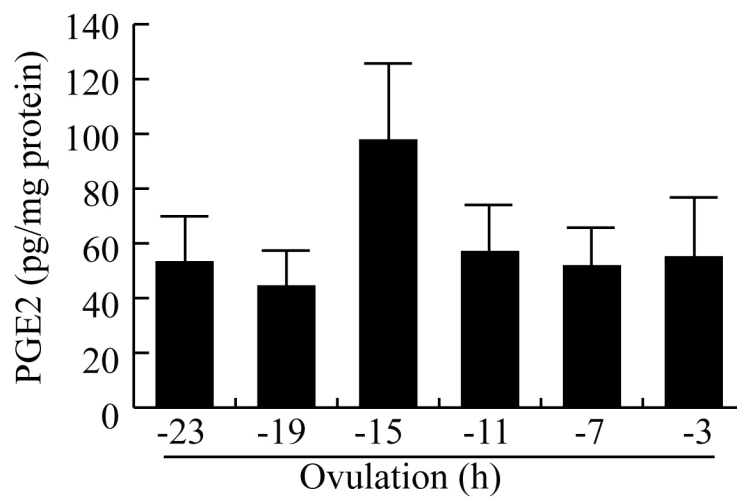
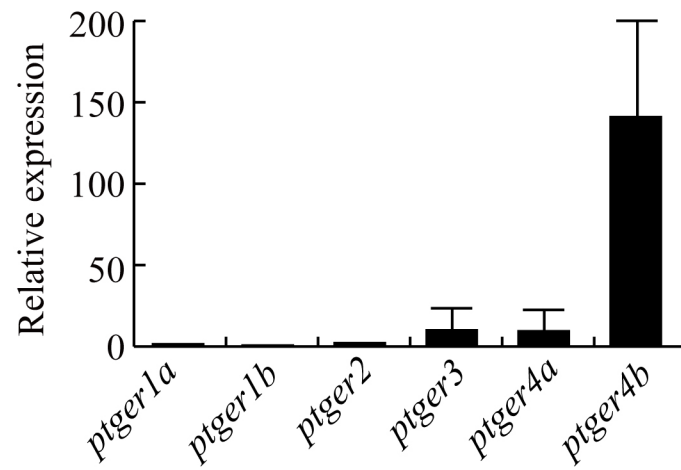


Figure 7 Fujimori et al.

(A)



(B)

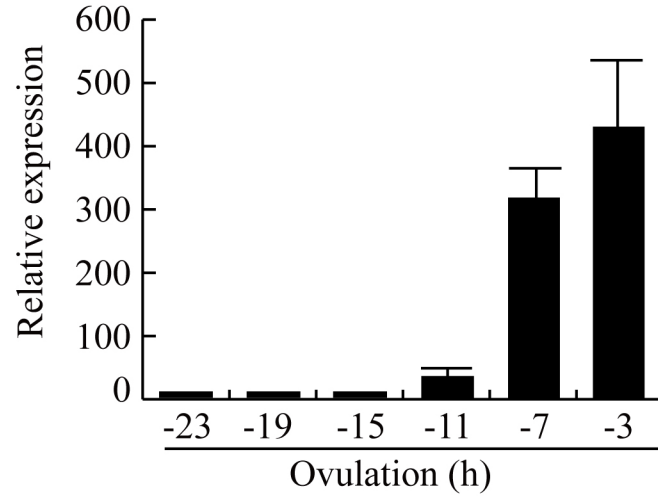


Figure 8 Fujimori et al.

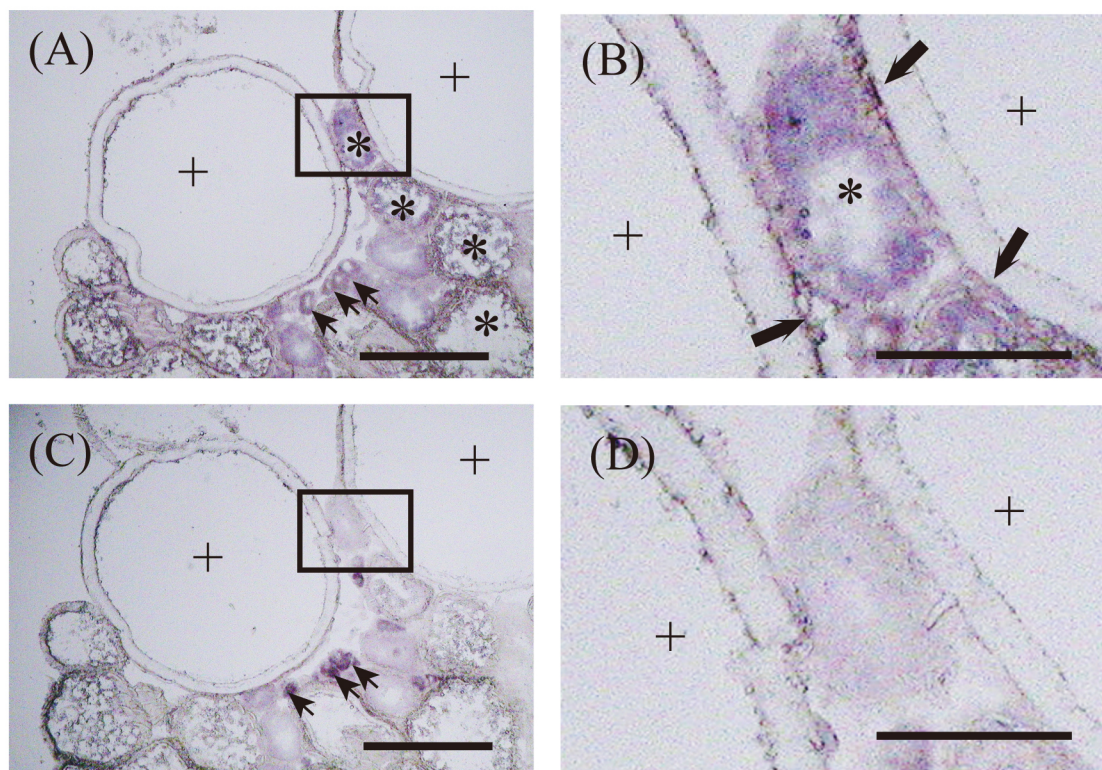


Figure 9 Fujimori et al.

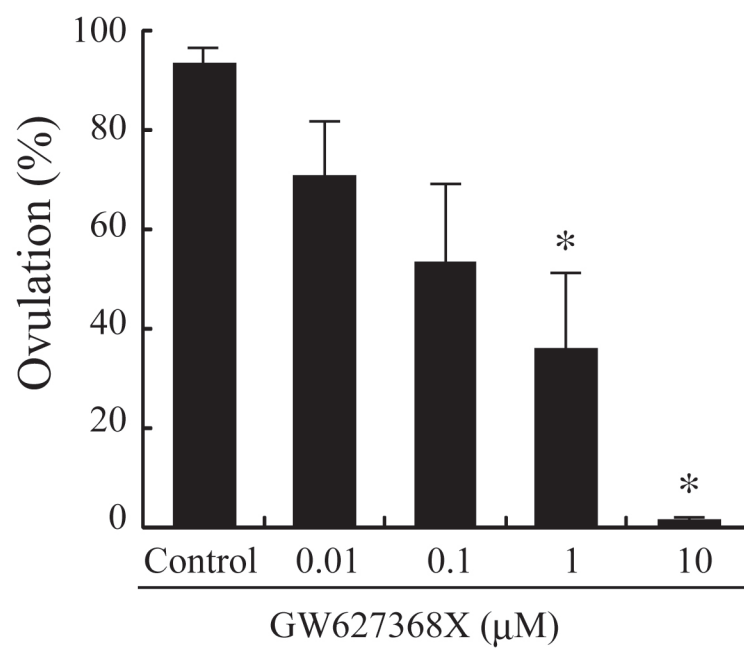


Figure 10 Fujimori et al.